



# Zolpidem Activation of Alpha 1-Containing GABA<sub>A</sub> Receptors Selectively Inhibits High Frequency Action Potential Firing of Cortical Neurons

## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Neuropharmacology,  
a section of the journal  
Frontiers in Pharmacology

Received: 09 April 2018

Accepted: 12 December 2018

Published: 09 January 2019

### Citation:

Neumann E, Rudolph U,  
Knutson DE, Li G, Cook JM,  
Hentschke H, Antkowiak B and  
Drexler B (2019) Zolpidem Activation  
of Alpha 1-Containing GABA<sub>A</sub>  
Receptors Selectively Inhibits High  
Frequency Action Potential Firing  
of Cortical Neurons.  
*Front. Pharmacol.* 9:1523.  
doi: 10.3389/fphar.2018.01523

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**Introduction:** High frequency neuronal activity in the cerebral cortex can be induced by noxious stimulation during surgery, brain injury or poisoning. In this scenario, it is essential to block cortical hyperactivity to protect the brain against damage, e.g., by using drugs that act as positive allosteric modulators at GABA<sub>A</sub> receptors. Yet, cortical neurons express multiple, functionally distinct GABA<sub>A</sub> receptor subtypes. Currently there is a lack of knowledge which GABA<sub>A</sub> receptor subtypes would be a good pharmacological target to reduce extensive cortical activity.

**Methods:** Spontaneous action potential activity was monitored by performing extracellular recordings from organotypic neocortical slice cultures of wild type and GABA<sub>A</sub>R- $\alpha$ 1(H101R) mutant mice. Phases of high neuronal activity were characterized using peri-event time histograms. Drug effects on within-up state firing rates were quantified via Hedges' g.

**Results:** We quantified the effects of zolpidem, a positive modulator of GABA<sub>A</sub> receptors harboring  $\alpha$ 1-subunits, and the experimental benzodiazepine SH-053-2'F-S-CH<sub>3</sub>, which preferably acts at  $\alpha$ 2/3/5- but spares  $\alpha$ 1-subunits. Both agents decreased spontaneous action potential activity but altered the firing patterns in different ways. Zolpidem reduced action potential firing during highly active network states. This action was abolished by flumazenil, suggesting that it was mediated by benzodiazepine-sensitive GABA<sub>A</sub> receptors. SH-053-2'F-S-CH<sub>3</sub> also attenuated neuronal activity, but unlike zolpidem, failed to reduce high frequency firing. To confirm that zolpidem actions were indeed mediated via  $\alpha$ 1-dependent actions, it was evaluated in slices from wild type and  $\alpha$ (H101R) knock-in mice. Inhibition of high frequency action potential firing was observed in slices from wild type but not mutant mice.

**Conclusion:** Our results suggest that during episodes of scarce and high neuronal activity action potential firing of cortical neurons is controlled by different GABA<sub>A</sub> receptor subtypes. Exaggerated firing of cortical neurons is reduced by positive modulation of  $\alpha$ 1-, but not  $\alpha$ 2/3/5-subunit containing GABA<sub>A</sub> receptors.

**Keywords:** zolpidem, benzodiazepines, GABA<sub>A</sub> receptor subtypes, neocortex, electrophysiology, GABA<sub>A</sub> receptor subtype specific drugs, neocortical activity

## INTRODUCTION

High frequency electroencephalographic activity is generated during sensory processing and cognition. In human subjects and in rodents sedation and anesthetic-induced unconsciousness correlates with a decrease in high frequency oscillations in the cerebral cortex and the hippocampus (Sleigh et al., 2001; Ma et al., 2002; Hudetz et al., 2011). In patients suffering from traumatic brain injury, exaggerated neuronal activity may lead to post-traumatic epilepsy (Cantu et al., 2015). Epileptic seizures *per se* are characterized by hypersynchronous high neuronal activity. Excessive neuronal activity causes extensive energy consumption and a breakdown of ion gradients across neuronal membranes. Furthermore, calcium ions enter the cytoplasm through different routes, including extra-synaptic NMDA receptors and internal calcium stores (Bano et al., 2005). These events potentially trigger apoptotic pathways, possibly resulting in long-lasting cognitive impairment (Parsons and Raymond, 2014). In these scenarios it seems desirable to limit cortical hyperactivity to prevent further damage. Most drugs currently used to depress cortical activity such as anesthetics or benzodiazepines act on the molecular level by enhancing the function of GABA<sub>A</sub> receptors. In cortical networks multiple GABA<sub>A</sub> receptor subtypes are abundant (Rudolph et al., 2001; Sieghart and Sperk, 2002; Whiting, 2003; Möhler, 2006). They differ in their subunit composition, cellular localization, and physiological function. However, it remains to be elucidated to what extent distinct receptor subtypes are involved in the control of high frequency neuronal activity.

In the cerebral cortex about 80% of neurons are glutamate-releasing PCs, which innervate different classes of GABAergic interneurons. About half of all GABA<sub>A</sub> receptors in the cerebral cortex harbor  $\alpha$ 1-subunits. They reside at high densities at the somata of PCs. Since action potentials are generated at the somata and axon hillock, the presence of  $\alpha$ 1-GABA<sub>A</sub> receptors at somatic sites makes it likely that this receptor subtype plays a major role in the control of action potential generation. We, therefore, hypothesized that high frequency action potential firing of cortical neurons can be attenuated by drugs which enhance the function of  $\alpha$ 1-subunits containing GABA<sub>A</sub> receptors. Consequently, we examined the effects of zolpidem, a positive modulator of  $\alpha$ 1-containing GABA<sub>A</sub> receptors (Sanna et al., 2002; Baur and Sigel, 2007). Studies on expressed receptors suggest that zolpidem's selectivity for  $\alpha$ 1-receptors is limited

to low concentrations. To clearly distinguish the drug's action at  $\alpha$ 1-receptors from effects via other targets, we studied zolpidem in slices prepared from both wild type and  $\alpha$ 1(H101R) knock-in mice (Rudolph et al., 1999). In these mutants, the physiological function of GABA<sub>A</sub> receptors harboring  $\alpha$ 1-subunits is not substantially compromised, but receptors are largely resistant to modulation by positive allosteric modulators of the benzodiazepine binding site, including zolpidem. To confirm the specificity of results obtained by  $\alpha$ 1-containing GABA<sub>A</sub> receptors preferring zolpidem in wild type and  $\alpha$ 1(H101R) cultured slices, we also tested the  $\alpha$ 2/3/5-preferring experimental benzodiazepine SH-053-2'F-S-CH3 (Fischer et al., 2010; Savic et al., 2010; Richetto et al., 2015). The major result is that positive modulation of  $\alpha$ 1-, but not  $\alpha$ 2/3/5-containing GABA<sub>A</sub> receptors effectively reduces action potential firing during phases of exaggerated neuronal activity.

## MATERIALS AND METHODS

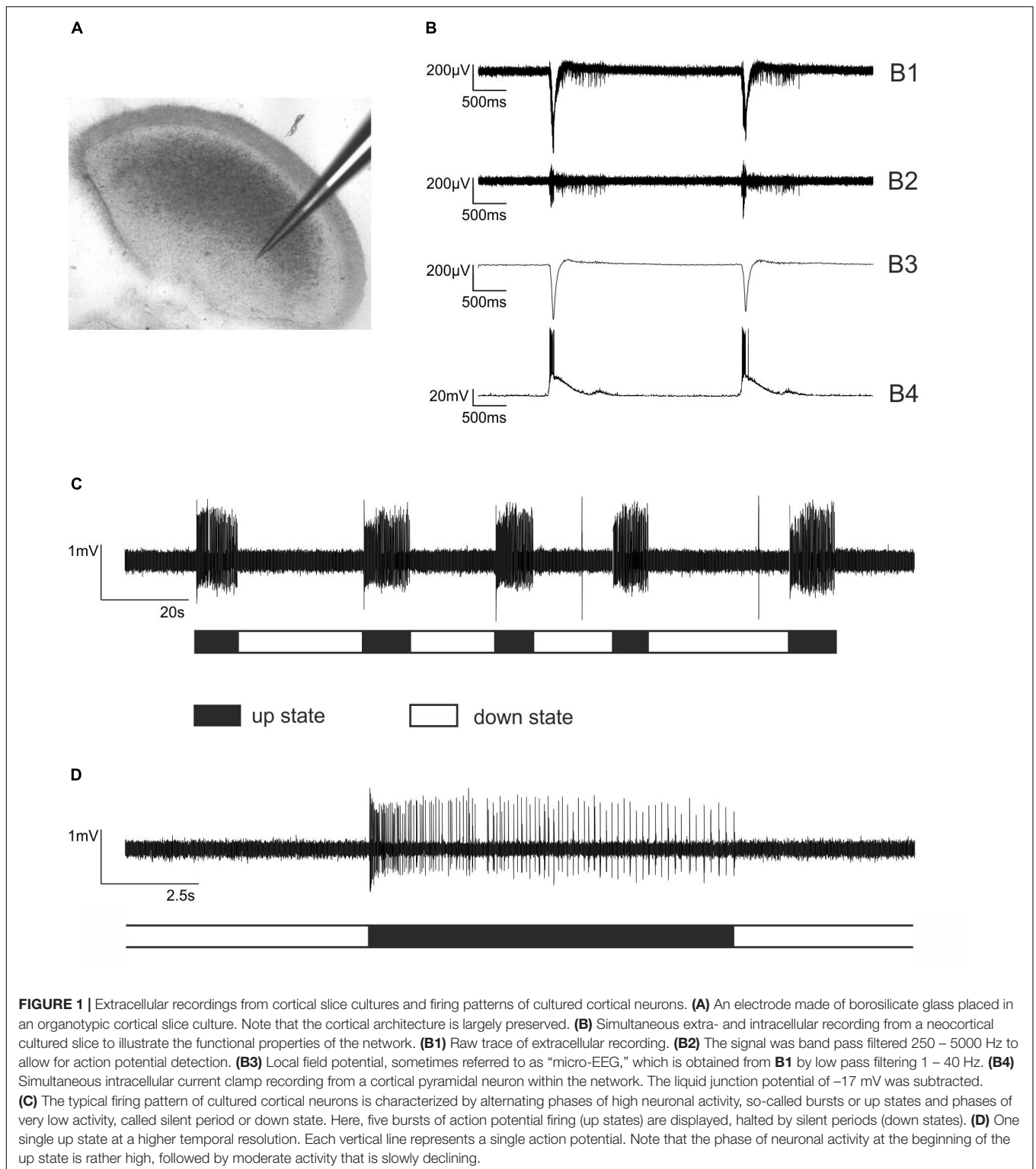
### Animals

For this study, we used 12 mice of both sexes, homozygous for an histidine to arginine point mutation at position 101 of the GABA<sub>A</sub> receptor  $\alpha$ 1-subunit (H101R) and 50 homozygous wild type controls of the same genetic background (C57BL/6J) (Rudolph et al., 1999). All procedures were approved by the Animal Care Committee (Eberhard-Karls-University, Tuebingen, Germany) and were in accordance with the institutional and federal guidelines of the German Animal Welfare Act (TierSchG). We put in a great deal of effort to reduce the number and suffering of animals.

### Organotypic Slice Cultures and Electrophysiology

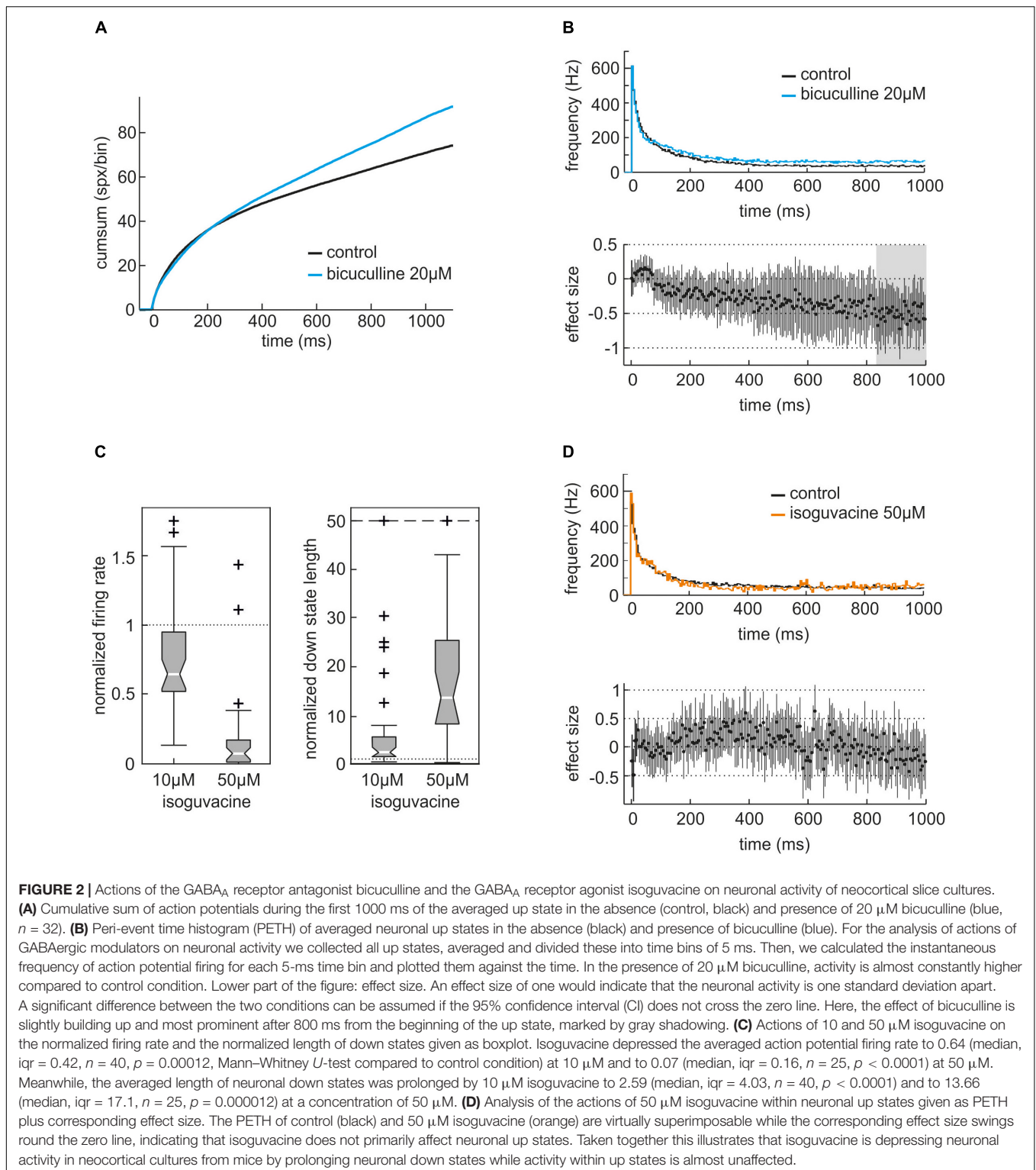
We prepared neocortical slice cultures from 2 to 5-day old mice as previously described (Gähwiler, 1981). For the preparation of the somatosensory cortex, mice were deeply anesthetized using isoflurane and decapitated. Cortical hemispheres were aseptically removed and after removal of the meninges, coronal slices were cut. These slices were fixed on glass coverslips using a plasma clot. Slices were cultured with nutrition medium in a roller drum at 37°C. For the current study we performed 305 extracellular network recordings of single- and multi-unit cortical activity in 173 slice cultures from wild type mice and 140 recordings in 81 cultures from  $\alpha$ 1(H101R) knock-in mice at 15 to 35 days *in vitro*. Recordings were performed with one or two extracellular electrodes in a recording chamber

**Abbreviations:** ACSE, artificial cerebrospinal fluid; CI, confidence interval; FS, fast-spiking cell; GABA,  $\gamma$ -amino-butyrac acid; iqr, interquartile range; NMDA, N-methyl-D-aspartate; OTC, organotypic culture; PC, pyramidal cell; PETH, peri-event time histogram.



mounted on an inverted microscope (**Figure 1A**). Further, we perfused slices with ACSF, consisting of (in mM) NaCl 120, KCl 3.3,  $\text{NaH}_2\text{PO}_4$  1.13,  $\text{NaHCO}_3$  26,  $\text{CaCl}_2$  1.8 and glucose 11, bubbled with 95% oxygen and 5% carbon dioxide. We advanced ACSF-filled glass electrodes with a resistance of about

3 to 5  $\text{M}\Omega$  into the tissue until extracellular spikes exceeding  $100 \mu\text{V}$  in amplitude were visible. We conducted all experiments at  $34^\circ\text{C}$  and acquired data on a personal computer, using a digidata 1200 AD/DA interface and Axoscope 9 software (Axon Instruments, Foster City, CA, United States). For **Figure 1B4**



whole-cell current clamp recording was performed from a visually identified pyramidal neuron. A borosilicate electrode was filled with a solution containing (in mM) K-gluconate 135, HEPES 10, EGTA 10, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2.0, Na<sub>2</sub>ATP 3.0, NaGTP 0.3, Na<sub>2</sub>phosphocreatine 10.0 (pH 7.3), and had

a resistance of  $\sim 3.5$  M $\Omega$ . The signal was acquired with a MultiClamp 700B amplifier, lowpass filtered at 6000 Hz, and digitized at 20 kHz via a Digidata 1440 interface and pClamp 10 software (Molecular Devices, San Jose, CA, United States).

## Preparation and Application of Test Solutions

We prepared a stock solution of zolpidem and flumazenil (both from Sigma, Taufkirchen, Germany) by diluting them in ethanol, while preparing a stock solution of SH-053-2'F-S-CH<sub>3</sub>, by diluting it in dimethyl sulfoxide. All drug-containing solutions were frozen, freshly dissolved in ACSF to the desired concentrations and filled into gastight glass syringes. The ethanol and dimethyl sulfoxide dilutions used for the current study had no effect on the firing patterns of cultured neocortical neurons (Drexler et al., 2013).

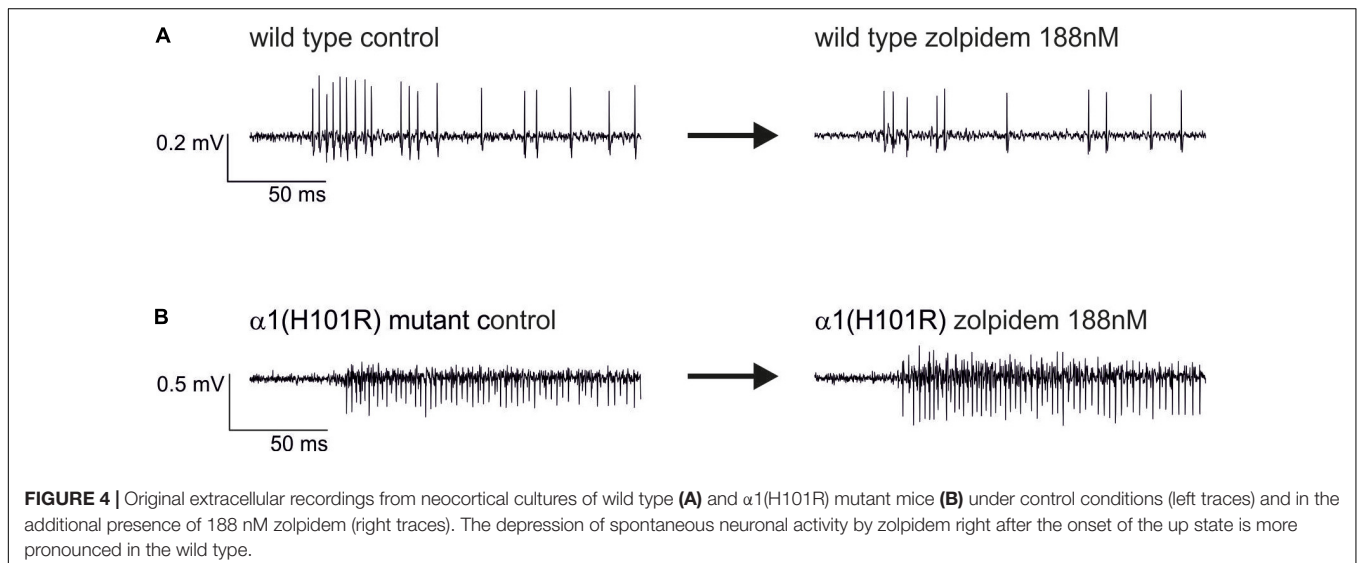
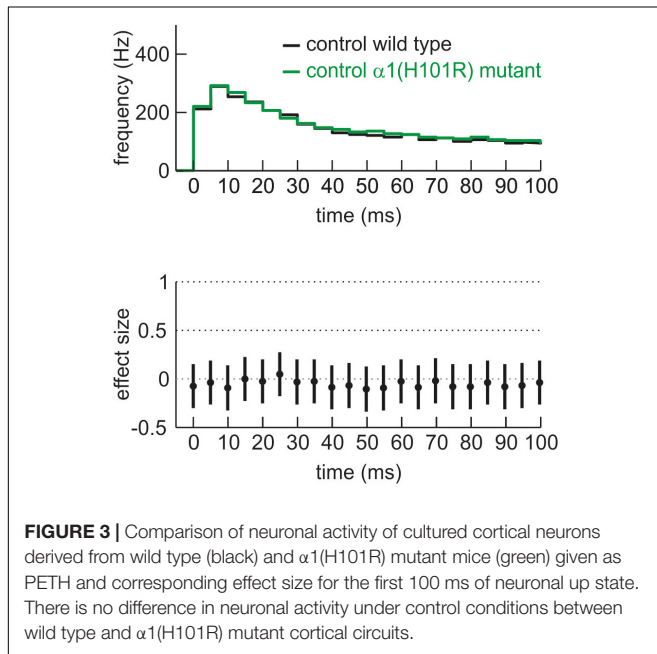
A drug containing ACSF was applied via bath perfusion, using syringe pumps (ZAK, Marktheidenfeld, Germany) connected to

the experimental chamber via Teflon tubing (Lee, Frankfurt, Germany). The flow rate was approximately 1 ml min<sup>-1</sup>. After switching from ACSF to drug-containing solutions, we replaced the medium in the experimental chamber by at least 95% within 2 min. To ensure steady state conditions, we performed recordings during drug treatment in 10-min intervals after changing the perfusate. This time interval proved to be sufficient for steady state conditions (Antkowiak, 1999), as diffusion times in slice cultures are considerably shorter compared to acute slice preparations (Gredell et al., 2004; Benkwitz et al., 2007). Slices were exposed to a maximum of two concentrations of the drug tested.

## Data and Statistical Analysis

We estimated group sizes based on earlier studies and analyzed recorded signals offline, using self-written programs in MATLAB (The MathWorks Inc., Natick, MA, United States). The typical firing pattern of neocortical slice cultures is characterized by bursts of spontaneous action potential firing (up state) separated by periods of neuronal silence (down state, see **Figure 1C**). An up state was defined as a group of action potentials with an initially high firing rate of 10 Hz, preceded by a down state. For further analysis, action potential activity in single cultures was binned and firing rates were averaged over all up states sampled during the standard recording interval of 180 s. We calculated grand averages, using data from different experiments to control unwanted sources of variation. Subsequently, we calculated PETHs by using time bins of 5 ms where  $t = 0$  marks the beginning of the burst.

For statistical quantification of bin-wise firing rates in PETHs, we report an effect statistic, Hedges'  $g$ , which is a standardized difference between two means, including 95% CIs (Nakagawa and Cuthill, 2007; Hentschke and Stuttgen, 2011). The higher the absolute value of  $d$ , the stronger the effect, and in cases in which the 95% CI does not encompass zero the effect can be considered significant ( $p < 0.05$ ) within the framework of



hypothesis testing (Nakagawa and Cuthill, 2007). All results except for *d* are presented as mean  $\pm$  SEM or as median and iqr, as appropriate. For comparison of drug versus control condition of all other parameters, we used the Mann-Whitney *U*-test followed by Bonferroni-Holm correction.

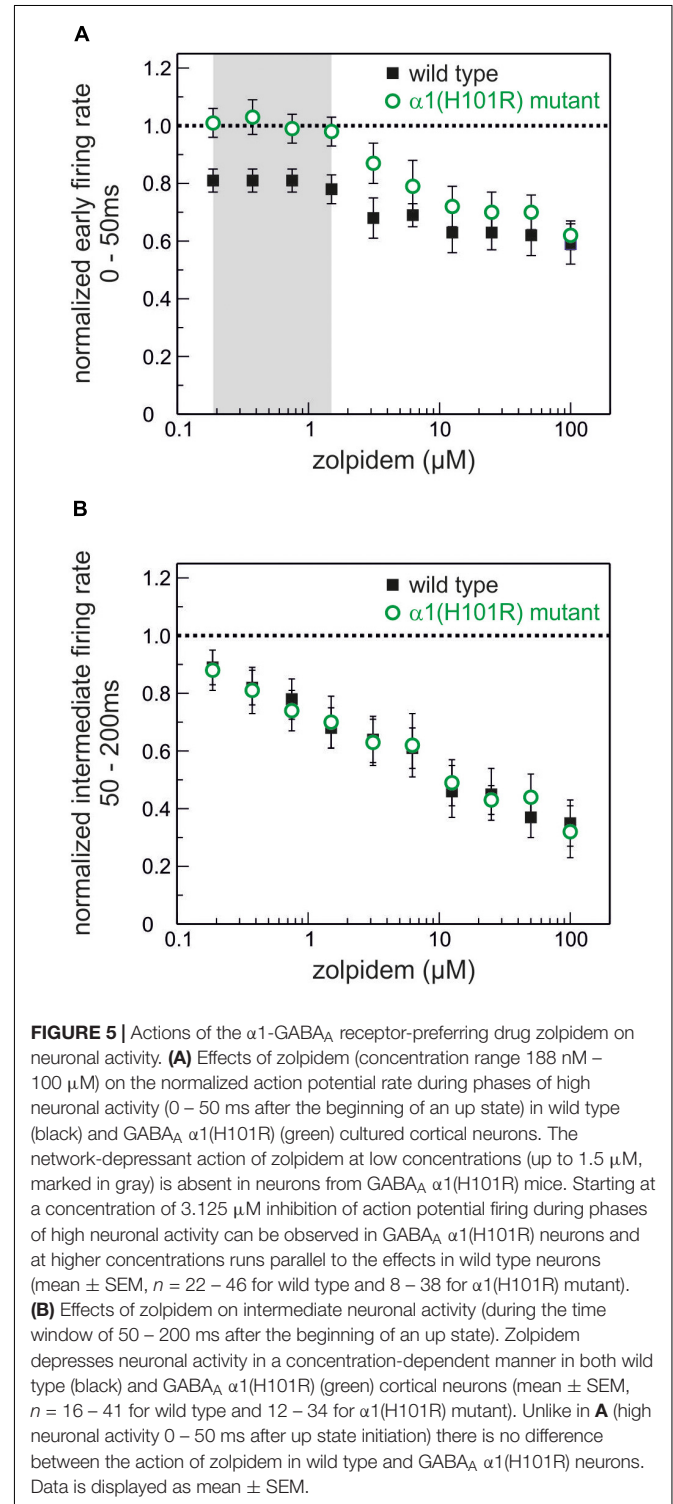
## RESULTS

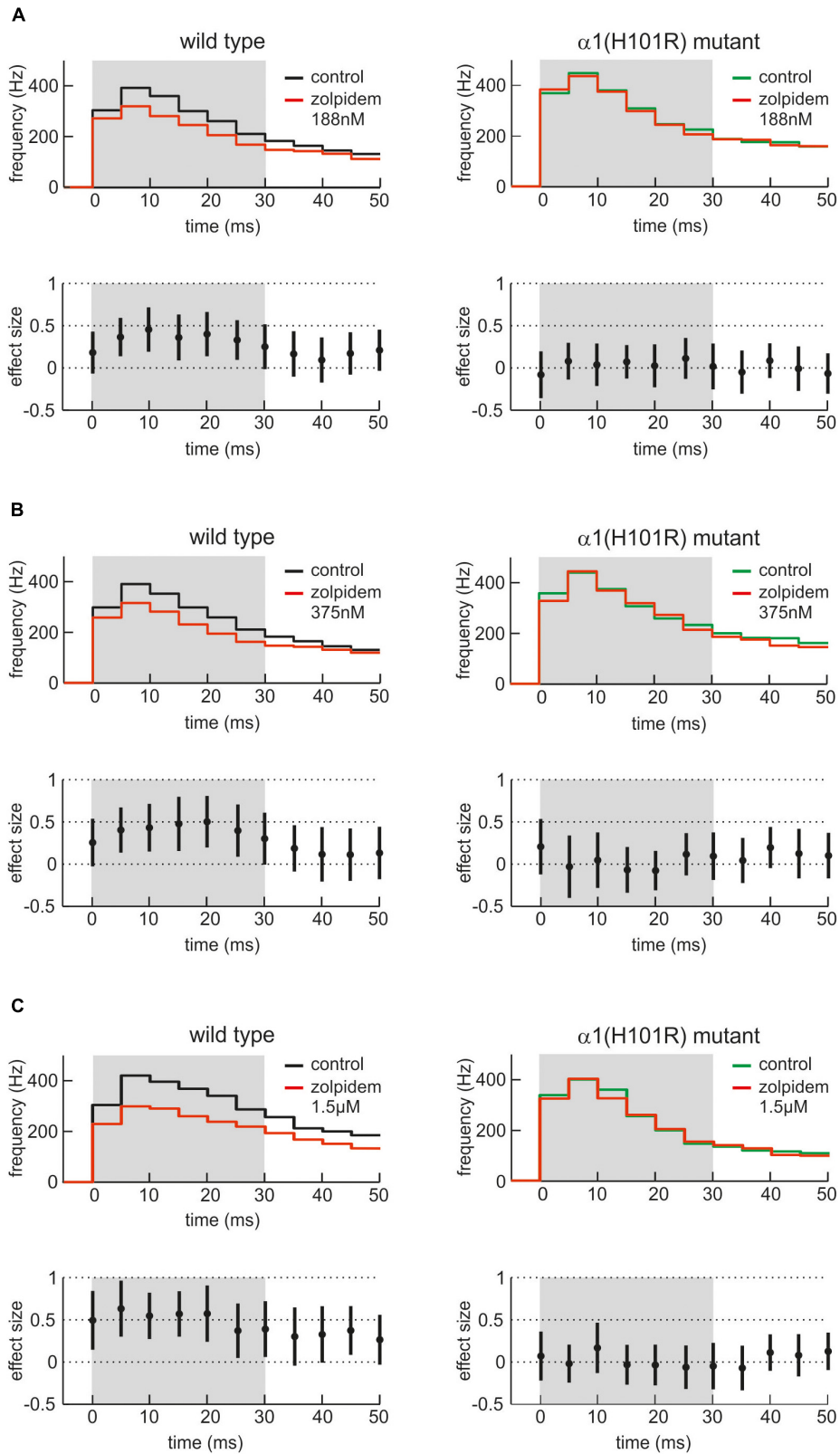
### Cortical Firing Patterns Under Control Conditions and in the Presence of Bicuculline and Isoguvacine

Typical neocortical activity patterns under drug-free conditions are displayed in **Figures 1B–D**. The dynamics of and the relation between population and single-neuron activity in the isolated cortical micro-network are exemplarily illustrated in **Figure 1B**, showing a combined extra- and intracellular recording from a cultured cortical slice. Single PC action potential firing (**Figure 1B4**) occurs synchronously with the appearance of up states of the network. In **Figure 1C** five up states separated by neuronal silence (down states) are visible. **Figure 1D** shows a single up state at higher temporal resolution. At the beginning of an up state, discharge rates are high, but then rapidly decline. To uncover the actions of interference within the GABAergic system we performed experiments with the GABA<sub>A</sub> receptor antagonist bicuculline and the GABA<sub>A</sub> receptor agonist isoguvacine. Bicuculline at a concentration of 20  $\mu$ M led to an accumulative increase of neuronal activity in neocortical cultures, slowly but continuously raising the activity a few hundred milliseconds after the beginning of neuronal up states (**Figures 2A,B**). By contrast, the GABA<sub>A</sub> receptor agonist isoguvacine depressed neuronal activity mainly by substantially prolonging the phases of neuronal silence (**Figures 2C,D**). Taken together these data illustrate that interventions within the cortical GABAergic system lead to differential and specific effects on neuronal firing patterns.

For the current study we aimed at detecting zolpidem's  $\alpha$ 1-mediated actions by comparing its effects in slices derived from wild type and  $\alpha$ 1(H101R) knock-in mice in which zolpidem does not bind to  $\alpha$ 1-containing GABA<sub>A</sub> receptors (Rudolph et al., 1999). However, this approach raised the question whether the mutation *per se* alters the firing properties of cortical neurons under drug-free conditions. **Figure 3** displays the PETH of firing rates in slices derived from wild type and  $\alpha$ 1(H101R) mutant mice during an interval of 100 ms following the onset of up states. During this time window, neurons discharge at their maximal frequencies. For statistical comparison we calculated effect sizes (Hedges' *g*) and 95% CIs for corresponding bins. Here, the GABA<sub>A</sub> receptor  $\alpha$ 1(H101R) point-mutation did not alter the discharge rate under drug free conditions. For additional confirmation that the mutation was silent, we calculated the frequency of action potentials, which we sampled during the total recording period of 180 s. This measure depends on the firing rates within up states and on the duration of up and down states. The mean action potential firing rate

under drug free conditions was  $14.6 \pm 0.9$  Hz ( $n = 136$ ) for wild type slices and  $14.7 \pm 0.9$  Hz ( $n = 81$ ,  $p > 0.5$ ) for  $\alpha$ 1(H101R) mutant mice slices, providing further evidence that the mutation did not alter the discharge properties of neocortical neurons.





**FIGURE 6 |** Actions of zolpidem on phases of high neuronal activity. **(A–C)** The effects of three low concentrations of zolpidem **(A: 188 nM; B: 375 nM; C: 1.5  $\mu$ M,**  $n = 29 - 41$ , presence of zolpidem indicated in red) are given as PETH with corresponding effect sizes for wild type cultured cortical neurons (left side, black) and *(Continued)*

**FIGURE 6 | Continued**

neurons from  $\alpha$ 1(H101R) mutant mice (right side, green). In each case, the upper half of the figure displays the instantaneous action potential firing rate under control condition and in the presence of zolpidem (red). The lower half of the figure shows the corresponding effect size, with a 95% CI not crossing the zero line being equivalent to a statistically significant effect of zolpidem. To reveal the action of zolpidem, PETH and effect size is shown for the first 50 ms after the beginning of averaged neocortical up states. Note that zolpidem induces a depression of neuronal activity between 5 and 30 ms after the beginning of the up state, i.e., when neuronal activity is particularly high. The effect of zolpidem was absent in neurons derived from  $\alpha$ 1(H101R) mutant mice (right side of the figure), indicating that the action of zolpidem is indeed mediated via  $\alpha$ 1-containing GABA<sub>A</sub> receptors.

## Zolpidem Depresses Peak Firing Rates in Slices From Wild Type, but Not From $\alpha$ 1(H101R) Mutant Mice

Original traces of the actions of zolpidem (188 nM) in wild type and  $\alpha$ 1(H101R) mutant cultured slices are given in **Figure 4**. **Figure 5** summarizes the concentration-dependent effects of zolpidem on the mean discharge rate in the time intervals 0 – 50 ms (**Figure 5A**) and 50 – 200 ms (**Figure 5B**), following up state onset. At concentrations of 188 nM – 1.5  $\mu$ M zolpidem reduced mean discharge rates only in wild type, but not in  $\alpha$ 1(H101R) mutant slices (marked by gray shading). However, at higher concentrations inhibition was also evident in slices from  $\alpha$ 1(H101R) mutant mice, suggesting that the drug increasingly lost its  $\alpha$ 1-selectivity. Zolpidem also reduced firing rates during a time window of 50 – 200 ms following up state onset. Interestingly, during this phase of neuronal activity depression of action potential firing was almost identical in slices from wild type and  $\alpha$ 1(H101R) mutant mice, indicating that  $\alpha$ 1-containing GABA<sub>A</sub> receptors are of minor or no importance in this phase of neuronal activity.

**Figure 6** presents the effects of three different concentrations of zolpidem in slices from wild type and  $\alpha$ 1(H101R) mutant mice. In all PETH firing rates peaked at the second bin, i.e., 5 – 10 ms after onset of up states. Zolpidem decreased firing rates in slices from wild type, but not in  $\alpha$ 1(H101R) mice. Statistically significant differences of discharge rates in the presence and absence of zolpidem in wild type slices are indicated by a gray background. It should be noted that effects are maximal while neurons discharge at their highest rates.

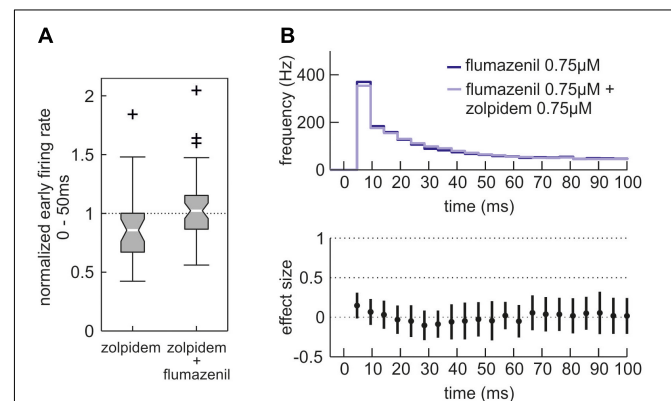
To confirm that the effects of zolpidem were mediated via the benzodiazepine binding site of the GABA<sub>A</sub> receptor, we applied the specific antagonist flumazenil. **Figure 7** demonstrates that flumazenil abolished zolpidem effects during phases of high neuronal activity.

## Effects of the $\alpha$ 2/3/5-Preferring Benzodiazepine SH-053-2'F-S-CH3

The results presented in **Figures 5,6** provide evidence for an important role of  $\alpha$ 1-containing GABA<sub>A</sub> receptors in controlling highly active network states. In a previous study (Drexler et al., 2013) we showed that positive modulation of  $\alpha$ 5-containing GABA<sub>A</sub> receptors affected the discharge patterns without altering high frequency action potential firing, suggesting that not all GABA<sub>A</sub> receptor subtypes may be suitable pharmacological targets for blocking cortical hyperactivity. High affinity binding sites for benzodiazepines are present on GABA<sub>A</sub> receptors incorporating  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-, or  $\alpha$ 5-subunits. Thus,

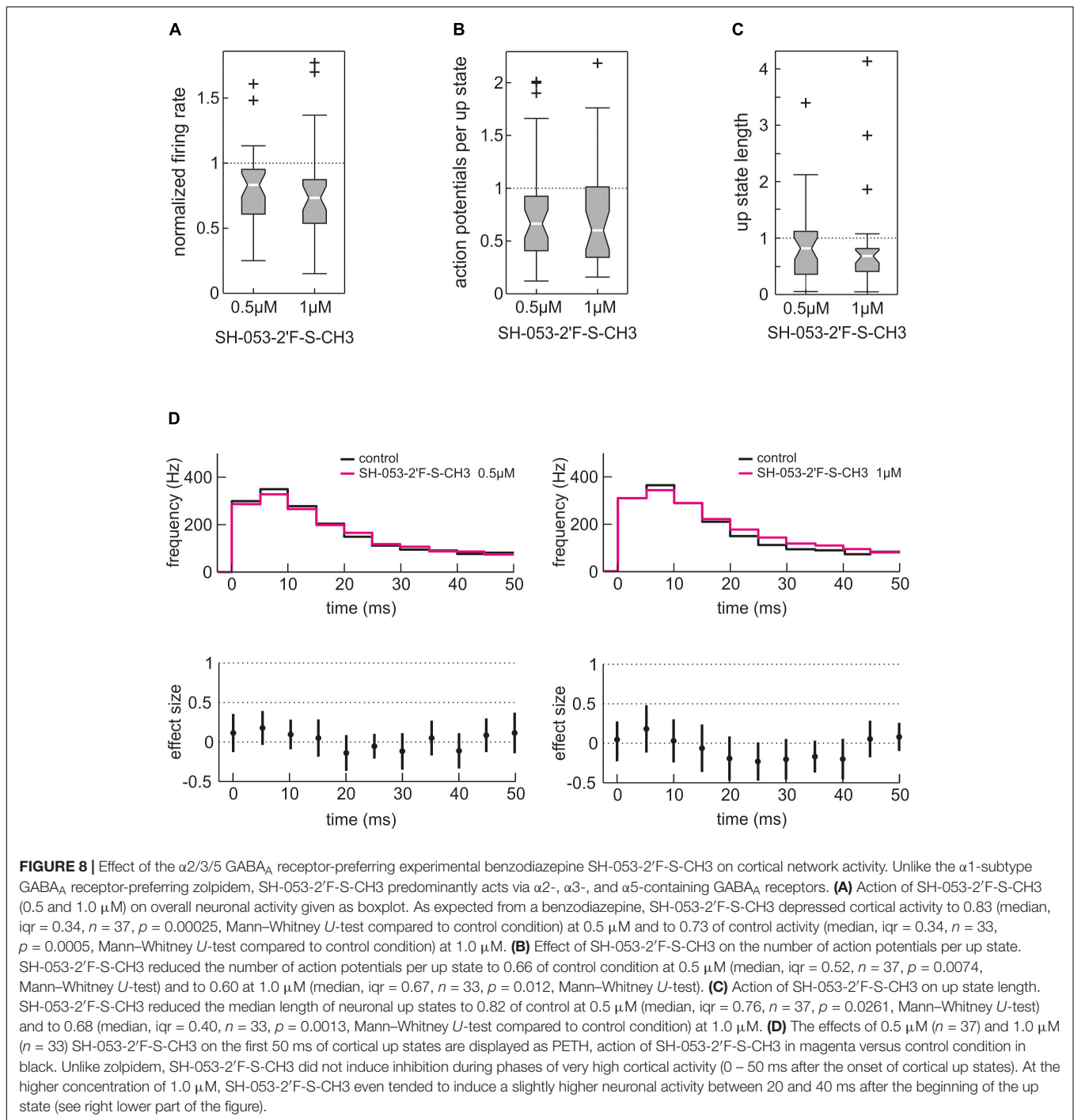
we wondered how the firing patterns of cortical neurons are affected by modulation of  $\alpha$ 2/3/5 receptors. It was previously demonstrated that the experimental benzodiazepine SH-053-2'F-S-CH3 preferably acts via  $\alpha$ 2-,  $\alpha$ 3-, and  $\alpha$ 5-containing GABA<sub>A</sub> receptors (Fischer et al., 2010; Savic et al., 2010), thus having a pharmacological GABA<sub>A</sub> receptor subtype target profile exactly opposite to that of the  $\alpha$ 1-preferring zolpidem.

As expected from a benzodiazepine, SH-053-2'F-S-CH3 depressed neuronal activity in cortical slice cultures (**Figures 8A–C**) in a concentration-dependent manner. This network depressing effect was evident for the overall event rate (**Figure 8A**) and can be explained by SH-053-2'F-S-CH3 reducing the number of action potentials per up state (**Figure 8B**) and by shortening the up state length (**Figure 8C**). However, in contrast to zolpidem, SH-053-2'F-S-CH3 (0.5 and 1.0  $\mu$ M) did not reduce action potential firing during highly active network states observed 0 – 100 ms after up state onset (**Figure 8D**). Overall, these findings suggest that among benzodiazepine-sensitive GABA<sub>A</sub> receptors mainly those containing  $\alpha$ 1-subunits are capable of limiting action potential firing during states of exaggerated neuronal activity.



**FIGURE 7 |** Effects of zolpidem in the additional presence of the benzodiazepine antagonist flumazenil. **(A)** Action of zolpidem (0.75  $\mu$ M) in the absence and presence of flumazenil (0.75  $\mu$ M) given as boxplot. While depression of high neuronal activity (0 – 50 ms after initiation of up state) by zolpidem is evident (left side,  $n = 30$ , median = 0.86, iqr = 0.33 of normalized activity) it is absent in the additional presence of flumazenil (right side,  $n = 47$ , median = 1.02, iqr = 0.29 of normalized activity,  $p = 0.0147$ , Mann-Whitney  $U$ -test). **(B)** Action of zolpidem (0.75  $\mu$ M) in the presence of flumazenil (0.75  $\mu$ M) given as PETH, with the dark blue curve showing the neuronal activity in the sole presence of flumazenil and the light blue curve showing a combined application of flumazenil and zolpidem. Within the first 100 ms after the beginning of neuronal up states the depressant action of zolpidem is absent in the additional presence of flumazenil.





## DISCUSSION

The neocortex is an important target for benzodiazepines and general anesthetic agents (Heinke et al., 2004; Hentschke et al., 2005). Furthermore, sedation with benzodiazepines predominantly involves cortical  $\alpha$ 1-containing GABA<sub>A</sub> receptors located on glutamatergic PCs (Zeller et al., 2008). These receptors also participate in seizure susceptibility (Kralic et al., 2002) and, in turn, mediate anticonvulsive drug actions (Rudolph

et al., 1999; Vlainic and Pericic, 2010). To elucidate the role of  $\alpha$ 1-containing GABA<sub>A</sub> receptors in shaping cortical activity patterns we opted for an *ex vivo* approach since it prevents active metabolites of the drugs under investigation from compromising the results. In OTCs derived from the rodent neocortex, patterns of neuronal activity are similar to those in the intact cerebral cortex, given that subcortical inputs are absent (Corner, 2008) and the development is similar to the corresponding *in vivo* situation (De Simoni et al., 2003;

Di Cristo et al., 2004). Similarity in development particularly includes the hyperpolarizing nature of GABA at the time OTC are used for recordings (Owens et al., 1996; Grasshoff et al., 2008), thereby representing an adult-like status of the neuronal network. The inhibitory nature of GABAergic drugs like intravenous anesthetics and benzodiazepines in cortical OTC has been shown in a number of previous studies, e.g., (Drexler et al., 2009, 2010; Razik et al., 2013; Balk et al., 2017). We, therefore, explored the actions of the GABA<sub>A</sub> receptor  $\alpha$ 1-preferring agent zolpidem in cultured slices from wild type and  $\alpha$ 1(H101) knock-in mice. It had previously been shown that the  $\alpha$ 1(H101R) point mutation renders this specific GABA<sub>A</sub> receptor subtype insensitive to benzodiazepines and zolpidem (Rudolph et al., 1999; Crestani et al., 2000).

The key finding from this study is that zolpidem inhibits neuronal activity, especially during phases of high network activity, an effect which is neither observed in neurons from  $\alpha$ 1(H101R) mutant mice nor is it produced by the  $\alpha$ 2/3/5-preferring benzodiazepine SH-053-2/F-S-CH3. This observation suggests that the activation of  $\alpha$ 1-receptors by GABA is largely restricted to episodes of high frequency action potential firing.

## Modulation of Neuronal Network Activity by Interventions in the GABAergic System

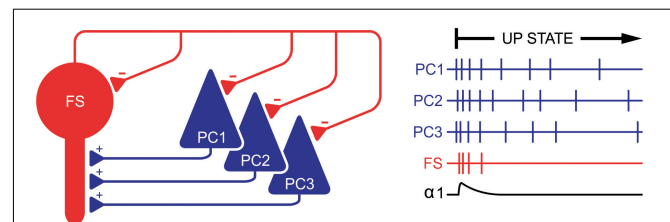
The firing patterns displayed in **Figure 1** are strongly shaped by GABA<sub>A</sub> receptor activity. This is indicated by the observation that the specific GABA<sub>A</sub> receptor antagonist bicuculline evoked rhythmic high frequency firing during bursts (Antkowiak and Hentschke, 1997) and significantly increased average firing rates of cultured cortical neurons about twofold (Grasshoff et al., 2007). Furthermore, bicuculline reduced the modulatory action of GABA-reuptake blockers (Razik et al., 2013) and drugs acting as positive allosteric modulators at GABA<sub>A</sub> receptors predominantly during bursts (Antkowiak, 1999). In the present study, we re-evaluated the effects of bicuculline (20  $\mu$ M) in cultured slices in order to confirm these previous reports. Consistent with previously published data, bicuculline enhanced action potential firing rates during neuronal up states (**Figures 2A,B**).

In addition, we investigated the effects of the GABA<sub>A</sub> receptor agonist isoguvacine. As expected, this agent depressed overall neuronal activity. However, this effect was mainly due to prolonged phases of neuronal down states, whereas up states remained almost unchanged (**Figures 2C,D**). These observations are in accordance with the effects of muscimol, another specific GABA<sub>A</sub>-receptor agonist (Razik et al., 2013). In cultured neocortical slices, muscimol prolonged neuronal down states, leaving up state activity almost unaffected. Taken together, the effects of bicuculline, isoguvacine and muscimol provide evidence that action potential firing during neuronal up states on the one hand, and during down states on the other hand is under the control of different types of GABA<sub>A</sub> receptor-mediated inhibition. Phasic inhibition mediated by synaptic GABA<sub>A</sub> receptors seems to dominate

during neuronal up states, whereas tonic activation of putative extrasynaptic GABA<sub>A</sub> receptors as caused by isoguvacine and muscimol is most effective during cortical down states (Razik et al., 2013).

## Zolpidem's $\alpha$ 1-Mediated Action Time-Locked to Highly Active Network States

Can the observation that zolpidem's  $\alpha$ 1-mediated action is time-locked to highly active network states be explained by current knowledge? Basically, GABA<sub>A</sub> receptor activation largely depends on the firing of GABA-releasing interneurons. In cortical microcircuits these interneurons display great diversity regarding their morphology, discharge properties, and synaptic wiring (Rudolph et al., 2001; Sieghart and Sperk, 2002; Fritschy and Brünig, 2003; Whiting, 2003; Möhler, 2006). Furthermore, different classes of GABAergic interneurons mediate their inhibitory action on excitatory PCs via different GABA<sub>A</sub> receptor subtypes, structural properties that are well-preserved in OTC (Di Cristo et al., 2004). In the cortex, fast-spiking parvalbumin positive basket cells (FS) constitute a major class of GABAergic interneurons. They are excited by pyramidal neurons and in turn release GABA onto  $\alpha$ 1-containing GABA<sub>A</sub> receptors located at high densities on the somata of PCs (Fritschy and Brünig, 2003). Thus, the activation of  $\alpha$ 1-receptors on PCs via FS-cells is part of a powerful inhibitory feedback loop. Unlike other GABAergic interneurons, action potential firing of FS-cells requires a strong excitatory drive, which is provided by PCs immediately after the onset of up states. Furthermore, FS-cells undergo prominent self-inhibition via autapses, a mechanism limiting the time for GABA being released by these neurons and activating  $\alpha$ 1-containing GABA<sub>A</sub> receptors on pyramidal neurons (Bacci et al., 2005). The



**FIGURE 9 |** Suggested mechanism that time-locks activation of  $\alpha$ 1-containing GABA<sub>A</sub> receptors to highly active network states. Right after the beginning of an up state, pyramidal cells (PCs) generate action potentials (vertical bars on the right) at a high frequency, thereby exciting fast-spiking basket cells (FS). This excitation is only strong and synchronous within the first approximate 50 ms of up states, although sufficient to induce action potential firing of FS cells. These FS cells in turn inhibit PCs via  $\alpha$ 1-containing GABA<sub>A</sub> receptors. However, this inhibition is transient since FS cells undergo self-inhibition via autapses, thereby limiting activation of  $\alpha$ 1-containing GABA<sub>A</sub> receptors and  $\alpha$ 1-mediated actions of zolpidem to a narrow time window (lowest trace on the right). Later, excitation of FS cells by PC is weaker and asynchronous, which is insufficient to induce action potential firing in FS cells (and subsequent activation of  $\alpha$ 1-containing GABA<sub>A</sub> receptors). During this phase of neuronal activity GABAergic inhibition is provided by non- $\alpha$ 1-containing GABA<sub>A</sub> receptors.

tight coupling between action potential firing of excitatory PCs, transient firing of inhibitory FS-cells, and subsequent activation of  $\alpha$ 1-containing GABA<sub>A</sub> receptors may be the mechanism linking zolpidem's  $\alpha$ 1-mediated effects to highly active network states (Figure 9).

## Actions of Zolpidem During Phases of Moderate Neuronal Activity

In the current study, peak firing rates of cortical neurons occurred within a narrow window of about 0 – 50 ms shortly after up state onset. Thereafter, action potential generation relaxed to a plateau of roughly 100 Hz. During this period of moderate neuronal activity, zolpidem significantly reduced its discharge rates. However, this effect was indistinguishable in wild type and  $\alpha$ 1(H101R) cultures. Our finding that zolpidem did not exclusively act via  $\alpha$ 1-containing GABA<sub>A</sub> receptors in phases of moderate neuronal activity is consistent with a behavioral study exploring the drugs' actions on the sleep time and EEG in wild type and  $\alpha$ 1(H101R) mice *in vivo* (Kopp et al., 2004). In this investigation zolpidem prolonged non-REM-sleep duration in wild type and  $\alpha$ 1(H101R) knock-in mice to a similar extent and the authors concluded that the hypnotic action of the drug could be mediated by  $\alpha$ 2- and/or  $\alpha$ 3-GABA<sub>A</sub> receptors (Kopp et al., 2004). However, to clearly identify non- $\alpha$ 1 GABA<sub>A</sub> receptors targeted by zolpidem, additional studies on  $\alpha$ 2,  $\alpha$ 3, and  $\alpha$ 5 knock-in mice would be required.

## Different Benzodiazepine-Sensitive GABA<sub>A</sub> Receptors Control Differential Forms of Neuronal Activity

We previously investigated the effects of the experimental  $\alpha$ 5-containing GABA<sub>A</sub> receptor-preferring benzodiazepine SH-053-2'F-R-CH<sub>3</sub> in cultured slices prepared from wild type and GABA<sub>A</sub> receptor  $\alpha$ 5(H105R) knock-in mice (Drexler et al., 2013). Effects on firing patterns of cortical neurons elicited via  $\alpha$ 5-containing GABA<sub>A</sub> receptors differed both from zolpidem's  $\alpha$ 1-mediated actions and from the effects of SH-053-2'F-S-CH<sub>3</sub>. Enhancing the function of  $\alpha$ 5-containing GABA<sub>A</sub> receptors did not alter peak discharge rates, but boosted action potential firing during phases of moderate neuronal activity. Unlike  $\alpha$ 1-containing GABA<sub>A</sub> receptors,  $\alpha$ 5-containing GABA<sub>A</sub> receptors are present in dendrites, but not in somata of PCs (Prenosil et al., 2006) and, as previously shown, in GABA-releasing interneurons (Rodgers et al., 2015), which may account for these differences. Overall, these findings suggest that those benzodiazepine-sensitive GABA<sub>A</sub> receptors containing  $\alpha$ 1 are most effective in reducing high frequency action potential firing.

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In summary, the findings from the current study may open novel ways to evaluate the use of drugs in clinical settings commonly associated with the occurrence of abnormal cortical hyperactivity, e.g., in patients suffering from traumatic brain injury or organophosphorus poisoning. In addition, we describe a system that may be used to evaluate pharmacological activity of substances at  $\alpha$ 1-containing GABA<sub>A</sub> receptors, using native receptors. This may support the screening process for activity in  $\alpha$ 1-containing GABA<sub>A</sub> receptors to also identify  $\alpha$ 1-sparing, i.e., non-sedative benzodiazepine-ligands which may be used to treat chronic pain, or in anxiety research and the treatment thereof. Furthermore, it is tempting to speculate that anesthetic agents which are highly effective in enhancing GABAergic inhibition via  $\alpha$ 1-containing GABA<sub>A</sub> receptors may minimize the incidence of intraoperative awareness by preventing states of high frequency cortical activity, e.g., induced by surgical stimulation.

## AUTHOR CONTRIBUTIONS

EN collected and analyzed the data and prepared the manuscript. UR helped to design the study and wrote the manuscript. DK, GL, and JC synthesized SH-053-2'F-S-CH<sub>3</sub> and helped to design the study. HH performed paired recordings and helped to analyze the data. BA and BD designed the study, analyzed the data, and wrote the manuscript.

## FUNDING

This work was supported by the German Research Foundation, grant no. AN 321/2-1 (BA). UR was supported by both the National Institute of General Medical Sciences (Award Number R01GM086448) and the National Institute of Mental Health (Award Number R01MH095905). JC was supported by the National Institute of Health (Award Numbers R01MH096463 and R01NS076517).

## ACKNOWLEDGMENTS

We wish to acknowledge the Shimadzu Laboratory for Advanced and Applied Analytical Chemistry at the University of Wisconsin–Milwaukee. We would also like to thank Claudia Holt and Ina Pappé for excellent technical assistance and Sandra Schmidt for her linguistic assistance. We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of University of Tübingen.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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