Bud extracts from Tilia tomentosa Moench inhibit hippocampal neuronal firing through GABAA and benzodiazepine receptors activation

Bud extracts from *Tilia tomentosa* Moench inhibit hippocampal neuronal firing through $GABA_A$ and benzodiazepine receptors activation

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Abstract

Ethnopharmacological relevance

Tilia tomentosa Moench bud extracts (TTBEs) is used in traditional medicine for centuries as sedative compound. Different plants belonging to the *Tilia* genus have shown their efficacy in the treatment of anxiety but still little is known about the mechanism of action of their bud extracts.

Aim of the study

To evaluate the action of TTBEs as anxiolytic and sedative compound on *in vitro* hippocampal neurons.

Material and methods

The anxiolytic effect of TTBEs was assayed by testing the effects of these compounds on $GABA_A$ receptor-activated chloride current of hippocampal neurons by means of the patch-clamp technique and microelectrode-arrays (MEAs).

Results

TTBEs acutely administered on mouse hippocampal neurons, activated a chloride current comparable to that measured in the presence of GABA (100 μM). Bicuculline (100 μM) and picrotoxin (100 μM) blocked about 90% of this current, while the remaining 10% was blocked by adding the benzodiazepine (BDZ) antagonist flumazenil (30 μM). Flumazenil alone blocked nearly 60% of the TTBEs activated current, suggesting that TTBEs binds to both $GABA_A$ and BDZ receptor sites. Application of high-doses of TTBEs on spontaneous active hippocampal neurons grown for 3 weeks on MEAs blocked the synchronous activity of these neurons. The effects were mimicked by GABA and prevented by picrotoxin (100 μM) and flumazenil (30 μM). At minimal doses, TTBEs reduced the frequency of synchronized bursts and increased the cross-correlation index of synchronized neuronal firing.

Conclusions

Our data suggest that TTBEs mimics GABA and BDZ agonists by targeting hippocampal GABAergic synapses and inhibiting network excitability by increasing the strength of inhibitory synaptic outputs. Our results contribute toward the validation of TTBEs as effective sedative and anxiolytic compound.

1. Introduction

Gemmotherapy aims at treating different kind of diseases by using extracts from buds, which are composed primarily of embryonic plant tissues particularly enriched of molecular compounds that regulate cell growth and differentiation (Henry, 1959). Flowers or leaves from *Tilia* genus are commonly used as mild antispasmodics, diaphoretics and sedatives (Aguirre-Hernandez et al., 2006; Alston, 1770; Cardenas-Rodriguez et al., 2014; Herrera-Ruiz et al., 2008; Loscalzo et al., 2009). Although the first evidence about the therapeutic effects of *Tilia* dates back to several hundred years ago (Alston, 1770), in more recent times most of the studies have been focused on animals and, surprisingly, clinical trials on humans have not yet been started (Sarris et al., 2013). These effects are attributable to the presence of pharmacologically active ligands of GABAergic and/or benzodiazepine receptors (Aguirre-Hernandez et al., 2006; Loscalzo et al., 2009; Viola et al., 1994).

The ability of *Tilia tomentosa* Moench to exert sedative effects on central nervous system is well described (Viola et al., 1994), but a detailed electrophysiological characterization of the effects of their bud extracts (TTBEs) is still missing. Flavonoids are probably among the most common compounds of plants used in therapy. They can be derived from leaves (Cardenas-Rodriguez et al., 2014) or flowers of plants belonging to *Tilia* genus (Aguirre-Hernandez et al., 2006; Herrera-Ruiz et al., 2008). Their anxiolytic action is well documented (Cardenas-Rodriguez et al., 2014; Herrera-Ruiz et al., 2008; Marder and Paladini, 2002; Viola et al., 1994), but not all the flavonoids contained in *Tilia* extracts act necessarily as GABAergic (Goutman et al., 2003) or benzodiazepine (BDZ) receptor agonists (Medina et al., 1997). Cardenas-Rodriguez *et al*. (2014) suggest that the flavonoid quercetin isolated from *Tilia Americana* is the main responsible of anxiolytic effect, while Goutman *et al*. (2003) show that the flavonoid inhibits rather than activate GABAergic currents. These apparently contradicting effects can be explained by considering that some flavonoids exert their anxiolytic effects by reducing oxidative damages without affecting or even stimulating GABAergic current (Cardenas-Rodriguez et al., 2014). Concerning this issue, a detailed electrophysiological study that bridges the gap between the sedative effect of TTBEs and the mechanism of action on central synapses is still missing. Since in mature neurons, γ-aminobutyric acid (GABA) is the main neurotransmitter mediating sedative and anxiolytic effects on cognitive brain regions, we have focused our experiments on the agonistic effects of TTBEs on postsynaptic type A GABAergic ($GABA_A$) receptors of hippocampal neurons.

Here, we tested the effects of TTBEs on GABAergic synapses by performing post-synaptic voltage-clamp and MEA recordings on hippocampal neurons in primary cultures. Direct application of TTBEs on post-synaptic terminals activates a chloride current comparable to that induced by GABA and largely blocked (90%) by bicuculline and picrotoxin. Since flumazenil, a BDZ receptor antagonist (Polc et al., 1981), blocks about 60% of the TTBEs induced chloride current we gained also evidence that bud extracts act on hippocampal neurons by activating both $GABA_A$ and BDZ receptor sites. We also tested the effects of TTBEs on the spontaneous synchronous activity of hippocampal neuronal network using MEAs (Gavello et al., 2012). We observed that similarly to GABA, low doses of TTBEs significantly inhibit neuron excitability and increase neuronal network synchronization, thus improving the efficiency of inhibitory synapses in mature hippocampal neurons (Bosman et al., 2012; Ellender et al., 2010; Ellender and Paulsen, 2010). Our data suggest that TTBEs mimics GABA and BDZ agonists by producing strong inhibitory effects on functional hippocampal networks. Thus, further purification and characterization of TTBEs would be of great interest to identify new compounds with sedative and anxiolytic effects.

2. Material and methods

2.1 Compound

The plant (*Tilia tomentosa* Moench) and family name (*Malvaceae*) have been checked with *http://www.theplantlist.org/* website accessing on April 1st 2015. *Tilia tomentosa* bud extracts (TTBEs) have been harvested from plants spontaneously growing in the valleys of Pellice, Chisone and Germanasca (Torino), Italy. A recollection of the plant has been done on April 7th 2015 and the voucher specimen (N° MRSN-PAO-9833) has been deposited in the Museum of Natural Sciences (Torino, Italy).

The extraction was performed by leaving the fresh buds in ethanol (95°) for ten days. The solution was then filtered and evaporated by means of a rotary evaporator at 35°C. Finally the TTBEs free from ethanol were dissolved in Tyrode's standard solution before performing the experiments. The concentration of TTBEs is expressed as mg of fresh buds/ml of Tyrode's solution.

2.2 Cell culture

All experiments were performed in accordance with the guidelines established by the National Council on Animal Care and approved by the local Animal Care Committee of Turin University (reference

ethical approval number 121/2015-PR). Hippocampal neurons were obtained from C57BL/6 mouse 18-day embryos. Hippocampus was rapidly dissected under sterile conditions, kept in cold HBSS (4ºC) with high glucose, and then digested with papain (0,5 mg/ml) dissolved in HBSS plus DNAse (0.1 mg/ml) as previously described (Gavello et al., 2012). Isolated cells were then plated at the final density of 1200 cells/mm^2 onto the MEA (previously coated with poly-DL-lysine and laminine). The cells were incubated with 1% penicillin/streptomycin, 1 % glutamax, 2.5 % fetal bovine serum, 2 % B-27 supplemented neurobasal medium in a humidified 5 % CO_2 atmosphere at 37ºC. Each MEA dish was covered with a fluorinated ethylene-propylene membrane (ALA scientific, Westbury, NY, USA) to reduce medium evaporation and maintain sterility, thus allowing repeated recordings from the same chip. Recordings were carried out at 18-28 DIV. Culture medium was partially (1/3) changed once a week, depending on the age of the culture (young cultures did not need weekly change of medium). Following experiments, MEA dishes were re-used by cleaning overnight in 1% Tergazyme (Sigma-Aldrich, St. Louis, MO), rinsing in distilled water and then sterilizing overnight under UV ray.

2.3 Current recordings, data acquisition and analysis

Patch electrodes, fabricated from thick borosilicate glasses (Hilgenberg, Mansifield, Germany), were pulled to a final resistance of 3-5 MΩ. Patch Clamp recordings were performed in whole-cell configuration using a Multiclamp 700-B amplifier connected to a Digidata 1440 and governed by the pClamp10 software (Axon Instruments, Molecular Devices Ltd, USA) (Marcantoni et al., 2009; Marcantoni et al., 2014; Marcantoni et al., 2010). Miniature and evoked inhibitory postsynaptic currents (mIPSCs, eIPSCs) were acquired with sample frequency of 10 KHz and filtered at half the acquisition rate with 8-pole low-pass Bessel filter. Recording with leak current >100pA or series resistance >20MΩ were discarded. All the experiments were performed at room temperature (22-24 °C). The eIPSCs analysis was performed with Clampfit software (Axon Instruments).

2.4 Solutions and drugs

mIPSCs and eIPSCs were recorded by perfusing the whole cell clamped postsynaptic neurons with Tyrode's solution containing (in mM): 2 $CaCl_2$, 130 NaCl, 2 $MgCl_2$, 10 Hepes, 10 glucose, 4 KCl (pH 7.4). D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 50μM) (Sigma Aldrich, St.Louis, MO, USA) and 6,7-Dinitroquinoxalone-2,3-Dione (DNQX, 20μM) (Sigma Aldrich) were added in all the experiments to block the excitatory transmission (Baldelli et al., 2005; Baldelli et al., 2002). Tetrodoxin

(0.3 μM) (Tocris Ltd, Bristol, UK) was added to block spontaneous action potential propagation. $GABA_A$ receptors blocker bicuculline methiodide and picrotoxin as well as the benzodiazepine receptor antagonist flumazenil were purchased from Sigma Aldrich. The standard internal solution was (in mM): 20 Cs-MSO_3, 90 CsCl, 10 Hepes, 5 EGTA, 2 $MgCl_2$, 4 ATP (disodium salt), 15 phosphocreatine.

2.5 MEA recordings

Multisite extracellular recordings were performed using the MEA-system, purchased from Multi-Channel Systems (Reutlingen, Germany). The 60 electrodes array (TiN/SiN) is composed by a 8 X 8 square grid with 200 μm inter-electrode spacing and 30 μm electrode diameter. Data acquisition was controlled through MC_Rack software (Multi-Channel Systems Reutlingen, Germany), setting the threshold for spike detection at -15 μV and sampling at 10 kHz (see Gavello et al. 2012). Experiments were performed in a non-humidified incubator at 37°C and with 5% CO_2, without replacing the culture medium.

Before starting the experiments, cells were allowed to stabilize in the nonhumified incubator for 90 seconds; then recordings of the spontaneous activity was carried out for 6 minutes.

2.6 Analysis of MEA activity

Bursts analysis was performed using Neuroexplorer software (Nex Technologies, Littleton, MA, USA) after spike sorting operations (see Gavello et al., 2012). A burst consists of a group of spikes with decreasing amplitude (Harris et al., 2001), thus we set a threshold of at least 3 spikes and a minimum burst duration of 10 ms. We set interval algorithm specifications such as maximum interval to start burst (0.17 sec) and maximum interval to end burst (0.3 sec) recorded in 0.02 s bins.

Cross-correlation probability vs. time diagrams were constructed by means of Neuroexplorer software (Nex Technologies, Littleton, MA, USA), using ± 0.5 s and and 5 ms bin size.

Data are expressed as means ± S.E.M, n indicates the number of MEA and statistical significance (p) was calculated by using Student's paired t-test. Values of $p< 0.05$ were considered significant.

3. Results

3.1 TTBEs activates a chloride current similar to GABA

Before testing TTBEs we first characterized the chloride currents of mouse embryo hippocampal inhibitory synapses after 2-3 weeks of culture by measuring the size and the reversal potential of

miniature GABAergic postsynaptic currents (mIPSCs) (Fig. 1a). Spontaneous mIPScs were continuously recorded at the soma of a voltage clamped neuron held at -70 mV (V_h) and perfused locally with a Tyrode's solution containing 0.3 µM TTX, D-APV (50 µM) and DNQX (20 µM) to block spontaneous action potentials and glutamatergic postsynaptic activity (see Methods). As shown in Fig. 1a, the mIPSCs were outward at -70 mV (mean amplitudes 13.9 ± 1.4 pA), decreased at more positive V_h and inverted at -10 mV, as expected from the Nernst equilibrium potential for a Cl^- permeable channel with 142 mM external [Cl^-] and assuming the internal [Cl^-] equal to 94 mM. The mIPSCs were rapidly and fully blocked by application of 40 µM bicuculline (not shown).

A similar dependence on V_h was observed for the postsynaptic currents evoked by brief applications (1-2 s) of 70.5 mg/ml TTBEs on hippocampal neurons at 18 DIV (Fig. 1b). The transiently activated currents had maximal negative amplitude at -70 mV (-1.9 ± 0.3 nA, n = 7), inverted at -10 mV and reached mean positive amplitudes of 1.3 ± 0.1 nA at +20 mV, thus suggesting that TTBEs evoke inhibitory post-synaptic Cl^- currents, from now on indicated as TTBE-eIPSCs. The TTBE-eIPSCs turned on rapidly (within one second) and returned completely to the baseline in less than 1 min. Repeated application of TTBEs at intervals of 2 minutes gave reproducible TTBE-eIPSCs with no evident amplitude reduction, suggesting reversible and stable effects of TTBEs over time in hippocampal GABAergic synapses (Fig. 1c). Increasing concentrations of bud extracts produced TTBE-eIPSCs of increasing size that nearly saturated within two decades of concentrations (Fig. 2a). The dose-response curve was fitted by a Hill equation: $I_{TTBEs} = [GABA]^n/([GABA]^n + K_d)$ with K_d = 7.1 mg/ml and n = 1.3.

To better evaluate the potency of TTBEs on hippocampal GABAergic synapses we next tested the effects of γ-aminobutyric acid (GABA) at concentration between 0.1 and 1 mM. The GABA-evoked IPSCs (GABA-eIPSCs) followed a dose-response curve fitted with a Hill equation, with K_d = 11.4 µM and n = 1.7 (Fig. 2b), which is similar to that previously reported in mouse (Fraser et al., 1995) and rat hippocampal neurons (Baldelli et al., 2002) with maximal currents at 100 µM GABA concentration. Comparing the size and rise time of the maximal TTBE-eIPSCs and GABA-eIPSCs we noticed that in both cases, after prolonged applications (40 s), approximately 10% of the maximal current still persisted (Fig. 2c, d), suggesting slow GABAergic desensitization during time as already observed in hippocampal granule cells (Kapur et al., 1999) and hippocampal neurons in primary culture (Mozrzymas et al., 2007). A more detailed analysis showed that maximal TTBE-eIPSCs (Fig. 2b, and

2c inset) were lower (1.7 ± 0.3 nA, n = 5 vs. 2.9 ± 0.4 nA, n = 5; * p< 0.05) but faster than GABA-eIPSCs. Time constant of activation (τ_{on}) was 0.4 ± 0.1 s (n = 5) for TTBEs and 1.3 ± 0.3 s (n = 4) for GABA (* p< 0.05), suggesting different mechanisms of activation of GABAergic receptors by TTBEs and GABA (Fig. 2c, inset). No significant differences were observed when the time constant of deactivation (τ_{off}) of evoked IPSCs were measured (not shown).

3.2 TTBEs effects are mediated by $GABA_A$ receptors

We next tested whether the $GABA_A$ receptor allosteric antagonist bicuculline (Andrews and Johnston, 1979; Ueno et al., 1997) was able to block the TTBE-eIPSCs when using maximal doses of TTBEs. Under these conditions, bicuculline was found to block 87.9 ± 1.5% (n = 9) of the TTBE-eIPSCs in a dose-dependent manner, with K_d = 37.7 μM, n = 1.1 and maximal block at 1 mM (Fig. 3a) .We hypothesized that the residual 12% of unblocked TTBE-eIPSCs by bicuculline could be inhibited by a non-competitive GABAergic receptor inhibitor. This hypothesis was supported by observing that, when TTBEs were administered at concentrations that produced half of the eIPSCs, corresponding to the K_d value, the effects of TTBEs were fully blocked by the allosteric inhibitor (Fig. 3c).

We then measured the ability of the noncompetitive $GABA_A$ receptor antagonist picrotoxin to block the TTBE-eIPSCs at maximal doses of TTBEs. Maximal blocking effects were observed with 1 mM picrotoxin (90.6% ± 2.5, n = 5; Fig. 3b) and were not significantly different from that induced by bicuculline. The dose-response curve of picrotoxin was fitted by a Hill equation with K_d = 29.4 μM and n = 1.5, in good agreement with what reported in rat hippocampal neurons where the K_d of picrotoxin was 14 μM for a current activated by saturating doses of GABA (100 μM); (Gibbs et al., 1997). Again, when saturating concentrations of picrotoxin (1 mM) were used, they could completely block the half-maximal TTBE-eIPSCs (Fig. 3d). By comparing the blocking action of the two $GABA_A$ receptors antagonist we observed that picrotoxin (Fig. 3b, black curve) was more effective than bicuculline (grey curve) (K_d 14 μM vs. 29.4 μM). We thus concluded that bicuculline and picrotoxin are potent antagonists of TTBE-induced chloride currents and that TTBEs act by mainly activating $GABA_A$ receptors.

3.3 TTBEs binds to benzodiazepine receptor sites

The remaining 10% of TTBE-eIPSCs insensitive to bicuculline and picrotoxin (Fig. 3c, d) suggests that TTBEs could act also by binding to BDZ receptors. Previous papers in fact suggest that different species of *Tillia* contain compounds with high-affinity for BDZ receptors (Aguirre-Hernandez et al.,

2006; Viola et al., 1994). They were identified as flavonoids (Medina et al., 1997; Viola et al., 1994) able to induce anxiolytic effects when tested in *in vivo* experiments (Herrera-Ruiz et al., 2008). We therefore tested the action of the BDZ antagonist flumazenil (30 μM) (Polc et al., 1981) on maximal TTBE-eIPSCs (Fig. 3e) and observed an inhibitory effect of 61.4 ± 4.9 % (n= 9). The block did not change significantly when flumazenil (30 μM) was tested on the half-maximal TTBE-eIPSCs (71.6 ± 3.0%; n= 8; p> 0.07), suggesting that, being flumazenil a competitive antagonist of BDZ receptors, its effect does not depend on TTBEs concentration.

Finally, when hippocampal neurons were previously treated with picrotoxin together with flumazenil, the maximal TTBE-eIPSCs was almost completely abolished (94.9 ± 0.4 %; n= 4) (Fig. 3e). The effect was significantly greater than that measured in the presence of TTBEs and flumazenil without picrotoxin (Fig. 3e; ** p<0.01). The same occurred when bicuculline was tested together with flumazenil. The inhibition of the TTBE-eIPSCs was 96.6 ± 0.6 % (n= 4), comparable to that measured in the presence of picrotoxin (Fig. 3e). We then concluded that TTBEs bind to both $GABA_A$ and BDZ receptor sites.

3.4 Effect of TTBEs on spontaneous firing of adult hippocampal neuronal network

Given the ability of TTBEs to activate GABAergic receptors, we were interested to test whether TTBEs were able to modify the spontaneous electrical activity of hippocampal networks. We administered the compound on hippocampal neurons previously plated on MEAs after 18 days in vitro (DIV). At this time the network can be considered completely established and characterized by synchronous burst activity (Ben-Ari et al., 2012; Crepel et al., 2007; Gavello et al., 2012). We noticed that when TTBEs were applied at the concentration corresponding to the K_d value (7.1 mg/ml), it always completely and reversibly inhibited the neuronal firing (Fig. 4a; n= 5). On the contrary, TTBEs were unable to reduce the spontaneous firing when neurons were previously treated with picrotoxin (100 μM) together with flumazenil (30 μM) (Fig. 4b). This confirms that TTBEs evoke IPSCs by activating both $GABA_A$ and BDZ receptors in neuronal networks. Given that this current is hyperpolarizing in adult neurons (Baldelli et al., 2005; Baldelli et al., 2002; Ben-Ari et al., 2012), its activation induced by TTBEs causes the inhibition of hippocampal firings.

We also measured the percentage of firing inhibition when TTBEs were administered at very low concentrations ($TTBEs_{min}$ = 1.7 mg/ml) (Fig. 5a). This value was derived from the dose response curve (Fig. 1a) and corresponds to the minimum concentration of TTBEs able to evoke detectable IPSCs. In

these conditions, we observed a significant reduction of firing frequency (from 2.1 ± 0.2 to 0.6 ± 0.1 Hz; n= 5, *** p<0.001 (Fig. 5a, b) and a reduced number of bursts (from 16.6 ± 1.2 to 4.8 ± 0.8 bursts/min, *** p<0.001) (Fig. 5a, c). Interestingly, the cross correlation index of the neuronal network increased significantly in the presence of the agonist (*** p< 0.001, Fig. 5d), suggesting that TTBEs increase the degree of network synchronization and thus the strength the synaptic output. This observation is in line with what recently reported about the role of GABAergic synapses in adult neurons, which drive neuronal network synchronization (Ellender et al., 2010; Ellender and Paulsen, 2010).

We then tested the effect of exogenous GABA on spontaneous neuronal firing (Fig. 5e). We found that, similarly to what observed in the presence of TTBEs, administration of minimal concentrations of GABA ($GABA_{min}$ = 0.5 µM) significantly decreased the firing frequency from 1.1 ± 0.1 Hz to 0.8 ± 0.1 Hz (n = 6, *** p< 0.001) (Fig. 5a, f), as well as the number of bursts (from 10.1 ± 1.2 to 7.3 ± 0.9 bursts/min; *** p< 0.001) (Fig. 5g). The cross correlation index nearly doubled (*** p< 0.001, Fig. 5h), raising from 0.09 ± 0.01 to 0.21 ± 0.01 (Fig. 5h). Finally, higher concentrations of GABA (11.4 µM), corresponding to the K_d value, completely abolished the neuronal firing (Fig. 5e). All this indicates that low doses of TTBEs, like GABA, slow down the firing frequency of the hippocampal network and increase the degree of neuronal synchronization confirming their efficacy to act as GABAergic agonists.

4. Discussion and conclusion

Unlike the extracts from flowers or leaves, buds are characterized by embryonic tissues that differentiate in leaves or flowers as a function of the molecular compounds produced and are responsible for regulating plant cell differentiation and proliferation processes. As such, buds are widely used as sedative (Aguirre-Hernandez et al., 2006; Loscalzo et al., 2009; Sarris et al., 2013; Viola et al., 1994) in gemmotherapy (Henry, 1959) but the molecular mechanisms at the basis of their action are far from being identified (Dekermendjian et al., 1999; Goutman et al., 2003; Marder and Paladini, 2002; Medina et al., 1998). Here, using a rigorous electrophysiological approach based on classical voltage-clamp and MEA recordings, we provided new evidences that TTBEs stimulate chloride currents at hippocampal synapses by binding to $GABA_A$ and BDZ receptors. The current is reversibly inhibited by bicuculline and picrotoxin and strongly attenuated by the BDZ specific

antagonist flumazenil. Focusing the attention on neuronal networks of hippocampus, we also showed that TTBEs have clear inhibitory effects on neuronal firing that is either blocked or attenuated by reducing the burst frequency with an accompanied increased synchronization of spontaneous firing. All this indicates that inhibition of neuronal firing network through GABAergic and BDZ receptors activation is likely to be the main mechanism by which the bud extracts from *Tilia* genus target brain activity (Aguirre-Hernandez et al., 2006; Loscalzo et al., 2009; Sarris et al., 2013).

4.1 TTBEs act as GABAergic and BDZ agonist

We have clearly shown that embryonic mouse hippocampal neurons at 18 DIV possess effective GABAergic neurons that respond with large postsynaptic currents to bath application of GABA concentrations from 0.1 μM to 1 mM (Fig. 2). These currents are largely blocked by competitive (bicuculline) and non competitive (picrotoxin) $GABA_A$ receptor antagonists (Fig. 3) and the concentration range of the agonist and antagonists is in good agreement with what reported on most central $GABA_A$ receptors (Macdonald and Olsen, 1994) and, particularly, with data on embryonic neuronal preparations of mouse (Baldelli et al., 2007) and rat hippocampus (Baldelli et al., 2005; Baldelli et al., 2002).

Given that 18 DIV embryonic mouse hippocampal neurons express high densities of functional $GABA_A$ receptors, we have also shown that, like GABA, TTBEs induce similarly fast activating currents (Fig. 2c). These currents are nearly 30% lower than those activated by GABA and are strongly inhibited (60%) by the BDZ competitive antagonist flumazenil. All this suggests an overall potentiating action of BDZs on $GABA_A$ receptors which derives from the presence of both GABA- and BDZ-mimetic compounds in the bud extracts of *Tilia tomentosa*. Given the strong inhibitory effect of flumazenil on the TTBE-induced chloride currents, it seems reasonable to hypothesize that the GABA-mimetic compounds activating the $GABA_A$ receptor are less potent than GABA or that the extracts contain some still uncovered compound that antagonizes GABA receptor openings, thus determining a net TTBE-eIPSCs of 30% smaller amplitude than the one activated by GABA. These issues will be solved when the molecular components contained in the buds will be purified and isolated. At present, our data support the view that TTBEs possess an overall strong BDZ potentiating action that when tested on electrically stimulated IPSCs could induce potentiation of GABAergic responses, thus mimicking classical anxiolytic effects. Indirectly, these findings suggest also that herbal medicines, like the bud

extracts of *Tilia tomentosa*, widely used in traditional therapies are rather potent and should be handled with care under the control of pharmacovigilance centres.

4.2 TTBEs inhibit hippocampal network firing

Our MEA recordings furnished also important indications on the effect of TTBEs on neurons that form spontaneously active hippocampal networks. Embryonic hippocampal neurons plated on MEAs develop functional inhibitory and excitatory synapses that reach a balance and give origin to spontaneous depolarizations that are usually synchronized in bursts of action potentials (Gavello et al., 2012; Hofmann and Bading, 2006; Li et al., 2007). Maturation of GABAergic synapses exert a critical role in the network development (Ben-Ari et al., 1989; Ben-Ari et al., 2012) and the excitation/inhibition balance is reached when GABAergic synapses switch from excitatory to inhibitory after about 18 DIV (Baldelli et al., 2005; Baldelli et al., 2002). We therefore set our experimental conditions to test the effect of TTBEs at 18-28 DIV, when GABAergic synapses are inhibitory (Ben-Ari et al., 2012), avoiding to assay the effect of TTBEs on excitatory GABAergic synapses during neuronal maturation because of minor interest for the present work.

Our findings show clearly that mouse hippocampal neurons cultured on MEAs fire spontaneously and their activity is highly synchronized (Fig. 4). TTBEs are able to either block the firing or reduce the frequency of synchronized bursts, most likely by potentiating the response of both synaptic and extrasynaptic $GABA_A$ receptors, similarly to GABA and other GABAergic agonists (Ellender and Paulsen, 2010; Semyanov et al., 2004). High doses of TTBEs block the firing (Fig. 4a) while minimal doses decrease the frequency of synchronized bursts (Fig. 5). These latter however increase the cross-correlation index (Fig. 5d, h) which is indicative of an increased firing synchronization and consequently improved efficiency of the hippocampal network in transferring synaptic stimuli (Bosman et al., 2012; Li et al., 2007). Thus, TTBEs possess all the features of GABA-mimetic drugs and could be used for the treatment of anxiety disorders. The chemical purification of the extracts and test of the isolated molecules will be the next step to fully understand TTBEs action.

The action of TTBEs may not be limited to the regulation of already formed inhibitory synapses. It should be considered in fact that under particular conditions, hippocampal dentate gyrus undergoes adult neurogenesis (Kheirbek and Hen, 2013) and may thus stimulate pattern separation mechanisms. Pattern separation is defined as a neuronal process that allows the distinction between two similar but not completely overlapping situations (Kheirbek et al., 2012). In patients affected by anxiety disorders,

pattern separation mechanisms are altered (Miller and Hen, 2015; Revest et al., 2009) and the identification of pharmacological compounds that stimulate adult neurogenesis in hippocampal dentate gyrus may represent an innovative therapy for the treatment of these disorders. To this regard, plants belonging to *Tilia* genus produce compounds, such as flavonoids, that are shown to stimulate adult neurogenesis (An et al., 2008; Zainuddin and Thuret, 2012). This seems a further good motivation to propose TTBEs as alternative compounds for the treatment of anxiety disorders.

A conclusive remark concerns the study of other specific excitatory parameters, such as spike frequency adaptation (SFA) (Benda and Herz, 2003; Marcantoni et al., 2014). This phenomenon is defined as a property by which neurons slow-down their firing frequency during prolonged depolarization and is correlated with the ability of neurons to synchronize their firing with surrounding networks (Fuhrmann et al., 2002; Ladenbauer et al., 2012). Recently we have demonstrated that, during the early onset of Alzheimer's disease, neurons from Tg2576 entorhinal cortex mice have altered excitable parameters, including SFA, even if mice still do not display macroscopic symptoms related to alteration of memory formation processes (Marcantoni et al., 2014). Given that TTBEs are able to increase the degree of network firing synchronization, it is likely that TTBEs could be effective to treat neurodegenerative diseases where neuronal synchronization is required.

Glossary

BDZ: benzodiazepine
D-APV: D-2-Amino-5-Phosphonovaleric acid
DIV: Days in Vitro
DNQX: 6,7-dinitroquinoxaline-2,3-dione
eIPSCs: evoked Inhibitory Post Synaptic Currents
GABA: γ-aminobutyric acid
GABA-eIPSCs: : γ-aminobutyric acid- evoked Inhibitory Post Synaptic Currents
MEA: Micro-Electrode Array
mIPSCs: miniature Inhibitory Post Synaptic Currents
SFA: Spike Frequency Adaptation
TTBEs: Tilia Tomentosa Bud Extracts
TTBE-eIPSCs: Tilia Tomentosa Bud Extract-evoked Inhibitory Post Synaptic Currents
TTX: Tetrodotoxin

Keywords

Tilia tomentosa Moench, anxiolytic drug, buds, $GABA_A$ receptors, benzodiazepines, microelectrode array (MEA)

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Authorship contribution

A.M, A.A. and E.C. designed the research; A.A, A.M., C.F. and C.C. performed experiments and analysed data; DG analysed data and revised the manuscript; A.M. and E.C. wrote the manuscript.

Figure legends

Figure 1

a) Miniature IPSCs (mIPSCs) recorded at +20, -10 and -70 mV holding potential in 18 DIV hippocampal neurons. At -10 mV, corresponding to the Nernst's equilibrium potential for chloride ions, the mIPSCs are completely abolished, while at +20 mV the chloride current is inward (positive deflection) and at -70 mV is outward and larger (negative deflection). This confirms the dependence on chloride ions of the recorded currents. **b**) Chloride currents activated by TTBEs (TTBEs-eIPSCs) at high concentration ($TTBEs_{max}$= 70.5 mg/ml) and plotted vs voltage (postsynaptic holding potential). **c**) An example of GABAergic chloride current activated by TTBEs at low concentration (17.2 mg/ml). A repeated administration of TTBEs (black bar) after about 2 min of wash produces an outward chloride current of comparable intensity.

Figure 2

Dose-response curve of the effect induced by TTBEs (**a**) and γ-aminobutyric acid, GABA (**b**) on GABAergic chloride current. The continuous curves are the result of a fit with a Hill equation described in the text. **c, d**) Examples of chloride currents activated by maximal concentration of TTBEs and GABA. The insets in (**c**) compare the maximum current and its rise time of activation (τ_{on}) measured during TTBEs and GABA administration at the highest concentration ($TTBEs_{max}$= 70.5 mg/ml; $GABA_{max}$= 100 μM).

Figure 3

Dose-response curves of the inhibitory effects of bicuculline **a)** and picrotoxin **b**) on the GABAergic chloride current activated by TTBEs at maximal saturating concentration. The grey curve in **b** is the sigmoidal dose response curve of bicuculline shown in **a**. **c**) About 90% of the GABAergic chloride current activated by TTBEs (max) (*1*) is blocked by bicuculline (*2*). When the concentration of TTBEs is reduced to the Kd value (7.1 mg/ml), bicuculline completely blocks the TTBEs-induced current (*3*). The bar graphs to the right represent the average of the two different inhibitory responses of bicuculline (3 mM) at different concentrations of TTBEs (max and Kd). **d**) About 90% of the GABAergic chloride current activated by TTBEs at saturating concentration (max) (*1*) is blocked by picrotoxin (*2*). When the concentration of TTBEs used is reduced up to the Kd value, picrotoxin completely blocks the TTBEs-induced current (*3*). The bar graphs to the right are the percentages of inhibition induced

by picrotoxin (1 mM) at different concentrations of TTBEs (max and Kd). **e**) About 60% of the GABAergic chloride current activated by TTBEs at saturating concentration (max) (*1*) is blocked by the BDZ receptor antagonist flumazenil (*2*). Concomitant administration of flumazenil and picrotoxin completely blocks the same chloride current (*3*). To the right are shown the percentages of inhibition induced by flumazenil alone and together with bicuculline or picrotoxin on TTBEs-activated GABAergic currents.

Figure 4

a) Extracellular electrical activity of embryonic hippocampal neurons after 18 DIV in culture recorded from three different electrodes of the same MEA. The hippocampal network electrical activity is characterized by silent phases alternated by synchronized and regular periods of bursts of action potentials. In these experiments TTBEs inhibited completely and reversibly the spontaneous firing when applied at concentration corresponding to the K_d (7.1 mg/ml). **b**) The inhibitory effect of TTBEs administration on spontaneous burst activity of hippocampal cultured neurons recorded by MEAs is completely abolished by pre-treating the neurons with picrotoxin and flumazenil. The insets show a burst of APs before *(1)* and after *(2)* TTBEs administration at an expanded time scale to resolve single APs.

Figure 5

a) Low TTBEs concentration, corresponding to the minimum concentration able to activate a GABAergic current (1.7 mg/ml) reduces the spontaneous firing frequency without causing a complete block. **b-d**) Mean values of the firing frequency (**b**) number of bursts/min (**c**) and cross-correlation index (**d**) induced by minimal concentration of TTBEs. Notice the decreased frequency and number of bursts and the increased cross-correlation index.

e) Minimal concentrations of GABA (0.5 µM) applied on hippocampal neurons decrease the firing frequency recorded by MEAs while higher concentrations of GABA, corresponding to the K_d (11.4 µM), completely abolish the spontaneous firing. **f-h**) Mean values of the firing frequency (f) number of bursts/min (**g**) and cross-correlation index (**h**). As for TTBEs, GABA decreased the firing frequency and number of bursts while increased the cross-correlation index.

References

Aguirre-Hernandez, E., Martinez, A.L., Gonzalez-Trujano, M.E., Moreno, J., Vibrans, H., Soto-Hernandez, M., 2006. Pharmacological evaluation of the anxiolytic and sedative effects of Tilia americana L. var. mexicana in mice. J. Ethnopharmacol. 109, 140-145.

Alston, C., 1770. Lectures on the Materia Medica, Edward and Charles Dilly, London

An, L., Zhang, Y.Z., Yu, N.J., Liu, X.M., Zhao, N., Yuan, L., Chen, H.X., Li, Y.F., 2008. The total flavonoids extracted from Xiaobuxin-Tang up-regulate the decreased hippocampal neurogenesis and neurotrophic molecules expression in chronically stressed rats. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 1484-1490.

Andrews, P.R., Johnston, G.A., 1979. GABA agonists and antagonists. Biochem. Pharmacol. 28, 2697-2702.

Baldelli, P., Fassio, A., Valtorta, F., Benfenati, F., 2007. Lack of synapsin I reduces the readily releasable pool of synaptic vesicles at central inhibitory synapses. J. Neurosci. 27, 13520-13531.

Baldelli, P., Hernandez-Guijo, J.M., Carabelli, V., Carbone, E., 2005. Brain-derived neurotrophic factor enhances GABA release probability and nonuniform distribution of N- and P/Q-type channels on release sites of hippocampal inhibitory synapses. J. Neurosci. 25, 3358-3368.

Baldelli, P., Novara, M., Carabelli, V., Hernandez-Guijo, J.M., Carbone, E., 2002. BDNF up-regulates evoked GABAergic transmission in developing hippocampus by potentiating presynaptic N- and P/Q-type Ca2+ channels signalling. Eur. J. Neurosci. 16, 2297-2310.

Baur, R., Sigel, E., 2005. Benzodiazepines affect channel opening of GABA A receptors induced by either agonist binding site. Mol. Pharmacol. 67, 1005-1008.

Ben-Ari, Y., Cherubini, E., Corradetti, R., Gaiarsa, J.L., 1989. Giant synaptic potentials in immature rat CA3 hippocampal neurones. J. Physiol. 416, 303-325.

Ben-Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist 18, 467-486.

Benda, J., Herz, A.V., 2003. A universal model for spike-frequency adaptation. Neural comput. 15, 2523-2564.

Bosman, C.A., Schoffelen, J.M., Brunet, N., Oostenveld, R., Bastos, A.M., Womelsdorf, T., Rubehn, B., Stieglitz, T., De Weerd, P., Fries, P., 2012. Attentional stimulus selection through selective synchronization between monkey visual areas. Neuron 75, 875-888.

Cardenas-Rodriguez, N., Gonzalez-Trujano, M.E., Aguirre-Hernandez, E., Ruiz-Garcia, M., Sampieri, A., 3rd, Coballase-Urrutia, E., Carmona-Aparicio, L., 2014. Anticonvulsant and antioxidant effects of Tilia americana var. mexicana and flavonoids constituents in the pentylenetetrazole-induced seizures. Oxid. Med. Cell. Longev. 2014: 759293.

Crepel, V., Aronov, D., Jorquera, I., Represa, A., Ben-Ari, Y., Cossart, R., 2007. A parturition-associated nonsynaptic coherent activity pattern in the developing hippocampus. Neuron 54, 105-120.

Dekermendjian, K., Kahnberg, P., Witt, M.R., Sterner, O., Nielsen, M., Liljefors, T., 1999. Structure-activity relationships and molecular modeling analysis of flavonoids binding to the benzodiazepine site of the rat brain GABA(A) receptor complex. J. Med. Chem. 42, 4343-4350.

Ellender, T.J., Nissen, W., Colgin, L.L., Mann, E.O., Paulsen, O., 2010. Priming of hippocampal population bursts by individual perisomatic-targeting interneurons. J. Neurosci.30, 5979-5991.

Ellender, T.J., Paulsen, O., 2010. The many tunes of perisomatic targeting interneurons in the hippocampal network. Front. Cell. Neurosci. 4.

Fraser, D.D., Duffy, S., Angelides, K.J., Perez-Velazquez, J.L., Kettenmann, H., MacVicar, B.A., 1995. GABAA/benzodiazepine receptors in acutely isolated hippocampal astrocytes. J.Neurosci. 15, 2720-2732.

Fuhrmann, G., Markram, H., Tsodyks, M., 2002. Spike frequency adaptation and neocortical rhythms. J.Neurophysiol. 88, 761-770.

Gavello, D., Rojo-Ruiz, J., Marcantoni, A., Franchino, C., Carbone, E., Carabelli, V., 2012. Leptin counteracts the hypoxia-induced inhibition of spontaneously firing hippocampal neurons: a microelectrode array study. PloS one 7, e41530.

Gibbs, J.W., 3rd, Sombati, S., DeLorenzo, R.J., Coulter, D.A., 1997. Physiological and pharmacological alterations in postsynaptic GABA(A) receptor function in a hippocampal culture model of chronic spontaneous seizures. J. Neurophysiol. 77, 2139-2152.

Goutman, J.D., Waxemberg, M.D., Donate-Oliver, F., Pomata, P.E., Calvo, D.J., 2003. Flavonoid modulation of ionic currents mediated by GABA(A) and GABA(C) receptors. Eur. J. Pharmacol. 461, 79-87.

Harris, K.D., Hirase, H., Leinekugel, X., Henze, D.A., Buzsaki, G., 2001. Temporal interaction between single spikes and complex spike bursts in hippocampal pyramidal cells. Neuron 32, 141-149.

Henry, P., 1959. Bases Biologiques de la Gemmothérapie, Saint-Norbert, Tongerlo A., Belgium.

Herrera-Ruiz, M., Roman-Ramos, R., Zamilpa, A., Tortoriello, J., Jimenez-Ferrer, J.E., 2008. Flavonoids from Tilia americana with anxiolytic activity in plus-maze test. J. Ehnopharmacol. 118, 312-317.

Hofmann, F., Bading, H., 2006. Long term recordings with microelectrode arrays: studies of transcription-dependent neuronal plasticity and axonal regeneration. J. Physiol., Paris 99, 125-132.

Kapur, J., Haas, K.F., Macdonald, R.L., 1999. Physiological properties of GABAA receptors from acutely dissociated rat dentate granule cells. J. Neurophysiol. 81, 2464-2471.

Kheirbek, M.A., Hen, R., 2013. (Radio)active neurogenesis in the human hippocampus. Cell 153, 1183-1184.

Kheirbek, M.A., Klemenhagen, K.C., Sahay, A., Hen, R., 2012. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. Nat. Neurosci. 15, 1613-1620.

Ladenbauer, J., Augustin, M., Shiau, L., Obermayer, K., 2012. Impact of adaptation currents on synchronization of coupled exponential integrate-and-fire neurons. PLoS Comput. Biol. 8, e1002478.

Li, Y., Zhou, W., Li, X., Zeng, S., Liu, M., Luo, Q., 2007. Characterization of synchronized bursts in cultured hippocampal neuronal networks with learning training on microelectrode arrays. Biosens. Bioelectron. 22, 2976-2982.

Loscalzo, L.M., Wasowski, C., Marder, M., 2009. Neuroactive flavonoid glycosides from Tilia petiolaris DC. extracts. Phytother. Res. : PTR 23, 1453-1457.

Macdonald, R.L., Olsen, R.W., 1994. GABAA receptor channels. Annu. Rev. Neurosci. 17, 569-602.

Marcantoni, A., Carabelli, V., Vandael, D.H., Comunanza, V., Carbone, E., 2009. PDE type-4 inhibition increases L-type Ca(2+) currents, action potential firing, and quantal size of exocytosis in mouse chromaffin cells. Pflugers Arch. 457, 1093-1110.

Marcantoni, A., Raymond, E.F., Carbone, E., Marie, H., 2014. Firing properties of entorhinal cortex neurons and early alterations in an Alzheimer's disease transgenic model. Pflugers Arch. 466, 1437-1450.

Marcantoni, A., Vandael, D.H., Mahapatra, S., Carabelli, V., Sinnegger-Brauns, M.J., Striessnig, J., Carbone, E., 2010. Loss of Cav1.3 channels reveals the critical role of L-type and BK channel coupling in pacemaking mouse adrenal chromaffin cells. J. Neurosci. 30, 491-504.

Marder, M., Paladini, A.C., 2002. GABA(A)-receptor ligands of flavonoid structure. Curr. Top. Med. Chem. 2, 853-867.

Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1997. Overview—Flavonoids: A New Family of Benzodiazepine Receptor Ligands. Neurochem Res 22, 419-425.

Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1998. Neuroactive flavonoids: new ligands for the Benzodiazepine receptors. Phytomedicine 5, 235-243.

Miller, B.R., Hen, R., 2015. The current state of the neurogenic theory of depression and anxiety. Curr. Opin. Neurobiol. 30, 51-58.

Moss, S.J., Smart, T.G., Blackstone, C.D., Huganir, R.L., 1992. Functional modulation of GABAA receptors by cAMP-dependent protein phosphorylation. Science 257, 661-665.

Mozrzymas, J.W., Cherubini, E., 1998. Changes in intracellular calcium concentration affect desensitization of GABAA receptors in acutely dissociated P2-P6 rat hippocampal neurons. J. Neurophysiol. 79, 1321-1328.

Mozrzymas, J.W., Wojtowicz, T., Piast, M., Lebida, K., Wyrembek, P., Mercik, K., 2007. GABA transient sets the susceptibility of mIPSCs to modulation by benzodiazepine receptor agonists in rat hippocampal neurons. J. Physiol. 585, 29-46.

Polc, P., Laurent, J.P., Scherschlicht, R., Haefely, W., 1981. Electrophysiological studies on the specific benzodiazepine antagonist Ro 15-1788. Naunyn Schmiedebergs Arch. Pharmacol. 316, 317-325.

Revest, J.M., Dupret, D., Koehl, M., Funk-Reiter, C., Grosjean, N., Piazza, P.V., Abrous, D.N., 2009. Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol. Psychiatry 14, 959-967.

Sarris, J., McIntyre, E., Camfield, D.A., 2013. Plant-based medicines for anxiety disorders, Part 1: a review of preclinical studies. CNS drugs 27, 207-219.

Semyanov, A., Walker, M.C., Kullmann, D.M., Silver, R.A., 2004. Tonically active GABA A receptors: modulating gain and maintaining the tone. Trends Neurosci. 27, 262-269.

Sigel, E., Steinmann, M.E., 2012. Structure, function, and modulation of GABA(A) receptors. J. Biol. Chem. 287, 40224-40231.

Ueno, S., Bracamontes, J., Zorumski, C., Weiss, D.S., Steinbach, J.H., 1997. Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABAA receptor. J. Neurosci. 17, 625-634.

Viola, H., Wolfman, C., Levi de Stein, M., Wasowski, C., Pena, C., Medina, J.H., Paladini, A.C., 1994. Isolation of pharmacologically active benzodiazepine receptor ligands from Tilia tomentosa (Tiliaceae). J. Ethnopharmacol. 44, 47-53.

Zainuddin, M.S., Thuret, S., 2012. Nutrition, adult hippocampal neurogenesis and mental health. Br. Med. Bull. 103, 89-114.

Fig.1

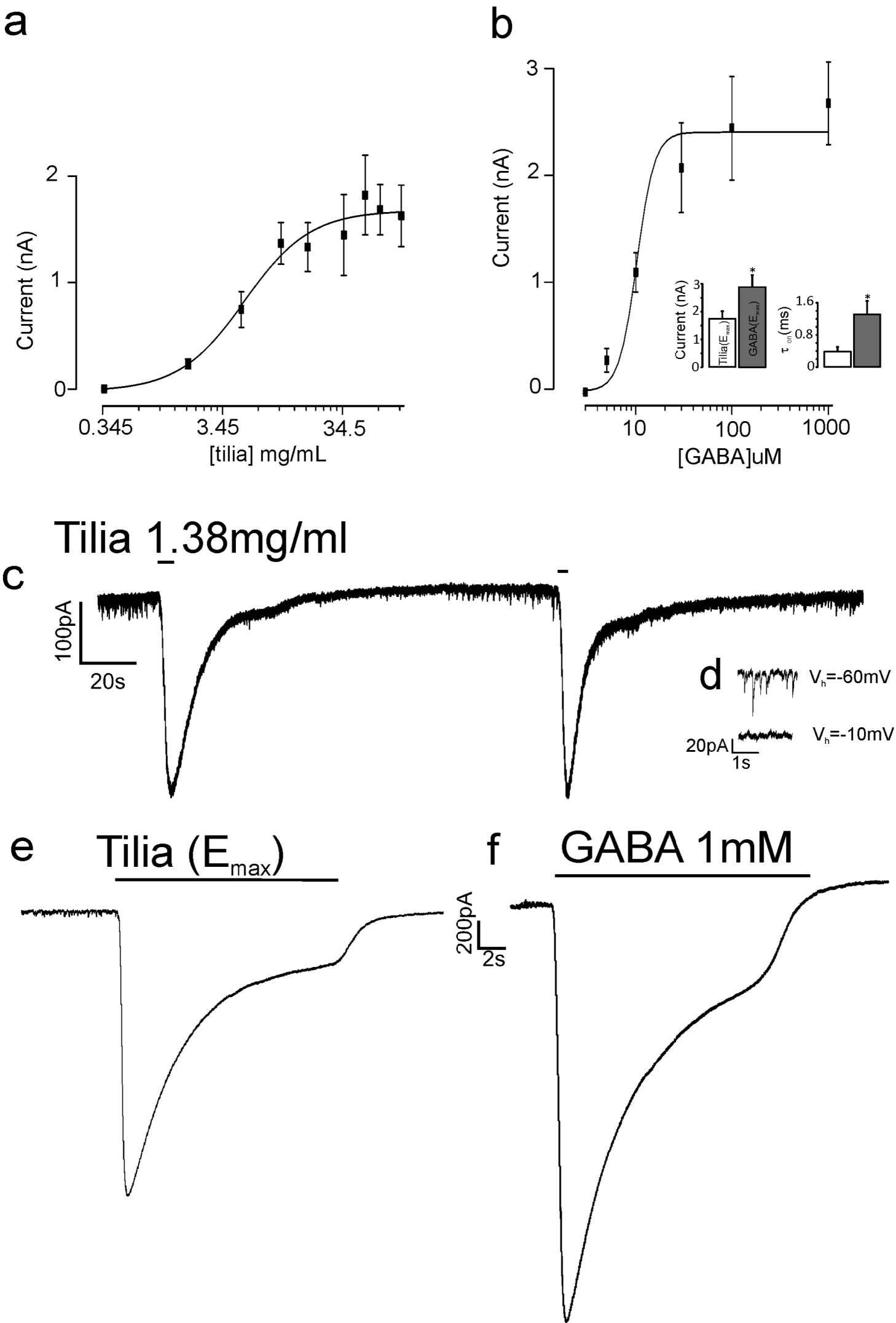
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Fig.2

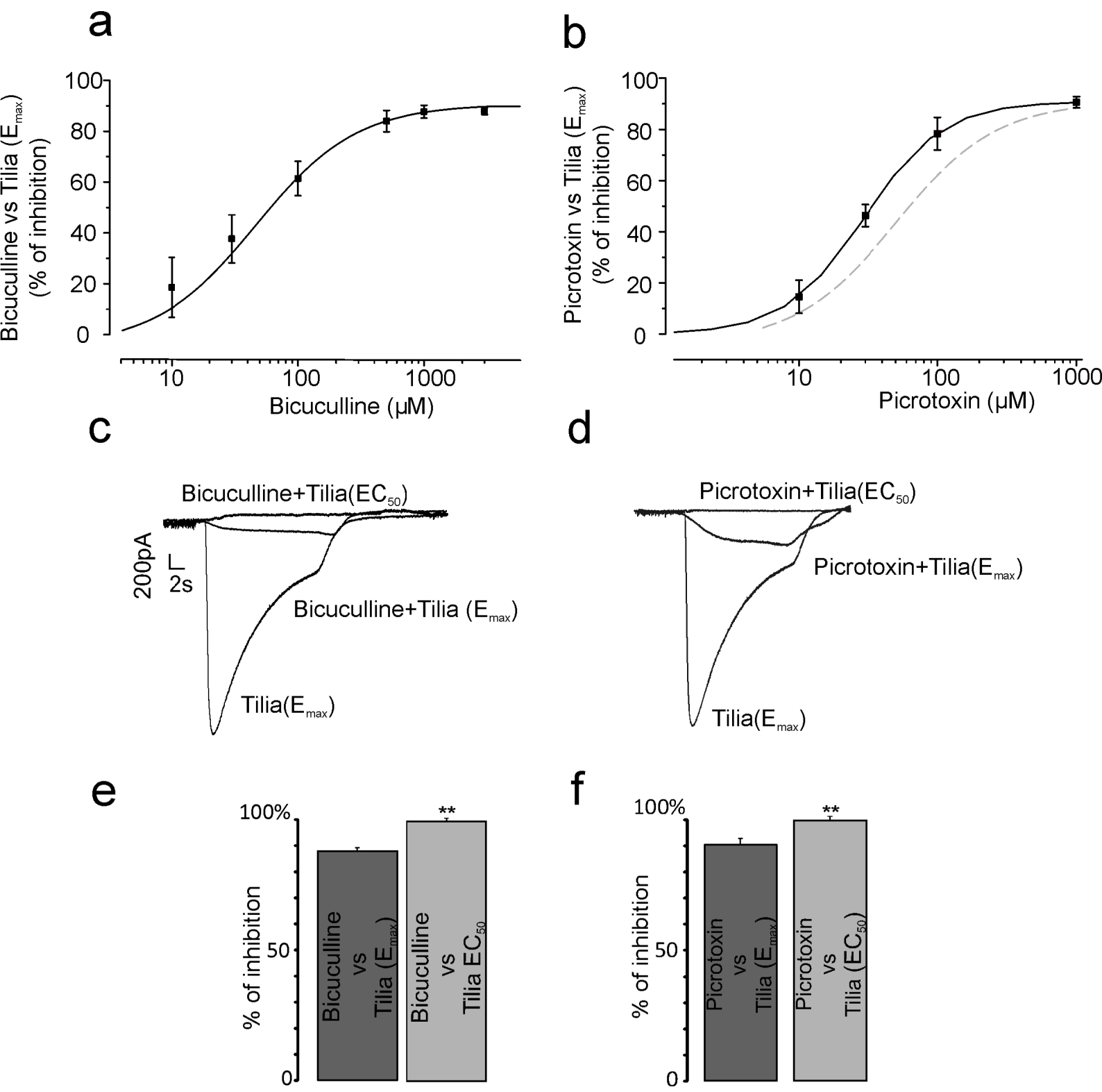
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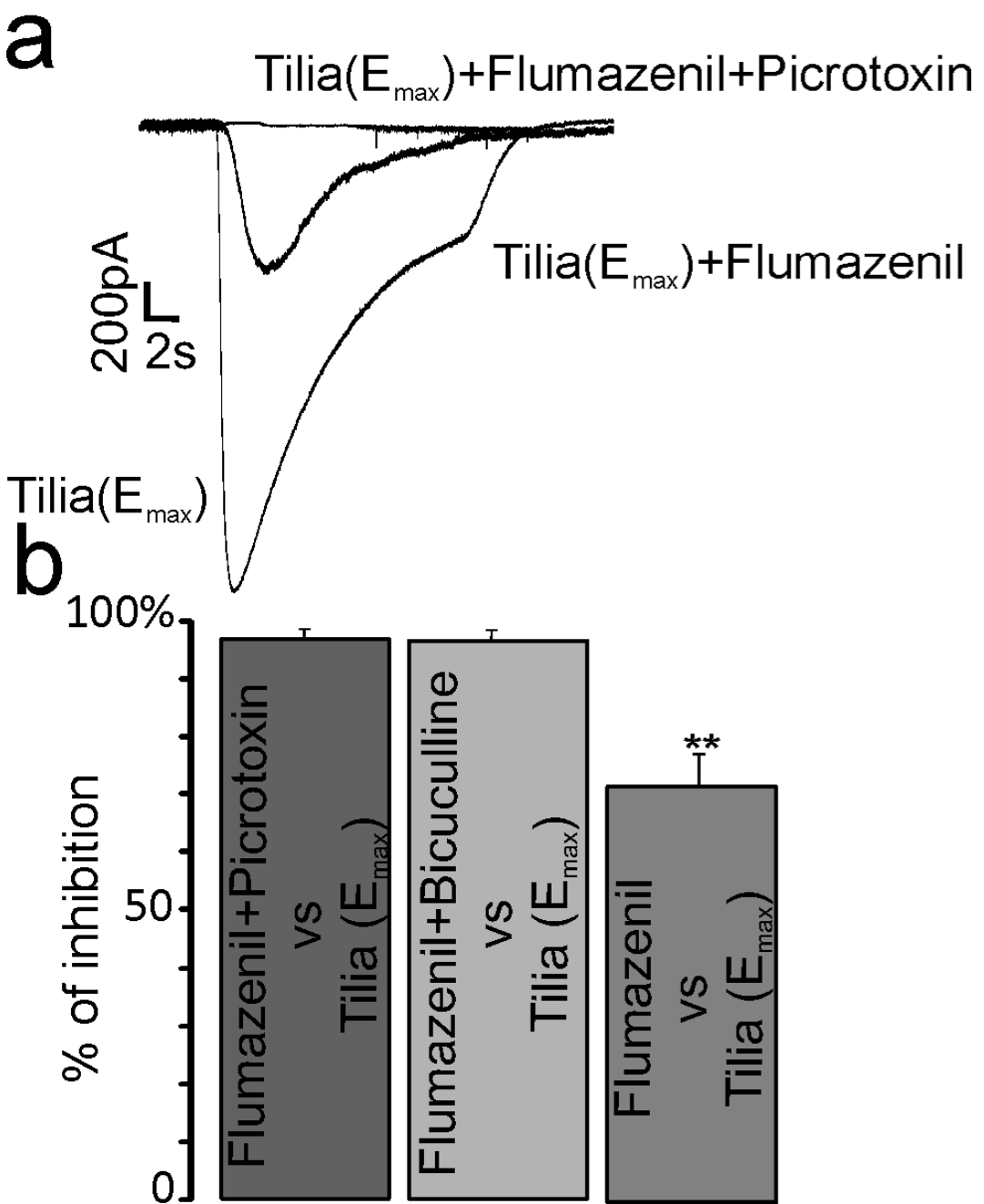
Fig.3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% of inhibition
Flumazenil+Picrotoxin vs Tilia (E_max)
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Fig.4

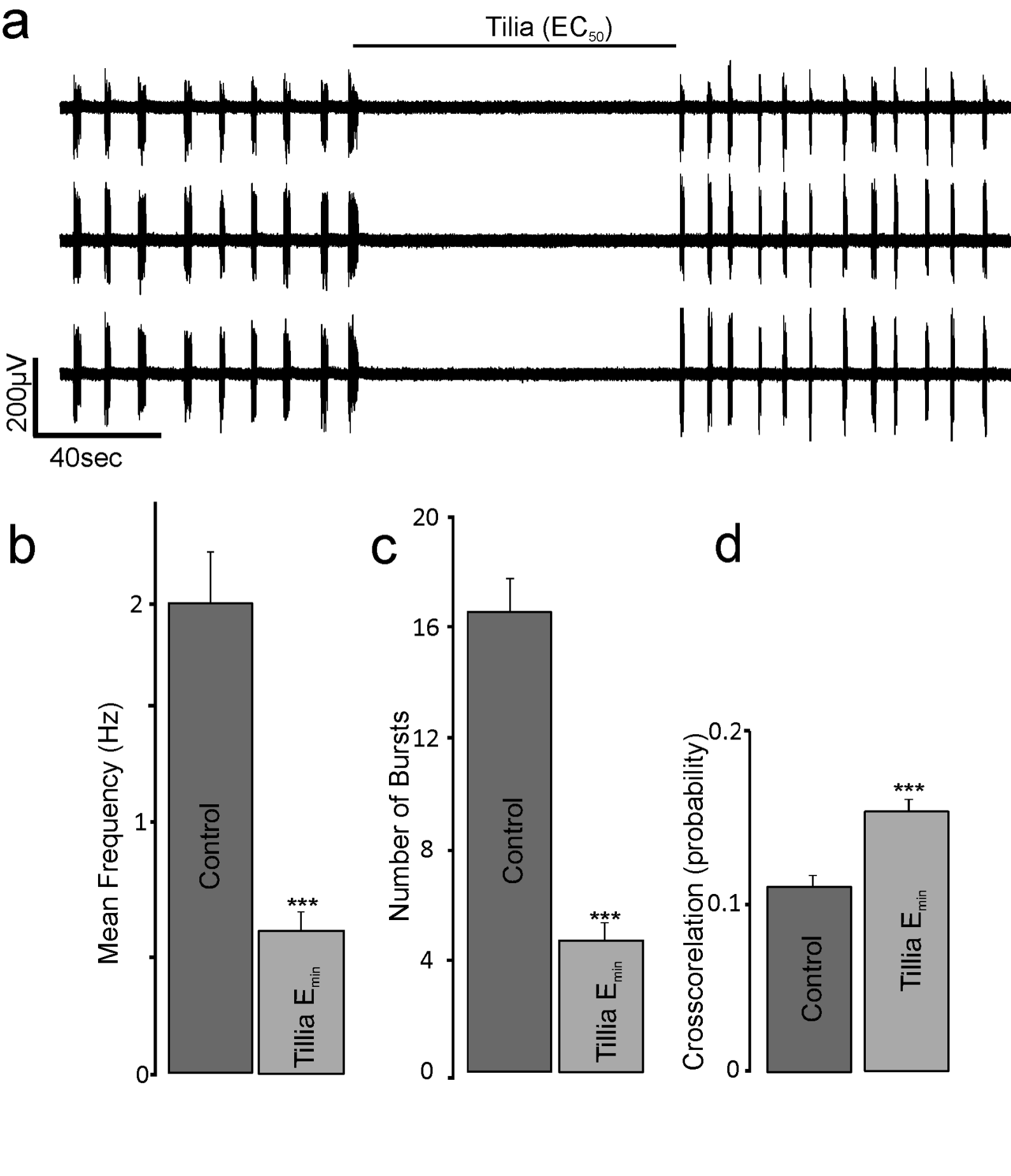
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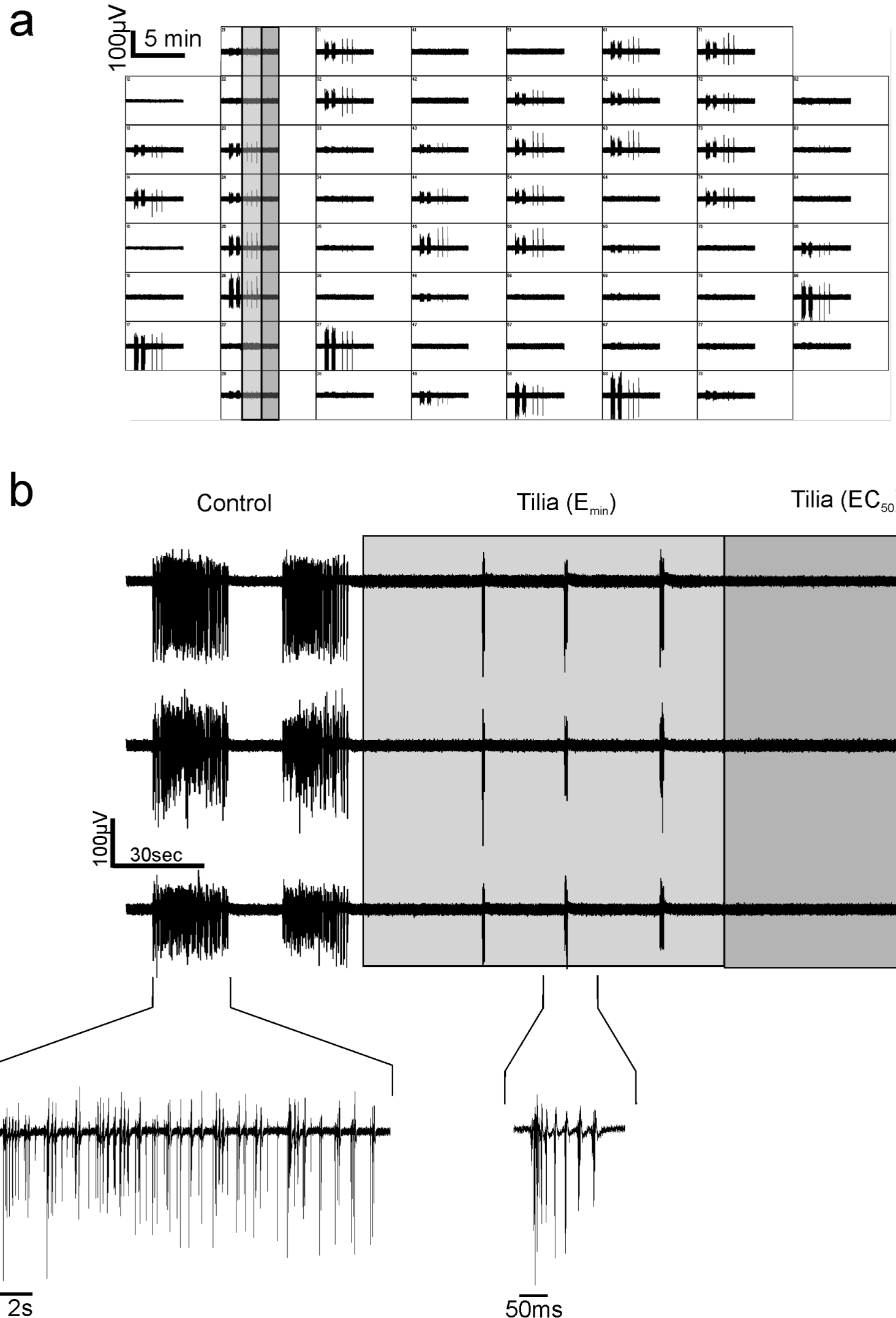
Fig.5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