# Kv3.1/Kv3.2 channel positive modulators enable faster activating kinetics and increase firing frequency in fast-spiking GABAergic interneurons

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# $K_v3.1/K_v3.2$ channel positive modulators enable faster activating kinetics and increase firing frequency in fast-spiking GABAergic interneurons

Kim Boddum [a, *], Charlotte Hougaard [b], Julie Xiao-Ying Lin [a], Nadia Lybøl von Schoubye [a], Henrik Sindal Jensen [b], Morten Grunnet [b], Thomas Jespersen [a]

[a] Cardiac Physiology Laboratory, University of Copenhagen, Faculty of Health Sciences, Department of Biomedical Sciences, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark
[b] Synaptic Transmission In vitro, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark

ABSTRACT

Due to their fast kinetic properties, $K_v3.1$ voltage gated potassium channels are important in setting and controlling firing frequency in neurons and pivotal in generating high frequency firing of interneurons. Pharmacological activation of $K_v3.1$ channels may possess therapeutic potential for treatment of epilepsy, hearing disorders, schizophrenia and cognitive impairments.

Here we thoroughly investigate the selectivity and positive modulation of the two small molecules, EX15 and RE01, on $K_v3$ channels. Selectivity studies, conducted in *Xenopus laevis* oocytes confirmed a positive modulatory effect of the two compounds on $K_v3.1$ and to a minor extent on $K_v3.2$ channels. RE01 had no effect on the $K_v3.3$ and $K_v3.4$ channels, whereas EX15 had an inhibitory impact on the $K_v3.4$ mediated current.

Voltage-clamp experiments in monoclonal $hK_v3.1b/HEK293$ cells (34 °C) revealed that the two compounds indeed induced larger currents and faster activation kinetics. They also decrease the speed of deactivation and shifted the voltage dependence of activation, to a more negative activation threshold. Application of action potential clamping and repetitive stimulation protocols of $hK_v3.1b$ expressing HEK293 cells revealed that EX15 and RE01 significantly increased peak amplitude, half width and decay time of $K_v3.1$ mediated currents, even during high-frequency action potential clamping (250 Hz).

In rat hippocampal slices, EX15 and RE01 increased neuronal excitability in fast-spiking interneurons in dentate gyrus. Action potential frequency was prominently increased at minor depolarizing steps, whereas more marginal effects of EX15 and RE01 were observed after stronger depolarizations.

In conclusion, our results suggest that EX15 and RE01 positive modulation of $K_v3.1$ and $K_v3.2$ currents facilitate increased firing frequency in fast-spiking GABAergic interneurons.

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## 1. Introduction

GABAergic interneurons are central in shaping communication and controlling excitability within the central nervous system. High frequency firing of GABAergic interneurons requires that both the action potentials and the afterhyperpolarizations to be short. Due to their fast activating properties, potassium channels of the $K_v3$ family are believed to be important in setting and controlling firing frequency in fast spiking neurons (Espinosa et al., 2008; Lenz et al., 1994; Lien and Jonas, 2003; Porcello et al., 2002; Rosato-Siri et al., 2015). The voltage-gated $K_v3$ potassium channels are responsible for the neurons ability for fast repolarization, thereby facilitating high-frequency action potential firing, which in some neurons can reach frequencies of several hundred Hz (Du et al., 1996; Erisir et al., 1999; Lien and Jonas, 2003; Wang et al., 1998; Wu and Kelly, 1993).

Four human $K_v3$ genes, named KCNC1-4, encoding $K_v3.1$-4, have been identified and all of these four genes produce several different splice variants, generating multiple protein isoforms (Joho and Hurlock, 2009; Weiser et al., 1994). $K_v3.1$ and $K_v3.2$ channels are delayed rectifier type channels with a high voltage threshold

(activating from −20 mV (Rudy and McBain, 2001; Taskin et al., 2015)). During membrane potential depolarization their conductance increases relatively fast: 10–90% rise time in 3–4 and 5–7 ms for $K_v3.1b$ and $K_v3.2a$, respectively (+20 mV, 20 °C (Rudy and McBain, 2001)). $K_v3.1b$ and $K_v3.2a$ show only minor inactivation, in contrast to $K_v3.3$ and $K_v3.4$, both mediate transient currents, with relatively fast activation and inactivation kinetics (Weiser et al., 1994).

$K_v3$ channels are necessary for the fast-spiking phenotype of certain GABAergic interneurons, and can deliver a repolarizing current sufficient to generate high-frequency activity in a neuron (Lien and Jonas, 2003). Especially $K_v3.1$ channels have been shown to be involved in the fast repolarization of interneuron action potentials and the generation of high frequency firing in numerous brain areas (Deuchars et al., 2001; Erisir et al., 1999; Johnston et al., 2010; Joho and Hurlock, 2009). $K_v3.1$ is also found in heteromultimers with the less abundant $K_v3.2$ subunits, which also can support fast frequency firing (Erisir et al., 1999; Rudy et al., 1999). The two main splice variants of $K_v3.1$ ($K_v3.1a$ and $K_v3.1b$) appear to have similar kinetic properties (Gu et al., 2012). However the two splice variants differ in their intracellular location, with $K_v3.1b$ mainly located in the axons and hence of significant importance for the fast spiking phenotype (Gu et al., 2012; Ozaita et al., 2002).

$K_v3.1$ channels as a therapeutic target has been suggested in the context of several disorders. Epileptic seizures has been found as a consequence of augmented $K_v3.1$ function in mouse models (Muona et al., 2015). The high expression of $K_v3.1$ channels in auditory brain stem is thought to facilitate the transmission of high-frequency temporal information and positive modulators might relieve hearing impairment (Parameshwaran et al., 2001; Wang et al., 1998). Moreover, cognitive dysfunction is a core feature in schizophrenia which has been linked to disturbances in the activity fast spiking GABAergic interneurons. Here $K_v3.1$ are essential for high-frequency repetitive activity (Lien and Jonas, 2003) and therefore, enhancing the fast spiking probabilities of interneurons holds a potential for therapeutic treatment of epilepsy, hearing disorders schizophrenia and cognitive impairments (Harte et al., 2014; Hernández-Pineda et al., 1999; Lewis et al., 2012; Nakazawa et al., 2012).

We have previously demonstrated the ability of the two compounds, example 15 (EX15) and reference 1 (RE01), patented by Autifony Therapeutics (Alvaro et al., 2011), to positively modulate the $K_v3.1a$ splice variant (Taskin et al., 2015). Later, Rosato-Siri and colleagues have shown RE01 (published under the name AUT1) to be able to rescue the fast spiking ability of interneurons, compromised by TEA treatment, in mouse somatosensory cortex slices (Rosato-Siri et al., 2015).

We therefore set out to investigate the relative specificity of the compounds between the four $K_v3$ channels of the two positive modulators (EX15 and RE01) as well as to make an in depth investigation of the biophysiological properties of the $K_v3.1$ channel and the impact of these two compounds. We further tested the effect of EX15 and RE01 on GABAergic interneurons in acute brain slices to evaluate how the positive modulation affects fast spiking abilities of these neurons.

## 2. Method

### 2.1. Oocyte electrophysiology

To obtain $hK_v3.1b$ DNA, a 252 bp DNA fragment (Eurofins Genomics, Germany) coding for the C-terminal of the $K_v3.1b$ splice variant, was subcloned into a $hK_v3.1a$-containing vector (pXOOM (Taskin et al., 2015),). pXOOM supports both mammalian transcription through the CMV promoter and includes a T7 bacterial promoter sequence for cRNA synthesis (Jespersen et al., 2002). Integrity of the $K_v3.1b$ cDNA was verified by sequencing (Macrogen Inc., Korea). mRNA transcripts of $K_v3.1a$ and $K_v3.1b$ together with $K_v3.2a$, $K_v3.3a$ and $3.4a$ (GenScript USA inc., USA) were synthesized with a mMESSAGE mMACHINE® SP7 Transcription Kit (Thermo Fisher Scientific inc., USA) according to manufacturer instructions.

Single *Xenopus laevis* oocytes (Lohmann Research Equipment, Germany) were injected with 50 nl cRNA solution ($K_v3.1a$, 1.35 pg; $K_v3.1b$, 2.03 pg; $K_v3.2a$, 0.04 pg; $K_v3.3a$, 0.12 pg and $3.4a$, 0.005 pg), using an automatic Nanoject microinjector (Drummond, USA), and incubated at 18 °C in Kulori solution, containing (in mM): 90 NaCl, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES (pH 7.4), at least 20 h prior to experiments.

Two-electrode voltage-clamp recordings were performed in Kulori solution at room temperature. For this purpose oocytes were impaled with 2 borosilicate glass pipettes with a tip resistance of 0.5–1 MΩ, containing a silver electrode and 2 M KCl.

Holding potential was set to −80 mV and the voltage dependent gating of the $K_v3$ channels was accessed with a step protocol, where 10 mV increments were applied from −70 mV to +20 mV in 100 ms duration.

Data was recorded using a Dagan CA-1B amplifier (Dagan Corp., USA), a HEKA EPC9 interface and HEKA Pulse software (HEKA electronics, Germany). The sampling rate was set at 25 kHz for all recordings.

### 2.2. Generation of monoclonal $K_v3.1b$-HEK239 cell line

HEK293 cells were maintained at 37 °C in a humidified 95% air/5% $CO_2$ environment in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin (Sigma-Adrich, USA). Cells were transfected with pXOOM-h$K_v3.1b$ using the transfecting agent, Lipofectamine (Lifetechnologies). To develop a stable monoclonal cell-line, the cells were first incubated 2 weeks in selection medium, containing 500 μg/ml of Geneticin, and sown into a 96 well plate (BG Falcon) after being diluted to 1:1.000.000. After 2 days, the wells were screened for single colonies of cells. Ten monoclonal cell lines were screened and one cell line with a stable current of ~10 nA at + 20 mV was selected for further characterization.

### 2.3. Whole-cell patch-clamp experiments in HEK239 cells

Whole cell patch clamp recordings were performed on monoclonal h$K_v3.1b$-HEK239 cells. Standard walled borosilicate glass pipettes with a resistance of 1.5–3 MΩ were used. Pipettes were filled with an intracellular pipette solution containing (in mM): 130 KCl, 10 HEPES, 5 EGTA, 1 $MgCl_2$ and 5 Mg-ATP, pH adjusted to 7.2. The series resistance was monitored throughout all experiments, using a −5 mV step command, and cells showing a >15% change, a resistance <15 MΩ or instable holding currents were not included in the analyses. During recordings, the cells were constantly perfused with 33–35 °C HEPES buffered solution, using a gravity driven perfusion system. The extracellular solution contained (in mM): 140 NaCl, 10 glucose, 4 KCl, 2 $CaCl_2$, 1 $MgCl$ and 10 HEPES.

Holding potential was set to −80 mV and the voltage protocols applied can be seen as inserts in Figs. 3–4. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, USA), filtered at 10 kHz, digitized at 50 kHz, and stored on a PC.

### 2.4. Brain slice electrophysiology

#### 2.4.1. Action potential clamping

The voltage trace used for action potential clamping (Fig. 4), was obtained from ex vivo recordings in acute slices from an adult (P63)
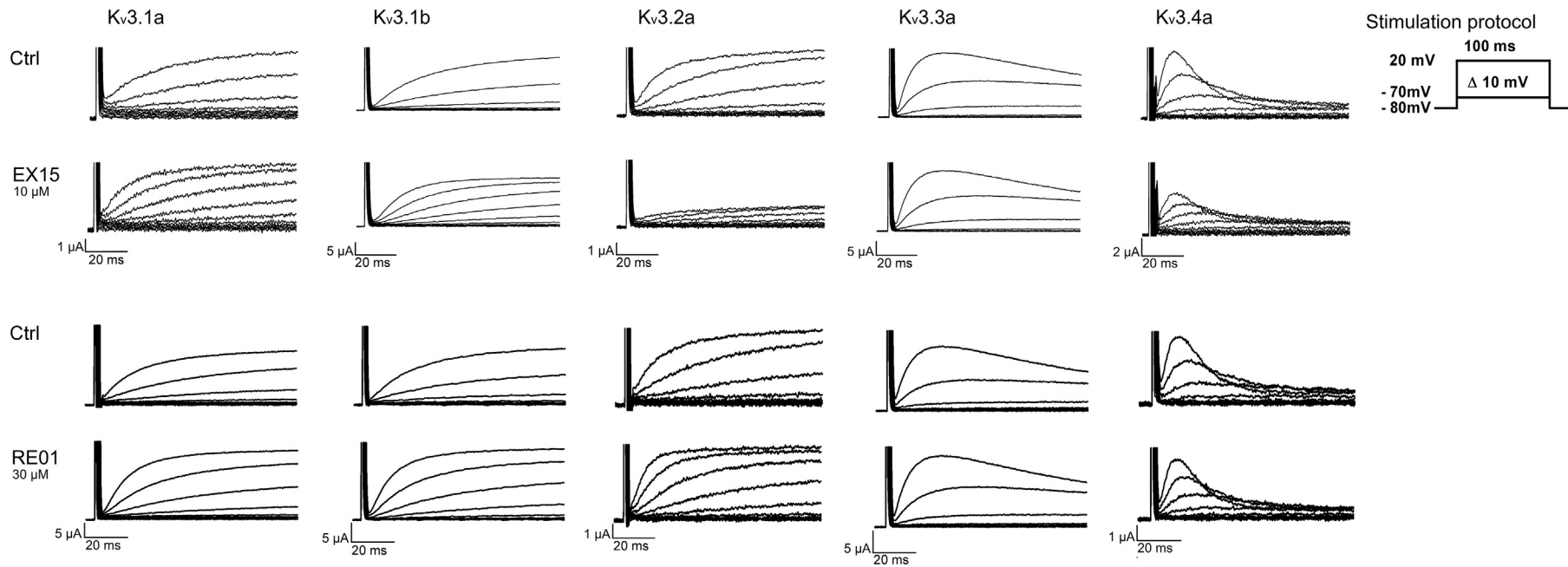
**Fig. 1. Voltage-dependent effect of EX15 and RE1 on Kv3 channel currents**. Representative voltage activated Kv3.1-4 currents expressed in *Xenopus laevis* oocytes recordings by two-electrode voltage-clamp. Current traces are shown in the absence (Crlt) and presence of 10 μM EX15 or 30 μM RE1. Currents were elicited from a holding potential of −80 mV by pulses applied in 10 mV increments to potentials ranging from −70 mV to +20 mV. The capacitance peaks are truncated. For quantitative characterization of the modulation by EX15 and RE1 of current amplitude and activation kinetics, see Table 1.

**Table 1**
**The impact of EX15 and RE01 on $K_v3$ subunits expressed in Oocytes.** Summary of compound induced changes. Both changes in current amplitude at 20 mV and activation time constant (Tau) are given as percentages of control. The shift in voltage dependent activation threshold in is reported as changes in conductance half-max in mV.

| Selectivity on Kv3 subunits | | | | | | | |
|---|---|---|---|---|---|---|---|
| EX15 | Concentration | | Kv3.1a | Kv3.1b | Kv3.2a | Kv3.3a | Kv3.4a |
| Current amplitude (at 20 mV) | 1 μM | % of control | 106.9 | 111.3 | N/A | N/A | N/A |
| | | SEM | 1.9 | 7.5 | | | |
| | | P | * | ** | | | |
| | 3 μM | % of control | 116.9 | 121.3 | 64.8 | 102.1 | 74.6 |
| | | SEM | 2.2 | 6.8 | 6.4 | 4.3 | 7.6 |
| | | P | ** | ** | *** | Ns | * |
| | 10 μM | % of control | 106.4 | 112.9 | 38.8 | 93.0 | 52.0 |
| | | SEM | 1.9 | 8.2 | 3.5 | 13.6 | 5.3 |
| | | P | ** | *** | *** | Ns | *** |
| Time-dependent activation (Tau) | 10 μM | % of control | 69.3 | 67.3 | 223.5 | N/A | N/A |
| | | SEM | 2.2 | 3.4 | 12.5 | | |
| | | P | *** | ** | ** | | |
| Conductance half-max (V½) | 10 μM | Shift in mV | -11.6 | -15.2 | -2.2 | -1.5 | 0.1 |
| | | SEM | 0.7 | 1.9 | 1.2 | 1.4 | 7.8 |
| | | P | *** | *** | ns | ns | ns |
| RE01 | Concentration | | Kv3.1a | Kv3.1b | Kv3.2a | Kv3.3a | Kv3.4a |
| Current amplitude (at 20 mV) | 3 μM | % of control | 110.4 | 133.0 | N/A | N/A | N/A |
| | | SEM | 7.9 | 10.1 | | | |
| | | P | * | ** | | | |
| | 10 μM | % of control | 116.3 | 120.3 | 99.7 | 106.3 | 101.2 |
| | | SEM | 8.4 | 8.8 | 13.4 | 13.6 | 6.1 |
| | | P | ** | *** | ns | ns | ns |
| | 30 μM | % of control | 120.4 | 119.6 | 99.3 | 109.6 | 94.5 |
| | | SEM | 9.7 | 8.6 | 13.3 | 13.8 | 5.8 |
| | | P | ** | *** | ns | ns | ns |
| Time-dependent activation (Tau) | 30 μM | % of control | 59.4 | 50.0 | 59.8 | N/A | N/A |
| | | SEM | 2.3 | 1.8 | 5.6 | | |
| | | P | *** | *** | ** | | |
| Conductance half-max (V½) | 30 μM | Shift in mV | -6.9 | -13.3 | -8.4 | -2.1 | -2.1 |
| | | SEM | 0.7 | 0.9 | 1.7 | 2.5 | 3.6 |
| | | P | *** | *** | *** | ns | ns |

WT mouse (dlx5/6-strain with a C57BL/6 and CD1 background), as previously described (Cho et al., 2015). Briefly: In order to identify GABAergic interneurons in the prefrontal cortex, a virus (AAV-Dlxl12b-mCherry) was injected 4 weeks prior to the experiment. The patch-clamp recording were obtained using Axon multiclamp 700B and a digidata 1440A (Axon Instruments, USA) at 32 °C (TC-324B, Warner Instrument Corporation, USA). 10 μM gabazine, 10 μM CNQX and 50 μM AP5 (Sigma-Aldrich, USA) was added to the extracellular solution, in order to block synaptic activity.

Effects of EX15 and RE01 on the current evoked in the $hK_v3.1b$-HEK239 cells were evaluated and significance tested, using a Wilcoxon Signed Rank Test.

### 2.4.2. Brain slice electrophysiology for evaluating the effect of EX15 and RE01 on firing frequency

Male Sprague Dawley rats (4—5 weeks of age, Taconics) were used for the preparation of acute brain slices. Experiments were carried out, and animals housed, in accordance with the Danish legislation regulating animal experiments; Law and Order on Animal experiments; Act No. 474 of 15/05/2014 and Order No. 12 of 07/01/2016.

After decapitation the brain was rapidly dissected out in ice-cold low-Na artificial (a)CSF, containing (in mM): 26 NaCl, 100 N-Methyl-D-glucamine-HCl, 2.5 KCl, 1 CaCl$_2$, 3 MgCl$_2$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 10 D-glucose, 0.3 ascorbate, 1 pyruvic acid and 2 kynurenic acid. The brain was glued to the platform of a vibratome (VT1200S, Leica Microsystems Inc.) and cut into 350 μm thick horizontal slices. After cutting, the slices were hemisected and stored in regular aCSF (in mM): 126 NaCl, 2.5 KCl, 2.5 CaCl$_2$, 1.3 MgCl$_2$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 10 D-glucose, 0.3 ascorbate, 1

pyruvic acid, at room temperature for at least 1 h prior to experiments. All solutions were equilibrated with carbogen (95% O$_2$/5% CO$_2$).

Cell identification and whole cell recordings: Basket cells, prototypic fast-spiking GABAergic interneurons, in the dentate gyrus were visualized using an Olympus BX51WI microscope equipped with oblique illumination. Basket cells were identified on the basis of their large size, fusiform or pyramidal shape of the soma and location of the soma at the border between the granule cell layer and hilus. Putative interneurons were only accepted for experiments if they fulfilled the following electrophysiological criteria: short duration action potentials (APs) (<1 ms), large afterhyperpolarizations, and, in response to sustained current injection, high frequency action potential firing (>80 Hz) with limited spike frequency adaptation.

Action potentials were evoked by injecting depolarizing currents (step of 800 ms duration). A minor depolarizing current step is defined as a step that evoked 10—15 action potential under control conditions whereas the stronger depolarization is defined as a step that evoked 30—50 action potentials in the absence of compound.

Somatic whole cell recordings in current-clamp mode (bridge-balanced) were performed using a Multiclamp 700B amplifier (Axon, Molecular Devices). Recordings were digitized at 20 kHz on a digidata 1440A digitizer (Axon, Molecular Devices) and lowpass filtered at 3 kHz. Recordings were not corrected for junction potential. Patch pipettes were pulled from borosilicated glass with filament (O.D. 1.5 mm, I.D: 1.1 mm, Sutter Instruments) using a P-97 Flaming/Brown puller (Sutter Instruments) and had resistances of 3—4 MΩ when filled with the intracellular solution (in mM): 110
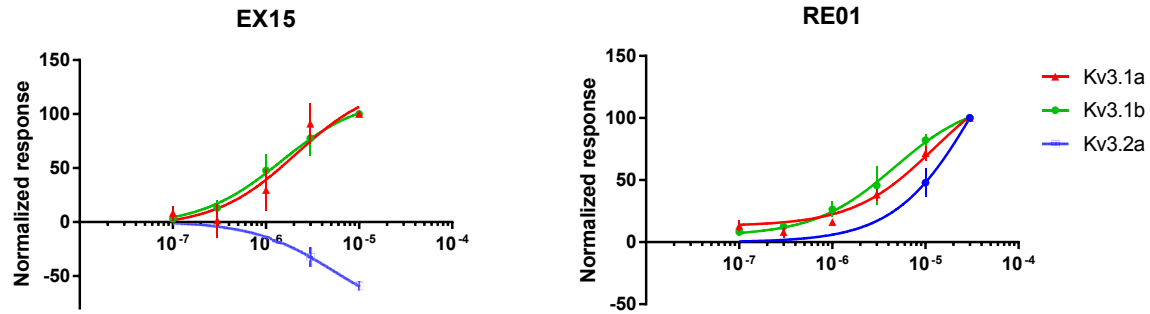
**Fig. 2. Concentration-dependence of RE1-and EX15-induced activation of $K_v3.1$ and $K_v3.2$ channels.** Concentration-response curve, recorted in oocytes, showing the modulating effect of RE1 or EX15 on max current mediated by $K_v3.1$ and $K_v3.2$ channels when depolarized to 10 mV. As expected, both EX15 and RE01 appear to modulate Kv3.1a and Kv3.1b in a similar fashion with no significant difference in the concentration-response relationship. RE01 did also positively modulate $K_v3.2a$, whereas the peak current amplitude of $K_v3.2a$ was reduced in the presence of EX15.
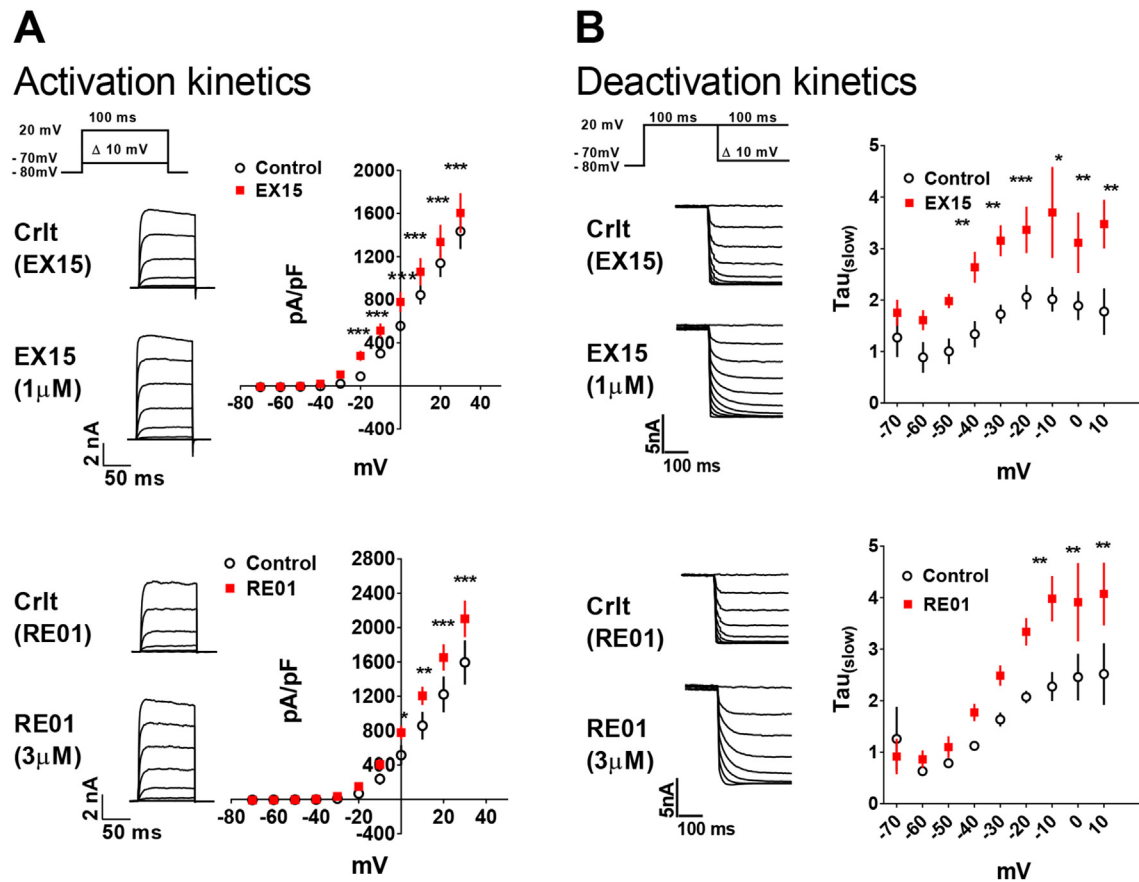


**Fig. 3. Modulation of $K_v3.1b$ channel kinetics by EX15 and RE1. A)** In h$K_v3.1b$/HEK cells, both EX15 (1 μM) and RE01 (3 μM) increased the Kv3.1b mediated current (at 20. mV: $117.3 \pm 3.6\%$ of control, $p < 0.001$ for EX15 and $127.7 \pm 11.7\%$ of control, $p < 0.001$ for RE01). Furthermore, the activation time constant of $K_v3.1b$ channels was decreased (at 20 mV) to $66.7 \pm 7.9\%$ of control for 3 μM EX15 and $56.9 \pm 9.2\%$ of control for 1 μM RE01 ($p < 0.05$ for both). **B)** The voltage dependent deactivation was slowed by EX15 and RE01. A two exponential fit revealed that the slow component of deactivation was increased (tau$_{slow}$ at 10 mV: $195.5 \pm 34\%$ and $222.6 \pm 51\%$ of control for EX15 and RE01 respectively, $p < 0.05$ for both).

$KCH_3SO_4$, 10 KCl, 2 $MgCl_2$, 1EGTA, 10 HEPES, 4 $Na_2$-ATP, 0.4 TRIS-GTP, 10 TRIS$_2$-Phosphocreatine, pH 7.3. During experiments slices were superfused (2 ml/min) with aCSF supplemented with 50 μM D-APV, 10 μM DNQX and 10 μM Gabazine to block synaptic transmission mediated by NMDA, AMPA and GABA$_A$ receptors. Experiments were conducted at 32–33 °C.

7 animals were used in the evaluation of each of the compounds and a maximum of two slices were included per animal. One cell was recorded in each slice, however, only the recordings meeting the above mentioned criteria were included in the data analysis.

### 2.5. Drugs

D-APV, DNQX and Gabazine were purchased from Tocris Bioscience (Bristol, United Kingdom). The two compounds, EX15 and RE01 (Rosato-Siri et al., 2015; Taskin et al., 2015) were
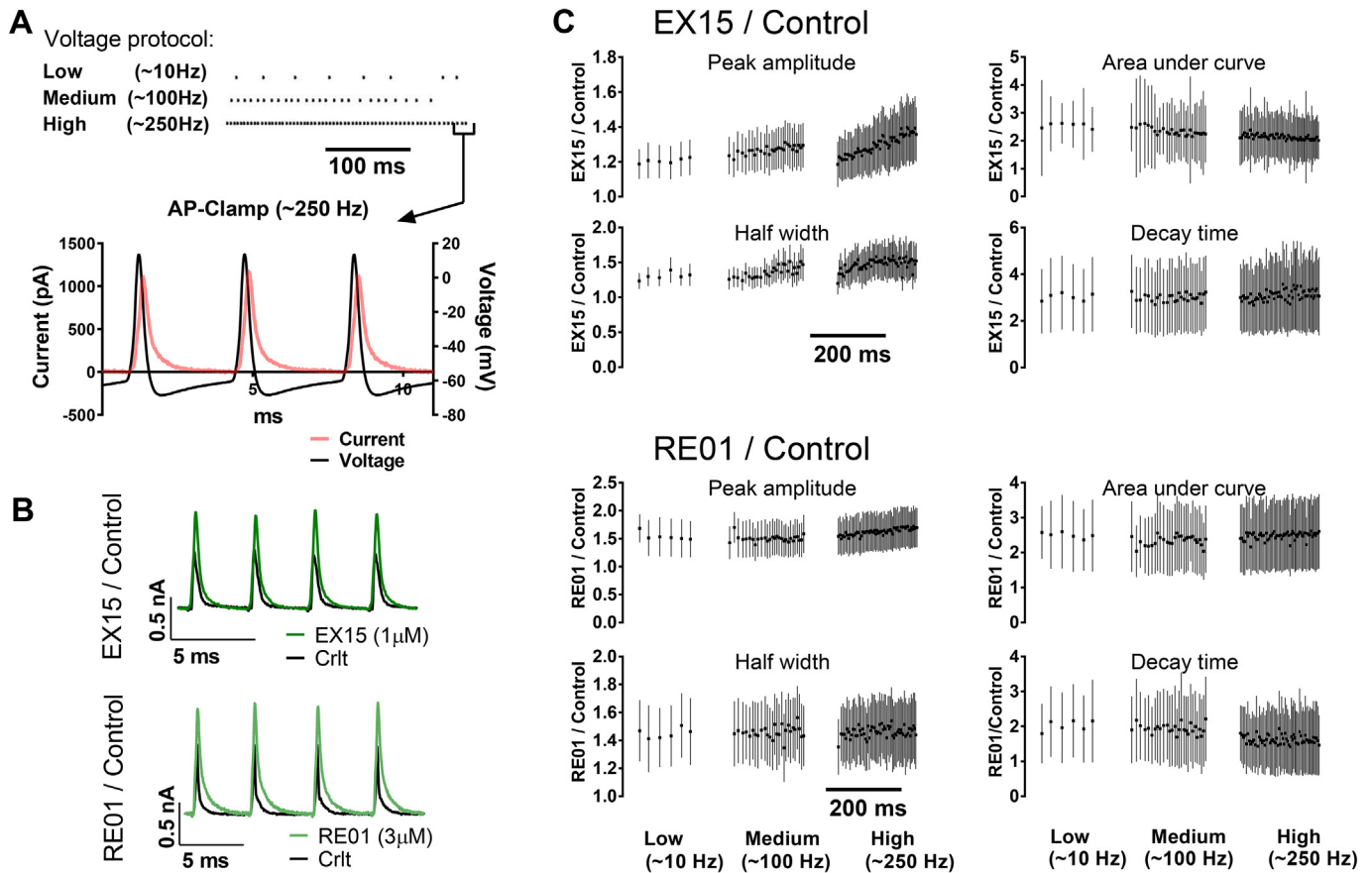
**Fig. 4. EX15 and RE01 increases K_v3.1b mediated repolarizing current.** Action potential clamping of hK_v3.1b/HEK293 cells with an action potential waveform previously recorded fast-spiking, paravalbumin-positive GABAergic neurons (slice recordings in mice). A) The major fraction of K_v3.1b current was elicited during the repolarizing phase. Additionally, no significant hyperpolarizing current was present at the beginning of the next action potential. B) Application of EX15 and RE01 had an impact on the K_v3.1b current, increasing peak amplitude, half width, decay time and area under the curve. C) EX15 and RE01 moderately increased the peak and half width of the K_v3.1b current. A drastic effect was observed on the decay time and consequently on the total current (p < 0.001 for all parameters, Wilcoxon Signed Rank Test).

synthesized by Lundbeck A/S (Copenhagen, Denmark).

## 2.6. Data analysis

Clampex 10.5 and Clampfit software (Molecular Devices, USA), was used for data acquisition and offline analysis, respectively. The activation time constant tau was calculated by fitting current traces into a single exponential function. Similarly, the two deactivating time constants $\text{tau}_{\text{fast}}$ and $\text{tau}_{\text{slow}}$ were determined by fitting current traces to a double exponential function. Activation time constant, amplitude, half width, decay time and area under the curve were estimated by Clampfit. Offset current was measured as the average current 0.5—0 ms prior to next stimulation.

Conductance half-max ($V_{1/2}$) was calculated in Graphpad Prism 7 software (Graphpad Software Inc., USA), by fitting the voltage-current relationship to a Boltzmann equation and drug induced leftward shift in voltage dependent activation was accessed by comparing the fitted $V_{1/2}$ - values.

Graphpad Prism 7 software (Graphpad Software Inc., USA) was used for statistical analysis and two-way ANOVA, Wilcoxon Signed Rank Test or T-Test was used to test for significance, except for evaluating the effect of EX15 and RE01 on firing frequency in brain slice (Fig. 6), where SigmaPlot (Systat Software Inc., USA) was used for performing a T-Test. Differences were considered significant when p < 0.05. Significance levels were designated with one, two or three asterisks (*) for p < 0.05, p < 0.001 and p < 0.0001,

respectively.

## 3. Results

### 3.1. K_v3 channel selectivity of EX15 and RE01 in oocytes

To characterize changes in kinetics of K_v3 channels following EX15 and RE01 application, and to determine the selectivity of EX15 and RE01 for the K_v3 channel family, we performed two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes, injected with cRNA coding for either K_v3.1a, K_v3.1b, K_v3.2a, K_v3.3a or K_v3.4a. The IV-relationships in the range −70 mV to 20 mV was accessed using a voltage protocol (holding potential of −80 mV, in 10 mV incrementing steps for 100 ms). Overall the two compounds had different impact on the biophysical parameters of the K_v3 channels (Fig. 1 and Table 1).

As expected, EX15 appear to modulate K_v3.1a and K_v3.1b in a similar fashion (Fig. 2) with no significant difference in the concentration-response relationship (p = 0.9). At the largest depolarization of the voltage protocol (20 mV for 100 ms), 3 μM EX15 increased the current amplitude to: 116.9 ± 2.2% (K_v3.1a) and 121.3 ± 6.8% (K_v3.1b) of control values (p < 0.01 for both). Conversely, the peak current amplitude of K_v3.2a (Fig. 2) and K_v3.4a was reduced in the presence of EX15 (at 3 μM it was 64.8 ± 6.4% and 74.6 ± 7.6%, respectively). EX15 had no significant effect on K_v3.3a currents.
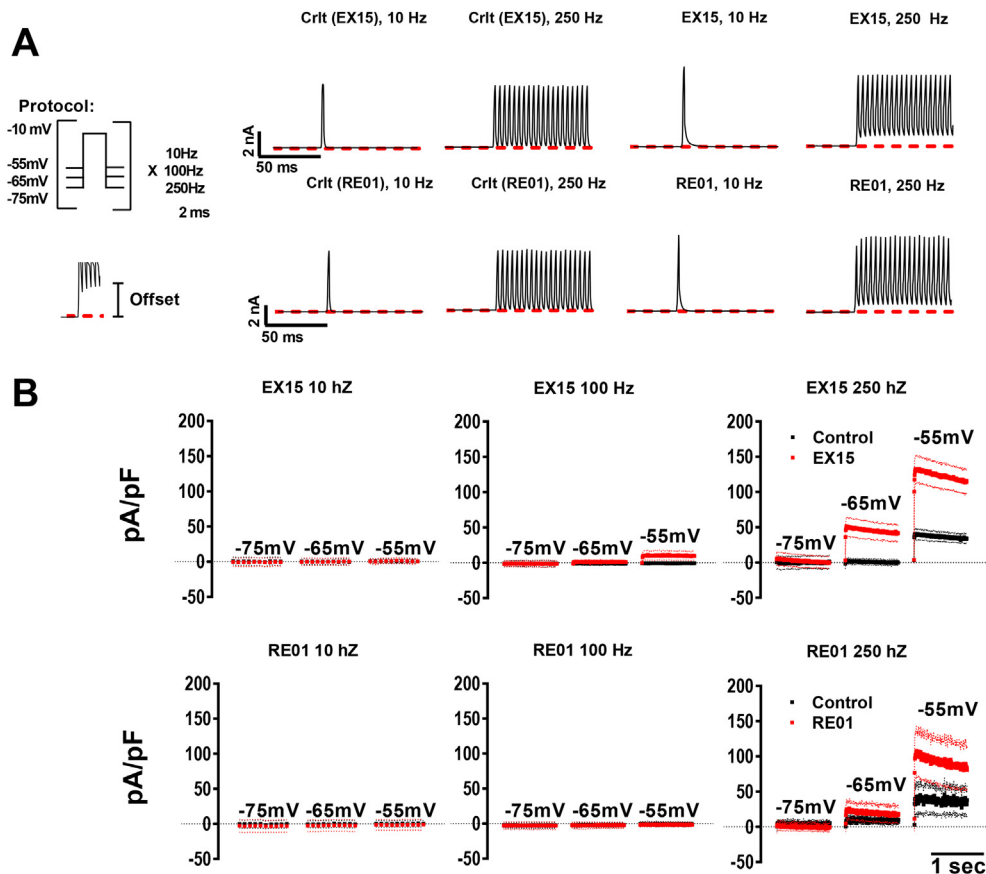
**Fig. 5. EX15 and RE01 increases offset current during high frequency firing.** hK$_v$3.1b/HEK293 cells clamped with repeated 2 ms depolarizing pulses to −10 mV at frequencies of 10 Hz, 100 Hz and 250 Hz from 3 different holding potentials (−55, −65 and −75 mV). Offset current: When a cell is firing at high frequencies, a fraction of K$_v$3.1 channels, opened during one action potential might remain open until the offset of the following action potential. A) Representative trace: 2 ms depolarizing pulses at frequencies of 10 Hz and 250 Hz from a holding a relative depolarized potential (−55 mV) with and without EX15 and RE01. B) Holding potential of −75 mV: no significant offset current, neither at control conditions nor in the presence of the two modulators. At −55 mV: the channels conducted a hyperpolarizing current at offset, during a 250 Hz stimulation protocol. The offset current was increased by both EX15 and RE01.

RE01 also increased the maximal current of K$_v$3.1a and K$_v$3.1b channels (at 10 μM 116.3 ± 8.4%, p < 0.01 and 120.3% ± 8.8%, p < 0.001), with no significant difference in the concentration-response relationship (p = 0.181). RE01 did also positively modulate K$_v$3.2a, however with less potency (Fig. 2), whereas no significant change in current amplitude was observed in K$_v$3.3a and K$_v$3.4a expressing oocytes.

As a change in time-dependent activation would have a major impact on the repolarization of neuronal action potentials, the effect of the compounds was analyzed. 10 μM EX15 positively changed the activation kinetics, i.e. it significantly decreased the activation time constant (making the channels activating faster) of K$_v$3.1a and K$_v$3.1b channels to 69.3 ± 2.2% and 67.3 ± 3.4% of control values. Interestingly, the activation time constant for K$_v$3.2a channels was increased to 223.5 ± 12.5%. RE01 at 30 μM had a positive effect on activation kinetics of K$_v$3.1a, K$_v$3.1b and K$_v$3.2a, where it decreased the activation time constant to 59.4% ± 2.3%, 50.0% ± 1.8% and 59.8% ± 5.6%, respectively.

For K$_v$3.1a and K$_v$3.1b channels, a significant left-shift of the voltage dependence of activation, to a more negative activation threshold (Table 1), was induced by 10 μM EX15 (K$_v$3.1a: −11.6 ± 0.7 mV and K$_v$3.1b: −15.2 ± 1.9 mV, p < 0.001) and 30 μM RE01 (K$_v$3.1a: −6.9 ± 0.7 mV and K$_v$3.1b: −13.3 ± 0.9 mV, p < 0.001). Additionally, 30 μM RE01 did also shift the activation threshold of K$_v$3.2a channels (−8.4 ± 1.7 mV, p < 0.001), enable the channels to open at more negative potentials.

## 3.2. Modulation of K$_v$3.1b channel kinetics by EX15 and RE1

As K$_v$3.1b is the splice variant, mainly responsible for the fast spiking phenotype (Gu et al., 2012; Ozaita et al., 2002), we performed a thorough evaluated of this channel. In order to analyze the fast changes in K$_v$3.1b kinetics at near physiological temperatures (34 °C) experiments were continued in monoclonal hK$_v$3.1b/HEK293 cells using whole-cell voltage-clamp. The analyses were performed on K$_v$3.1b channels as these are the subunits primarily responsible for the spiking frequency ability of interneurons (Gu et al., 2012; Lien and Jonas, 2003). We evaluated 1 μM EX15 and 3 μM RE01, concentrations previously shown to be close to the EC$_{50}$ values of the two compounds (Taskin et al., 2015) and as high compound concentrations has been found to cause a use-dependent inhibition of the Kv3.1 current (Taskin et al., 2015).

The results confirmed the ability of the two compounds to positively modulate the K$_v$3.1b channel properties (Fig. 3a), as both 1 μM EX15 and 3 μM RE01 increased K$_v$3.1b mediated currents (at 20 mV: 17.3 ± 3.6%, p < 0.001 for EX15 and 27.7 ± 11.7%, p < 0.001 for RE01). Again, we found that EX15 and RE01 decreased the activation time constant of K$_v$3.1b channels (at 20 mV: 33.3 ± 7.9% for 3 μM EX15 and 43.4 ± 9.2% for 1 μM RE01, p < 0.05 for both).

Next, we examined the voltage dependent deactivation to assess deactivation kinetics. A two exponential fit revealed that EX15 and RE01 increased the slow component of deactivation (tau$_{slow}$ at 10 mV: 262 ± 34%, p < 0.05 for EX15 and 223 ± 51%, p < 0.05 for
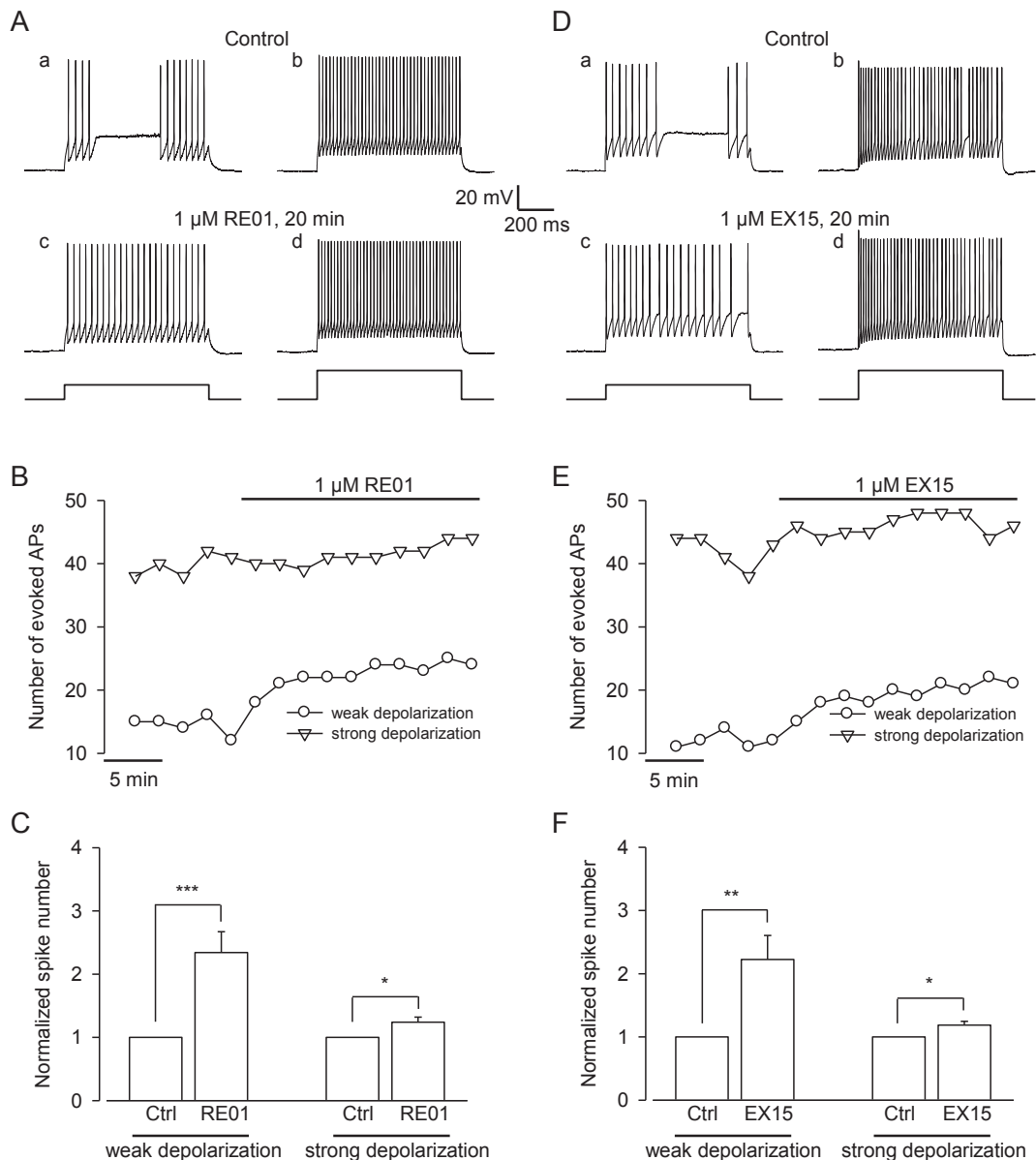
**Fig. 6.** . **EX15 and RE01 increases neuronal excitability in fast-spiking interneurons located in dentate gyrus in rat hippocampal slices.**(A) Action potential firing recorded upon application of 800 ms long depolarizing current pulses before (a + b) and after (c + d) application of 1 μM RE01 (Aa and Ac, 150 pA depolarizing current injection; Ab and Ad, 350 pA depolarizing current). (B) Number of evoked action potentials a function of time, data is from the same cell as in (A). 1 μM RE01 was applied to the perfusing aCSF as indicated by the bar. Circles are the number of action potential evoked upon minor (150 pA) and triangles the number of action potential at stronger (350 pA) depolarizing current injections. (C) Effect of RE01 on neuronal excitability in the absence (Ctrl) or presence of 1 μM RE01 following minor or strong depolarizations. Data are depicted relative to the average number of action potential recorded the last 5 min before compound application (average of 3 data points). Spike number in the presence of RE01 is the average of data obtained during the last 5 min of compound application (likewise average of 3 data points), average ± SEM of 5 experiments. (D—F) Same type of experiments but with 1 μM EX15 instead of RE01 in the perfusate (n = 4, 150 and 250 pA currents were injected for minor and strong depolarizations, respectively). In C and F, minor depolarizations are defined as the depolarizing pulse that evoked 10—15 action potential under control conditions whereas strong depolarizations is the pulse that elicited 30—50 action potentials.

RE01, Fig. 3b) showing that the modulators decrease the speed of deactivation. The compounds did not significantly change the fast component of deactivation, tau$_{fast}$ (data not shown).

### 3.3. EX15 and RE01 increase the K$_v$3.1b repolarizing current during an action potential

Knowing that EX15 and RE01 were able to modulate the fast kinetics of K$_v$3.1 channels, we evaluated the potential of the two compounds to modulate K$_v$3.1b channel currents during high frequency action potential firing as seen in fast-spiking GABAergic interneurons. To do so, we clamped the membrane potential of

K$_v$3.1b-transfected cells by a train of action potentials to investigate the temporal relationship between K$_v$3.1b channel conductance and the different phases of an action potential. The input voltage traces used for this experiment were obtained from previously recorded fast-spiking, paravalbumin-positive GABAergic neurons (slice recordings in mice), where we injected steps of increasing current to collect 250 ms recordings with firing frequencies of approximately 10 Hz, 100 Hz and 250 Hz, respectively (Fig. 4a).

When depolarizing hK$_v$3.1b/HEK293 cells with an action potential waveform, the major fraction of K$_v$3.1 current was elicited after the action potential had reached its peak, thus during the repolarizing phase. Additionally, the fast deactivation properties of

the $K_v3.1b$ channel ensured that no significant hyperpolarizing current was present at the beginning of the next action potential, even during presentation of high frequency action potential trains.

Application of EX15 and RE01 had a significant impact on the $K_v3.1b$ current (Fig. 4b and c). While both compounds moderately increased the peak and half width, a drastic effect was observed on the decay time and consequently on the total current measured as area under the curve (p < 0.001 for all parameters).

### 3.4. $K_v3.1b$ conduct during high frequency firing

As our experiments revealed that EX15 and RE01 increased the deactivation time constants of $K_v3.1b$ channels, it can be speculated that high frequency firing combined with relatively depolarized potentials just prior to the action potential (the offset potential) may produce an accumulation of $K_v3.1$ channels in the open state following compound application. To investigate the frequency-dependence of the potassium current conducted through $K_v3.1$ channels as a function of the offset potential 2 ms depolarizing pulses at frequencies of 10 Hz, 100 Hz and 250 Hz, were applied (Fig. 5). This was performed at 3 different holding potentials ($-55$, $-65$ and $-75$ mV) to mimic physiological relevant potentials of GABAergic interneurons during the interspike interval. At a holding potential of $-75$ mV no significant offset current was measured at the 3 frequencies, neither at control conditions nor in the presence of the two modulators. However, under these conditions, the holding potential is near the potassium reversal potential and the electrochemical driving force is small. When setting the holding potential at $-55$ mV, affecting both $K_v3.1b$ deactivation kinetics and electrochemical driving force, the cells conducted a hyperpolarizing current at offset, during a 250 Hz stimulation protocol. This shows that a fraction of the $K_v3.1b$ channels were not deactivated at offset. This offset current was indeed magnified by both EX15 and RE01. At an intermediate holding potential of $-65$ mV, only cells treated with EX15 and stimulated at 250 Hz, conducted a significant different current.

### 3.5. EX15 and RE01 increase firing frequency in fast-spiking GABAergic interneurons

The findings indicate that EX15 and RE01 can potentiate $K_v3.1b$-mediated hyperpolarizing currents, which will be expected to facilitate action potential repolarization and thereby shorten the action potential and refractory period. Conversely, the modulators also prolong the channel deactivation and can increase the hyperpolarizing offset current at high frequencies and hereby prolong the refractory period.

To gain insight into whether the observed difference in kinetics of EX15 and RE01 affected the spike ability of fast-spiking GABAergic interneurons, whole cell patch clamp experiments were conducted on basket cells in the dentate gyrus of rat brain slices (Fig. 6, see methods for cell identification). To evaluate the $K_v3$ modulators ability to modify interneuron firing frequency, action potential firing was evoked by depolarizing current steps with different amplitude either in the absence or presence of 1 μM EX15 (Fig. 6A) or 1 μM RE01 (Fig. 6D).

In the present experiments, a minor depolarizing current step is defined as a step that evoked 10—15 action potential under control conditions whereas the stronger depolarization is defined as a step that evoked 30—50 action potentials in the absence of compound. After 20 min of exposure to either RE01 or EX15, the number of evoked action potentials were prominently increased at the minor depolarizing steps, whereas more marginal, although significant, effects of EX15 and RE01 are seen after stronger depolarizations (Fig. 6B,C and 6E,F).

At minor depolarizing currents, the GABAergic interneurons displayed a stuttering firing pattern (Fig. 6A and D), which was changed into a continuous firing pattern at strong depolarizations. When incubated with either RE01 or EX15, the firing pattern was changed from stuttering firing towards a more continuous firing pattern, hence the relative large increase in action potentials fired (Fig. 6C and F).

When comparing the first action potential after injection of the depolarizing current, there was no measurable effect of the two compounds on neither action potential duration ($APD_{50}$), upstroke velocity (dv/dt), amplitude nor after hyperpolerization (p > 0.05 for all, data not shown). When comparing the last action potential of the run or the average of all the action potentials, a clear difference was seen in the morphology in the presence of each of the compounds. These effects are, at least partly, consequences of the change from a stuttering firing pattern to continuous firing, where the adaptation to higher firing frequency per se changes the morphology. Hence, a possible effect of the compounds are masked and evaluation therefore not possible (data not included).

## 4. Discussion

The current work present selectivity, positive modulation and increase of firing frequency induced by the two positive modulators RE1 and EX15, which indicate that these compounds can act as excellent tool compounds in investigating the therapeutic potential of $K_v3.1$ and $K_v3.2$ activation.

The selectivity of EX15 and RE01 was studied in Xenopus laevis oocytes expressing human $K_v3.1a$, $K_v3.1b$, $K_v3.2a$, $K_v3.3a$, and $K_v3.4a$ channels, using the two electrode voltage clamp technique. RE01 was found to specifically alter the activation threshold and both the activation- and deactivating kinetics for $K_v3.1a$, $K_v3.1b$ and $K_v3.2a$ channels. EX15 did similarly exert a positive modulation of $K_v3.1a$ and $K_v3.1b$, however, the compound inhibited currents of both $K_v3.2a$ and $K_v3.4a$. Neither RE1 nor EX15 was found to have a significant effect on the $K_v3.3a$ channels.

Whereas $K_v3.1$ has been shown to be necessary for the fast spiking phenotype of GABAergic interneurons, $K_v3.2$ has been shown to possess a supporting role in high frequency firing (Chow et al., 1999; Erisir et al., 1999; Rudy et al., 1999; Tansey et al., 2002). Therefor the lack of compounds selectivity between the two channels types might be beneficial in a therapeutic context. Conversely, the fact that EX15 inhibited currents of both $K_v3.2$ and $K_v3.4$ might have negative consequences for neuronal excitability and survival (Pannaccione et al., 2007; Tavian et al., 2011; Yeung et al., 2005).

### 4.1. EX15 and RE01 modulates $K_v3.1b$ kinetics in a fashion favoring high frequency firing

Experiments with recombinant human $K_v3.1b$ receptors expressed in HEK293 cells show that both EX15 and RE01 enhance the activity of the channel, confirming the finding in oocytes and in consistence with previous studies (Brown et al., 2016; Rosato-Siri et al., 2015; Taskin et al., 2015).

As the two compounds are capable of increasing the $K_v3.1$ channel mediated current, and RE01 also increasing the $K_v3.2$ current, they are indeed able to increase the potassium current enabling interneurons to fire in a fast spiking manner (Erisir et al., 1999; Rudy et al., 1999; Wang et al., 1998), which possibly explains the positive effect on high frequency firing. Similarly, modulation of the $K_v3.1$ channel mediated current has in a dynamic clamp experiment been shown able to increase firing frequency in interneurons (Lien and Jonas, 2003) indicating that the modulation by the two compounds may have a similar effect. The same study,

however, showed that both a leftward shift of the voltage dependent activation and a longer deactivation time may reduce neuronal firing frequency. As EX15 and RE01 are influencing the activation and deactivation kinetics of the $K_v3.1b$ channel, we made an in depth investigation of the biophysical properties of the $K_v3.1b$ channel, mimicking physiological relevant conditions.

### 4.2. Modulation during high frequency firing

From *in silico* modulation studies it has been demonstrated that $K_v3.1$ channels enable faster firing frequencies by ensuring faster repolarizing and a larger hyperpolarization (Lien and Jonas, 2003). These properties will allow sodium channels to be released faster from inactivation, the refractory period will be reduced giving faster spiking ability (Lien and Jonas, 2003). Using an action potential waveform as voltage-command revealed that the gating properties of $K_v3.1b$ ensure that the repolarizing current was conducted during the peak of the action potential wave form, which is optimal in regard to shortening the action potential. Additionally, the deactivation kinetics of the $K_v3.1b$ channel ensured that a major fraction of the channels were still open during the hyperpolarizing phase, enlarging the hyperpolarizing and hence increasing sodium channel availability at the beginning of the next action potential. Deactivation was still sufficient fast to secure that there was no significant current at the offset of the next action potential, thus avoiding a negative impact on the upstroke and hence the firing capability. This was true even when the $K_v3.1b$/HEK cells were clamped to action potential trains above 200 Hz. Both EX15 and RE01 significantly increased the $K_v3.1b$ mediated current elicited during the action potential, which in a neuron would result in an even faster repolarization and shorter action potential. Furthermore, the currents were prolonged, which in neurons would translate into more pronounced hyperpolarization. These abilities support the notion of the compounds being capable of increasing neuronal firing rates.

However, the increase in $K_v3.1b$ decay time induced by EX15 and RE01, which is elongating the repolarizing current, will at high frequencies, cause a fraction of the channels to remain open at the offset of the next action potential. Such a current will result in longer refractory period and hence reduced fast spiking ability. The size of the offset current is both time and voltage dependent, and to characterize the current we clamped the $K_v3.1b$/HEK cells to trains of 2 ms square pulses of increasing frequencies and from different physiological relevant resting potentials. The two compounds did indeed prolong the deactivation in a fashion, giving rise to a hyperpolarizing offset current at high firing rates. This was more pronounced for EX15, whereas for RE01, only relatively depolarized resting potential ($-55$ mV) in combination with very high frequency firing (250 Hz) could elicit an offset current. The induced delay in deactivation and the following increase in offset current are expected to have a negative effect on firing frequency and as such could suggest that EX15 would have a smaller window of effect relative to RE01. Conversely, at medium to high frequencies (before the offset current arises), the delayed deactivation will only increase the hyperpolarization, increase sodium channel availability and reduce refractoriness.

### 4.3. Functional significance of modulated $K_v3.1$ kinetics

The consequences of changed gating properties and thereby impact on action potential firing caused by EX15 and RE01 will be a sum of increased repolarization current, leftward shift of voltage gated activation and slower deactivation kinetics, thereby being highly voltage and frequency dependent. Our evaluation of EX15 and RE01 in fast-spiking GABAergic interneurons from the dentate

gyrus showed, that they could indeed increase the firing frequencies at low stimulation, however, the effect was significantly reduced at higher stimulations (inducing 50—80 Hz firing). At frequencies above 100 Hz there was no effect (neither positive nor negative, data not shown), as also reported by (Rosato-Siri et al., 2015). At such high rates, the firing frequencies are possibly limited by other physiological factors than $K_v3$ channels.

This study, show for the first time, that $K_v3.1$ and $K_v3.2$ positive modulatory compounds can increase the firing rate of fast spiking interneurons. EX15 and RE01 were both able to increase the firing frequency in response to both weak and strong depolarizing stimuli. To which extend the different $K_v3$ subtypes and splice variants contribute to this finding is not possible to estimate, as they are all present in fast spiking interneurons and $K_v3.1$ channels are likely to exist in heteromultimers with the less abundant $K_v3.2$ subunits (Erisir et al., 1999; Rudy et al., 1999).

The therapeutic potential of EX15 may be reduced due to its inhibitory modulation of $K_v3.2$ and $K_v3.4$, whereas RE01 displayed a highly desirable selectivity profile amongst the $K_v3$ receptors. Additionally, work by Rosato-Siri show that RE01 does not interact with a wide range of ion channels, receptors and transporters (Rosato-Siri et al., 2015).

## 5. Conclusion

The consequences of changed gating properties of $K_v3.1b$ caused by EX15 and RE01 will be a sum of increased repolarization current, leftward shift of voltage gated activation and slower deactivation kinetics, hence highly voltage and frequency dependent. EX15 and RE01 can indeed increase the intrinsic firing frequencies at low stimulation in fast-spiking GABAergic interneurons from the hippocampus, however the effect was significantly reduced at higher stimulations.

In conclusion, due to the positive modulation of $K_v3.1$/$K_v3.2$ and its high selectivity, compounds like RE01 may be beneficial in treatment of schizophrenia, epilepsy, hearing disorders and cognitive impairments.

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