Kynurenic acid and zaprinast induce analgesia by modulating HCN channels through GPR35 activation

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Abstract: Hyperpolarization-activated cyclic nucleotide- gated (HCN) channels have a key role in the control of cellular excitability. HCN2, a subgroup of the HCN family channels, are heavily expressed in small dorsal root ganglia (DRG) neurons and their activation seems to be a important in the determination of pain intensity. Intracellular elevation of cAMP levels activates HCN-mediated current (Ih) and small DRG neurons excitability. GPR35, a Gi/o coupled receptor, is highly expressed in small DRG neurons, and we hypothesized that its activation, mediated by endogenous or exogenous ligands, could lead to pain control trough a reduction of Ih current. Patch clamp recordings were carried out in primary cultures of rat DRG neurons and the effects of GPR35 activation on Ih current and neuronal excitability were studied in control conditions and after adenylate cyclase activation with either forskolin (FRK) or prostaglandin E2 (PGE2). We found that both kynurenic acid (KYNA) and zaprinast, the endogenous and synthetic GPR35 agonist respectively, were able to antagonize the FRK-induced depolarization of resting membrane potential by reducing Ih-mediated depolarization. Similar results were obtained when PGE2 was used to activate adenylate cyclase and to increase Ih current and the overall neuronal excitability. Finally, we tested the analgesic effect of both GPR35 agonists in an in vivo model of PGE2-induced thermal hyperalgesia. In accord with the hypothesis, both KYNA and zaprinast showed a dose dependent analgesic effect. In conclusion, GPR35 activation leads to a reduced excitability of small DRG neurons in vitro and causes a dose-dependent analgesia in vivo. GPR35 agonists, by reducing adenylate cyclase activity and inhibiting Ih in DRG neurons may represent a promising new group of analgesic drugs.

Cover letter

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PSICOLOGIA, AREA DEL FARMACO
E SALUTE DEL BAMBINO

11/5/2015

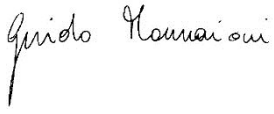
Dear Prof. Josef Kittler,

please consider the manuscript entitled “Kynurenic acid and zaprinast induce analgesia by modulating HCN channels through GPR35 activation” for publication as a research article in Neuropharmacology Special issue “kynurenine pathway”.

In this manuscript, we found that both kynurenic acid (KYNA) and zaprinast, the endogenous and synthetic GPR35 agonist respectively, were able to antagonize FRK and PGE2-induced depolarization of resting membrane potential by reducing Ih-mediated depolarization. GPR35 agonists analgesic effect was also tested in an in vivo model of PGE2-induced thermal hyperalgesia showing a dose dependent analgesic effect. We conclude that GPR35 activation by KYNA and zaprinast leads to a reduced excitability of small DRG neurons in vitro and causes a dose-dependent analgesia in vivo. Therefore, KYNA and zaprinast, by reducing adenylate cyclase activity and inhibiting Ih in DRG neurons through GPR35 activation may represent a promising new group of analgesic drugs.

Sincerely,

Prof. Guido Mannaioni



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*Title page

Kynurenic acid and zaprinast induce analgesia by modulating HCN channels through GPR35 activation

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Abstract

Hyperpolarization-activated cyclic nucleotide- gated (HCN) channels have a key role in the control of cellular excitability. HCN2, a subgroup of the HCN family channels, are heavily expressed in small dorsal root ganglia (DRG) neurons and their activation seems to be a important in the determination of pain intensity. Intracellular elevation of cAMP levels activates HCN-mediated current (Ih) and small DRG neurons excitability. GPR35, a Gi/o coupled receptor, is highly expressed in small DRG neurons, and we hypothesized that its activation, mediated by endogenous or exogenous ligands, could lead to pain control trough a reduction of Ih current. Patch clamp recordings were carried out in primary cultures of rat DRG neurons and the effects of GPR35 activation on Ih current and neuronal excitability were studied in control conditions and after adenylate cyclase activation with either forskolin (FRK) or prostaglandin E_2 (PGE_2). We found that both kynurenic acid (KYNA) and zaprinast, the endogenous and synthetic GPR35 agonist respectively, were able to antagonize the FRK-induced depolarization of resting membrane potential by reducing Ih-mediated depolarization. Similar results were obtained when PGE_2 was used to activate adenylate cyclase and to increase Ih current and the overall neuronal excitability. Finally, we tested the analgesic effect of both GPR35 agonists in an *in vivo* model of PGE2-induced thermal hyperalgesia. In accord with the hypothesis, both KYNA and zaprinast showed a dose dependent analgesic effect. In conclusion, GPR35 activation leads to a reduced excitability of small DRG neurons *in vitro* and causes a dose-dependent analgesia *in vivo*. GPR35 agonists, by reducing adenylate cyclase activity and inhibiting Ih in DRG neurons may represent a promising new group of analgesic drugs.

1. Introduction

The hyperpolarization-activated cyclic nucleotide gated (HCN) channel family mediates an inward current called Ih which is activated at low membrane potentials (between -60 and -90 mV) and has a key role in controlling cellular excitability and the rhythmic activity of spontaneously firing cells (DiFrancesco, 1993; He et al., 2014). In mammals, the HCN ion channel family is composed of four homologous members (HCN1 to HCN4) with different biophysical properties and different expression pattern. While cAMP significantly shifts the activation curves of HCN2 and HCN4 toward more positive potentials, HCN1 and HCN3 are not particularly sensitive to the cyclic nucleotide (Biel et al., 2002; Biel et al., 2009). Interestingly, HCN2 channels are particularly expressed in small DRG neurons, playing an important role in pain transmission (Emery et al., 2011; Momin et al., 2008). The G protein coupled receptor 35 (GPR35) was identified almost 20 years ago (O'Dowd et al., 1998), but its endogenous ligand is still not clear and the basic information available on the role this receptor may play in physiology and pharmacology are still unclear (Smith, 2012). GPR35 may be activated by kynurenic acid, an endogenous tryptophan metabolite and by zaprinast, an high affinity ligand previously studied as a cGMP phosphodiesterase inhibitor (Taniguchi et al., 2006; Wang et al., 2006). Its activation may cause a significant decrease of cAMP levels in different cellular models (Berlinguer-Palmini et al., 2013; Martin et al., 2015; Ohshiro et al., 2008) and it is well demonstrated that GPR35 is expressed in DRG and in spinal cord neurons (Taniguchi et al., 2006; Wang et al., 2006; Ohshiro et al., 2008). It has never been studied, however, whether or not the same DRG cell type express both HCN2 and GPR35 or if GPR35 activation may affect nociceptor excitability by acting on HCN2 channels.

We previously showed that kynurenine administration increases plasma and spinal cord levels of KYNA and that this increase may attenuate inflammatory pain in rats. Indeed, we

showed a close correlation between plasma KYNA levels and the degree of analgesia (Cosi et al., 2011). Moreover zaprinast and other GPR35 agonists such as cromolyn disodium (Leza et al., 1992; Yang et al., 2010) are provided with analgesic actions comparable with those of KYNA and since the maximal effects of KYNA and zaprinast are not additive, we proposed that GPR35 activation could be the main mechanism whereby KYNA and zaprinast cause analgesia (Cosi et al., 2011; Moroni et al., 2012).

However, since the electrophysiological actions of KYNA and of other GPR35 agonists in isolated DRG neurons have not been studied in detail, in the present research we evaluated the role of GPR35 agonists on the function of HCN. Our aim was to obtain further information on the previously observed analgesic actions of GPR35 agonists and to clarify the role of GPR35 expressed in DRG neurons. The possibility of obtaining analgesia by acting in DRG seems particularly interesting and may lead to pain treatment with pharmacological agents unable to penetrate the central nervous system thus avoiding most of the side effects of the currently available pain therapies.

2. Methods

2.1 Animal models.

All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and the local bioethics committee approved all experiments. Housing, handling and killing were in compliance with the Council Directive 2010/63EU and the Council of 22 September 2010. The experimental protocols were approved by the Animal Care Committee of the Department of Neurofarba, University of Florence.

2.2 Primary sensory neuronal cultures.

Rat dorsal root ganglion (DRG) neurons were isolated from neonatal Wistar rats (P7-P10) killed by cervical dislocation followed by decapitation. DRG neurons were prepared as previously described (Vellani et al., 2004). Briefly, 20–30 ganglia were isolated, incubated in collagenase (2.5 mg ml^{-1}) for 1 h at 37° C and mechanically triturated with a 45 µm sterile needle. The cell suspension was filtered in 40 µm Nylon filter (BD Falcon) then centrifuged and re-suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 50 u ml^{-1} penicillin and 0.05 mg ml^{-1} streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 10 % fetal bovine serum (FBS, Gibco), 50 ng ml^{-1} nerve growth factor (NGF, Promega) and 1.25 µg ml^{-1} cytosine β-D-arabinofuranoside (Ara-C, Sigma). DRG neurons were plated onto 13 mm borosilicate cover glass previously coated with polyL-lysine (100 µg ml^{-1} ,Sigma) and laminin (10 µg ml^{-1}, Sigma). The medium was changed after 24h. All electrophysiology recordings were made within 72 h from dissociation. DRG neurons were classified based on diameter of somata as follows: small (<26 µm), medium (26 – 35µm) and large (> 35µm).

2.3 Immunocytochemistry.

24h cultured DRG neurons were fixed in 4% formaldehyde and incubated in phosphate buffered saline (PBS) containing 0.1% Triton X-100, 1% BSA, and rabbit anti-GPR35 antibody (1:200 dilution; Abcam) and rabbit anti-HCN2 antibody (1:500 dilution; Abcam) for 2h at RT. After washing neurons were incubated with secondary antibodies conjugated to AlexaFluor-488 or Cy3 (1:1000 dilution; invitrogen) for 1h at RT. Relative fluorescence was quantified using ImageJ software (NIH, Bethesda, USA).

2.4 Whole-cell recordings.

Cultured DRG were incubated with vehicle, KYNA or zaprinast for 20' at 37 C° in culture medium, than were moved to the patch clamp setup for electrophysiology recordings. Experiments with KYNA were performed in presence of the NMDA antagonist D-APV (50 μM) and the $\alpha 7$ nicotinic receptor antagonist MLA (100 nM). Experiments with zaprinast were performed in presence of the phosphodiesterase inhibitor 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX) (1 mM). Voltage clamp experiments were performed using a PC-505B amplifier (Warner, Handen, CT, USA) and digitalized with a Digidata 1440 A and Clampex 10 (Axon, Sunnyvale, CA,USA). Pipettes, resistance 3-4.5 MΩ, were pulled from borosilicate capillaries (Harvard Apparatus, London, UK) using a Narishige PP830 vertical puller (Narishige International Ltd, London, UK). Access resistance was monitored during whole-cell voltage clamp recordings throughout experiments with short, -10 mV steps. Recordings undergoing $\geq$ 10% drift in access resistance were discarded. Pipette capacitance transients were cancelled while no whole-cell compensation was used. Signals were sampled at 10 kHz, low-pass filtered at 1 kHz. All recordings were

made at 21-22°C. Ih was activated with a two step protocol, consisting of a sequence of hyperpolarizing voltage steps (1.5 s) from -50 to -140 mV, followed by a step at -140 mv (holding potential -60 mV). Ih activation curves were obtained plotting the Ih tail currents against each imposed potential and were fitted with a Boltzmann equation of the following form:

$$I_t / I_{t(max)} = 1 / (1 + \exp [(V_m - V_{½}) / k]$$

Where Vm is the membrane potential, $V_{½}$ is the membrane potential at which Ih is half-activated, k is the slope factor, I_t is the current amplitude of the tail current recorded for a given pre-pulse and $I_{t(max)}$ is the maximum current amplitude of the tail current.

Neuronal excitability was studied performing current clamp protocol, consisting of increasing steps of depolarizing current (500 ms, 50pA), until generation of a single Action Potential (AP). To study AP threshold dVm/dt was plotted against voltage. AP threshold was estimated as previously reported (Sekerli et al., 2004).

Solutions. Extracellular solution contained (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 3 KCl, 10 Hepes, 10 D(+) glucose, adjusted to pH 7.3 with NaOH, osmolarity 300-310 mosmol l^{-1}. Pipette were filled with an intracellular solution containing (in mM): 140 K+-gluconate, 10 HEPES, 5 EGTA, 2 $MgCl_2$, 5 Na2Pcreatine, 0.3 NaGTP, 2 MgATP and adjusted to pH 7.3 with KOH, osmolarity 310-315mosmol l^{-1}. Cells were continuously perfused with extracellular solution using gravity-fed perfusion system. All substances used were stocked 1000 fold concentrated to avoid freeze/thaw circles. All substances was solved in dimethyl sulfoxide (DMSO) unless otherwise specified. Final concentration was respectively: forskolin (Sigma) 50 µM, PGE2 (Sigma) 20 µM, Zaprinast (Tocris) 1 µM, KYNA (tocris) 30 µM, D-APV (Tocris) 50 µM solved in water, Methyllycaconitine citrate (MLA) (tocris) 100 nM solved in water and ZD7288 (Tocris) 50 µM solved in water.

2.5 Behavioral test

Hot plate. Wistar rats were injected intraplantar (i.pl.) with 50 µl of PGE2 (0.75 nmol), or vehicle, into the left hind paw. A third group of animals were treated intraperitoneal (i.p.) with vehicle or Zaprinast (1; 5; 10 mg/Kg) or KYNA (100; 200; 300 mg/Kg) 20 min before receiving PGE2. 20 min after PGE2 injection, animals were placed on the hot plate device set to 47.5 °C. The latency of licking or flinching response of the left paw was measured in all groups. The cut-off time was set to 60 s to minimize skin damage.

2.6 Statistical analysis.

All datasets were tested for approximation to a normal distribution using Graphpad Prism software (D'Agostino test). Datasets were then analyzed using parametric statistical tests (t test for single comparisons or one-way analysis of variance [ANOVA] with Tukey post-hoc correction for multiple comparisons). Mean differences were considered significant for $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

3. Results

3.1 GPR35 and HCN2 are co-expressed in small primary sensory neurons from dissociated rat DRG

In the first series of experiments, we used immunofluorescence to study the expression of GPR35 and HCN in the different population of DRG sensory neurons. We found that GPR35 and HCN2 immunoreactivity was present in small, medium and large with the highest relative florescence for both GPR35 and HCN2 in the small DRG neurons (Fig. 1A).

We then used whole-cell patch clamp technique and the standard two-step voltage clamp protocol (DiFrancesco et al., 1986) to characterize Ih currents (Fig. 1B). According with the literature (Kouranova et al., 2008; Mayer and Westbrook, 1983; Scroggs et al., 1994), we found that in our preparation the Ih amplitude was 125 ± 52 pA at the test step of -110 mV (Fig. 1B, red trace) and that the Ih activation curve had a V½ of -100,6 ± 1,9 mV. The HCN blocker ZD7288 (50 μM for 10') fully abolished Ih current (Fig. 1B *top-right*). Interestingly, Ih pharmacological suppression had no discernible effect on RMP (-54,8 ± 2,0 mV to -55,1 ± 3,2 mV, n = 6, p = 0.7, ctrl vs ZD7288, respectively; Fig. 1B *bottom-right*) showing that the control of the RMP in small DRG neurons differs from that of other neuronal cell types (Biel et al., 2009; Masi et al., 2013).

3.2 GPR35 agonists counteract forskolin effect on RMP and Ih activation curve

When DRG neurons were exposed to forskolin (FRK, an activator of adenylate cyclase), Ih activation curves were shifted towards more positive potentials leading to RMP depolarization and increasing cell excitability (Momin et al., 2008). As shown in Fig. 2A, FRK application caused a mean RMP depolarization of 5 mV (-56,5 ± 1,1 to -51,6 ± 1,2 mV, n =

8, p = 7,5 E-4, ctrl vs FRK, respectively) and induced a ~ +15 mV shift of Ih activation curve (V½ -100,6 ± 1,9 mV to -85,4 ± 1,6 mV, n = 12, ctrl vs FRK, respectively). These effects were completely prevented by pre-incubation with Ih blocker ZD7288 (-57,2 ± 1,9 to -57,3 ± 2,1 mV, n = 6, ZD7288 vs ZD7288 + FRK, respectively).

In order to study the effects of GPR35 activation on forskolin-induced Ih changes we used KYNA and zaprinast. Since the effects observed after KYNA exposure could be mediated by NMDA (Stone et al., 2013) or α7 nicotinic acetylcholine receptor antagonism (Albuquerque and Schwarcz, 2013), all the experiments were performed in presence of maximally active concentrations of the NMDA receptor antagonist D-APV and of the α7 nicotinic antagonist MLA. To avoid that the effects observed after zaprinast exposure could be mediated by its phosphodiesterase inhibitory activity, all the experiments involving zaprinast were performed in presence of high concentrations of the phosphodiesterase inhibitor IBMX.

Then we tested the modulation of GPR35 agonists on RMP and Ih activation curve, *per se* and we noticed that neither KYNA nor zaprinast directly affected RMP or Ih activation curve. On the contrary, both KYNA and zaprinast were able to counteract FRK effects (Fig. 2B and 2C). Indeed, both GPR35 agonists significantly reduced FRK-induced depolarization (-56,4 ± 1,2 mV to -54,3 ± 1,5 mV, n = 8, p = 0.14 KYNA vs KYNA + FRK, respectively; -57,0 ± 1,2 mV to -56,1 ± 1,0 mV, n = 8, p = 0.08 zaprinast vs zaprinast + FRK respectively) and FRK-induced rightward-shift of the activation curve (V½ -98,3 ± 0,7 mV to -95,4 ± 0,9 mV, n = 8, KYNA vs KYNA + FRK, respectively; -98,3 ± 0,7 mV to -95,4 ± 0,9 mV, n = 8, zaprinast vs zaprinast + FRK, respectively). Fig. 2C reports the quantitative effects of GPR35 agonists on both RMP (top) and Ih activation curve V½ (bottom).

3.3 GPR35 agonists counteract the effect of PGE2

PGE2 is a powerful endogenous pro-inflammatory mediator and it increases primary sensory neuron excitability by increasing intracellular cAMP levels. It has been demonstrated that application of PGE2 in small DRG neurons leads to an HCN-mediated depolarization (Momin et al., 2008). Therefore, we determined the ability of GPR35 agonists to prevent, or reduce, the electrophysiological effects of PGE2 in small DRG neurons. We observed that PGE2 20 μM induced RMP depolarization (from -59,8 ± 1,8 mV to -54,4 ± 1,5 mV, n = 8, p = 0.003) and caused a positive shift of Ih activation curve ($V_{½}$ from -98,7 ± 0,4 mV to -87,1 ± 0,8 mV, n=8) (Fig. 3A). Interestingly, both PGE2-induced RMP depolarization and the shift of Ih activation curve were significantly reduced by pre-application of KYNA (RMP from -57,9 ± 0,6 mV to -57 ± 0,9 mV, n = 8, p = 0.08, ; $V_{½}$ from 102,3 ± 2,2 mV to -100,5 ± 1,7 mV, n = 8, KYNA vs KYNA + PGE2, respectively) or of zaprinast (RMP from -57,2 ± 0,8 mV to -57,7 ± 0,9 mV, n = 8, p = 0.13; $V_{½}$ from-101,3 ± 1,7 mV to -100,5 ± 1,5 mV, n = 8, zaprinast vs zaprinast + FRK, respectively), thus indicating that GPR35 activation was able to prevent PGE2-dependent RMP depolarization by counteracting the positive shift of Ih activation curve.

3.4 GPR35 agonists counteract the effect of PGE2 on neurons excitability

We then studied the small DRG neurons intrinsic excitability by performing voltage recordings (see methods). We observed that PGE2 did not affect neither AP firing threshold (-26,9 ± 2,3 mV to 29,8 ± 2,0 mV, n = 8 , p = 0.52, ctrl vs PGE2, respectively; Fig. 4A, inset #2) nor AP amplitude or duration (data not shown). Application of PGE2 led to depolarization of the RMP (Fig. 4A, inset #1) that ultimately reduced AP latency (10,76 ± 1,5 ms 7,1 ± 1,0 ms, n = 8, p = 0.01, ctrl vs PGE2 respectively; Fig. 4A, inset #3) and the depolarization needed to reach AP threshold (ΔmV, -28,3 ± 3.3 mV to -21,5 ± 3,6 mV, n = 8, p = 0.002, ctrl vs PGE2, respectively; Fig. 4B bottom). GPR35 activation prevented small DRG neurons

PGE2-induced depolarization therefore no effect on either AP latency (9.3 ± 0.6 ms to 9.5 ± 2.1 ms, n = 8, p = 0.91; KYNA vs KYNA + PGE2, respectively; 10,9 ± 1,3 ms to 10,3 ± 1,4 ms, n = 8, p = 0.51, zaprinast vs zaprinast + PGE2, respectively) or on the depolarization needed to reach AP threshold (-33,0 ± 6,4 mV to -33,5 ± 4,7 mV, n = 8, p = 0.73, KYNA vs KYNA + PGE2, respectively; -30,9 ± 5,7 mV to -32,0 ± 5,0 mV, n = 8, p = 0.083, zaprinast vs zaprinast + PGE2) was observed following PGE2.

3.5 Analgesic effect of GPR35 agonists in a model of PGE2-induced thermal hyperalgesia

Finally, we assessed if GPR35 activation *in vivo* could lead to analgesia in a model of thermal hyperalgesia induced by injecting PGE2 into the hind paw (see methods). Intradermal injection of PGE2 0.75 nmol reduced the paw withdrawal latency in response to heat stimuli (vehicle 58.2 ± 4.9 s n = 13; PGE2 15.6 ± 1.6 s n = 17 p = 7.8 E-17). As shown in Fig. 5A, animals pretreated (20’ before) with KYNA i.p. showed a dose dependent increase in withdrawal latency (PGE2 + KYNA 100 mg/Kg: 22.4 ± 2.6 s, n = 8, p = 1; PGE2 + KYNA 200 mg/Kg: 30.8 ± 5.9 s, n = 9, p = 0.002; PGE2 + KYNA 300 mg/Kg: 44.4 ± 5.7 s, n = 8, p = 1.1 E-7. All p values are vs PGE2). Similarly, zaprinast showed a significant analgesic effect on PGE2-induced hyperalgesia (PGE2 + zaprinast 1 mg/Kg: 19.2 ± 3.3 s, n = 10, p = 0.96; PGE2 + zaprinast 5 mg/Kg: 32.3 ± 6.1 s, n = 8, p = 0.01; PGE2 + zaprinast 10 mg/Kg: 23.6 ± 4.4 s, n = 9, p = 0.5. All p values are vs PGE2 + veh).

4. Discussion

Our results show that the small neurons of the DRG (nociceptors) express significant amount of GPR35 a Gi/o coupled protein and of HCN2 a hyperpolarization activated cyclic nucleotide gated channel protein able to control cellular excitability. It is possible to increase cellular excitability by exposing cultured nociceptors to either FSK or PGE2 by increasing the local cAMP availability, are able to shift toward more positive potentials the Ih activation curve thus leading to cellular depolarization (see Fig 2 and 3) and possibly to an increased excitability of these neurons (Emery et al., 2011; Momin et al., 2008). KYNA and zaprinast, two different GPR35 agonists, prevented these effects suggesting that the previously observed analgesic actions of these (Cosi et al., 2011) and of other GPR35 agents, for instance as chromalyn (Leza et al., 1992), may be mediated by changes in HCN2 channel function. Indeed, neither FRK nor PGE2 were able to modify nociceptor RMP or shift the HCN activation curve toward more positive potentials when KYNA or zaprinast were present. We also found that the local increase of cAMP induced by either FRK or PGE2 was not associated to changes in AP amplitude, duration or threshold, thus ruling out the involvement of voltage gated Na+ channel in the observed effects. These results are in line with previous observation arguing against a major effect of cAMP increase on voltage-dependent Na+ or K+ currents (Momin et al., 2008). The application of FRK or PGE2 increased neuronal excitability by shifting HCN activation curve and by causing RMP depolarization (Fig 4). Under those conditions, the latency and the further depolarization needed to reach the AP threshold were reduced. In the presence of GPR35 agonists, the HCN-mediated effect of FRK and PGE2 were prevented and the neurons maintained the physiological excitability. In performing the present experiments we were aware that the two GPR35 agonists we used are not specific and therefore appropriate controls were carried

out. When we used KYNA we ruled out the possibility that the effects were mediated through the NMDA or α7 nicotinic receptors by adding supra-maximal concentrations of appropriate antagonists and all the experiments with zaprinast were performed in the presence of high concentrations of IBMX another phosphodiesterase inhibitor (see methods). We also attempted to antagonize GPR35 activation by using CID 2745687 (1-10 μM) one of the few available receptor antagonist. Unfortunately, the compound had non-expected actions in the system: in particular it reduced Ih amplitude possibly because of a direct interaction with HCN channels. The need of better and less species specific GPR35 agonists and antagonists (Jenkins et al., 2012) could be important in order to better characterize the GPR35 mediated activation of Ih modulation (Mackenzie and Milligan, 2015). In this line, it is interesting to note that we considered KYNA a possible endogenous GPR35 ligand (Cosi et al., 2011; Moroni et al., 2012; Wang et al., 2006). It has been recently reported, however, that the main endogenous GPR35 agonist could be the chemokine CXCL17 (Maravillas-Montero et al., 2015). In spite of this proposal, we observed that low micromolar concentration of KYNA may activate GPR35 in different systems (Berlinguer-Palmini et al., 2013) and we are still convinced that GPR35 is an interesting target for a new generation of analgesic agents.

In order to further study *in vivo* the analgesic action of GPR35 agonists we used PGE2 to induce of thermal hyperalgesia. Previous experiments have shown that KYNA precursor L-kynurenine was able to exert antinociceptive effects in an *in vivo* model of inflammatory pain by increasing KYNA content in plasma and in the CNS (Cosi et al., 2011). Kynurenine was used because KYNA itself poorly cross the blood brain barrier (Lou et al., 1994). However, since the DRG are located outside the blood–brain barrier (Sapunar et al., 2012) we directly administered KYNA to stimulate GPR35 located in the DRG neurons. We observed a significant analgesic effect even if the doses of KYNA were relatively high. This could be explained by the fact that KYNA is rapidly eliminated in the urine and its plasma

concentrations rapidly declines (Chiarugi et al., 1996; Moroni et al., 1988). We also tested zaprinast a more potent GPR35 agonist, which had significance antinociceptive effect at dose of 5 mg/Kg. The bell shaped dose-response curve of zaprinast in vivo is reasonably to be ascribed to phosphodiesterase inhibition with a significant local cAMP accumulation at the highest doses (Taniguchi et al., 2006). Interestingly, aspirin has been recently proposed to exert part of its anti-inflammatory effect through GPR35 modulation (Dodd et al., 2013). Indeed, 2,3,5-trihydroxybenzoic acid, salicyluric acid and gentisuric acid, which are metabolites of aspirin, were reported to activate human GPR35 in both dynamic mass redistribution and βarrestin - 2 recruitment assays (Deng and Fang, 2012). Therefore, since aspirin metabolites and kynurenic acid increase the adhesion of leukocytes to vascular endothelial cells and shedding of neutrophil L-selectin from human peripheral monocytes (Barth et al., 2009), it is interesting to speculate upon an involvement of aspirin metabolites and agonism of GPR35 in this process (Mackenzie and Milligan, 2015). Taking together, GPR35 modulation in immune system, calcium channels and HCN channels in nociceptors, provide the evidence of a promising new target in inflammatory and neuropathic pain. In conclusions, our data may suggest that GPR35 is an interesting target for new analgesic agents. These agents may act in the DRG neurons and are not required to penetrate the blood brain barrier thus lacking most of the side effects of currently available centrally acting analgesics.

5. Acknowledgements

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Figure legends

Figure 1. GPR35 and HCN2 are co-expressed in rat DRG small primary sensory neurons. A. Immunofluorescent detection of GPR35 (top left) and HCN2 (top right) in rat DRG neurons. Small double-labeled neurons are indicated by arrows. (Scale bar = 50 μm). Histogram, mean fluorescence intensity of GPR35 and HCN2 signal in small (n = 50), medium (n = 42) and large (n = 35) DRG neurons . **B**. Characterization of Ih current in small rat DRG neurons. Top, whole cell current traces before (ctrl) and after application of Ih-blocker ZD7288. Black arrow indicates tail currents at the -140 mV step. Inset, protocol scheme, scale: 250 ms, 150 pA. Bottom left, Ih activation curve obtained plotting the tail currents amplitude against each imposed potential. Bottom right, scatter graph representing the resting membrane potential before and after the ZD7288 mediated HCN channels blockade, no changing in RMP are observed.

Figure 2. GPR35 agonists counteract forskolin effect on RMP and Ih activation curve. A. top, Representative traces of forskolin-induced RMP depolarization, recorded in current–clamp configuration in control condition (left) and in presence of ZD7288 (right). Middle, RMP depolarization quantitative analysis. Bottom, Ih activation curve. In control condition, forskolin shifts the Ih activation curve towards more depolarized potentials, on the contrary in presence of ZD7288 no alteration are observed after forskolin application. Inset, representative traces of Ih current at the voltage step of -90 mV, before and after forskolin application, scale: 250 ms / 50 pA. **B**. top, Representative traces of forskolin-induced depolarization, in presence of KYNA (left) or zaprinast (right). Middle, RMP depolarization quantitative analysis. Bottom, forskolin effect on the Ih activation curve, in presence of KYNA or zaprinast. In presence of both GPR35 agonists, no alterations of Ih activation curve are observed after application of forskolin. Inset, representative traces of Ih current at the

voltage step of -90 mV, before and after forskolin application, scale: 250 ms / 50 pA. **C**. top, quantification of the relative depolarization after application of forskolin in control condition, in presence of ZD7288 or in presence of GPR35 agonists. Bottom, ΔV½ of Ih activation curve after forskolin application in all conditions.

Figure 3. GPR35 agonists counteract the effect of PGE2. A Top, PGE2 50 µM for 4' induced RMP depolarization in control condition (left) in presence of kynA (middle) or zaprinast (right). Bottom, PGE2 induced shift of the Ih activation in control condition in presence of kynA or zaprinast. **B**. top, quantification of the relative depolarization after application of PGE2 in control condition or in presence of GPR35 agonists. Bottom, ΔV½ of Ih activation curve after PGE2 application in the same conditions.

Figure 4. GPR35 agonists counteract the effect of PGE2 on neuronal excitability. Small DRG neuron excitability was studied by applying increasing steps of depolarizing current until generation of a single action potential (AP). **A**. Example traces of an AP evoked injecting 200 pA of depolarizing current, before and after PGE2 application, scale: 10 ms / 15 mV. In square, magnification of the traces showing the resting potential (1), AP threshold (2) and the latency to reach the threshold (3), scale: 2 ms / 5 mV. Bottom, example traces of the same experiment performed in presence of zaprinast. **B**. Scatter graphs showing the PGE2 effect on AP threshold, latency and the Δmv to reach the threshold, in control condition (circle), in presence of KYNA (square) or zaprinast (triangle).

Figure 5. In vivo analgesic effect of GPR35 agonists in a PGE2 model of thermal hyperalgesia. In adult wistar rats hid paw injection of PGE2, generate a hyperalgesic effect in the hot plate test set at 47,5 C°. The latency of the hind paw withdrawal is reduced from 58,2 ± 0,9 s in animals treated with vehicle, to 15,6 ± 1,6 s in animals treated

with PGE2. Animals treated with KYNA (left) or zaprinast (right) show a dose dependent analgesic effect.

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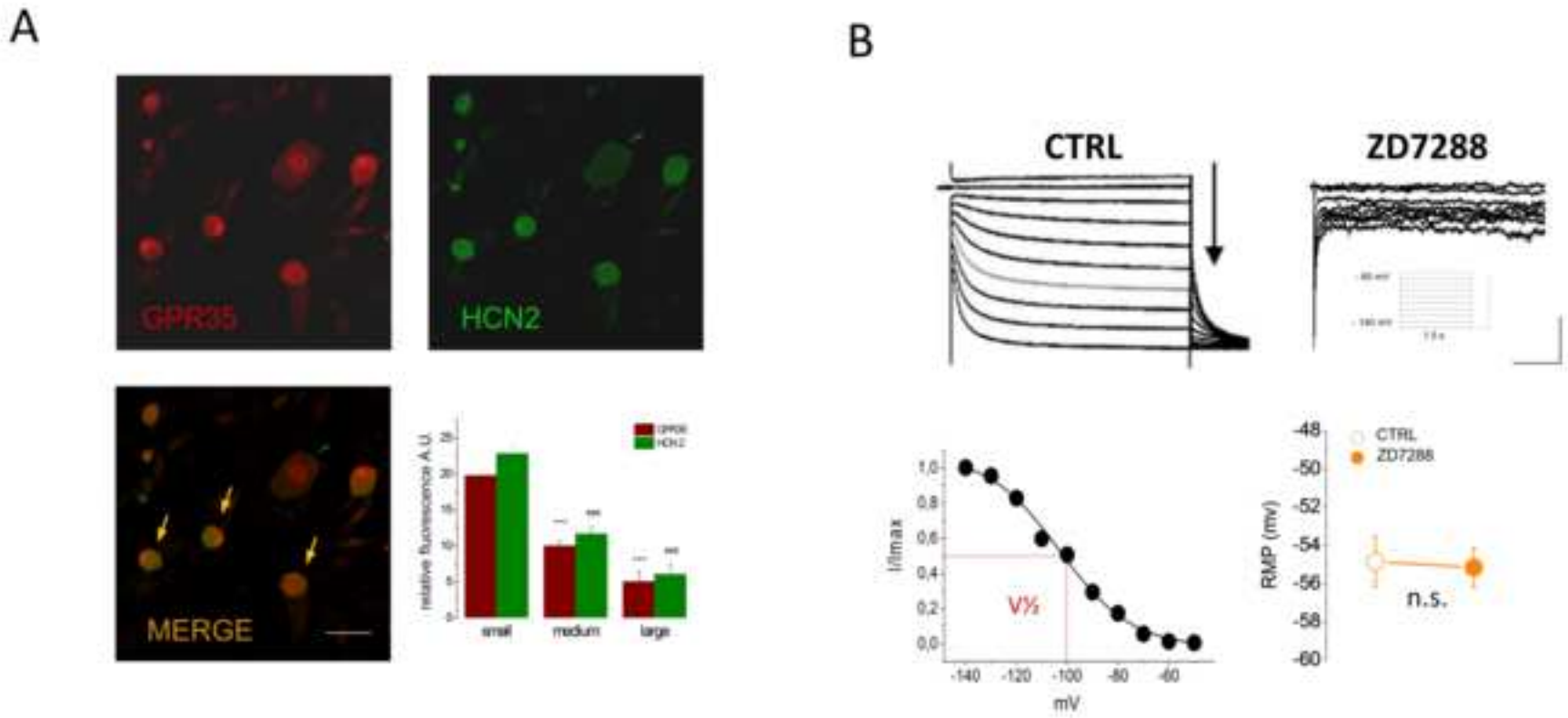

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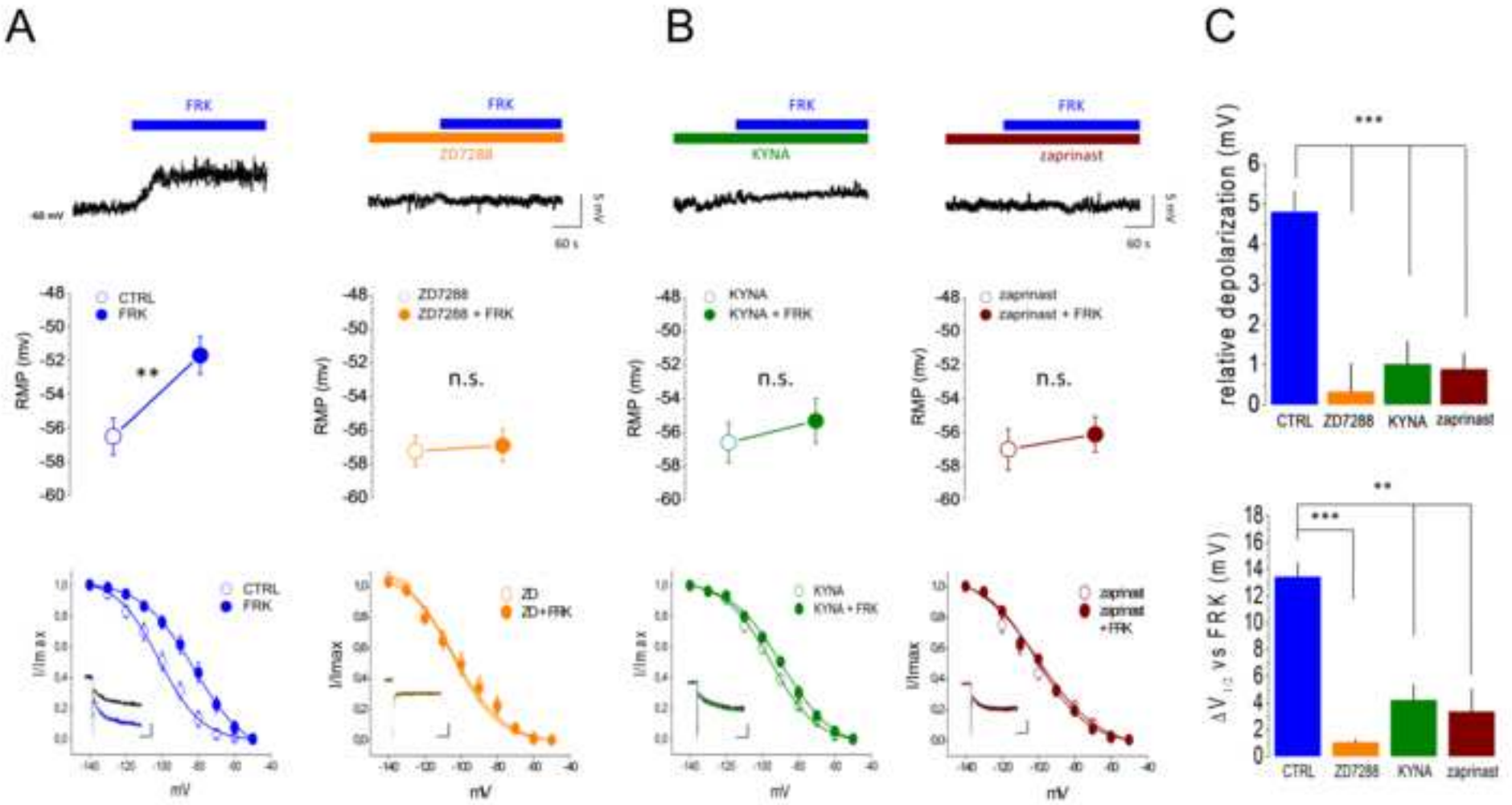

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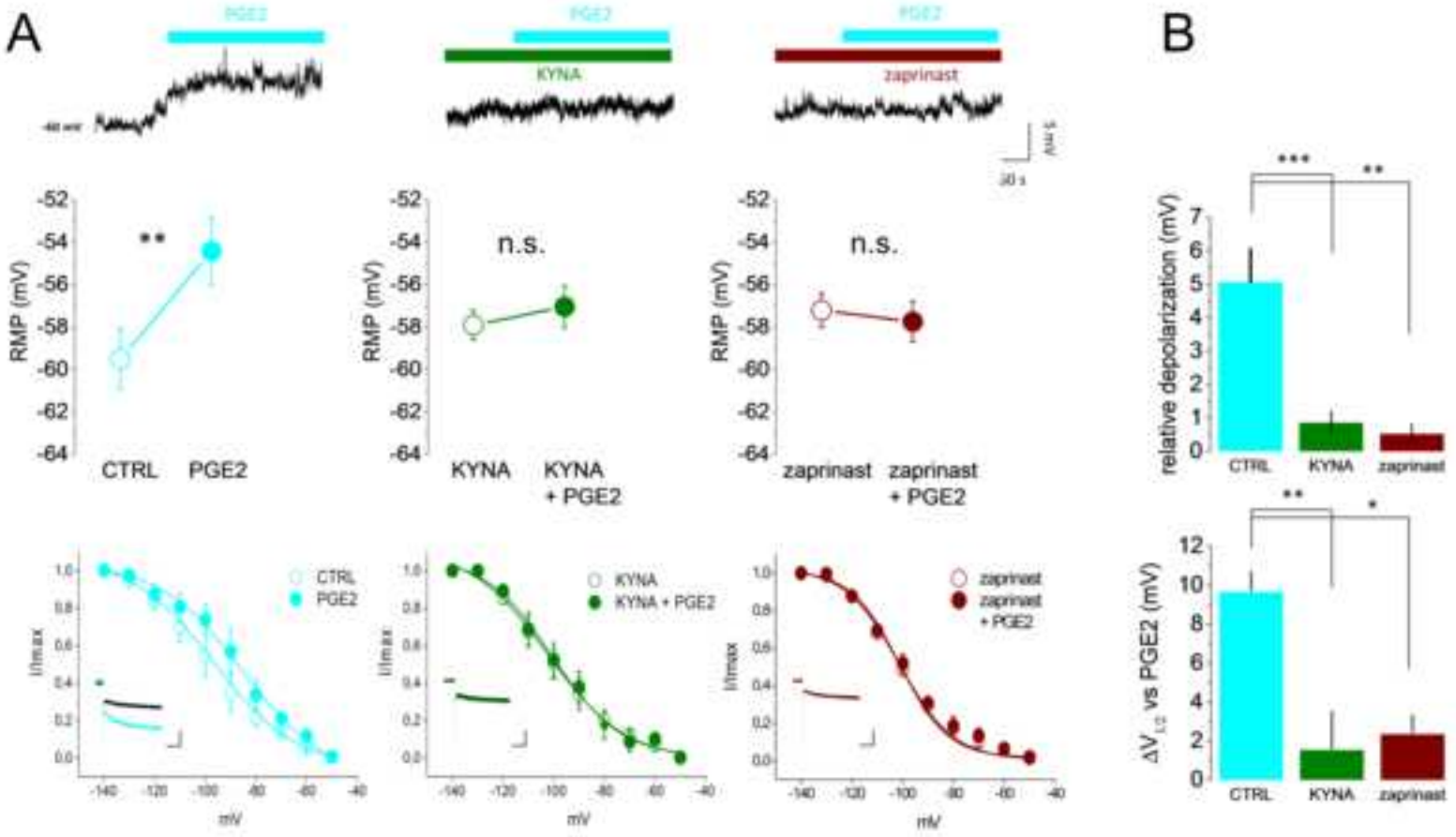

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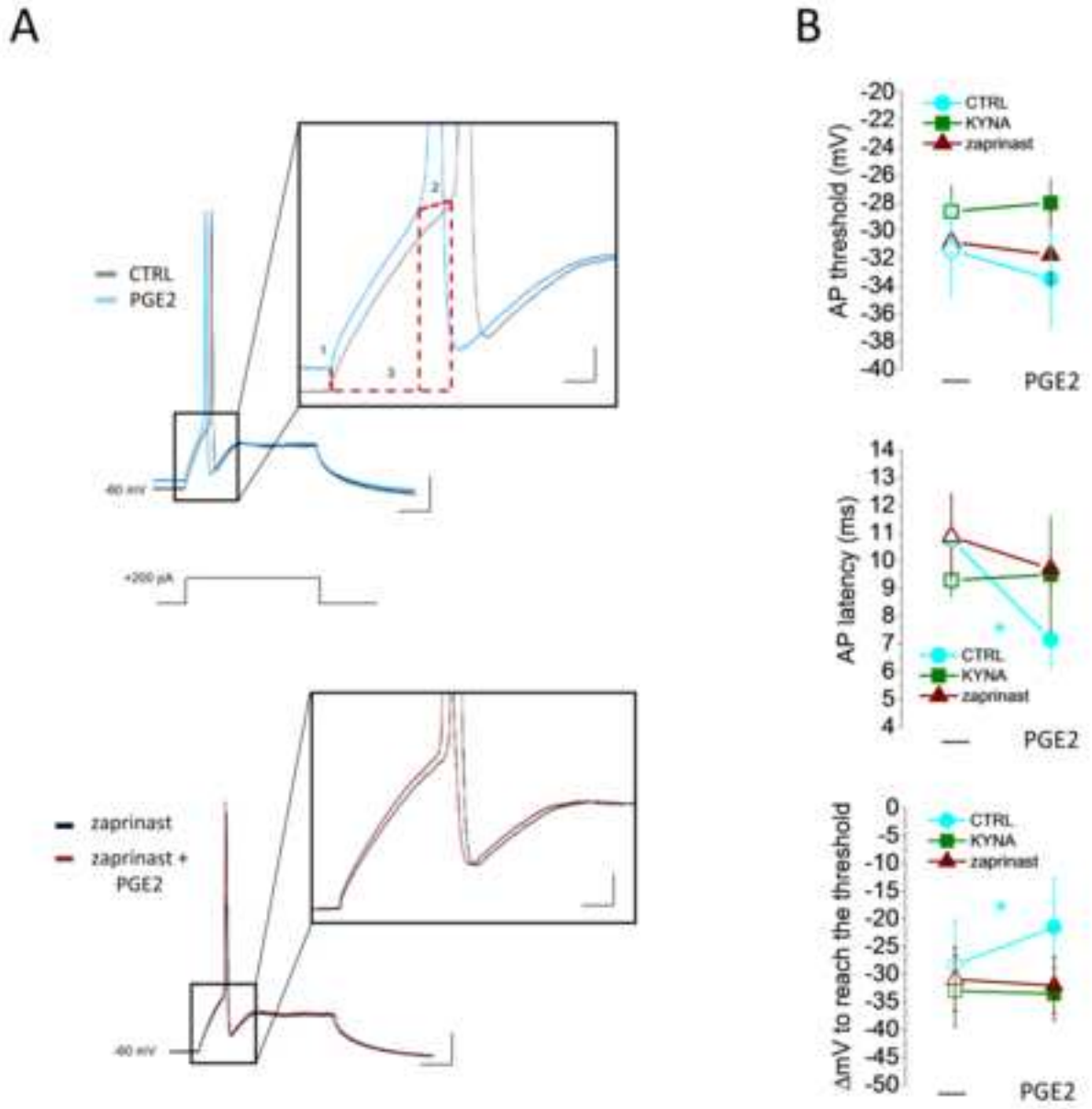

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