β3-Adrenergic receptor-dependent modulation of the medium afterhyperpolarization in rat hippocampal CA1 pyramidal neurons

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Abstract

Action potential firing in hippocampal pyramidal neurons is regulated by generation of an afterhyperpolarization (AHP). Three phases of AHP are recognised, with the fast AHP regulating action potential firing at the onset of a burst, and the medium and slow AHPs supressing action potential firing over 100s of milliseconds and seconds respectively. Activation of β-adrenergic receptors suppresses the slow AHP by a protein kinase A-dependent pathway. However, little is known regarding modulation of the medium AHP. Application of the selective β-adrenergic receptor agonist isoproterenol suppressed both the medium and slow AHPs evoked in rat CA1 hippocampal pyramidal neurons recorded from slices maintained in organotypic culture. Suppression of the slow AHP was mimicked by intracellular application of cAMP, with the suppression of the medium AHP by isoproterenol still being evident in cAMP-dialysed cells. Suppression of both the medium and slow AHPs was antagonised by the β-adrenergic receptor antagonist propranolol. The effect of isoproterenol to suppress the medium AHP was mimicked by two β3-adrenergic receptor agonists: BRL37344 and SR58611A. The medium AHP was mediated by activation of SK and deactivation of H channels at the resting membrane potential. Suppression of the medium AHP by isoproterenol was reduced by pre-treating cells with the H-channel blocker ZD7288. These data suggest that activation of β3-adrenergic receptors inhibits H-channels, which suppresses the medium AHP in CA1 hippocampal neurons by utilising a pathway that is independent of a rise of intracellular cAMP. This finding highlights a potential new target in modulating H-channel activity, and thereby neuronal excitability.

New & Noteworthy

The noradrenergic input into the hippocampus is involved in modulating long-term synaptic plasticity and is implicated in learning and memory. We demonstrate that activation of functional β3-adrenergic receptors suppresses the medium AHP in hippocampal pyramidal

neurons. This finding provides an additional mechanism to increase action potential firing frequency, where neuronal excitability is likely to be crucial in cognition and memory.

Introduction

Action potential firing is regulated by the generation of an afterhyperpolarization (AHP) following a single or train of action potentials (Alger and Nicoll, 1980; Madison and Nicoll, 1984; Alger and Williamson, 1988; Storm, 1990; Gu et al., 2005). The AHP influences both the frequency and patterning of neuronal firing. An increase in action potential firing is observed after pharmacologically inhibiting AHPs (Lancaster and Adams, 1986; Pedarzani and Storm, 1993; Stocker et al., 1999; Gu et al., 2005; Tombaugh et al., 2005), while a decrease in action potential firing results from potentiation of the AHP (Pedarzani et al., 2005; Gu et al., 2008). Hippocampal CA1 pyramidal neurons exhibit an AHP that has three main components, based on both kinetics and pharmacology. The fast AHP results from activation of BK channels and regulates firing at the onset of a burst (Storm, 1989). In contrast, the medium AHP regulates firing 50-200 ms after a burst of action potentials and the slow AHP affects firing over many seconds following a burst of action potentials (Storm, 1989; Stocker, 2004; Gu et al., 2005; Vatanparast and Janahmadi, 2009; Chen et al., 2014). The medium AHP in hippocampal CA1 neurons is mediated by 3 different ion channel subtypes, small-conductance, calcium-activated K^+ (SK) channels (Stocker et al., 1999; Bond et al., 2004; Church et al., 2014), voltage-dependent M-channels (K_V7/KCNQ) (Gu et al., 2005; Tzingounis et al., 2007), and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (Gu et al., 2005; Kaczorowski, 2011). There is still controversy regarding the channel(s) underlying the slow AHP in hippocampal pyramidal neurons, with the intermediate-conductance Ca^{2+}-dependent K^+ (IK) channel (King et al., 2015; Turner et al., 2016), voltage-dependent K_V7 channel (Tzingounis et al., 2007), and ATPase-Na^+/K^+ pump (Gulledge et al., 2013) all been proposed to mediate this slow afterpotential.

The slow AHP is unusual in being suppressed by different monoamines, such as noradrenaline (Madison and Nicoll, 1982; Pedarzani and Storm, 1993), dopamine (Malenka and Nicoll, 1986), serotonin (Pedarzani and Storm, 1993), and histamine (Haas and Greene, 1986; Pedarzani and Storm, 1993). Monoamine neurotransmitters suppress the slow AHP by the canonical $G\alpha_s$ G-protein pathway, which results in the formation of cAMP by the stimulation of adenylate cyclase and subsequent activation of protein kinase A (Pedarzani and Storm, 1993). For example, suppression of the slow AHP by noradrenaline is antagonised by the β-adrenergic receptor (βAR) antagonist propranolol (Madison and Nicoll, 1982). Immunohistochemistry has identified that β1- and β2-adrenergic receptors are expressed throughout the hippocampus, with both subtypes being located in both the soma and dendrites of CA1 pyramidal neurons (Guo and Li, 2007; Cox et al., 2008). β3-adrenergic receptor mRNA has also been identified in the rodent and human hippocampus (Rodriguez et al., 1995; Evans et al., 1996), albeit a lower expression than seen for β1 and β2 receptors (Evans et al., 1996; Claustre et al., 2008). The functional role of the β3AR in the hippocampus is unclear; however β3AR agonists demonstrate anti-depressant and anxiolytic effects in rodents (Simiand et al., 1992; Consoli et al., 2007; Claustre et al., 2008; Tamburella et al., 2010; Tanyeri et al., 2013).

In this study we show that non-selective pharmacological activation of β-adrenergic receptors suppressed both the medium and slow AHPs, an effect antagonised by propranolol. The effect of β receptor activation on the slow AHP was occluded by intracellular dialysis of cAMP, whereas suppression of medium AHP persisted. The medium AHP was generated by SK and HCN channels in CA1 neurons held at -75 mV, with the effect of isoproterenol reduced by prior block of HCN-mediated H-current. Suppression of the medium AHP was mimicked by two different selective β3-adrenergic receptor agonists. These data suggest that the activation of β3-adrenergic receptors suppresses the medium AHP by inhibiting HCN channel activity using a cAMP-independent pathway. These findings

110 illustrate an additional mechanism to modulate hippocampal neuron excitability utilising a
111 novel pathway.
112

Materials and Methods

Organotypic hippocampal slice cultures. Organotypic slice cultures from the hippocampus were prepared from 18 to 20-day-old male Wistar rats as described previously (Stoppini et al., 1991). Rats were killed by cervical dislocation in accordance with Schedule 1 of the UK home office guidelines set out in the Animals (Scientific Procedures) Act 1986. All procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU. All experimental procedures were reviewed by the University of Bristol Ethical Review Group (reference: UB/12/006). Brains were removed and horizontal brain slices (300 μM) were cut in ice-cold (~4 ˚C) sucrose-based cutting solution containing (in mM): 189 sucrose, 10 D-glucose, 26 $NaHCO_3$, 3 KCL, 5 $MgSO_4.7H_2O$, 0.1 $CaCl_2$, 1.25 NaH_2PO_4, saturated with 95% O_2 and 5% CO_2 using a VT1000 S vibrating blade microtome (Leica Microsystems Ltd, Milton Keynes, UK). After sectioning, hippocampal slices were transferred to a storage chamber filled with artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 24 $NaHCO_3$, 1.25 $NaH_2PO_4.H_2O$, 1 $MgSO_4.7H_2O$, 2 $CaCl_2$, 10 D-glucose saturated with 95% O_2 and 5% CO_2. Under aseptic conditions, slices were washed twice with culture media containing Minimum Essential Medium (Gibco) supplemented with (in mM): 50 $NaHCO_3$, 75 HEPES, 0.437 glutamine, 0.625 $CaCl_2$, 1.25 $MgSO_4.7H_2O$, 0.425 ascorbic acid, 32 D-glucose, with 12.5% heat-inactivated horse serum, 1 mg/lL insulin, and 100 units/ml penicillin with 100 μg/ml streptomycin (pH 7.28 with NaOH, osmolarity ~ 320 mOsm). Slices were washed a further two times in culture media without supplemented penicillin/streptomycin. Slices were cultured on a porous (0.4 μM) membrane (Millicell inserts, Millipore) and maintained at 37˚C in 5% CO_2 for 3 days before slices were used for electrophysiological recordings. The characteristics of the medium and slow AHPs recorded in CA1 pyramidal neurons from slices maintained in short-term organotypic culture were comparable with those reported in CA1 neurons from acute hippocampal slice preparations taken from similar aged animals (Maccaferri et al., 1993; Stocker et al., 1999; Lancaster et al., 2001; Kaczorowski et al., 2007; Kaczorowski, 2011).

Electrophysiology. Slices were continuously perfused (2-3 ml/min) with aCSF supplemented with NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) (10 μM) to inhibit spontaneous AMPA receptor-mediated excitatory post-synaptic currents. The aCSF was continuously oxygenated and temperature was maintained at ~33˚C using an HPT-2 inline heater (Scientifica, UK). Whole-cell current-clamp recordings were made from visually identified pyramidal neurones from the CA1 region of the hippocampus using an infrared-light emitting diode mounted on an Axioskop2 microscope (Carl Zeiss). Fire-polished electrodes (3-5 MΩ) were fabricated from borosilicate glass (1.5 mm O.D., 0.86 mm I.D.) containing (in mM): 125 $KMeSO_4$, 10 KCl, 10 NaCl, 20 HEPES, 2 MgATP, 0.3 Na_2GTP, 0.2 EGTA, pH 7.3, osmolarity 280-285 mOsm. A liquid junction potential error was experimentally measured (+13 mV) and was compensated for during recording. The membrane voltage for all recordings was recorded in the bridge-balance mode of the MultiClamp 700A amplifier (Molecular Devices, CA, USA). Voltage responses were filtered at 1.2 kHz (eight-pole low-pass Bessel filter) and sampled at 5 kHz using Pulse (HEKA Electronics, Lambrecht, Germany).

Data analysis. AHPs were elicited by evoking a train of 15 action potentials by brief (2 ms) 2 nA somatic current injections delivered at 50 Hz. Any cell that did not fire the correct number of APs was discarded. Analysis of the medium AHP and slow AHPs were carried out using custom-written MatLab scripts (The MathWorks Company). The mAHP were measured as the peak negative membrane deflection between 0 to 100 ms after the cessation of the last AP action potential fired. The slow AHP was measured 1 second after the last AP action potential was fired. The overlapping kinetic profiles of the medium AHP and slow AHP was minimized by measuring the AHP components within these time points. Finally, the hyperpolarization induced H-current-mediated depolarizing sag was measured as the percentage difference between the peak negative deflection and the membrane voltage

once at steady state in response to a hyperpolarizing current injection (100 pA amplitude, 500 ms duration) (Tamagnini et al., 2015).

Cell input resistance was determined from a -100 pA current pulse (500 ms) delivered 1 second before a train of action potentials to generate AHPs. The non-conductive anion methylsulphate ($MeSO_4$) is associated with a time-dependent rise in input resistance (Zhang et al., 1994; Velumian et al., 1997; Kaczorowski et al., 2007). We observed that the cell input resistance rose by 13.4 ± 4.3% during a 5-minute baseline recording period (105.8 ± 5.2 vs 121.0 ± 12.9 MΩ; n = 6; t = -3.0, P = 0.03; paired two-tailed Student's t-test). During this same 5-minute baseline recording, the medium AHP amplitude did not change (-4.4 ± 0.3 vs -4.5 ± 0.2 mV; t = 0.2, P = 0.84, paired two-tailed Student's t-test), while the slow AHP increased by 25.3 ± 21.6% (-2.2 ± 0.2 vs -2.6 ± 0.2 mV; $t_{(5)}$ = 1.0, P = 0.4). All recordings used cells with a stable resting membrane potential more negative than -60 mV.

Drugs. All salts were purchased from Sigma-Aldrich except HEPES, which was obtained from Merck Serono (Fletham, UK). Isoproterenol and propranolol were purchased from Sigma-Aldrich, whereas NBQX, ZD7288, XE991, BRL37344, SR58611A and apamin were purchased from Tocris Biosciences (Bristol, UK). NBQX and ZD7288 were both prepared as stock solutions in dimethylsulfoxide (DMSO) and diluted in aCSF when required. BRL37344, SR58611A, apamin, isoproterenol and propranolol were prepared as stock solutions in water. All drugs were prepared as 1000x stock solutions and stored at -20 ˚C until required, except for isoproterenol and propranolol which were prepared daily. All drugs were applied to the perfusion system.

Statistics. Statistical analysis was performed using SPSS (v21, IBM) and representative traces were drawn using Origin 9 (Microcal Software). All data is presented as mean ± SEM. Paired two-tailed Student's t-tests were used to compare the means between control and drug treatment groups. A repeated measures ANOVA was used to compare AHP

196 amplitudes after the addition of two or more drugs. Fisher's least significant difference (LSD)
197 post-hoc tests were performed to assess between-drug treatment differences.
198

Results

The β-adrenergic receptor agonist isoproterenol inhibits both the medium and slow AHPs

AHPs were evoked from a membrane potential of -75 mV by a train of 15 action potentials evoked by brief (2 ms) current injections (2 nA) fired at 50 Hz (Fig. 1A). As previously reported (Pedarzani and Storm, 1993), bath application of the selective β-adrenergic receptor agonist isoproterenol (1 μM; n = 7) reduced the amplitude of the slow AHP by 71.6 ± 20.6% (Fig. 1A, D & F) (-1.1 ± 0.2 vs 0.4 ± 0.2; t = -3.8, *P* = 0.009, paired two-tailed Student's *t*-test). We are the first to report that activation of β-adrenergic receptors also reduced the amplitude of the medium AHP, with the peak medium AHP amplitude being reduced by 37.6 ± 6.5% (Fig. 1A-B, C & E) (-3.1 ± 0.4 vs -1.9 ± 0.3 mV; t = -4.6, *P* = 0.004, paired two-tailed Student's *t*-test). Both responses were antagonised by the selective competitive β-adrenergic receptor antagonist, propranolol. Pre-treatment of slices with propranolol (10 μM) for 10 minutes prevented the effect of isoproterenol in suppressing either the medium (Fig. 1G) (control -5.2 ± 1.3 mV, propranolol -5.6 ± 1.7 mV, propranolol + isoproterenol -5.4 ± 1.8 mV; overall effect of drugs $F_{(2, 6)} = 0.2$, *P* = 0.81, repeated measures ANOVA) or slow (Fig. 1H) (control -3.6 ± 1.5 mV, propranolol -4.1 ± 1.8 mV, propranolol + isoproterenol -4.4 ± 2.0 mV; n = 4; overall effect of drugs $F_{(2,6)} = 1.4$, *P* = 0.33, repeated measures ANOVA) AHPs. These data indicate that activation of β-adrenergic receptors suppresses both the medium and slow AHPs.

β-adrenergic receptor-mediated modulation of the medium AHP is independent of a rise of intracellular cAMP

The time-course of the medium and slow AHPs overlap (Stocker et al., 1999; Gerlach et al., 2004), making it difficult to resolve whether each AHP component can be modulated independently. The slow AHP was suppressed by inclusion of cAMP (1 mM) in the whole-cell electrode solution (Pedarzani and Storm, 1993) (non-cAMP, *n* = 80, cAMP, n = 20; overall effect of cAMP $F_{(1, 98)} = 13.4$, *P* = 0.0004; two-way repeated measures ANOVA. Fig.

2A), and was observed together with a significant reduction in the amplitude of the medium AHP (Fig. 2B) (n = 80, cAMP, n = 20; overall effect of cAMP $F_{(1, 98)}$ = 12.6, *P* = 0.0006; two-way repeated measures ANOVA). It is most likely that these observations reflect an overlap in time-course. Therefore, it is crucial to determine whether the medium AHP is sensitive to isoproterenol in neurons dialyzed with cAMP. Addition of isoproterenol (1 μM) to neurons dialyzed with cAMP (1 mM) reduced the amplitude of the medium AHP by 58.2 ± 8.2% (Fig. 2C, D, E & F) (-1.9 ± 0.3 vs -0.9 ± 0.3 mV; *n* = 5; t = -8, *P* = 0.001, paired two-tailed Student's *t*-test). The magnitude of reduction of the mAHP by isoproterenol was the same between control and cells dialyzed with cAMP (t = 1.9, p = 0.073). These data demonstrate that suppression of the medium AHP by isoproterenol is not the result of an overlapping time-course of afterpotentials and suppression of the slow AHP. It is apparent that the effect of isoproterenol is mediated by β-adrenergic receptors and that suppression of the medium AHP is independent of a rise of intracellular cAMP.

Blocking HCN channels with ZD7288 reduced suppression of the medium AHP by isoproterenol

The medium AHP in hippocampal CA1 pyramidal neurons is proposed to be mediated by activation of SK and deactivation of HCN channels at hyperpolarized voltages (Gu et al., 2005; Kaczorowski, 2011; Church et al., 2015). This was confirmed by observing that addition of the HCN channel blocker ZD7288 (1 μM) blocked the medium AHP by 52.2 ± 16.5% (*P* = 0.047) (Fig. 3A-D), with the sequential addition of the SK channel inhibitor apamin (100 nM) blocking the remaining afterpotential by a further 43.3 ± 11.8% (*P* = 0.054) (Fig. 3A-D) (control -4.3 ± 1.5 mV, ZD7288 -2.0 ± 0.7 mV, ZD7288 + apamin -0.1 ± 0.3 mV; n = 4; overall effect of drugs $F_{(2, 6)}$ = 19.8, *P* = 0.002, repeated measures ANOVA). No effect of either ZD7288 or apamin on the slow AHP was observed (Fig. 3D) (control -1.8 ± 0.1 mV, ZD7288 -2.4 ± 0.2 mV, ZD7288 + apamin -2.2 ± 0.4 mV; n = 4; overall effect of drugs $F_{(2, 6)}$ = 1.9, *P* = 0.23, repeated measures ANOVA).

These data pose the question of which channel subtype(s) is modulated by β-adrenergic receptor activation. Blocking HCN channels with ZD7288 (1 μM) reduced the medium AHP by 74.5 ± 14.5% (*P* = 0.06) (Fig. 4Ai-iv) and reduced the effect of subsequent concomitant addition of isoproterenol (1 μM) (Fig. 4Ai-iv) (control -4.4 ± 0.5 mV, ZD7288 -1.0 ± 0.5 mV, ZD7288 + isoproterenol -0.8 ± 0.6 mV; n = 3; overall effect of drugs $F_{(2, 4)}$ = 15.1, P = 0.014; main effect of ZD7288 P = 0.06; main effect of ZD7288 + isoproterenol P = 0.21; repeated measures ANOVA). In contrast, application of apamin (100 nM) blocked the medium AHP by 32.7 ± 6.3% (*P* = 0.007) (Fig. 4Bi-iv), with the concomitant addition of isoproterenol (1 μM) still suppressing the remaining medium AHP by 37.2 ± 8.4% (Fig. 4Bi-iv) (P = 0.003) (control -4.9 ± 0.8 mV; apamin -3.4 ± 0.7 mV; apamin + isoproterenol -1.5 ± 0.6 mV; n = 4; overall effect of drugs $F_{(2, 6)}$ = 22.8, *P* = 0.002; repeated measures ANOVA). Activation of HCN channels by membrane hyperpolarization results in a depolarizing sag in the electrotonic potential. This sag is reduced by HCN channel blockers, such as ZD7288 (Day et al., 2005; Thuault et al., 2013). Application of a sub-maximal concentration of ZD7288 (1 μM) reduced the sag by 96%, reducing the sag from contributing 29.6 ± 2.1% of the electrotonic potential to contributing 2.1 ± 1.2% (t = 13.8; p < 0.0001, paired two-tailed Student's t-test). Application of isoproterenol (1 μM) resulted in a 27.6 ± 8.8% reduction in depolarizing sag (25.6 ± 1 vs 18.6 ± 2.4%; n = 7; t = 3.3 *P* = 0.02; paired two-tailed Student's *t*-test). These data strongly suggest that suppression of the medium AHP by isoproterenol is mediated by modulating HCN channel activity in hippocampal CA1 pyramidal neurons.

The selective β3-adrenergic receptor agonists BRL37344 and SR58611A suppress the medium AHP

Low levels of β3-adrenergic receptor mRNA is expressed in the hippocampus of mouse and rat (Evans et al., 1996; Claustre et al., 2008). β3-adrenergic receptors display a low binding affinity for propranolol (Hoffmann et al., 2004), and we observed that pre-treating hippocampal slices with 1 μM propranolol (n = 8) failed to antagonise the effect of isoproterenol on the medium AHP (data not shown). The β3-adrenergic receptor selective

agonist BRL37344 (10 μM; *n* = 4) (Hoffmann et al., 2004) suppressed the medium AHP by 42.2 ± 9.3% (Fig. 5Ai-ii) (-3 ± 0.4 vs -1.8 ± 0.5 mV; t = -4.2, *P* = 0.025, paired two-tailed Student's *t*-test). Application of a second β3-adrenergic receptor selective agonist SR58611A (10 μM) (Bianchetti and Manara, 1990; Simiand et al., 1992) also suppressed the medium AHP by 33.7 ± 6.5% (Fig. 5Bi-ii) (-3.1 ± 0.4 vs -2.1 ± 0.4 mV; n = 7; t = -3.6, *P* = 0.012). The magnitude of suppression of the medium AHP by BRL37344 and SR58611A were not significantly different (t = -1.1, *P* = 0.312; unpaired two-tailed Student's *t*-test). In contrast to BRL37344 which exhibited no significant reduction on the H-current-mediated depolarizing sag (25.8 ± 2.4 vs 18.1 ± 2.6%; *n* = 4; t = 1.9; *P* = 0.151; paired two-tailed Student's *t*-test), SR58611A reduced the H-current-mediated depolarizing sag by 17.5% (23.2 ± 3.0 vs 19.0 ± 2.9%; *n* = 10; t = 3.0; *P* = 0.014; paired two-tailed Student's *t*-test). BRL37344 (10 μM) failed to affect the medium AHP when applied in the presence of propranolol (10 μM) (Fig. 5C & D) (control -4.7 ± 0.9 mV, propranolol -5.0 ± 1.5 mV, propranolol + BRL37344 -4.9 ± 1.6 mV; $F_{(2,4)}$ = 0.1, *P* = 0.93, repeated measures ANOVA; n = 3). These findings indicate the presence of functional β3-adrenergic receptors in hippocampal CA1 pyramidal neurons that selectively suppress the medium AHP, and not the slow AHP. Finally, BRL37344 (10 μM) suppressed the medium AHP by 37.8 ± 8.2% in CA1 pyramidal neurons dialyzed with cAMP (1 mM) (Fig. 5E & F) (-2.9 ± 0.2 vs -1.8 ± 0.2 mV; t = -4.3, *P* = 0.003; *n* = 9, paired two-tailed Student's *t*-test), There was no significant difference in the magnitude of block produced by BRL37344 (10 μM) in cAMP-dialysed cells (1 μM) compared with non-cAMP dialysed neurons (0 mM cAMP 42.2 ± 9.3%; 1 mM cAMP 37.8 ± 8.2% t = 0.3, *P* = 0.76). These data confirm that activation of β3-adrenergic receptors suppresses the medium AHP by a cAMP-independent signalling pathway.

Discussion

The slow AHP is unusual in being a potassium current subject to modulation by GPCR activation (Pedarzani and Storm, 1993). This slow afterpotential is sensitive to activation of GPCRs that are coupled via G_s to activation of adenylate cyclase, a rise of intracellular cAMP and subsequent activation of protein kinase A (Pedarzani and Storm, 1993). We have confirmed the sensitivity of the slow AHP to activation of β-adrenergic receptors and a rise of intracellular cAMP. In contrast, evidence of the medium AHP being modulated by metabotropic receptor activation in hippocampal pyramidal cells is lacking. It was noted that the medium AHP was reduced when the slow AHP was suppressed by forskolin, but this was suggested to result from an overlap of the time-courses of the two components of the AHP (Gu et al., 2005). We have carefully separated the amplitudes of the two afterpotentials, by plotting of the AHP time-course on a log time base (Figure 1B). In addition, we have presented data of the reduction of the medium AHP by isoproterenol in cells where the slow AHP has been abolished by intracellular dialysis of cAMP (Figure 2C-F). These data confirm that the medium AHP is a substrate for modulation and that suppression of the afterpotential is not mediated by a rise of intracellular cAMP. The effect of isoproterenol was mimicked by two selective β3-adrenergic receptor agonists, BRL37344 and SR58611A. The mRNA encoding the β3-adrenergic receptor has been detected in various brain regions including the hippocampus in rat, mouse and humans (Rodriguez et al., 1995; Evans et al., 1996; Claustre et al., 2008). The precise role of the β3-adrenergic receptor in brain is unclear, but BRL37344 and SR58611A possess anti-depressant and anxiolytic-like effects in rodents (Simiand et al., 1992; Claustre et al., 2008; Stemmelin et al., 2008; Tanyeri et al., 2013). Activation of hippocampal β3-adrenergic receptors by norepinephrine and isoproterenol mediate an increase in hippocampal neural precursor activity by enhancing the proliferation of multipotent neuronal stem (Jhaveri et al., 2010).

BRL37344 displays a 90-fold and 20-fold higher selectivity towards rat β3-adrenergic receptors compared with β1- and β2-adrenergic receptors respectively (Hoffmann et al.,

2004), whereas SR58611A exhibits a 280-fold and 140-fold higher selectivity for rat β3-adrenergic receptors compared with β1- and β2-adrenergic receptors respectively (Gauthier et al., 1996; Stemmelin et al., 2008). The selectivity of these agonists is better than that reported for available β3-adrenergic receptor antagonists. For example, L-748337 displays less than a 20-fold selectivity and L-748328 exhibits only a 45-fold selectivity for β3-adrenergic receptors over β2-adrenergic receptors (Candelore et al. 1999). In addition, SR 59230A displays only a 10-fold selectivity for β3-adrenergic receptors over β1- and β2-adrenergic receptors (Manara et al., 1996). In absence of attempting full concentration-response relationships in the absence and presence of increasing concentrations of antagonist(s), we elected to use β3-adrenergoic receptor-selective agonists to elucidate the role of these receptors in hippocampal neurons. A very small reduction in the amplitude of the slow AHP was seen in the presence of BRL37344, but not when SR58611A was applied (Figure 5Aii & 5Bii), which might result from the low affinity binding of BRL37344 to β1- and β2-adrenergic receptors. These findings indicate that activation of β3-adrenergic receptors suppresses the medium and not the slow AHP in hippocampal CA1 pyramidal neurons.

SK and HCN channels underlie the medium AHP in CA1 pyramidal neurons at hyperpolarized potentials

There is some controversy regarding the channel subtypes that contribute to the medium AHP, with activation of SK (Stocker et al. 1999) and deactivation of HCN channels (Gu et al. 2005) being proposed to underlie the afterpotential at hyperpolarized potentials. The role of SK channel activation is unclear, as it has been suggested only to result from non-physiological activation protocols used in voltage clamp (Gu et al. 2005). The data presented in this study shows that the HCN channel blocker ZD7288 (1 μM) reduced the medium AHP by 55-75% in neurons held at -75 mV, confirming deactivation of HCN channel current is a significant component of the medium AHP (Gu et al., 2005; Kaczorowski, 2011). Apamin (100 nM) blocked the medium AHP remaining in ZD7288 (1 μM), confirming that SK channel activation mediates a significant component of the medium AHP (Stocker et al.,

1999; Bond et al., 2004; Kaczorowski et al., 2007; Church et al., 2014). Both SK and HCN channels can be either homomeric or heteromeric. For example, the current underlying the medium AHP is inhibited by apamin with an IC_{50} of 450 pM (Stocker et al., 1999), which is reminiscent of the sensitivity of heteromeric SK1-SK2 channel current to the toxin (Church et al. 2015). Homo- and hetero-tetrameric HCN channels display different electrophysiological and kinetic properties when expressed in heterologous cell lines (Santoro et al., 2000; Robinson and Siegelbaum, 2003; Wahl-Schott and Biel, 2009). The expression of HCN channel subunits varies throughout the brain, with only HCN1 and HCN2 mRNA and protein abundantly expressed in the hippocampus (Santoro et al., 2000). Native H-current is thought to be mediated by a population of heteromeric HCN1-2 and homomeric HCN1 subunit channels because the biophysical properties of H-current recorded in CA1 pyramidal reflect a population of heteromeric HCN1-2 channels and possible HCN1 homomeric channels (Santoro et al. 2000).

Non-selective actions of ZD7288 have been reported, with T-type calcium channel current blocked with an IC_{50} of 100 μM (Felix et al., 2003), ionotropic glutamate receptor currents reduced by 40% using 20 μM ZD7288 (Chen, 2004), and inhibition of sodium channel current in dorsal root ganglion neurons with an IC_{50} close to 10 μM (Wu et al., 2012). In addition, relatively long-term application of ZD7288 can produce non-selective effects, with application of ZD7288 (30 μM) producing a significant depression of glutamatergic-mediated synaptic activity at hippocampal mossy fibre synapses (Chevaleyre and Castillo, 2002; Gill et al., 2006). We circumvented the possibility of non-selective effects of ZD7288 by blocking AMPA receptor-mediated synaptic potentials with NBQX, using only 1 μM of the compound, and finally recording the effect of the HCN channel blocker within 10 minutes of application. Pre-block of HCN channels with ZD7288 reduced suppression of the remaining medium AHP by isoproterenol (Fig. 4Aiii & iv). These data suggest that β3-adrenergic receptor-mediated suppression of the medium AHP occurs by inhibition of HCN channel activity. HCN channel activation undergoes a depolarizing shift from an increase in the intracellular

391 cAMP concentration (Wainger et al., 2001; Santoro et al., 2004; Chen et al., 2007; Zong et
392 al., 2012). However, activation of β3-adrenergic receptors suppresses the medium AHP in
393 neurons dialyzed with cAMP. These data show that modulation of HCN channels is
394 independent of a rise of intracellular cAMP.
395
396 **Do β3ARs functionally couple with HCN channels?**
397 The medium AHP was retained in cells dialyzed with cAMP. Furthermore, the reduction of
398 the medium AHP by isoproterenol was independent of intracellular cAMP levels. In addition,
399 the medium AHP was reduced in amplitude by the β3 adrenergic receptor-selective agonist
400 BRL37344, both in control cells and cells dialyzed with cAMP. The isoproterenol- and
401 BRL37344-mediated suppression of the medium AHP in the presence of raised intracellular
402 cAMP indicates that the inhibitory effect on the medium AHP is not mediated by activation of
403 either $G\alpha_s$- or $G\alpha_i$ G-protein affecting cAMP levels. The β3-adrenergic receptor signalling
404 pathway is ill-defined, with the receptor being reported to couple to $G\alpha_s$ or $G\alpha_i$ (Collins,
405 2012). Other studies report β3-adrenergic receptors signal in a biphasic manner by coupling
406 to both $G\alpha_s$ and $G\alpha_i$ (Begin-heick, 1995; Gauthier et al., 1996; Hadi et al., 2013).
407 Regardless of possible second messenger pathways, activation of β3-adrenergic receptors
408 causes relaxation of rat bladder, an effect blocked by ZD7288. This observation indicates
409 that coupling can occur between activation of β3-adrenergic receptors and HCN channels
410 (Kashyap et al., 2015). The reported anti-depressant and anti-anxiolytic properties of β3-
411 adrenergic receptor agonists make this pathway an attractive one to target, with further
412 investigation being needed.
413
414 HCN channels, which underlie H-current, are a key regulator of intrinsic excitability in CA1
415 pyramidal neurons. H-current has a direct influence on neuronal input resistance, the resting
416 membrane potential and membrane time constant. H-current filters out low frequency
417 fluctuations in membrane voltage in a self-regulating feedback mechanism to regulate
418 synaptic input (He et al., 2014). Inhibition of H-current by ZD7288 increased firing frequency

in hippocampal neurons (Gasparini & DiFrancesco, 1997), an effect demonstrating the contribution of the H-current to the medium AHP. It is interesting to note that this current can be modulated to affect firing by activation of a receptor that is expressed at low levels in hippocampal neurons. It will be useful to resolve the relative subcellular locations of HCN and β3-adrenergic receptors in hippocampal neurons to determine if coupling is localized within the cell.

Afterhyperpolarizations have a profound impact on hippocampal neuronal excitability. Action potential firing frequency increases with inhibition of either the medium (Stocker et al., 1999) or the slow AHP (Madison and Nicoll, 1982, 1986; Pedarzani and Storm, 1993; Pedarzani et al., 1998; Stocker et al., 1999). The amplitude of the AHP is correlated with learning and memory with larger AHPs being observed in aged animals that have difficulty in learning. Pharmacological suppression of the AHP in aged animals improves learning of a simple associative task (Moyer and Disterhoft, 1994; Tombaugh et al., 2005). The identification of a novel mechanism regulating the medium AHP provides additional plasticity to how membrane excitability might change to affect these processes. The identity of functional β3-adrenergic receptors in hippocampal CA1 neurons highlights a potential new target in modulating HCN channels and thereby synaptic plasticity.

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668 **Figure 1. Isoproterenol suppresses the medium and slow AHPs in hippocampal CA1**
669 **pyramidal neurons.**

670 **A.** Representative membrane voltage traces of the AHP evoked by 15 action potentials
671 elicited by 2 ms current injections delivered at 50 Hz before (control, black) and after the
672 addition of isoproterenol (1 μM; grey). The action potentials are truncated for clarity.

673 **B.** Evoked medium (mAHP) and slow afterhyperpolarizations (sAHP) plotted on a $\log_{10}$ time
674 scale to illustrate how the mAHP and sAHP were discriminated. The mAHP amplitude was
675 measured from the peak voltage deflection recorded at 0-100 ms post-burst, and the sAHP
676 amplitude was measured as the amplitude at 1 s post-burst.

677 **C.** Diary plot of the amplitude of the medium AHP. Application of isoproterenol (1μM)
678 reduced the amplitude of the evoked medium AHP (n=7).

679 **D.** Diary plot of the amplitude of the slow AHP. Application of isoproterenol (1μM) largely
680 abolished the amplitude of the evoked slow AHP (n=7).

681 Bar charts depicting the reduction in the amplitudes of the evoked medium (mAHP) (**E**) and
682 slow (sAHP) AHPs (**F**) amplitude by isoproterenol (1 μM). (n=7, paired two-way Student's *t*-
683 test, ** $P < 0.01$).

684 Bar charts depicting the lack of effect on the amplitudes of the evoked medium (mAHP) (**G**)
685 and slow (sAHP) AHPs (**H**) amplitude by isoproterenol (1 μM), when applied in the presence
686 of propranolol (10 μM) (n=4, repeated measures ANOVA, NS $P > 0.05$).

687

688

Figure 2. Suppression of the medium AHP by isoproterenol in cells dialysed with cAMP.

A. Bar chart showing the amplitude of the slow AHP (sAHP) in control cells (black bars, n=80) and in cells dialysed with cAMP (1 mM) (hollow bars, n=20). The slow AHP was measured after being evoked by 5 to 25 action potentials, with the amplitude of the slow afterpotential increasing with increasing number of action potentials. The slow AHP was occluded in cells dialysed with cAMP (1 mM).

B. Bar chart showing that dialysis with cAMP (1 mM) (n=20) reduces the amplitude but does not occlude the medium AHP. The medium AHP was measured after being evoked by 5 to 25 action potentials, with the amplitude of the medium AHP increasing with increasing number of action potentials in control (black bars, n=80) and in the presence of intracellular cAMP (hollow bars, n=20).

C. Effect of isoproterenol (1 μM; grey) on the medium AHP evoked by 15 APs fired at 50 Hz. Note the lack of a slow AHP in both control (black) and in the presence of isoproterenol. Action potentials have been truncated for clarity.

D. Evoked medium (mAHP) displayed on a $\log_{10}$ time scale, showing the lack of a slow AHP. Application of isoproterenol (1 μM) supressed the medium AHP (grey trace).

E. Diary plot of the amplitude of the medium AHP. Application of isoproterenol (1μM) suppressed the medium AHP amplitude in cells dialysed with cAMP (1 mM) (n=5).

F. Bar chart showing the amplitude of the medium AHP in control (black bar) and in the presence of isoproterenol (1 μM) (hollow bar). Isoprotenerol (1 μM) suppressed the medium AHP in cells dialyzed with cAMP (n=5, paired two-way Student's *t*-test, *** $P < 0.001$).

Figure 3. SK and HCN channels mediate the medium AHP in hippocampal CA1 pyramidal neurons held at -75 mV.

A. Representative membrane voltage traces of AHPs evoked by a train of 15 action potentials fired at 50 Hz in control conditions (black), ZD7288 (1 μM; dark grey), and apamin (100 nm) + ZD7288 (1 μM, light grey). Action potentials have been truncated for clarity.

B. Evoked medium AHP and slow AHP from A displayed on a $\log_{10}$ time scale to illustrate the complete block on the medium AHP following the sequential inhibition of HCN and SK channels by ZD7288 and apamin, respectively.

C. Diary plot of the amplitude of the medium AHP before and after the sequential addition of ZD7288 (1 μM) and ZD7288 + apamin (100 nM). Note that the medium AHP is completely inhibited by the combination of channel blockers.

D. Bar chart illustrating the reduction of the medium AHP by ZD7288 (1 μM), and ZD7288 + apamin (100 nM), with no effect of either blocker on the amplitude of the slow AHP.

Figure 4. Block of HCN channels reduces suppression of the medium AHP by isoproterenol.

Ai, Representative membrane voltage trace of the AHP evoked by a train of 15 action potentials fired at 50 Hz in the absence (black trace) and presence of the H-current blocker ZD7288 (1 μM, dark grey), and ZD7288 + isoprotenerol (1 μM, light grey). The medium AHP was largely blocked by ZD7288, with the addition of isoproterenol having little effect.

Aii. Evoked medium (mAHP) and slow (sAHP) AHPs displayed on a $\log_{10}$ time scale, showing the large block of the medium AHP by ZD7288 (dark grey) occluding subsequent suppression by isoproterenol (light grey).

Aiii. Diary plot of the amplitude of the medium AHP, showing the dominating effect of block of H-current by ZD7288 (1 μM) and minor effect of subsequent addition of isoproterenol (1 μM).

Aiv. Bar chart showing the amplitude of the medium AHP, significant block of that amplitude by ZD7288 and minor additional effect of the subsequent addition of isoprotenerol.

Bi. Representative membrane voltage trace of the AHP evoked by a train of 15 action potentials fired at 50 Hz in the absence (black trace) and presence of the SK channel inhibitor apamin (100 nM, dark grey), and apamin + isoprotenerol (1 μM, light grey). Only a small effect of apamin on the medium AHP was observed, with subsequent addition of isoproterenol significantly suppressing both the medium and slow AHPs.

Bii. Evoked medium (mAHP) and slow (sAHP) AHPs displayed on a $\log_{10}$ time scale, showing the minor block of the medium AHP by apamin (dark grey), with a much greater suppression of both the medium and slow AHPs by subsequent addition of isoproterenol (light grey).

Biii. Diary plot of the amplitude of the medium AHP, showing the minor block by apamin (100 nM) and the significant suppression produced by the subsequent addition of isoproterenol (1 μM).

Biv. Bar chart showing the amplitude of the medium AHP. Addition of apamin (100 nM) blocked only a small component of the medium AHP, with a much greater suppression observed on the subsequent addition of isoprotenerol.

Figure 5. β3 adrenergic receptor activation suppresses the medium AHP.

Ai. Representative membrane voltage trace of AHPs evoked by a train of 15 action potentials fired at 50 Hz in the absence (black) and presence of the β3AR selective agonist BLR37344 *(*10 μM*;* grey).

Aii. Evoked medium AHP and slow AHP from **Ai** displayed on a $\log_{10}$ time scale to illustrate a reduction in the medium and slow AHPs by addition of BRL37344 (10 μM).

Aiii. Bar chart showing suppression of both AHP components by BRL37344 (10 μM) (n=4; paired two-tailed Student's t-test).

Bi. Representative membrane voltage trace of AHPs evoked by a train of 15 action potentials fired at 50 Hz in the absence (black) and presence of the β3AR selective agonist

SR58611A (10 μM; grey) in CA1 pyramidal neurons. Action potentials were truncated for clarity.

Bii. Evoked medium AHP and slow AHP from **Bi** displayed on a $\log_{10}$ time scale to illustrate a reduction in the medium AHP by addition of SR58611A (10 μM). Note the lack of effect of the β3-adrenergic receptor agonist on the slow AHP (see Discussion).

Biii. Bar chart showing suppression of both AHP components by SR58611ABRL37344 (10 μM) (n=7; paired two-tailed Student's t-test).

C. Diary plot of the amplitude of the medium AHP and the lack of effect of addition of BRL37344 (10 μM) in the continued presence of propranolol (10 μM).

D. Bar chart showing the amplitude of the medium AHP in control conditions, after 10 minute incubation with propranolol (10 μM), and after addition of BRL37344 (10 μM) in the continued presence of propranolol.

E. Evoked mAHP and sAHP plotted on a $\log_{10}$ time scale from a cell dialyzed with cAMP (1 mM)(black trace). Addition of BRL37344 (10 μM) suppressed the mAHP in cAMP-dialyzed cells (grey trace).

F. Summary bar chart showing small but robust block of the mAHP by BRL37344 (10 μM) (*n* = 9, paired two-tailed Student's t-test).

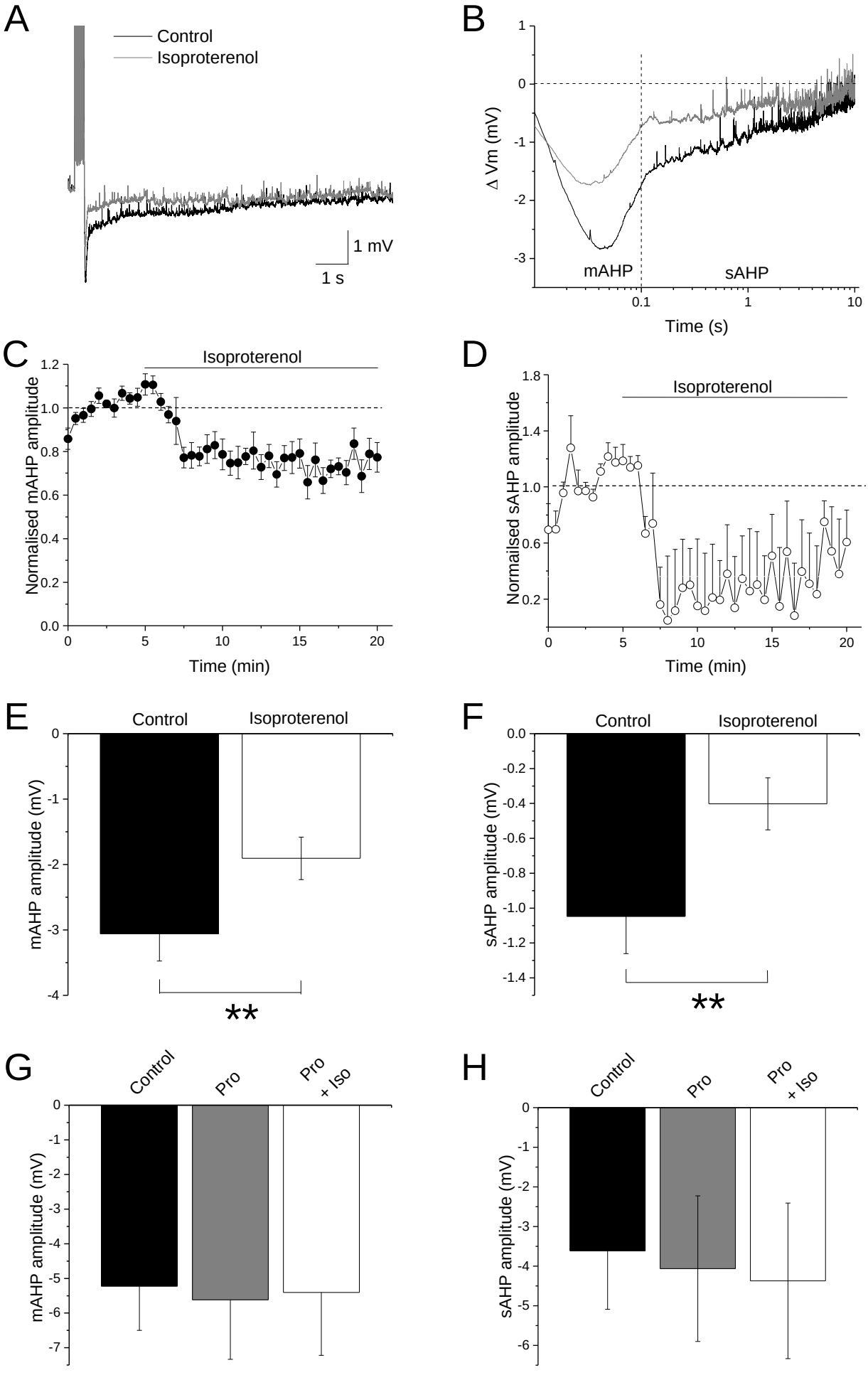

Figure 1
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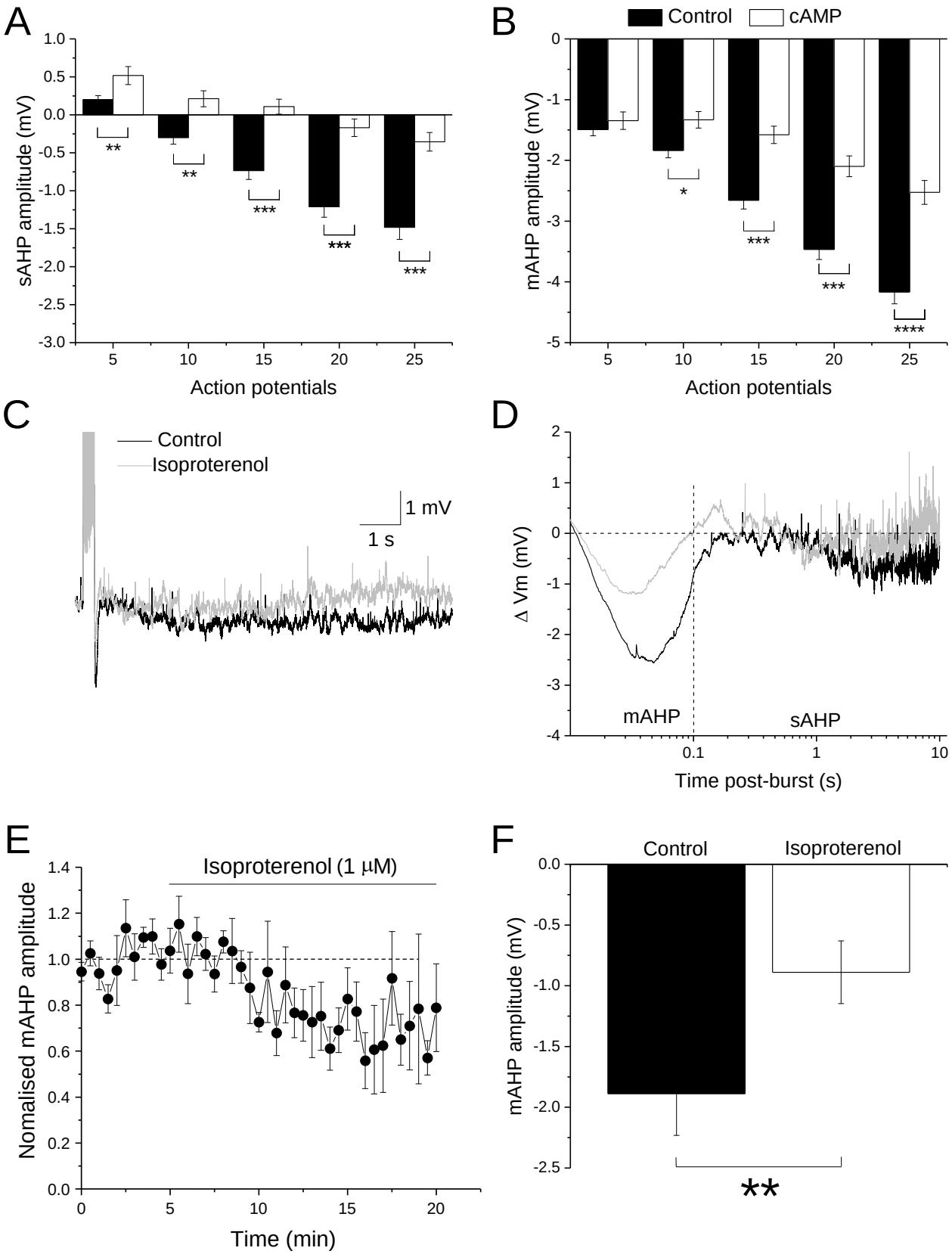

Figure 2
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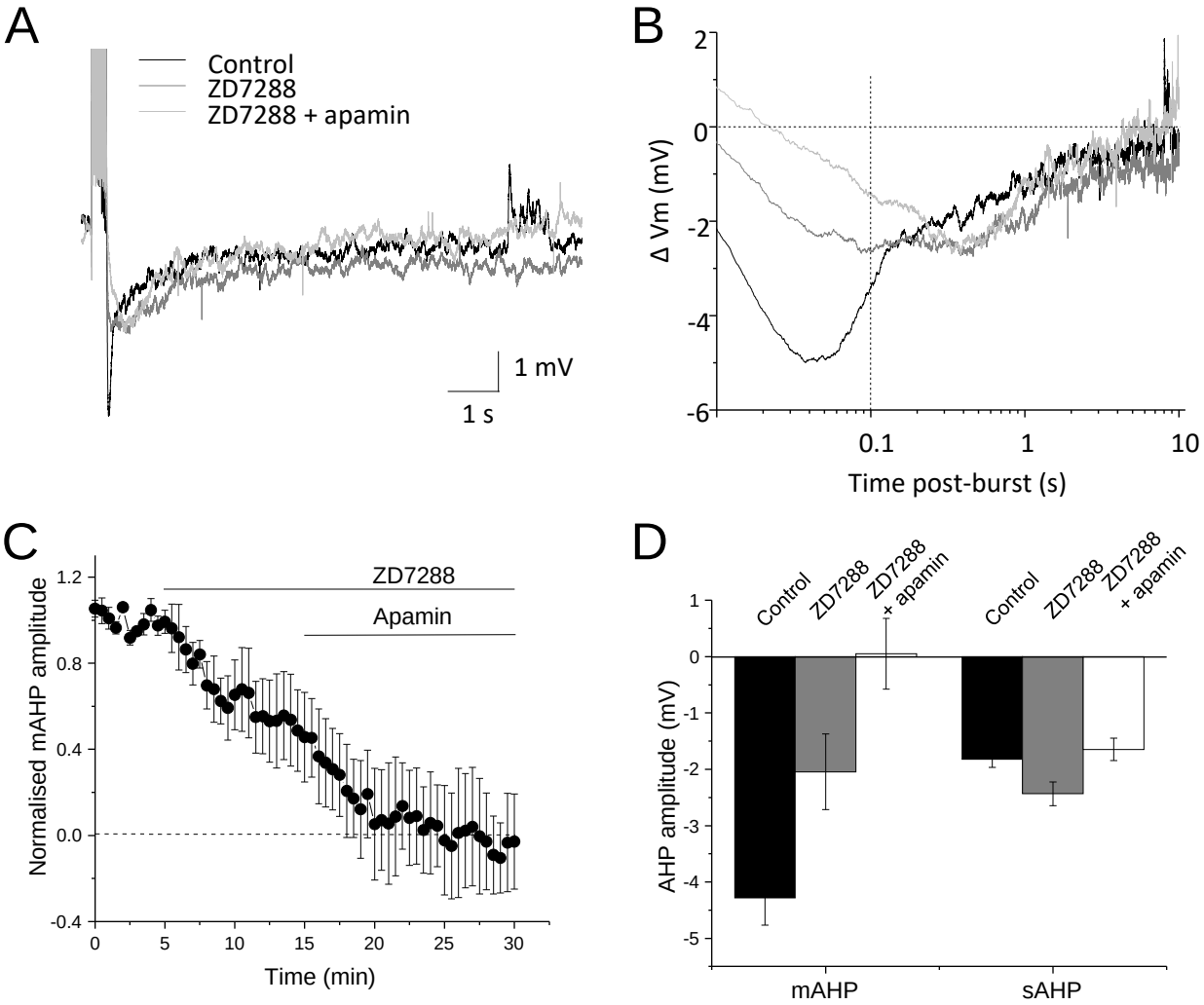

Figure 3
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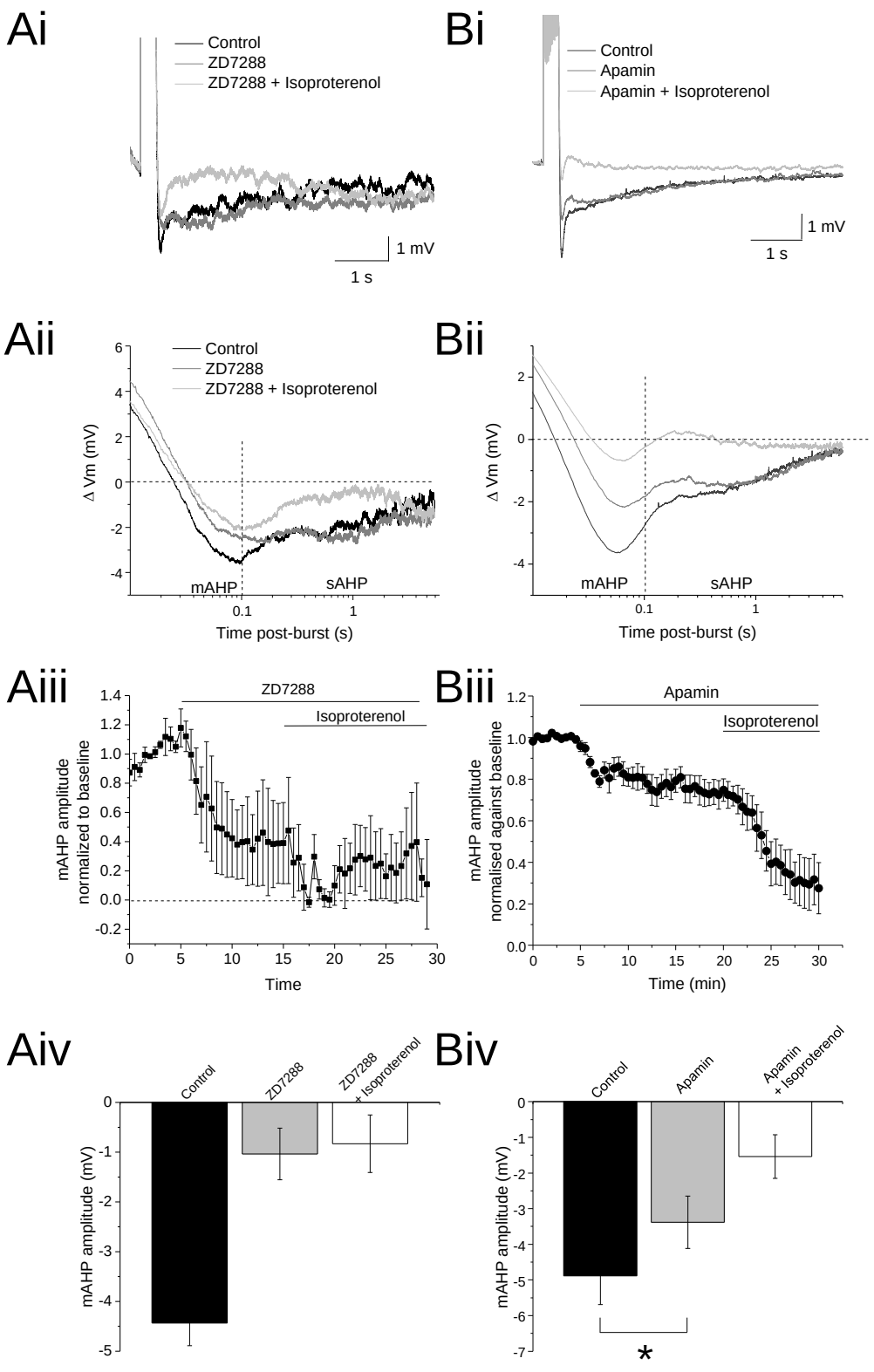

Figure 4
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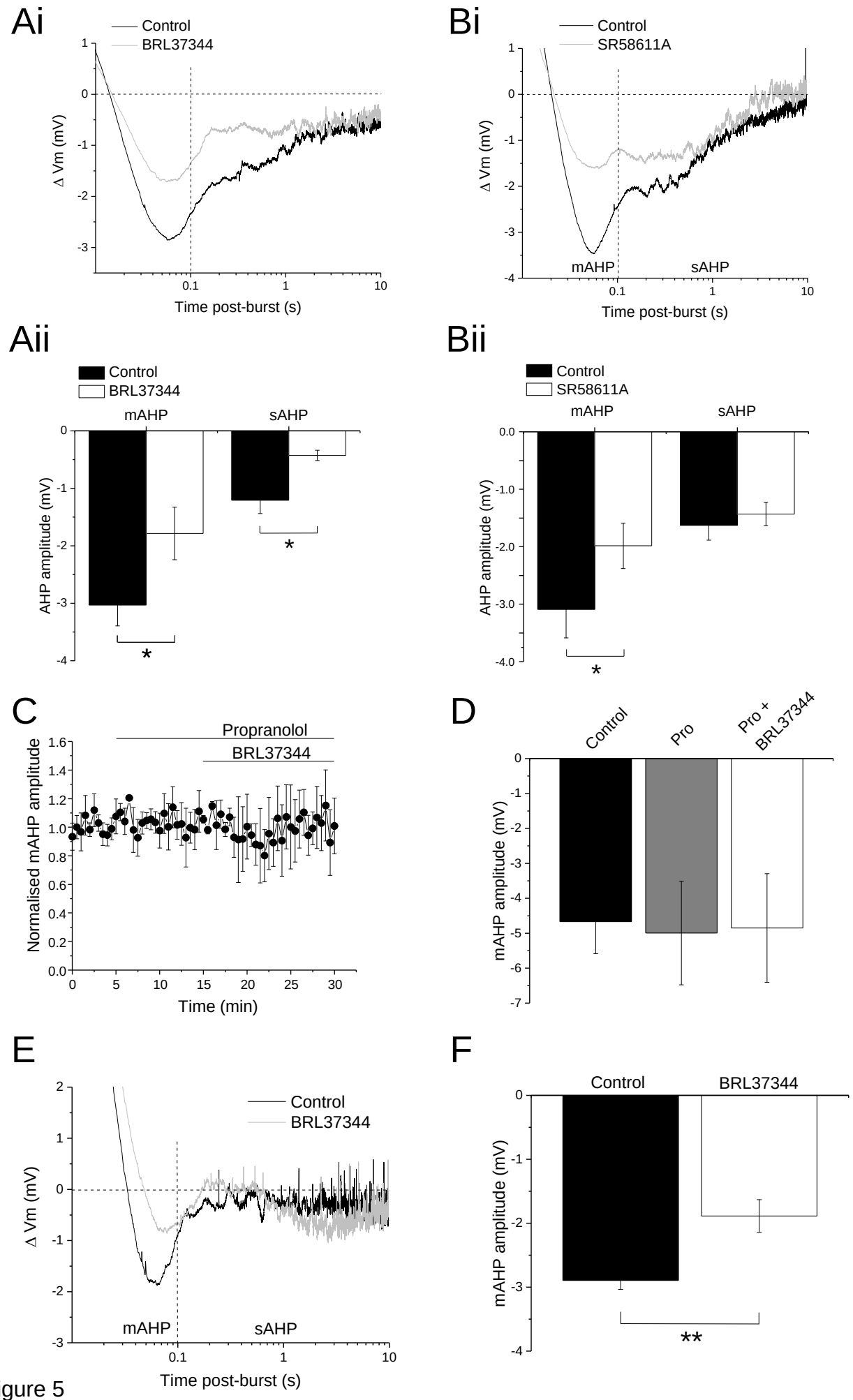

Figure 5
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