

RESEARCH ARTICLE | *Cellular and Molecular Properties of Neurons*

## $\beta_3$ -Adrenergic receptor-dependent modulation of the medium afterhyperpolarization in rat hippocampal CA1 pyramidal neurons

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Submitted 21 May 2018; accepted in final form 7 January 2019

**Church TW, Brown JT, Marrion NV.**  $\beta_3$ -Adrenergic receptor-dependent modulation of the medium afterhyperpolarization in rat hippocampal CA1 pyramidal neurons. *J Neurophysiol* 121: 773–784, 2019. First published January 9, 2019; doi:10.1152/jn.00334.2018.— Action potential firing in hippocampal pyramidal neurons is regulated by generation of an afterhyperpolarization (AHP). Three phases of AHP are recognized, with the fast AHP regulating action potential firing at the onset of a burst and the medium and slow AHPs suppressing action potential firing over hundreds of milliseconds and seconds, respectively. Activation of  $\beta$ -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  $\beta$ -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-dialyzed cells. Suppression of both the medium and slow AHPs was antagonized by the  $\beta$ -adrenergic receptor antagonist propranolol. The effect of isoproterenol to suppress the medium AHP was mimicked by two  $\beta_3$ -adrenergic receptor agonists, BRL37344 and SR58611A. The medium AHP was mediated by activation of small-conductance calcium-activated  $K^+$  channels and deactivation of H channels at the resting membrane potential. Suppression of the medium AHP by isoproterenol was reduced by pretreating cells with the H-channel blocker ZD7288. These data suggest that activation of  $\beta_3$ -adrenergic receptors inhibits H channels, which suppresses the medium AHP in CA1 hippocampal neurons by utilizing a pathway that is independent of a rise in 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  $\beta_3$ -adrenergic receptors suppresses the medium afterhyperpolarization 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.

afterhyperpolarization; hippocampus; modulation

### INTRODUCTION

Action potential firing is regulated by the generation of an afterhyperpolarization (AHP) after a single action potential or a train of action potentials (Alger and Nicoll 1980; Alger and Williamson 1988; Gu et al. 2005; Madison and Nicoll 1984; Storm 1990). The AHP influences both the frequency and patterning of neuronal firing. An increase in action potential firing is observed after pharmacological inhibition of AHPs (Gu et al. 2005; Lancaster and Adams 1986; Pedarzani and Storm 1993; Stocker et al. 1999; Tombaugh et al. 2005), whereas a decrease in action potential firing results from potentiation of the AHP (Gu et al. 2008; Pedarzani et al. 2005). 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 after a burst of action potentials (Chen et al. 2014; Gu et al. 2005; Stocker 2004; Storm 1989; Vatanparast and Janahmadi 2009). The medium AHP in hippocampal CA1 neurons is mediated by three different ion channel subtypes, small-conductance calcium-activated  $K^+$  (SK) channels (Bond et al. 2004; Church et al. 2015; Stocker et al. 1999), 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  $Na^+$ - $K^+$ -ATPase pump (Gulledge et al. 2013) all proposed to mediate this slow afterpotential.

The slow AHP is unusual in being suppressed by different monoamines, such as norepinephrine (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

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norepinephrine is antagonized by the  $\beta$ -adrenergic receptor antagonist propranolol (Madison and Nicoll 1982). Immunohistochemistry has identified that  $\beta_1$ - and  $\beta_2$ -adrenergic receptors are expressed throughout the hippocampus, with both subtypes being located in both the soma and dendrites of CA1 pyramidal neurons (Cox et al. 2008; Guo and Li 2007).  $\beta_3$ -Adrenergic receptor mRNA has also been identified in the rodent and human hippocampus (Evans et al. 1996; Rodriguez et al. 1995), albeit a lower expression than seen for  $\beta_1$  and  $\beta_2$  receptors (Claustre et al. 2008; Evans et al. 1996). The functional role of the  $\beta_3$ -adrenergic receptor in the hippocampus is unclear; however,  $\beta_3$ -adrenergic receptor agonists demonstrate antidepressant and anxiolytic effects in rodents (Claustre et al. 2008; Consoli et al. 2007; Simiand et al. 1992; Tamburella et al. 2010; Tanyeri et al. 2013).

In this study we show that nonselective pharmacological activation of  $\beta$ -adrenergic receptors suppressed both the medium and slow AHPs, an effect antagonized by propranolol. The effect of  $\beta$ -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  $\beta_3$ -adrenergic receptor agonists. These data suggest that the activation of  $\beta_3$ -adrenergic receptors suppresses the medium AHP by inhibiting HCN channel activity using a cAMP-independent pathway. These findings illustrate an additional mechanism to modulate hippocampal neuron excitability utilizing a novel pathway.

## 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 United Kingdom (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 European Union Directive 2010/63/EU. All experimental procedures were reviewed and approved by the University of Bristol Ethical Review Group (reference: UB/12/006). Brains were removed, and horizontal brain slices (300  $\mu$ m) were cut in ice-cold ( $-4^\circ\text{C}$ ) sucrose-based cutting solution containing (in mM) 189 sucrose, 10 D-glucose, 26 NaHCO<sub>3</sub>, 3 KCl, 5 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 CaCl<sub>2</sub>, and 1.25 NaH<sub>2</sub>PO<sub>4</sub>, saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>, with a VT1000 S vibrating blade microtome (Leica Microsystems, 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<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 1 MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 CaCl<sub>2</sub>, and 10 D-glucose, saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Under aseptic conditions, slices were washed twice with culture medium containing minimum essential medium (Gibco) supplemented with (in mM) 50 NaHCO<sub>3</sub>, 75 HEPES, 0.437 glutamine, 0.625 CaCl<sub>2</sub>, 1.25 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.425 ascorbic acid, and 32 D-glucose, with 12.5% heat-inactivated horse serum, 1 mg/l insulin, and 100 U/ml penicillin with 100  $\mu$ g/ml streptomycin (pH 7.28 with NaOH, osmolarity  $\sim$ 320 mosM). Slices were washed a further two times in culture medium without supplemented penicillin-streptomycin. Slices were cultured on a porous (0.4  $\mu$ m) membrane (Millicell inserts; Millipore) and maintained at 37°C in 5% CO<sub>2</sub> 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 (Kaczorowski 2011; Kaczorowski et al. 2007; Lancaster et al. 2001; Maccaferri et al. 1993; Stocker et al. 1999).

**Electrophysiology.** Slices were continuously perfused (2–3 ml/min) with aCSF supplemented with 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX; 10  $\mu$ M) to inhibit spontaneous  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory postsynaptic currents. The aCSF was continuously oxygenated, and temperature was maintained at  $\sim$ 33°C with an HPT-2 inline heater (Scientifica). Whole cell current-clamp recordings were made from visually identified pyramidal neurons from the CA1 region of the hippocampus with an infrared light-emitting diode mounted on an Axioskop2 microscope (Carl Zeiss). Fire-polished electrodes (3–5 M $\Omega$ ) were fabricated from borosilicate glass (1.5-mm OD, 0.86-mm ID) containing (in mM) 125 KMeSO<sub>4</sub>, 10 KCl, 10 NaCl, 20 HEPES, 2 MgATP, 0.3 Na<sub>2</sub>GTP, and 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). Voltage responses were filtered at 1.2 kHz (8-pole low-pass Bessel filter) and sampled at 5 kHz with 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 action potentials was discarded. Analyses of the medium and slow AHPs were carried out with custom-written MATLAB scripts (MathWorks). The medium AHPs were measured as the peak negative membrane deflection between 0 and 100 ms after the cessation of the last action potential fired. The slow AHPs were measured 1 s after the last action potential was fired. The overlapping kinetic profiles of the medium and slow AHPs was minimized by measuring the AHP components within these time points. Finally, the hyperpolarization-induced H-current-mediated depolarizing sag was measured as the percent 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).

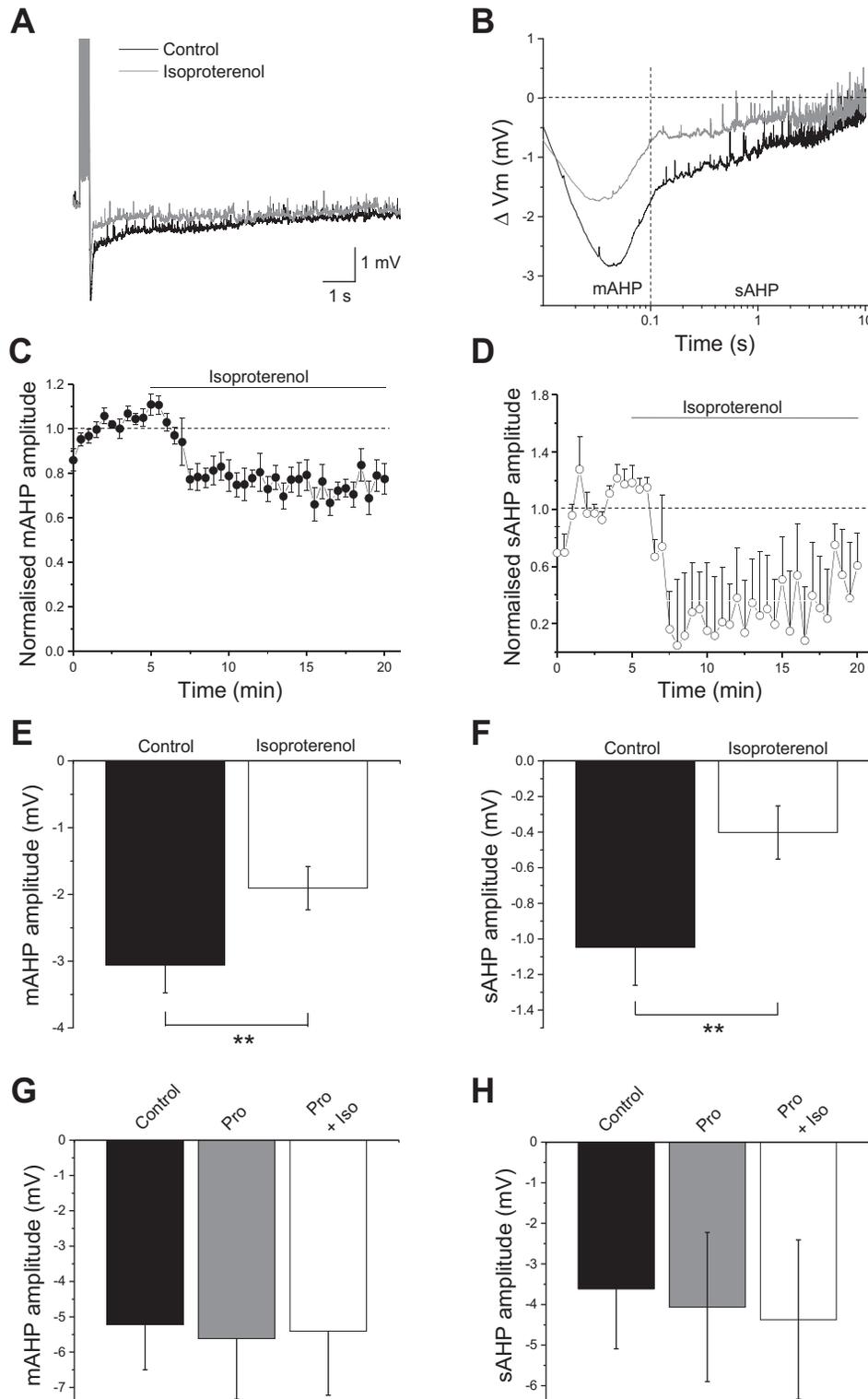
Cell input resistance was determined from a  $-100$ -pA current pulse (500 ms) delivered 1 s before a train of action potentials to generate AHPs. The nonconductive anion methylsulfate (MeSO<sub>4</sub>) is associated with a time-dependent rise in input resistance (Kaczorowski et al. 2007; Velumian et al. 1996; Zhang et al. 1994). We observed that the cell input resistance rose by  $13.4 \pm 4.3\%$  during a 5-min baseline recording period ( $105.8 \pm 5.2$  vs.  $121.0 \pm 12.9$  M $\Omega$ ;  $n = 6$ ;  $t = -3.0$ ,  $P = 0.03$ , paired 2-tailed Student's *t*-test). During this same 5-min baseline recording, the medium AHP amplitude did not change ( $-4.4 \pm 0.3$  vs.  $-4.5 \pm 0.2$  mV;  $t = 0.2$ ,  $P = 0.84$ , paired 2-tailed Student's *t*-test), whereas the slow AHP increased by  $25.3 \pm 21.6\%$  [ $-2.2 \pm 0.2$  vs.  $-2.6 \pm 0.2$  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 (Feltham, 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 dimethyl sulfoxide and diluted in aCSF when required. BRL37344, SR58611A, apamin, isoproterenol, and propranolol were prepared as stock solutions in water. All drugs were prepared as 1,000 $\times$  stock solutions and stored at  $-20^\circ\text{C}$  until required, except for isoproterenol and propranolol, which were prepared daily. All drugs were applied to the perfusion system.

**Statistics.** Statistical analysis was performed with SPSS (v21; IBM), and representative traces were drawn with Origin 9 (Microcal Software). All data are presented as means  $\pm$  SE. 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 amplitudes after the addition of two or more drugs. Fisher's least significant difference post hoc tests were performed to assess between-drug treatment differences.

## RESULTS

*$\beta$ -Adrenergic receptor agonist isoproterenol inhibits both 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  $\beta$ -adrenergic receptor agonist iso-



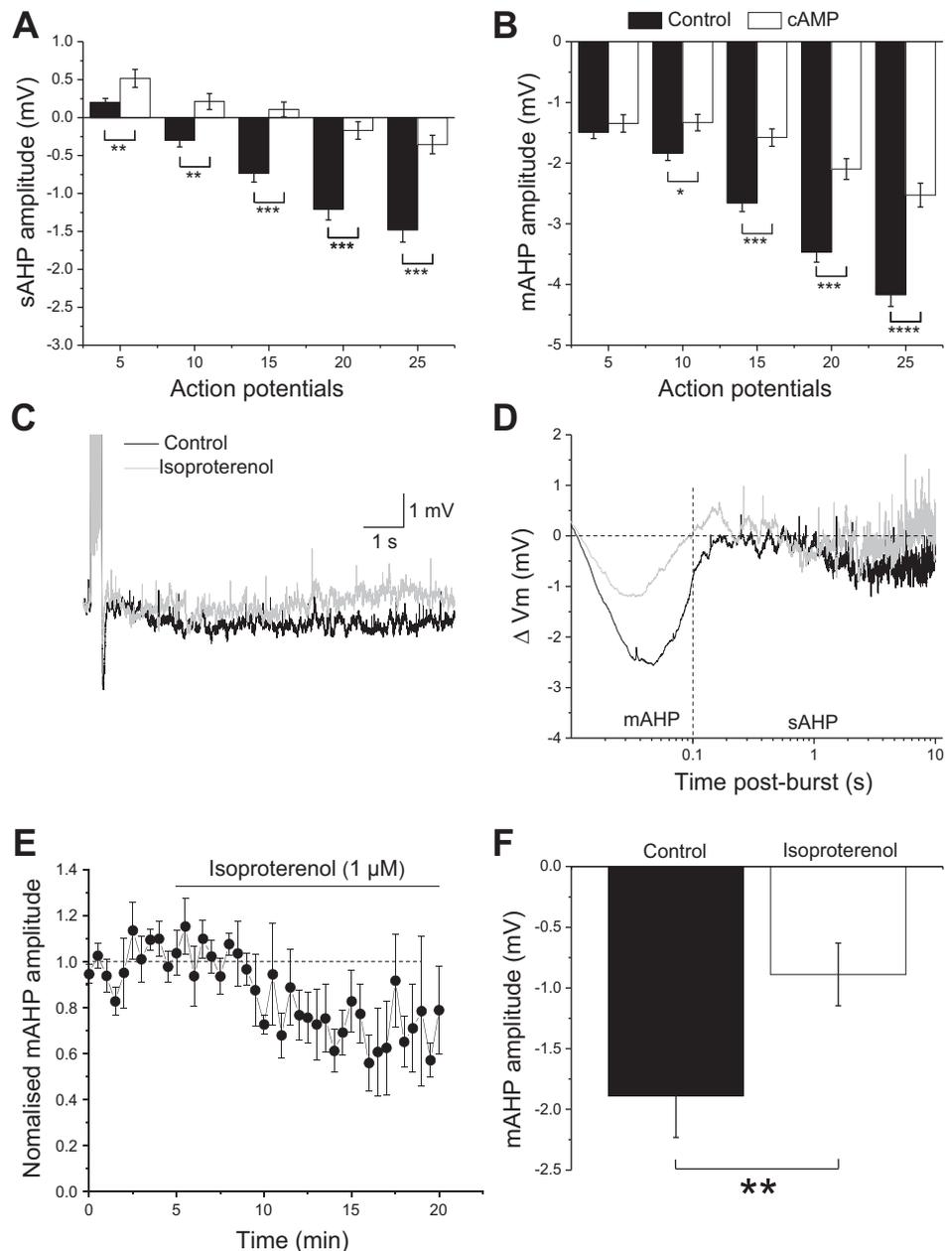
**Fig. 1.** Isoproterenol suppresses the medium (mAHP) and slow (sAHP) afterhyperpolarizations in hippocampal CA1 pyramidal neurons. **A:** representative membrane voltage traces of the AHP evoked by 15 action potentials elicited by 2-ms current injections delivered at 50 Hz before (control) and after the addition of isoproterenol (1  $\mu$ M). Action potentials are truncated for clarity. **B:** evoked mAHP and sAHP plotted on a log<sub>10</sub> timescale to illustrate how the mAHP and sAHP were discriminated. mAHP amplitude was measured from the peak voltage deflection recorded at 0–100 ms after burst, and sAHP amplitude was measured as the amplitude at 1 s after burst.  $V_m$ , membrane voltage. **C:** diary plot of the amplitude of the mAHP. Application of isoproterenol (1  $\mu$ M) reduced the amplitude of the evoked mAHP ( $n = 7$ ). **D:** diary plot of the amplitude of the sAHP. Application of isoproterenol (1  $\mu$ M) largely abolished the amplitude of the evoked sAHP ( $n = 7$ ). **E** and **F:** bar charts depicting the reduction in the amplitudes of the evoked mAHP (**E**) and sAHP (**F**) amplitude by isoproterenol (1  $\mu$ M).  $n = 7$ ; paired 2-way Student's *t*-test, \*\* $P < 0.01$ . **G** and **H:** bar charts depicting the lack of effect on the amplitudes of the evoked mAHP (**G**) and sAHP (**H**) by isoproterenol (Iso; 1  $\mu$ M) when applied in the presence of propranolol (Pro; 10  $\mu$ M).  $n = 4$ ; repeated-measures ANOVA, not significant ( $P > 0.05$ ).

proterolol ( $1 \mu\text{M}$ ;  $n = 7$ ) reduced the amplitude of the slow AHP by  $71.6 \pm 20.6\%$  (Fig. 1, A, D, and F) ( $-1.1 \pm 0.2$  vs.  $-0.4 \pm 0.2$  mV;  $t = -3.8$ ,  $P = 0.009$ , paired 2-tailed Student's  $t$ -test). We are the first to report that activation of  $\beta$ -adrenergic receptors also reduced the amplitude of the medium AHP, with the peak medium AHP amplitude being reduced by  $37.6 \pm 6.5\%$  (Fig. 1, A–C and E) ( $-3.1 \pm 0.4$  vs.  $-1.9 \pm 0.3$  mV;  $t = -4.6$ ,  $P = 0.004$ , paired 2-tailed Student's  $t$ -test). Both responses were antagonized by the selective competitive  $\beta$ -adrenergic receptor antagonist propranolol. Pre-treatment of slices with propranolol ( $10 \mu\text{M}$ ) for 10 min prevented the effect of isoproterenol in suppressing either the medium [Fig. 1G; control  $-5.2 \pm 1.3$  mV, propranolol  $-5.6 \pm 1.7$  mV, propranolol + isoproterenol  $-5.4 \pm 1.8$  mV; overall effect of drugs  $F_{(2,6)} = 0.2$ ,  $P = 0.81$ , repeated-measures ANOVA] or slow [Fig. 1H; control  $-3.6 \pm 1.5$  mV, propranolol  $-4.1 \pm 1.8$  mV, propranolol + isoproterenol

$-4.4 \pm 2.0$  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  $\beta$ -adrenergic receptors suppresses both the medium and slow AHPs.

*$\beta$ -Adrenergic receptor-mediated modulation of medium AHP is independent of a rise in intracellular cAMP.* The time courses of the medium and slow AHPs overlap (Gerlach et al. 2004; Stocker et al. 1999), making it difficult to resolve whether each AHP component can be modulated independently. The slow AHP was suppressed by inclusion of cAMP ( $1 \text{ mM}$ ) in the whole cell electrode solution (Pedarzani and Storm 1993) [non-cAMP,  $n = 80$ , cAMP,  $n = 20$ ; overall effect of cAMP  $F_{(1,98)} = 13.4$ ,  $P = 0.0004$ , 2-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  $F_{(1,98)} = 12.6$ ,  $P = 0.0006$ , 2-way repeated-measures ANOVA]. It

Fig. 2. Suppression of the medium afterhyperpolarization (mAHP) by isoproterenol in cells dialyzed with cAMP. A: bar chart showing the amplitude of the slow AHP (sAHP) in control cells ( $n = 80$ ) and in cells dialyzed with cAMP ( $1 \text{ mM}$ ;  $n = 20$ ). The sAHP was measured after being evoked by 5–25 action potentials, with the amplitude of the slow afterpotential increasing with increasing number of action potentials. The sAHP was occluded in cells dialyzed with cAMP ( $1 \text{ mM}$ ). B: bar chart showing that dialysis with cAMP ( $1 \text{ mM}$ ;  $n = 20$ ) reduces the amplitude but does not occlude the mAHP. The mAHP was measured after being evoked by 5–25 action potentials, with the amplitude of the mAHP increasing with increasing number of action potentials in control ( $n = 80$ ) and in the presence of intracellular cAMP ( $n = 20$ ). C: effect of isoproterenol ( $1 \mu\text{M}$ ) on the mAHP evoked by 15 action potentials fired at 50 Hz. Note the lack of a sAHP both in control and in the presence of isoproterenol. Action potentials have been truncated for clarity. D: evoked mAHP displayed on a  $\log_{10}$  timescale, showing the lack of a sAHP. Application of isoproterenol ( $1 \mu\text{M}$ ) suppressed the mAHP (gray trace).  $V_m$ , membrane voltage. E: diary plot of the amplitude of the mAHP. Application of isoproterenol ( $1 \mu\text{M}$ ) suppressed mAHP amplitude in cells dialyzed with cAMP ( $1 \text{ mM}$ ) ( $n = 5$ ). F: bar chart showing the amplitude of the mAHP in control and in the presence of isoproterenol ( $1 \mu\text{M}$ ). Isoproterenol suppressed the mAHP in cells dialyzed with cAMP.  $n = 5$ ; paired 2-way Student's  $t$ -test,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $****P < 0.0001$ .



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  $\mu$ M) to neurons dialyzed with cAMP (1 mM) reduced the amplitude of the medium AHP by  $58.2 \pm 8.2\%$  (Fig. 2, C–F;  $-1.9 \pm 0.3$  vs.  $-0.9 \pm 0.3$  mV;  $n = 5$ ;  $t = -8$ ,  $P = 0.001$ , paired 2-tailed Student's  $t$ -test). The magnitude of reduction of the medium AHP by isoproterenol was the same in control cells 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  $\beta$ -adrenergic receptors and that suppression of the medium AHP is independent of a rise in intracellular cAMP.

**Blocking HCN channels with ZD7288 reduced suppression of medium AHP by isoproterenol.** The medium AHP in hippocampal CA1 pyramidal neurons is proposed to be mediated by activation of SK channels and deactivation of HCN channels at hyperpolarized voltages (Church et al. 2015; Gu et al. 2005; Kaczorowski 2011). This was confirmed by observing that addition of the HCN channel blocker ZD7288 (1  $\mu$ M) blocked the medium AHP by  $52.2 \pm 16.5\%$  ( $P = 0.047$ ; Fig. 3), with the sequential addition of the SK channel inhibitor apamin (100 nM) blocking the remaining afterpotential by a further  $43.3 \pm 11.8\%$  [ $P = 0.054$ ; Fig. 3D; control  $-4.3 \pm 1.5$  mV, ZD7288  $-2.0 \pm 0.7$  mV, ZD7288 + apamin  $-0.1 \pm 0.3$  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

$-1.8 \pm 0.1$  mV, ZD7288  $-2.4 \pm 0.2$  mV, ZD7288 + apamin  $-2.2 \pm 0.4$  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  $\beta$ -adrenergic receptor activation. Blocking HCN channels with ZD7288 (1  $\mu$ M) reduced the medium AHP by  $74.5 \pm 14.5\%$  ( $P = 0.06$ ; Fig. 4A) and reduced the effect of subsequent concomitant addition of isoproterenol (1  $\mu$ M) [Fig. 4A; control  $-4.4 \pm 0.5$  mV, ZD7288  $-1.0 \pm 0.5$  mV, ZD7288 + isoproterenol  $-0.8 \pm 0.6$  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  $32.7 \pm 6.3\%$  ( $P = 0.007$ ; Fig. 4B), with the concomitant addition of isoproterenol (1  $\mu$ M) still suppressing the remaining medium AHP by  $37.2 \pm 8.4\%$  [Fig. 4B;  $P = 0.003$ ; control  $-4.9 \pm 0.8$  mV; apamin  $-3.4 \pm 0.7$  mV; apamin + isoproterenol  $-1.5 \pm 0.6$  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 submaximal concentration of ZD7288 (1  $\mu$ M) reduced the sag by 96%, reducing the sag from contributing  $29.6 \pm 2.1\%$  of the electrotonic potential to contributing  $2.1 \pm 1.2\%$  ( $t = 13.8$ ;  $P < 0.0001$ , paired 2-tailed Student's  $t$ -test). Application of isoproterenol (1  $\mu$ M) resulted in a  $27.6 \pm 8.8\%$  reduction in depolarizing sag ( $25.6 \pm 1\%$  vs.  $18.6 \pm 2.4\%$ ;  $n = 7$ ;  $t = 3.3$ ,  $P = 0.02$ , paired 2-tailed Student's  $t$ -test). These data strongly suggest that suppression of the medium AHP by isoproterenol is mediated by modulat-

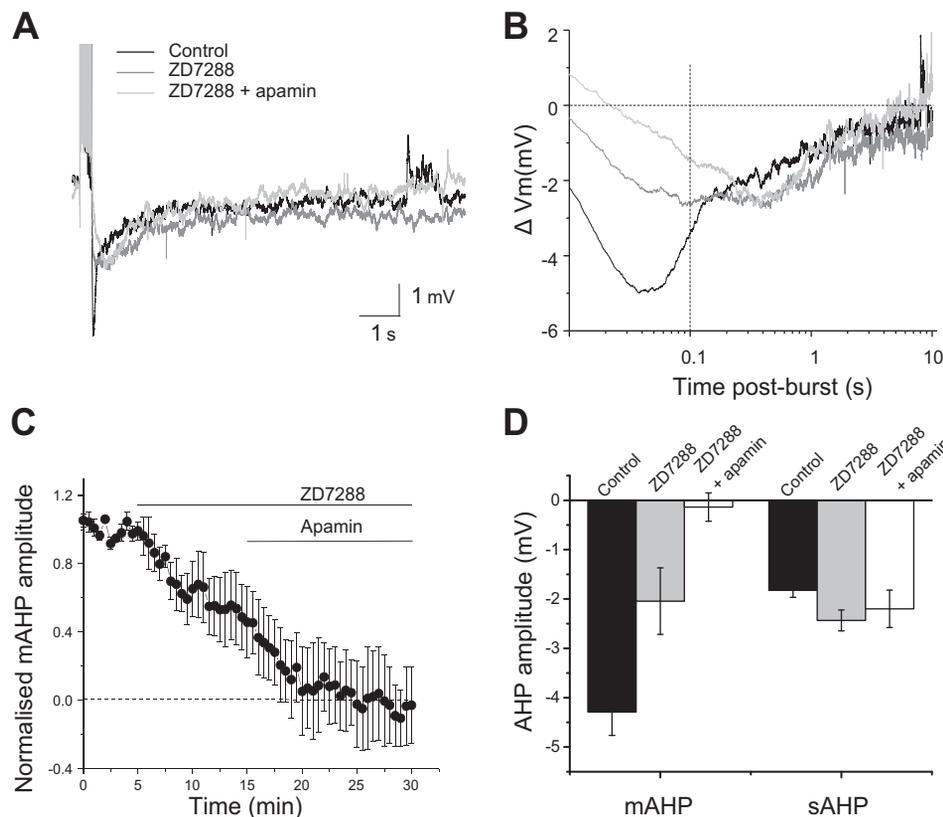
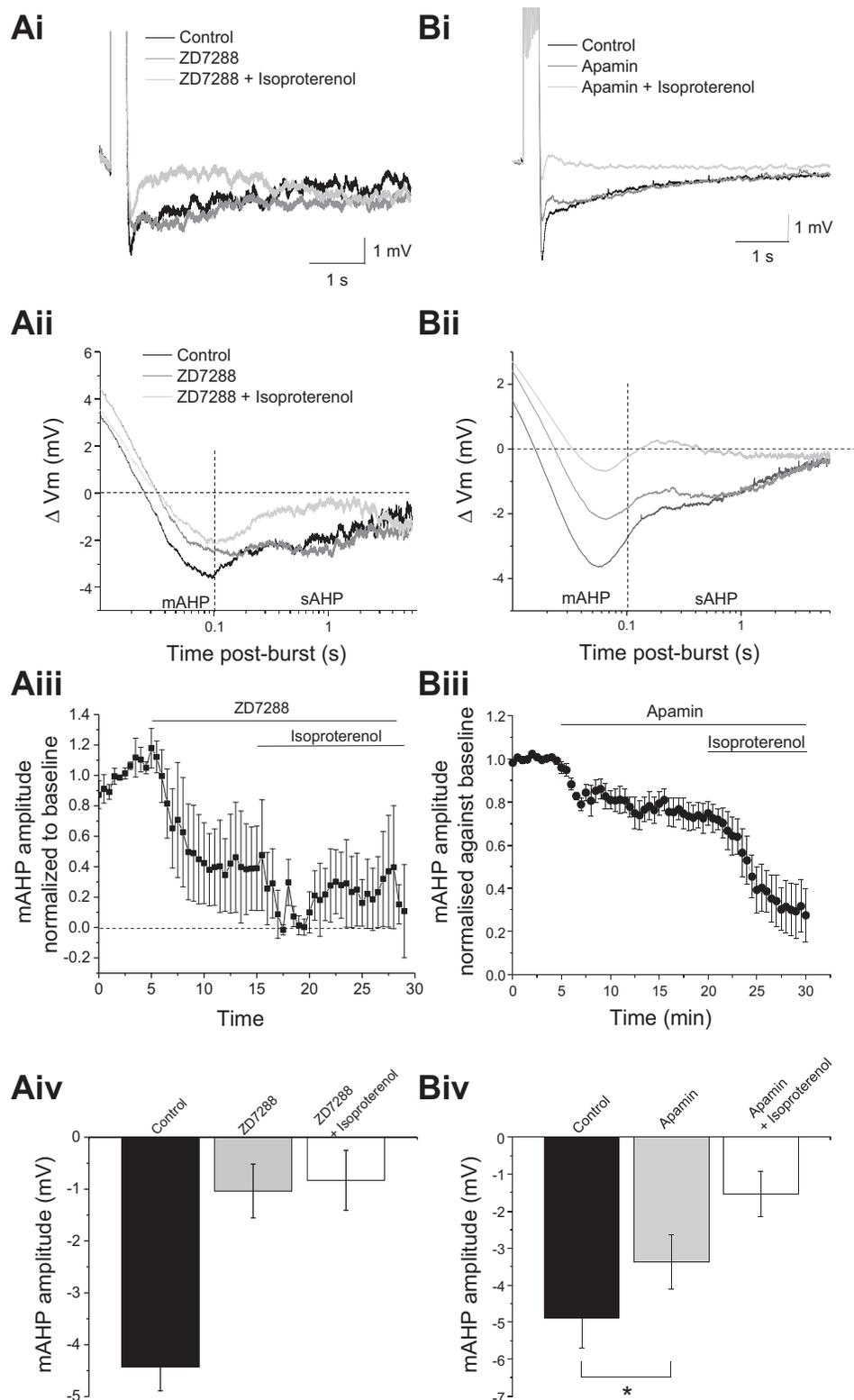


Fig. 3. Small-conductance, calcium-activated  $K^+$  (SK) and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels mediate the medium afterhyperpolarization (mAHP) in hippocampal CA1 pyramidal neurons held at  $-75$  mV. *A*: representative membrane voltage ( $V_m$ ) traces of AHPs evoked by a train of 15 action potentials fired at 50 Hz in control conditions, with ZD7288 (1  $\mu$ M), and with apamin (100 nM) + ZD7288 (1  $\mu$ M). Action potentials have been truncated for clarity. *B*: evoked mAHP and slow AHP (sAHP) from *A* displayed on a  $\log_{10}$  timescale to illustrate the complete block on the mAHP following the sequential inhibition of HCN and SK channels by ZD7288 and apamin, respectively. *C*: diary plot of the amplitude of the mAHP before and after the sequential addition of ZD7288 (1  $\mu$ M) and ZD7288 + apamin (100 nM). Note that the mAHP is completely inhibited by the combination of channel blockers. *D*: bar chart illustrating the reduction of the mAHP by ZD7288 (1  $\mu$ M) and ZD7288 + apamin (100 nM), with no effect of either blocker on the amplitude of the sAHP.

ing HCN channel activity in hippocampal CA1 pyramidal neurons.

*Selective  $\beta_3$ -adrenergic receptor agonists BRL37344 and SR58611A suppress medium AHP.* Low levels of  $\beta_3$ -adrenergic receptor mRNA are expressed in the hippocampus of mouse and rat (Claustre et al. 2008; Evans et al. 1996).  $\beta_3$ -

Adrenergic receptors display a low binding affinity for propranolol (Hoffmann et al. 2004), and we observed that pretreatment of hippocampal slices with 1  $\mu$ M propranolol ( $n = 8$ ) failed to antagonize the effect of isoproterenol on the medium AHP (data not shown). The  $\beta_3$ -adrenergic receptor-selective agonist BRL37344 (10  $\mu$ M;  $n = 4$ ) (Hoff-



mann et al. 2004) suppressed the medium AHP by  $42.2 \pm 9.3\%$  (Fig. 5A;  $-3 \pm 0.4$  vs.  $-1.8 \pm 0.5$  mV;  $t = -4.2$ ,  $P = 0.025$ , paired 2-tailed Student's  $t$ -test). Application of a second  $\beta_3$ -adrenergic receptor-selective agonist, SR58611A ( $10 \mu\text{M}$ ) (Bianchetti and Manara 1990; Simiand et al. 1992), also suppressed the medium AHP by  $33.7 \pm 6.5\%$  (Fig. 5B;  $-3.1 \pm 0.4$  vs.  $-2.1 \pm 0.4$  mV;  $n = 7$ ;  $t = -3.6$ ,  $P = 0.012$ ). The magnitudes of suppression of the medium AHP by BRL37344 and SR58611A were not significantly different ( $t = -1.1$ ,  $P = 0.312$ , unpaired 2-tailed Student's  $t$ -test). In contrast to BRL37344, which exhibited no significant reduction on the H-current-mediated depolarizing sag ( $25.8 \pm 2.4\%$  vs.  $18.1 \pm 2.6\%$ ;  $n = 4$ ;  $t = 1.9$ ;  $P = 0.151$ , paired 2-tailed Student's  $t$ -test), SR58611A reduced the H-current-mediated depolarizing sag by  $17.5\%$  ( $23.2 \pm 3.0\%$  vs.  $19.0 \pm 2.9\%$ ;  $n = 10$ ;  $t = 3.0$ ;  $P = 0.014$ , paired 2-tailed Student's  $t$ -test). BRL37344 ( $10 \mu\text{M}$ ) failed to affect the medium AHP when applied in the presence of propranolol ( $10 \mu\text{M}$ ) [Fig. 5, C and D; control  $-4.7 \pm 0.9$  mV, propranolol  $-5.0 \pm 1.5$  mV, propranolol + BRL37344  $-4.9 \pm 1.6$  mV;  $F_{(2,4)} = 0.1$ ,  $P = 0.93$ , repeated-measures ANOVA;  $n = 3$ ]. These findings indicate the presence of functional  $\beta_3$ -adrenergic receptors in hippocampal CA1 pyramidal neurons that selectively suppress the medium AHP and not the slow AHP. Finally, BRL37344 ( $10 \mu\text{M}$ ) suppressed the medium AHP by  $37.8 \pm 8.2\%$  in CA1 pyramidal neurons dialyzed with cAMP ( $1 \text{ mM}$ ) (Fig. 5, E and F;  $-2.9 \pm 0.2$  vs.  $-1.8 \pm 0.2$  mV;  $t = -4.3$ ,  $P = 0.003$ , paired 2-tailed Student's  $t$ -test;  $n = 9$ ). There was no significant difference in the magnitude of block produced by BRL37344 ( $10 \mu\text{M}$ ) in cAMP-dialyzed cells ( $1 \mu\text{M}$ ) compared with non-cAMP-dialyzed neurons ( $0 \text{ mM}$  cAMP  $42.2 \pm 9.3\%$ ,  $1 \text{ mM}$  cAMP  $37.8 \pm 8.2\%$ ;  $t = 0.3$ ,  $P = 0.76$ ). These data confirm that activation of  $\beta_3$ -adrenergic receptors suppresses the medium AHP by a cAMP-independent signaling pathway.

## DISCUSSION

The slow AHP is unusual in being a potassium current subject to modulation by G protein-coupled receptor activation (Pedarzani and Storm 1993). This slow afterpotential is sensitive to activation of G protein-coupled receptors that are coupled via  $G_s$  to activation of adenylate cyclase, a rise in 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  $\beta$ -adrenergic receptors and a rise in intracellular cAMP. In contrast, evi-

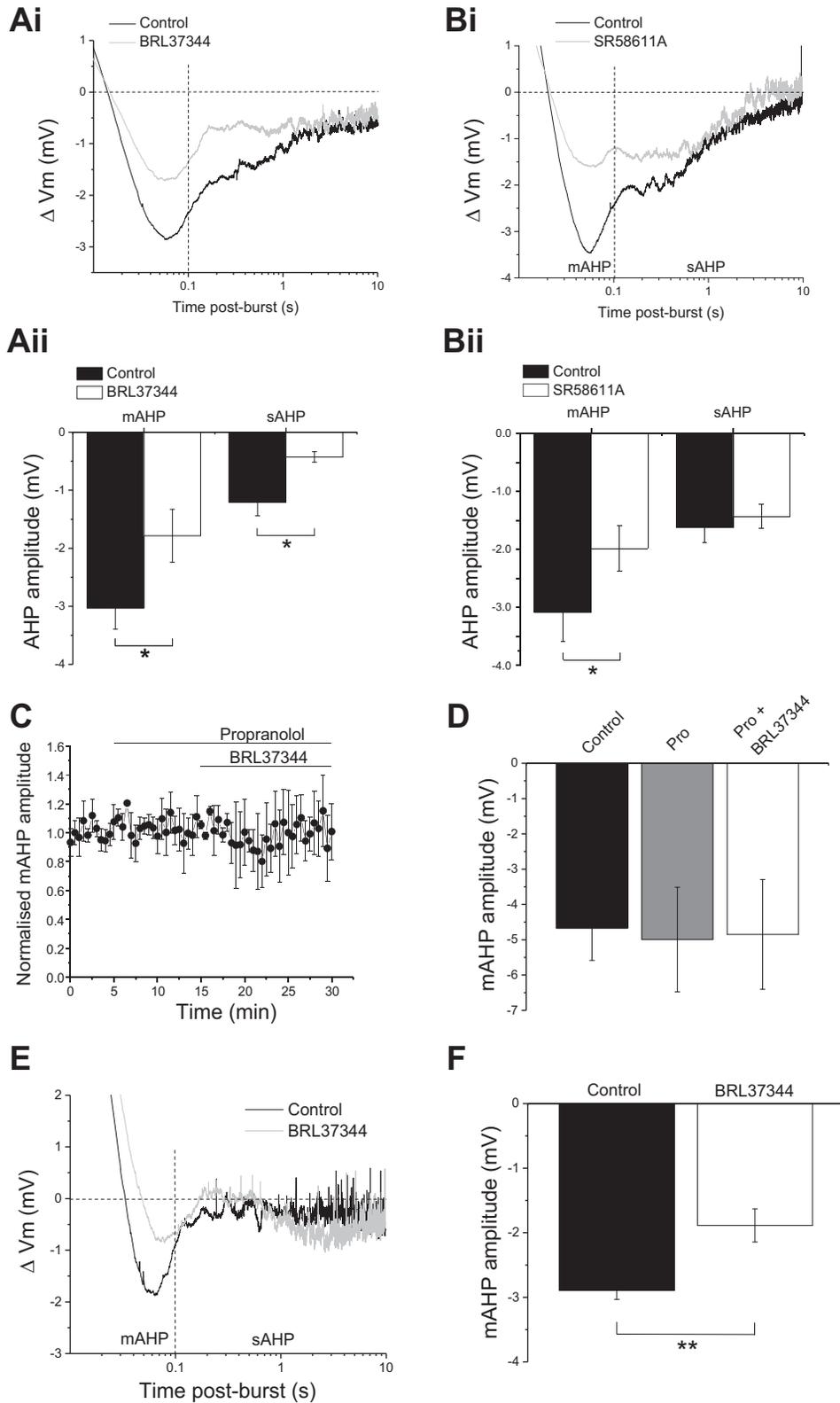
dence of the medium AHP being modulated by metabotropic receptor activation in hippocampal pyramidal cells is lacking. It was noted that the medium AHP was reduced when the slow AHP was suppressed by forskolin, but this was suggested to result from an overlap of the time courses of the two components of the AHP (Gu et al. 2005). We have carefully separated the amplitudes of the two afterpotentials, by plotting the AHP time course on a log time base (Fig. 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 (Fig. 2, C–F). These data confirm that the medium AHP is a substrate for modulation and that suppression of this afterpotential is not mediated by a rise in intracellular cAMP. The effect of isoproterenol was mimicked by two selective  $\beta_3$ -adrenergic receptor agonists, BRL37344 and SR58611A. The mRNA encoding the  $\beta_3$ -adrenergic receptor has been detected in various brain regions including the hippocampus in rat, mouse, and human (Claustre et al. 2008; Evans et al. 1996; Rodriguez et al. 1995). The precise role of the  $\beta_3$ -adrenergic receptor in brain is unclear, but BRL37344 and SR58611A possess antidepressant and anxiolytic-like effects in rodents (Claustre et al. 2008; Simiand et al. 1992; Stemmelin et al. 2008; Tanyeri et al. 2013). Activation of hippocampal  $\beta_3$ -adrenergic receptors by norepinephrine and isoproterenol mediates an increase in hippocampal neural precursor activity by enhancing the proliferation of multipotent neuronal stem cells (Jhaveri et al. 2010).

BRL37344 displays 90-fold and 20-fold higher selectivity toward rat  $\beta_3$ -adrenergic receptors compared with  $\beta_1$ - and  $\beta_2$ -adrenergic receptors, respectively (Hoffmann et al. 2004), whereas SR58611A exhibits 280-fold and 140-fold higher selectivity for rat  $\beta_3$ -adrenergic receptors compared with  $\beta_1$ - and  $\beta_2$ -adrenergic receptors, respectively (Gauthier et al. 1996; Stemmelin et al. 2008). The selectivity of these agonists is better than that reported for available  $\beta_3$ -adrenergic receptor antagonists. For example, L-748337 displays less than a 20-fold selectivity and L-748328 exhibits only a 45-fold selectivity for  $\beta_3$ -adrenergic receptors over  $\beta_2$ -adrenergic receptors (Candelore et al. 1999). In addition, SR 59230A displays only a 10-fold selectivity for  $\beta_3$ -adrenergic receptors over  $\beta_1$ - and  $\beta_2$ -adrenergic receptors (Manara et al. 1996). In the absence of attempting full concentration-response relationships in the absence and presence of increasing concentrations of antagonist(s), we elected to use  $\beta_3$ -adrenergic 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

Fig. 4. Block of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels reduces suppression of the medium afterhyperpolarization (AHP) by isoproterenol. *Ai*: representative membrane voltage ( $V_m$ ) trace of the AHP evoked by a train of 15 action potentials fired at 50 Hz in the absence (control) and presence of the H-current blocker ZD7288 ( $1 \mu\text{M}$ ) and ZD7288 + isoproterenol ( $1 \mu\text{M}$ ). The mAHP was largely blocked by ZD7288, with the addition of isoproterenol having little effect. *Aii*: evoked mAHP and slow AHP (sAHP) displayed on a log<sub>10</sub> timescale, showing the large block of the mAHP by ZD7288 (dark gray) occluding subsequent suppression by isoproterenol (light gray). *Aiii*: diary plot of the amplitude of the mAHP, showing the dominating effect of block of H current by ZD7288 ( $1 \mu\text{M}$ ) and the minor effect of subsequent addition of isoproterenol ( $1 \mu\text{M}$ ). *Aiv*: bar chart showing the amplitude of the mAHP, significant block of that amplitude by ZD7288, and minor additional effect of subsequent addition of isoproterenol. *Bi*: representative membrane voltage trace of the AHP evoked by a train of 15 action potentials fired at 50 Hz in the absence (control) and presence of the small-conductance, calcium-activated  $K^+$  (SK) channel inhibitor apamin ( $100 \text{ nM}$ ) and apamin + isoproterenol ( $1 \mu\text{M}$ ). Only a small effect of apamin on the mAHP was observed, with subsequent addition of isoproterenol significantly suppressing both mAHP and sAHP. *Bii*: evoked mAHP and sAHP displayed on a log<sub>10</sub> timescale, showing the minor block of the mAHP by apamin (dark gray), with a much greater suppression of both mAHP and sAHP by subsequent addition of isoproterenol (light gray). *Biii*: diary plot of the amplitude of the mAHP, showing the minor block by apamin ( $100 \text{ nM}$ ) and the significant suppression produced by the subsequent addition of isoproterenol ( $1 \mu\text{M}$ ). *Biv*: bar chart showing the amplitude of the mAHP. Addition of apamin ( $100 \text{ nM}$ ) blocked only a small component of the mAHP, with a much greater suppression observed on the subsequent addition of isoproterenol.

SR58611A was applied (Fig. 5, *Aii* and *Bii*), which might result from the low-affinity binding of BRL37344 to  $\beta_1$ - and  $\beta_2$ -adrenergic receptors. These findings indicate that activation of  $\beta_3$ -adrenergic receptors suppresses the medium and not the slow AHP in hippocampal CA1 pyramidal neurons.

*SK and HCN channels underlie 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 channels (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 nonphysiological activation protocols used in voltage clamp (Gu et al. 2005). The data presented in this study show that the HCN channel blocker ZD7288 (1  $\mu$ M) reduced the medium AHP by 55–75% in neurons held at  $-75$  mV, confirming that 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  $\mu$ M), confirming that SK channel activation mediates a significant component of the medium AHP (Bond et al. 2004; Church et al. 2015; Kaczorowski et al. 2007; Stocker et al. 1999). 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 heterotetrameric HCN channels display different electrophysiological and kinetic properties when expressed in heterologous cell lines (Robinson and Siegelbaum 2003; Santoro et al. 2000; 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).

Nonselective actions of ZD7288 have been reported, with T-type calcium channel current blocked with an  $IC_{50}$  of 100  $\mu$ M (Felix et al. 2003), ionotropic glutamate receptor currents reduced by 40% with 20  $\mu$ M ZD7288 (Chen 2004), and inhibition of sodium channel current in dorsal root ganglion neurons with an  $IC_{50}$  close to 10  $\mu$ M (Wu et al. 2012). In addition, relatively long-term application of ZD7288 can produce nonselective effects, with application of ZD7288 (30  $\mu$ M) producing a significant depression of glutamatergic-mediated synaptic activity at hippocampal mossy fiber synapses (Chevalyere and Castillo 2002; Gill et al. 2006). We circumvented the possibility of nonselective effects of ZD7288 by blocking AMPA receptor-mediated synaptic potentials with NBQX, using only 1  $\mu$ M of the compound, and finally recording the effect of the HCN channel blocker within 10 min of application. Preblock of HCN channels with ZD7288 reduced suppression of the remaining medium AHP by isoproterenol (Fig. 4A, *iii* and *iv*). These data suggest that  $\beta_3$ -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 cAMP concentration (Chen et al. 2007; Santoro et al. 2004; Wainger et al. 2001; Zong et al. 2012). However, activation of  $\beta_3$ -adrenergic receptors suppresses the medium AHP in neurons dialyzed with cAMP. These data show that modulation of HCN channels is independent of a rise in intracellular cAMP.

*Do  $\beta_3$ -adrenergic receptors functionally couple with HCN channels?* The medium AHP was retained in cells dialyzed with cAMP. Furthermore, the reduction of the medium AHP by isoproterenol was independent of intracellular cAMP levels. In addition, the medium AHP was reduced in amplitude by the  $\beta_3$ -adrenergic receptor-selective agonist BRL37344, in both control cells and cells dialyzed with cAMP. The isoproterenol- and BRL37344-mediated suppression of the medium AHP in the presence of raised intracellular cAMP indicates that the inhibitory effect on the medium AHP is not mediated by activation of either  $G_{\alpha_s}$  or  $G_{\alpha_i}$  G protein-affecting cAMP levels. The  $\beta_3$ -adrenergic receptor signaling pathway is ill defined, with the receptor being reported to couple to  $G_{\alpha_s}$  or  $G_{\alpha_i}$  (Collins 2012). Other studies report that  $\beta_3$ -adrenergic receptors signal in a biphasic manner by coupling to both  $G_{\alpha_s}$  and  $G_{\alpha_i}$  (Bégin-Heick 1995; Gauthier et al. 1996; Hadi et al. 2013). Regardless of possible second messenger pathways, activation of  $\beta_3$ -adrenergic receptors causes relaxation of rat bladder, an effect blocked by ZD7288. This observation indicates that coupling can occur between activation of  $\beta_3$ -adrenergic receptors and HCN channels (Kashyap et al. 2015). The reported antidepressant and antianxiolytic properties of  $\beta_3$ -adrenergic receptor agonists make this pathway an attractive one to target, with further investigation being needed.

HCN channels, which underlie H current, are a key regulator of intrinsic excitability in CA1 pyramidal neurons. H current has a direct influence on neuronal input resistance, the resting membrane potential, and membrane time constant. H current filters out low-frequency fluctuations in membrane voltage in a self-regulating feedback mechanism to regulate synaptic input (He et al. 2014). Inhibition of H current by ZD7288 increased firing frequency in hippocampal neurons (Gasparini and 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  $\beta_3$ -adrenergic receptors in hippocampal neurons to determine whether coupling is localized within the cell.

Fig. 5.  $\beta_3$ -Adrenergic receptor ( $\beta_3$ -AR) activation suppresses the medium afterhyperpolarization (mAHP). *Ai*: mAHP and slow AHP (sAHP) evoked by a train of 15 action potentials fired at 50 Hz in the absence (control) and presence of the  $\beta_3$ -AR-selective agonist BRL37344 (10  $\mu$ M) displayed on a  $\log_{10}$  timescale to illustrate a reduction in the mAHP and sAHP by addition of BRL37344.  $V_m$ , membrane voltage. *Aii*: bar chart showing suppression of both AHP components by BRL37344 (10  $\mu$ M).  $n = 4$ ; paired 2-tailed Student's  $t$ -test. *Bi*: mAHP and sAHP evoked by a train of 15 action potentials fired at 50 Hz in the absence (control) and presence of the  $\beta_3$ -AR-selective agonist SR58611A (10  $\mu$ M) in CA1 pyramidal neurons displayed on a  $\log_{10}$  timescale to illustrate a reduction in the mAHP by addition of SR58611A. Note the lack of effect of the  $\beta_3$ -AR agonist on the sAHP (see DISCUSSION). *Bii*: bar chart showing suppression of both AHP components by SR58611A (10  $\mu$ M).  $n = 7$ ; paired 2-tailed Student's  $t$ -test. *C*: diary plot of the amplitude of the mAHP and the lack of effect of addition of BRL37344 (10  $\mu$ M) in the continued presence of propranolol (10  $\mu$ M). *D*: bar chart showing the amplitude of the mAHP in control conditions, after 10-min incubation with propranolol (Pro; 10  $\mu$ M), and after addition of BRL37344 (10  $\mu$ M) in the continued presence of propranolol. *E*: evoked mAHP and sAHP plotted on a  $\log_{10}$  timescale from a cell dialyzed with cAMP (1 mM; control). Addition of BRL37344 (10  $\mu$ M) suppressed the mAHP in cAMP-dialyzed cells. *F*: summary bar chart showing small but robust block of the mAHP by BRL37344 (10  $\mu$ M).  $n = 9$ ; paired 2-tailed Student's  $t$ -test \* $P < 0.05$ , \*\* $P < 0.01$ .

AHPs 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 (Madison and Nicoll 1982, 1986; Pedarzani et al. 1998; Pedarzani and Storm 1993; Stocker et al. 1999) AHP. 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  $\beta_3$ -adrenergic receptors in hippocampal CA1 neurons highlights a potential new target in modulating HCN channels and thereby synaptic plasticity.

#### ACKNOWLEDGMENTS

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#### GRANTS

This work was funded by the Medical Research Council.

#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

T.W.C. and N.V.M. conceived and designed research; T.W.C. performed experiments; T.W.C. analyzed data; T.W.C., J.T.B., and N.V.M. interpreted results of experiments; T.W.C. and N.V.M. prepared figure; N.V.M. drafted manuscript; T.W.C., J.T.B., and N.V.M. edited and revised manuscript; T.W.C. and N.V.M. approved final version of manuscript.

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