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Ketamine inhibits synaptic transmission and nicotinic acetylcholine receptor-mediated responses in rat intracardiac ganglia *in situ*

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Running title: Ketamine inhibition of intracardiac ganglion transmission

Conflict of interests

The authors declare no conflict of interests.

Highlights:

- Ketamine attenuated the excitatory postsynaptic responses evoked by nerve stimulation in a concentration-dependent manner.
- This reduction was significant at clinically relevant concentrations at high frequencies.
- Ketamine inhibits nicotinic acetylcholine receptor-mediated currents in dissociated rat intracardiac ganglion (ICG) neurons.
- Ketamine inhibits cholinergic synaptic transmission in the rat ICG accounting for attenuation of vagal bradycardia.

Abstract

The intravenous anaesthetic ketamine, has been demonstrated to inhibit nicotinic acetylcholine receptor (nAChR)-mediated currents in dissociated rat intracardiac ganglion (ICG) neurons (Weber et al., 2005). This effect would be predicted to depress synaptic transmission in the ICG and would account for the inhibitory action of ketamine on vagal transmission to the heart (Inoue and König, 1984). This investigation was designed to examine the activity of ketamine on (i) postsynaptic responses to vagal nerve stimulation, (ii) the membrane potential, and (iii) membrane current responses evoked by exogenous application of ACh and nicotine in ICG neurons *in situ*. Intracellular recordings were made using sharp intracellular microelectrodes in a whole mount ICG preparation. Preganglionic nerve stimulation and recordings in current- and voltage-clamp modes were used to assess the action of ketamine on ganglionic transmission and nAChR-mediated responses. Ketamine attenuated the postsynaptic responses evoked by nerve stimulation. This reduction was significant at clinically relevant concentrations at high frequencies. The excitatory membrane potential and current responses to focal application of ACh and nicotine were inhibited in a concentration-dependent manner by ketamine. In contrast, ketamine had no effect on the directly-evoked action potential or excitatory responses evoked by focal application of γ-aminobutyric acid (GABA). Taken together, ketamine inhibits synaptic transmission and nicotine- and ACh-evoked currents in adult rat ICG. Ketamine inhibition of synaptic transmission and nAChR-mediated responses in the ICG contributes significantly to its attenuation of the bradycardia observed in response to vagal stimulation in the mammalian heart.

Keywords: anaesthesia; intrinsic cardiac ganglia; ketamine; nicotinic acetylcholine receptor; synaptic transmission; action potential; calcium channels; vagal nerve stimulation

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium ion concentration; dSEVC, discontinuous single electrode voltage clamp; EPSP, excitatory postsynaptic potential; I_{ACh}, ACh-evoked current; IC_{50}, half-maximal inhibitory concentration; ICG, intracardiac ganglion; nAChR, nicotinic acetylcholine receptor; PSS, physiological salt solution; WHBP, working heart-brainstem preparation.

Introduction

Ketamine is used as an anaesthetic and analgesic in clinical and veterinary practice, and similarly in animal research. Since its activity is rapid in onset and short lived, it is ideal for brief surgical operations and is used in conflict zones and disaster situations (Trelles Centurion et al., 2017). In addition, the risk of overdose is small and hence few complications are associated with its use. Ketamine is used in human surgery in adults and pediatric patients, being listed in the WHO Essential Medicine List since 1985 (WHO Model List of Essential Medicines, 2017). The principal target for the action of ketamine is generally held to be its antagonist action at the N-methyl-D-aspartate (NMDA) receptor (Anis et al., 1983; Lodge and Mercier, 2015). Ketamine, however, has been demonstrated to interact with several other neurotransmitter receptors and ion channels (Zanos et al., 2018) including opioid and muscarinic acetylcholine receptors (in particular, M_1) and can have anaesthetic effects on voltage-gated Na^+ channels (Sinner and Graf, 2008).

The intravenous (i.v.) dissociative anaesthetic ketamine is known to affect cardiac parameters such as heart rate and cardiac output under clinical conditions (Tweed et al., 1972) and in chronically instrumented animals (Akine et al., 2001; Blake and Korner, 1981, 1982; Inoue and Arndt, 1982). Ketamine also has a direct effect on cardiac muscle, decreasing myocardial contractility, but only at supratherapeutic ketamine doses (~100 μM) (Sprung et al., 1998). Considering autonomic regulation, excitatory neurotransmission in guinea-pig sympathetic ganglia is blunted by ketamine (Juang et al., 1980; Mahmoodi et al., 1980). This effect is overridden by the stimulation of a central sympathetic response sustaining or increasing blood pressure (Kurdi et al., 2014). However, ketamine inhibition of parasympathetic neurons involved in the regulation of cardiac function may contribute to the increase in heart rate (Inoue and König, 1988). Ketamine has been shown to inhibit nicotinic excitation in cardiac preganglionic parasympathetic neurons of the nucleus ambiguus of the brainstem (Irnaten et al., 2002). Postganglionic intracardiac neurons have also been shown to modulate heart rate in a nicotine-dependent manner, demonstrating the involvement of neuronal nicotinic acetylcholine receptors (nAChRs) (Bibevski et al., 2000; Ji et al., 2002).

Ketamine inhibits reversibly and stereo-specifically neuronal nAChRs in rat PC12 cells (Sasaki et al., 2000) and human SH-SY5Y cells (Friederich et al., 2000). Furthermore, bath application of ketamine has been shown to cause a concentration-dependent inhibition of ACh-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat ICG neurons (Weber et al., 2005). At a clinically relevant concentration, ketamine (10 μM) reversibly

inhibited ACh-induced increases in $[Ca^{2+}]_i$ but failed to inhibit increases in $[Ca^{2+}]_i$ evoked by focal application (100 μM) of either muscarine or ATP. Taken together, ketamine would be predicted to reduce the excitatory postsynaptic potential (EPSP) amplitude and depress synaptic transmission in the ICG and would account for the action of ketamine on peripheral vagal transmission to the heart (Inoue and König, 1988). We have directly tested this proposal by investigating the actions of ketamine on postsynaptic responses to vagal nerve stimulation and exogenous ACh and nicotine in ICG neurons in a whole mount ganglion preparation of the excised right atrial ganglion plexus from the adult rat. A preliminary account of the results has been given in a published abstract (Harper et al., 2006).

Material and Methods

Preparation

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010). The whole mount ICG preparation has been described previously (Rimmer and Harper, 2006). Briefly, twenty young, non-pregnant, female adult Wistar rats (≥ 6 wk, 125–220 g) were used. The University of Dundee is a designated scientific establishment (certificate of designation no. 60/2602) under the Animals (Scientific Procedures) Act 1986 ('the Act'). Rats were obtained from a designated supplier in the UK (Harlan UK Ltd., Oxon, UK) and were housed and cared for according to Home Office Guidelines on the Operation of the Act. Animals were killed by concussion and cervical dislocation, as authorised in Schedule 1 to the Act. No formal prior approval is required for Schedule 1 kill. The Ethics Committee does, however, conduct a Census of Schedule 1 procedures.

A whole mount preparation comprising the right atrial ganglion plexus and underlying myocardium was pinned out in a recording chamber (~1.0 ml volume) lined with Sylgard 184 silicone elastomer (Dow Corning) and superfused with bicarbonate buffered physiological salt solution (PSS) at ~2 ml/min (Gilson Minipuls 3; Gilson, Middleton WI, USA). The temperature of the superfusing solution was controlled by a Peltier heating device (PDMI-2 micro incubator; Medical Systems Corp., Greenvale NY, USA) to 36°C, monitored by an independent thermistor probe in the recording chamber. The tissue was left to resuscitate in these conditions for ~30 min before commencing electrophysiological recording. ICG neurons were visualized using differential interference contrast (DIC) optics on a fixed stage microscope. Recordings were normally made from the sino-atrial ganglion, the largest located at the junction of the right superior vena cava and right atrium (Rimmer

and Harper, 2006).

Electrophysiological recording, data acquisition and analysis

Intracellular recordings from postganglionic ICG somata were made using sharp microelectrodes pulled from borosilicate glass (GC120F; Harvard Apparatus, Holliston MA, USA) with DC resistances of ~120 MΩ when filled with 0.5 M KCl (0.5 M K acetate for GABA procedures). Membrane voltage and current responses were recorded with single microelectrode clamp amplifiers, Axoclamp 2A (Axon Instruments Inc., Foster City, CA USA) and NPI SEC-05X (npi electronic GmbH, Tamm, Germany). Voltage and current signals were digitized at 50 kHz and 10 kHz, respectively, and transferred to a dual-core Pentium computer using an analog-to-digital converter [Micro 1401 Mk II interface; Cambridge Electronic Design (CED), Cambridge, UK] and Spike 2, version 6/7 software (CED).

Branches of the vagus and interganglionic nerve trunks were stimulated using a glass suction electrode fabricated from borosilicate glass (GC150F; Harvard Apparatus) connected to a constant voltage isolated stimulator (Digitimer DS2; Digitimer Ltd., Welwyn Garden City, UK). Nerve trunks were stimulated using stimulus pulses of 0.02 to 0.2 ms width and 5 – 50 V amplitude. ACh, nicotine and GABA (100 μM) were focally applied using a pressure-ejection device (~150 kPa; Picospritzer II, General Valve Corp. Fairfield, NJ, USA), and the pressure ejection pipette was positioned <50 μm from the neuronal soma to maximize the response to agonist application. The pulse was adjusted to the shortest possible duration, consistent with the peak voltage or current responses being unchanged with a rapid recovery to control levels.

Solutions and pharmacological agents

PSS contained (in mM): 118 NaCl, 25 $NaHCO_3$, 1.13 NaH_2PO_4, 4.7 KCl, 1.8 $CaCl_2$, 1.3 $MgCl_2$, 11.1 D-glucose and was gassed with carbogen (95% O_2–5% CO_2) to pH 7.4. All reagents were of analytical grade. Vetalar ((±)-ketamine hydrochloride, 100 mg/ml, also containing 0.1% w/v benzthonium chloride as a preservative) obtained from Pfizer (Sandwich, Kent, UK) was dissolved immediately before application in PSS at the concentrations stated.

Data and statistical analysis

Data were acquired at two time points in control conditions, and following 20 mins superfusion of ketamine or upon recovery to control PSS solution. The reported actions of ketamine were investigated in no more than one ICG neuron in each preparation. Data are presented as the means ± SD of the number of observations (individual neurons, n) indicated, and were compared using ANOVA, paired and unpaired *t*-tests as indicated in the text (Prism 8, GraphPad Software, Inc., San Diego CA, USA) using a statistical significance (*) of $P < 0.05$. Our experiments were conducted on ICG neurons of *in situ* preparations each from individual adult rats (N = 20), and animals were not randomized.

Results

The action of ketamine on the passive membrane properties of the postganglionic ICG neurons

All ICG neurons investigated had a resting membrane potential ≥ –40 mV, overshooting somatic action potentials evoked by short depolarizing current pulses (2-3 ms) and received excitatory input due to spontaneous transmitter release from the vagus or interganglionic nerve terminals. Recordings were stable for at least 15 mins before readings were taken and the superfusing solution was altered. The passive membrane properties, resting membrane potential and input resistance, were in good agreement with previous reports for adult rat ICG neurons *in situ* (Rimmer and Harper, 2006).

Ketamine evoked a concentration-dependent hyperpolarization (< 5 mV) of the resting membrane potential. Superfusion of 100 μM ketamine, the maximum concentration tested, significantly produced a hyperpolarizing shift in the mean resting membrane potential from –49.6 ± 4.2 mV to –51.6 ± 3.9 mV (n = 10, *paired t-test). The input resistance of ICG neurons was also reversibly increased by ~30% in the presence of 100 μM ketamine from a mean value of 111.5 ± 47.6 MΩ to 146.7 ± 58.1 MΩ (n = 10, *paired t-test) (see Figure 1). The directly-evoked action potential overshoot and duration at the 0 mV level were not significantly affected by 100 μM ketamine (12.7 ± 4.2 mV and 0.87 ± 0.10 ms, control and 11.1 ± 3.4 mV and 0.90 ± 0.17 ms in the presence of ketamine, n = 8, *paired t-test). The effect of 100 μM ketamine was also investigated on depolarization-activated Ca^{2+} currents mediated by human Cav2.1 (P/Q-type), Cav2.2 (N-type) and Cav2.3 (R-type) channels expressed in HEK293 cells (n = 3 for each) (Figure S1). Ketamine (100 μM) did not inhibit Ca^{2+} current amplitude mediated by any of the neuronal Cav2 channels which is consistent

with its lack of effect on the action potential.

Effect of ketamine on synaptic transmission in ICG

All ICG neurons investigated received a secure/suprathreshold excitatory input following nerve stimulation (Rimmer and Harper, 2006). Nerve stimuli were normally delivered at 0.2 Hz at twice threshold intensity (voltage). A synaptic potential was normally observed as an inflection on the falling phase and after-hyperpolarization of the action potential. The impact of ketamine on the ability of the postganglionic neuron to follow the activity of pre-ganglionic stimuli at different frequencies (normally 5-100 Hz) was studied. Trains of stimuli (20), with intervening recovery periods of 30 s, were applied and action potential discharge monitored in the postganglionic neuronal soma. Synaptic efficacy was used to provide an objective index of the frequency dependence of synaptic transmission. This was determined as the percentage of postsynaptic action potentials as a function of nerve stimulation frequency plotted on a logarithmic scale (Smith et al., 2016). The number of successful somatic action potentials (normally constant to 20 Hz) decreased as the frequency of the train of stimuli increased (see Figure 2). Figure 2 A (i-vi) presents progressive block of ganglionic transmission at 50 Hz by superfusion of increasing concentrations of ketamine. The concentration-dependence of ketamine's action on synaptic efficacy for 2-100 Hz for a representative neuron is presented in Figure 2B. Given that a fraction of neurons within the ICG have been reported to be afferent in nature (Edwards et al., 1995), $CdCl_2$ (100 μM) was applied at the end of the experimental manoeuvres to test and substantiate that the response was synaptically mediated, and not due to antidromic conduction (Rimmer and Harper, 2006).

Ganglionic transmission is progressively decreased with increasing concentrations of ketamine, however, statistical analysis relationship is obscured by the distinct frequency dependence of synaptic efficacy between individual ICG neurons. Whereas for all neurons studied synaptic efficacy was 100% at 5 Hz and this was reduced to 68.4 ± 34.1% (n = 8; range 15-100 %) at 50 Hz (approximating maximum cardiac vagal efferent frequency, Kunze 1972). To circumvent this issue, the data were expressed as a percentage of that recorded in control conditions for each test frequency as shown in Figure 3. Synaptic transmission was unaffected by 10 μM ketamine at low frequencies but was significantly blunted at 50 and 100 Hz (one-way ANOVA, Tukey's multiple comparison test). Stimulus frequencies of 20 and 10 Hz required ketamine concentrations of 50 and 100 μM, respectively, to achieve significant

reduction of synaptic efficacy. Even at the highest concentration of ketamine tested (100 μM), ganglionic transmission was not completely blocked, the first stimulus of the train evoking an action potential in the postganglionic neuron, for example see Figure 2A(v).

Action of ketamine on excitatory ACh- and nicotine-evoked membrane potential responses in adult rat ICG neurons.

The action of ketamine on nAChRs was assayed by focal application of ACh and nicotine (100 μM, 20 or 50 ms). Under control conditions, this challenge evoked a transient depolarizing membrane potential response and multiple, adapting, action potential discharge. The peak depolarization evoked by either ACh (mean 20.9 mV, median 21.2 ± 9.3 mV, n = 5) or nicotine (mean 16.8 mV, median 15.1 ± 6.4 mV, n = 5) was blunted by ketamine. Figure 4A and B illustrate the decrease of the peak transient depolarization evoked by ACh and nicotine by superfusion of ketamine (50 and 100 μM). Figure 4C shows the reduction of depolarization with ketamine concentration. The extent of inhibition by 50 μM ketamine was similar for both ACh- and nicotine-evoked depolarizations reduced to 0.63 ± 0.08 and 0.74 ± 0.09 (n = 5) of control values for ACh and nicotine, respectively. The effect of 100 μM ketamine was significantly greater for ACh than for nicotine with peak depolarization reduced to 0.31 ± 0.11 and 0.54 ± 0.13 (n = 5) of control values, respectively (*unpaired t-test).

Immunohistochemical staining using antibodies against GABA demonstrated that GABA is present in some processes but primarily in the somata of ICG neurons (Fischer et al., 2005). However, there is no evidence for GABAergic pathways projecting to the heart. Therefore, this suggests that putative GABA release from ICG neurons may act in an autocrine or paracrine fashion to regulate ganglionic transmission. Focal application of GABA (100 μM) evoked a transient membrane depolarization of ~10 mV amplitude mediated by $GABA_A$ receptors expressed in adult rat ICG neurons *in situ* (Fischer et al., 2005). In the present study, the depolarization induced by focal application of GABA was observed in all neurons studied of adult rat ICG preparations (N = 3) and bath application of ketamine (100 μM) had no effect on the GABA-mediated excitatory responses in those neurons (Figure 4D).

The action of ketamine on nicotine- and ACh-evoked currents.

Membrane current responses to focal nicotine (and ACh) application were recorded using the discontinuous single electrode voltage clamp (dSEVC). The somatic $I_{nicotine/ACh}$ were taken after balancing the bridge and neutralising electrode capacitance in discontinuous current clamp mode. Gain was adjusted such that the membrane potential response during agonist application was ≤ 2 mV. The peak membrane current evoked by focal application of nicotine (100 μM) under control conditions at a holding potential of −50 mV was −0.85 ± 0.37 nA (n = 5). Representative current recordings in response to nicotine in the absence (control, recovery) and presence of ketamine (50 and 100 μM) are shown in Figure 5A. Ketamine reduced the ACh- and nicotine-evoked currents in a concentration-dependent manner (Figure 5B). The mean half-maximal inhibitory concentration (IC_{50}) obtained for ketamine inhibition of nicotine-evoked currents was 44.1 μM ($n \geq 5$). Ketamine inhibition of ACh-evoked membrane currents exhibited a similar IC_{50} as shown in Figure 5B.

Families of currents elicited by brief pulses of ACh recorded from a voltage-clamped neuron at various membrane potentials in the absence (control) and presence of ketamine (20, 50 and 100 μM) are shown in Figure 6A. The inhibition of ACh-evoked currents by ketamine is consistent with the previously reported effect of ketamine on nAChR-mediated Ca^{2+} transients in dissociated rat ICG neurons (Weber et al., 2005). Increasing concentrations of ketamine reduced the amplitude of ACh-evoked currents recorded at various holding potentials is shown in Figure 6B. The voltage-dependence of ketamine block is reflected in that the inhibition of ACh-evoked peak current amplitude by 100 μM ketamine was more than threefold greater at −90 mV compared to −30 mV. The extent of ketamine inhibition as a function of membrane potential is similar to that observed for α3β4 nAChRs expressed in *Xenopus* oocytes (Yamakura et al., 2000). Voltage-dependent inhibition of nAChR-mediated currents whereby the degree of inhibition increased with membrane hyperpolarization, suggests that ketamine is entering the membrane electric field and blocking the open nAChR-channel pore (Scheller et al., 1996).

Discussion

The increasing use of ketamine as a potential rapid-onset antidepressant (Zanos and Gould, 2018; Villas Boas et al., 2019) necessitates a better understanding of its effects on blood pressure and heart rate, well-known side effects at higher doses. Furthermore, it is recognised that abuse of or chronic treatment with ketamine cause ventricular structural and functional alterations which lead to alterations in cardiac electrophysiological properties and

increase the susceptibility to arrhythmias (Li et al., 2012). In this study, we have directly tested the proposal that ketamine inhibits nAChR-mediated currents in rat ICG neurons. Ketamine blunted cholinergic ganglionic transmission which accounts for the action of ketamine on vagal transmission to the heart (Inoue and König, 1988). Ketamine block was progressive, with secure transmission being recorded before ensuing subthreshold EPSPs. This could be the result of ketamine suppressing transmitter release at the preganglionic nerve terminals or block of the postganglionic nAChRs. However, ketamine (100 µM) had no significant effect on action potential overshoot or duration in ICG neurons and we have previously been shown that depolarization-activated calcium channel currents in rat ICG neurons were unaffected in the presence of 10 µM ketamine (Weber et al., 2005). Furthermore, 10 µM ketamine has been shown to have no effect on high voltage-activated (HVA) Ca^{2+} currents in rat hippocampal neurons and at 100 µM reduced the peak HVA Ca^{2+} currents by only 25.5 ± 6.9% and shifted the inactivation curve (~5 mV) to more hyperpolarized potentials (Tan et al., 2010). Our results also show that the predominant presynaptic calcium channels mediating synaptic transmission, Cav2.1, Cav2.2 and Cav2.3, were not significantly inhibited by 100 µM ketamine (Figure S1). Ketamine reduced excitability in rat superficial dorsal horn neurons by inhibiting voltage-gated Na^+ and K^+ currents with IC_{50}'s >100 µM (Schnoebel et al., 2005) and, in differentiated hippocampal H19-7 cells, 100 µM ketamine reduced Na^+ current amplitude by <20% and inhibited Ca^{2+}-activated K^+ currents with an IC_{50} of 274 µM (Huang et al., 2012). In identified cardiac parasympathetic preganglionic neurons in nucleus ambiguus of the rat brainstem slice preparation, the current-voltage relations for the transient K^+ current and delayed rectifier K^+ current were unaltered by ketamine (10 µM -1 mM) whereas the peak Na^+ current amplitude was reversibly inhibited (Irnaten et al., 2002). Ketamine inhibited tetrodotoxin (TTX)-sensitive and TTX-resistant Na^+ channels in rat dorsal root ganglion neurons with IC_{50}s of 147 µM and 866 µM, respectively (Zhou and Zhao, 2000), and human neuronal Na^+ channels natively expressed in SH-SY5Y cells with an IC_{50} of 1140 µM (Reckziegel et al., 2002). Taken together, these findings indicate that ketamine inhibition of presynaptic Ca^{2+} or Na^+ channels is unlikely to make any substantive contribution to ketamine inhibition of synaptic transmission in ICG.

Ketamine has been shown to inhibit several subtypes of neuronal nAChRs (Coates and Flood, 2001; Flood and Krasowski, 2000; Furuya et al., 1999; Yamakura et al., 2000), although the homomeric α7 nAChR appears less sensitive (Tassonyi et al., 2002). nAChRs

principally comprised of β subunits have been held to be more sensitive than those mainly of α subunits (Zanos et al., 2018; Yamakura et al., 2000). Ketamine, a non-competitive antagonist at neuronal nAChRs, has been shown to be most potent (IC_{50} = 9.5 μM) at rat α3β4 nAChRs expressed in *Xenopus* oocytes (Yamakura et al., 2000). The α3β4 nAChR subtype is the predominant nAChR subtype expressed in cardiac parasympathetic ganglion neurons (Bibevski et al., 2000; Poth et al., 1997), and most likely to contribute significantly to vagal synaptic transmission in the mammalian heart. However, human α3β4 nAChRs exhibit a different stoichiometry in mammalian HEK293 cells ($(\alpha3)_3(\beta4)_2$) compared to that when expressed in *Xenopus* oocytes ($(\alpha3)_2(\beta4)_3$) which is reflected in a lower ACh sensitivity and higher single channel conductance (Krashia et al., 2010). This may contribute to a different potency of ketamine inhibition of neuronal ACh-evoked currents and vagal synaptic transmission in mammalian ICG neurons. Furthermore, the present study was limited to postganglionic neurones receiving strong/secure inputs having a high safety factor for transmission of impulses (Rimmer and Harper, 2006).

The inhibition of EPSP amplitude was significant at clinically relevant concentrations at high stimulation frequencies. Ketamine used at clinical half-maximal effective concentration had previously been shown to inhibit nAChR-mediated increases in $[Ca^{2+}]_i$ by ~40% (Weber et al., 2005). As the ketamine concentration was increased, block of ganglionic transmission was apparent at successively lower frequencies. Inspecting presynaptic nerve stimulation frequencies reveals a broad range in the efficacy of ganglionic transmission in ICG neurons at ≥ 20 Hz (Smith et al., 2016). To our knowledge, there is little data available on the frequency of parasympathetic cardiac efferents targeting ICG neurons in the rat. In the Working Heart-Brainstem Preparation (WHBP) application of strong reflex stimuli (baroreceptor and peripheral chemoreceptor) evoked a substantial increase in synaptic activity in ICG neurons, maximum EPSP frequency of 55 and 65 Hz for baro- and chemo-receptor stimuli, respectively (McAllen et al., 2011). However, since the perfusion temperature in the WHBP preparation was 32°C these frequencies are, in all likelihood, an underestimation of those relevant to physiological temperature.

The excitatory membrane potential and current responses to focal application of exogenous ACh and nicotine were reversibly inhibited in a concentration-dependent manner by ketamine. Ketamine (100 μM) had no effect on the excitatory responses evoked by focal application of GABA in rat ICG neurons *in situ*. Taken together, ketamine inhibits ACh-evoked responses and synaptic transmission in rat ICG and therefore attenuates the

bradycardia observed in response to vagal stimulation in the mammalian heart. Impaired parasympathetic control is a powerful independent prognostic indicator of arrhythmia. Reduction of transmission in the ICG will produce a sympatho-vagal imbalance, resulting in a dominance of sympathetic pro-arrhythmic activity (Kalla et al., 2016). The role of the ICG in modulating extrinsic autonomic input to the heart and atrial fibrillation initiation is being increasingly recognised (Choi et al., 2017; Gibbons et al., 2012; Hou et al., 2007). Ketamine has also been demonstrated to reduce transmission in mammalian sympathetic ganglia (Juang et al., 1980; Mahmoodi et al., 1980) and both tachycardia and bradycardia have been described with ketamine administration (Akine et al., 2001; Blake and Korner, 1981, 1982). These differences may be due to the impact of ketamine on ganglionic transmission on the prevailing balance of sympatho-vagal drive regulating cardiac rhythm.

At physiological pH (7.4) there will be approximately equal amounts of the protonated and uncharged forms of the racemic mixture of ketamine, given its experimental pKa being 7.5. The ionization of ketamine influences its pH-dependent potency whereby the potency for (±) ketamine inhibition of recombinant NMDA (GluN1/GluN2A) receptors expressed in *Xenopus* oocytes is increased 6.9-fold at pH 6.9 compared to pH 7.6 (Dravid et al., 2007). Processes which lower extracellular pH, for example in myocardial ischaemia (approx. pH 6.1; Clarke et al., 1993) will enhance the fraction of charged ketamine. If the pH dependence of inhibition of NMDA receptor-channels extends to nAChRs, it would be anticipated to enhance block of nAChRs and synaptic transmission in mammalian ICG. The cardiovascular effects of ketamine, in particular, its positive chronotropic action by inhibiting nAChRs in postganglionic ICG neurons, may well be exacerbated in cardiovascular disease states. Clinically, the plasma concentration of ketamine peaks at 10-60 µM during general anesthesia after intravenous administration of 2 $mg.kg^{-1}$ (Idvall et al., 1979) which is consistent with the concentration-dependence of ketamine inhibition of nAChRs and synaptic transmission in mammalian ICG. In conclusion, our results indicate that ketamine inhibition of synaptic transmission in rat ICG is concentration- and frequency-dependent primarily due to inhibition of postsynaptic nAChRs and not the voltage-gated Na^+ or Ca^{2+} channels mediating the presynaptic action potential and transmitter (ACh) release.

Authors contributions

Performed experiments and analysed the data: A.A.H., K.R., J.R.D., J.R.M., D.J.A.

Conceived and designed the studies, supervised the project and wrote the manuscript: A.A.H., D.J.A.

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Appendix A. Supplementary data

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Figure Legends

Figure 1. Current-voltage relationship of adult rat intracardiac ganglion (ICG) neurons in the absence (control) and presence of ketamine. A, voltage responses obtained in response to long depolarizing and hyperpolarizing current pulses (+0.1, –0.1 to –0.5 nA) are shown for control conditions (i) and in the presence of ketamine (ii, 100 μM). B, current-voltage (I-V) relations for each condition plotted for the peak voltage response (○,▽) and the steady state response measured at the end of the current step (●,▼).

Figure 2. The action of ketamine on ganglionic transmission in adult rat ICG neurons. Waterfall displays of postsynaptic responses to 20 stimuli at 50 Hz applied at time "s" are shown. Responses to a train of such stimuli delivered to the preganglionic nerve trunk in control conditions, A(i) and progressive block of ganglionic transmission by superfusion of ketamine A(ii), 10 μM; A(iii), 20 μM; A(iv), 50 μM; A(v), 100 μM, and A(vi) washout, recovery are presented. B, frequency dependence (synaptic efficacy) of ganglionic transmission in control conditions and increasing concentrations of ketamine. Data presented for the exemplar neuron shown in A (i-vi).

Figure 3. The action of ketamine on the frequency dependence of ganglionic transmission. Ketamine attenuated synaptic efficacy in a concentration-dependent manner. The results are expressed as a percentage of that recorded in control conditions for each test frequency (log scale). Evidence of blunting of synaptic efficacy at low concentrations of ketamine was apparent at high frequencies (data shown as mean ± SD, $n \geq 5$ neurons except for 100 μM ketamine, n = 4). Synaptic transmission was significantly decreased by 10 μM ketamine at 50 and 100 Hz (n = 7, *one-way ANOVA, Tukey's multiple comparison test).

Figure 4. Effect of ketamine on ACh- and nicotine-evoked voltage (E_m) responses in ICG neurons. A (i-iii) example of the action of 50 and 100 μM ketamine on focal application of ACh (20 ms), and B (i-iii) nicotine, application indicated by arrowhead. Focal application of these agents in control conditions evoked transient depolarizing responses and action potential discharge. C, plots of the action of ketamine (log scale) on the peak, with time, depolarizing responses to ACh and nicotine. Data expressed as a fraction of that recorded in control conditions. D, Focal application of GABA (100 μM, 50 ms) evoked membrane

depolarization and action potential firing. Ketamine (100 μM) had no effect on the GABA-evoked excitatory responses.

Figure 5. The action of ketamine on nAChR-mediated currents activated by focal application of nicotine at a holding potential of –50 mV. A(i) control, (ii) 50 and (iii) 100 μM ketamine, respectively, and (iv) washout/ recovery. B, log concentration-response relationship of the action of ketamine on ACh- and nicotine-evoked membrane currents. Data expressed as mean ± SD ($n \geq 5$ for each [ACh], n indicated for each [nicotine]).

Figure 6. The action of ketamine on ACh-evoked currents (I_{ACh}) in rat ICG neurons. A, families of membrane currents evoked by focal application of ACh recorded from a voltage-clamped (dSEVC) neuron at various membrane potentials, –30, –50, –70 and –90 mV in the absence (control) and presence of 20 and 100 μM ketamine (Ket). B, current-voltage relationships for (I_{ACh}) amplitude in absence (control, washout) and presence of ketamine (20, 50, and 100 μM).

Figure 1
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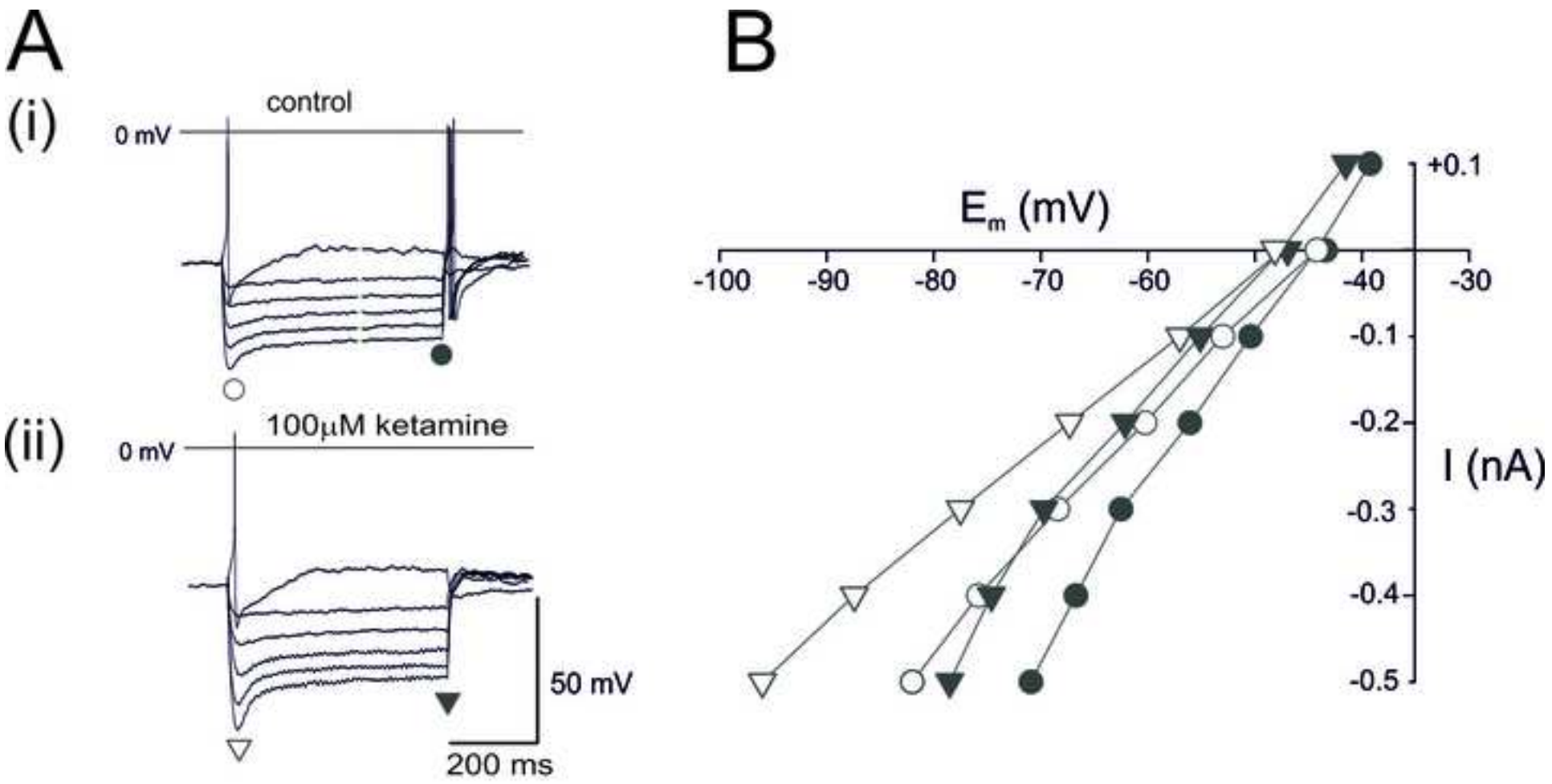

Figure1

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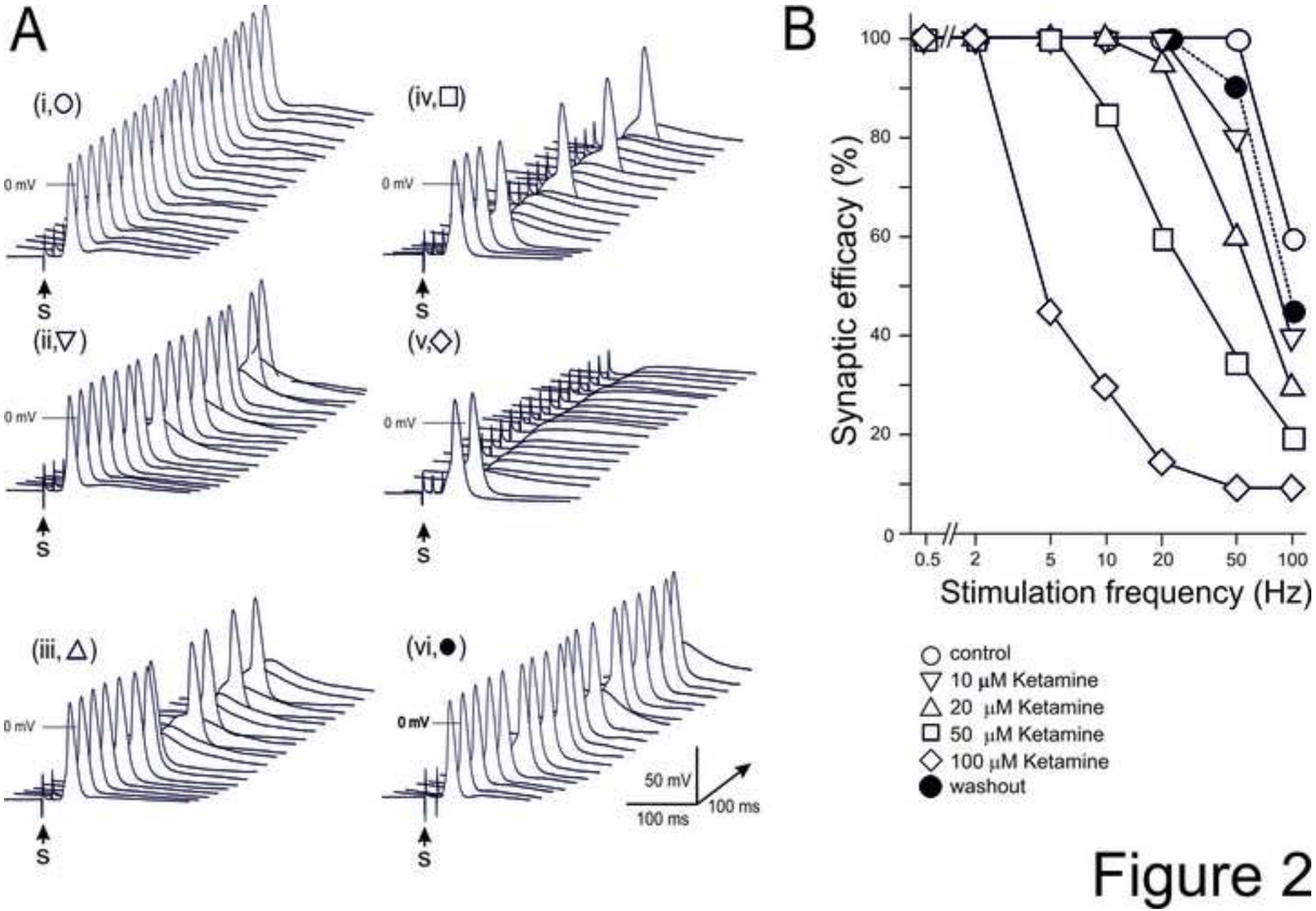

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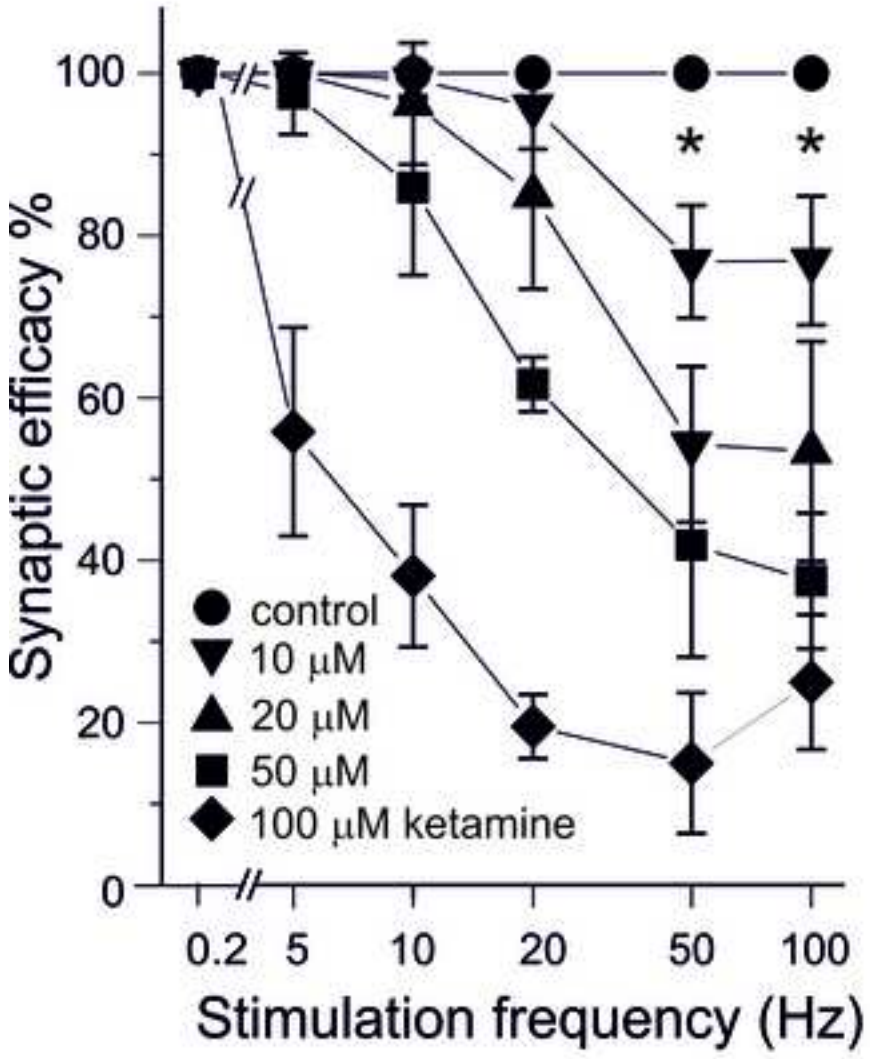

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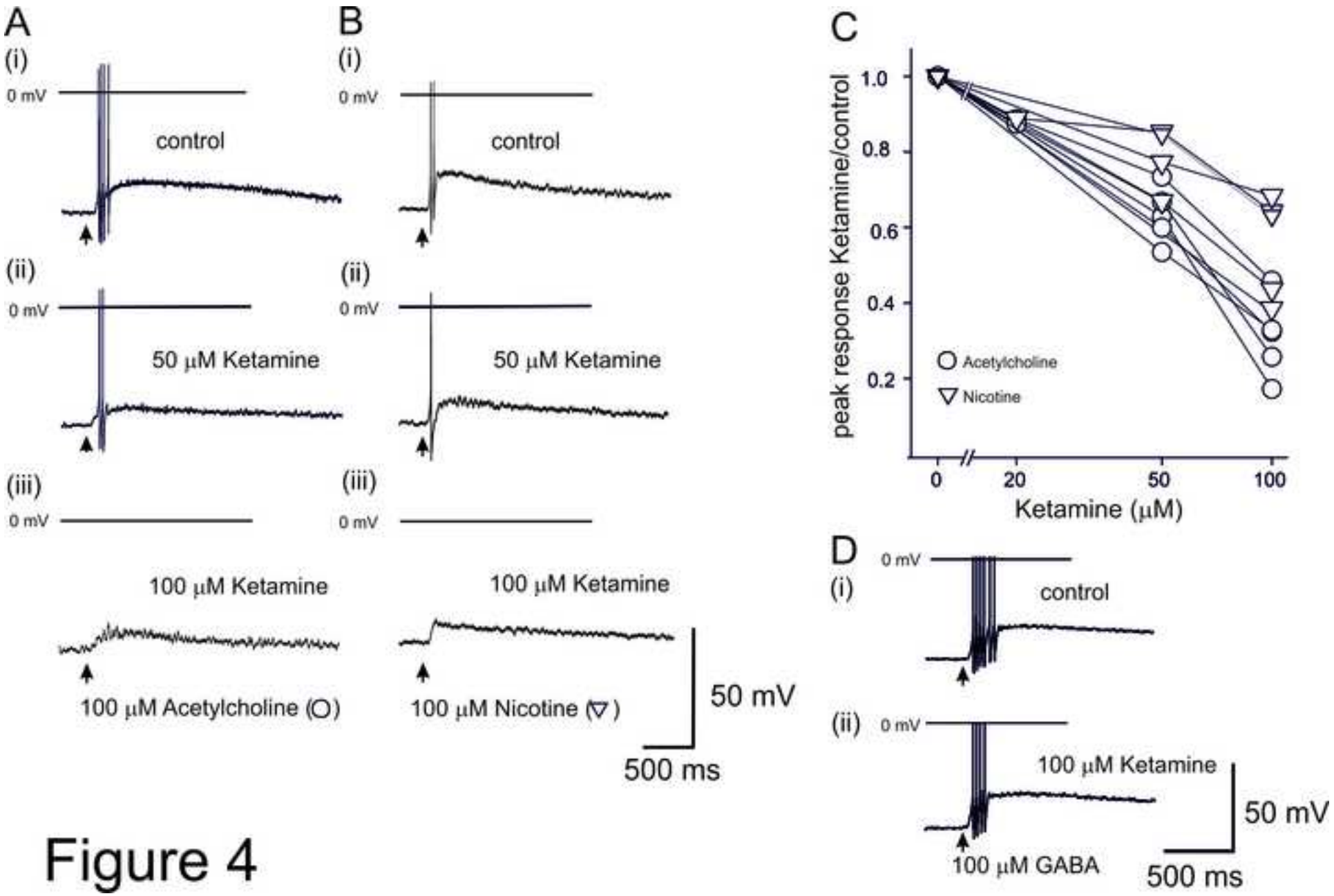

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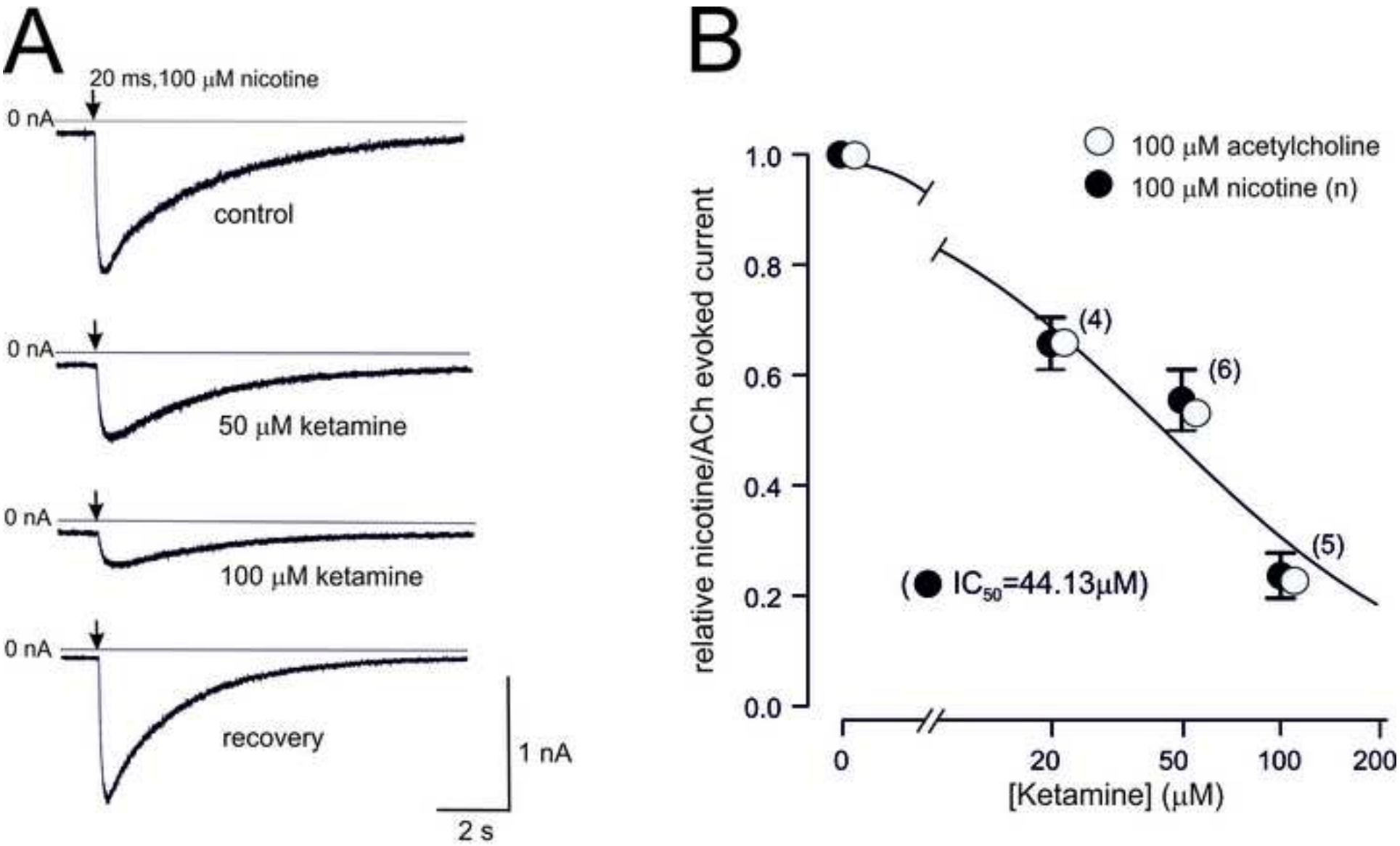

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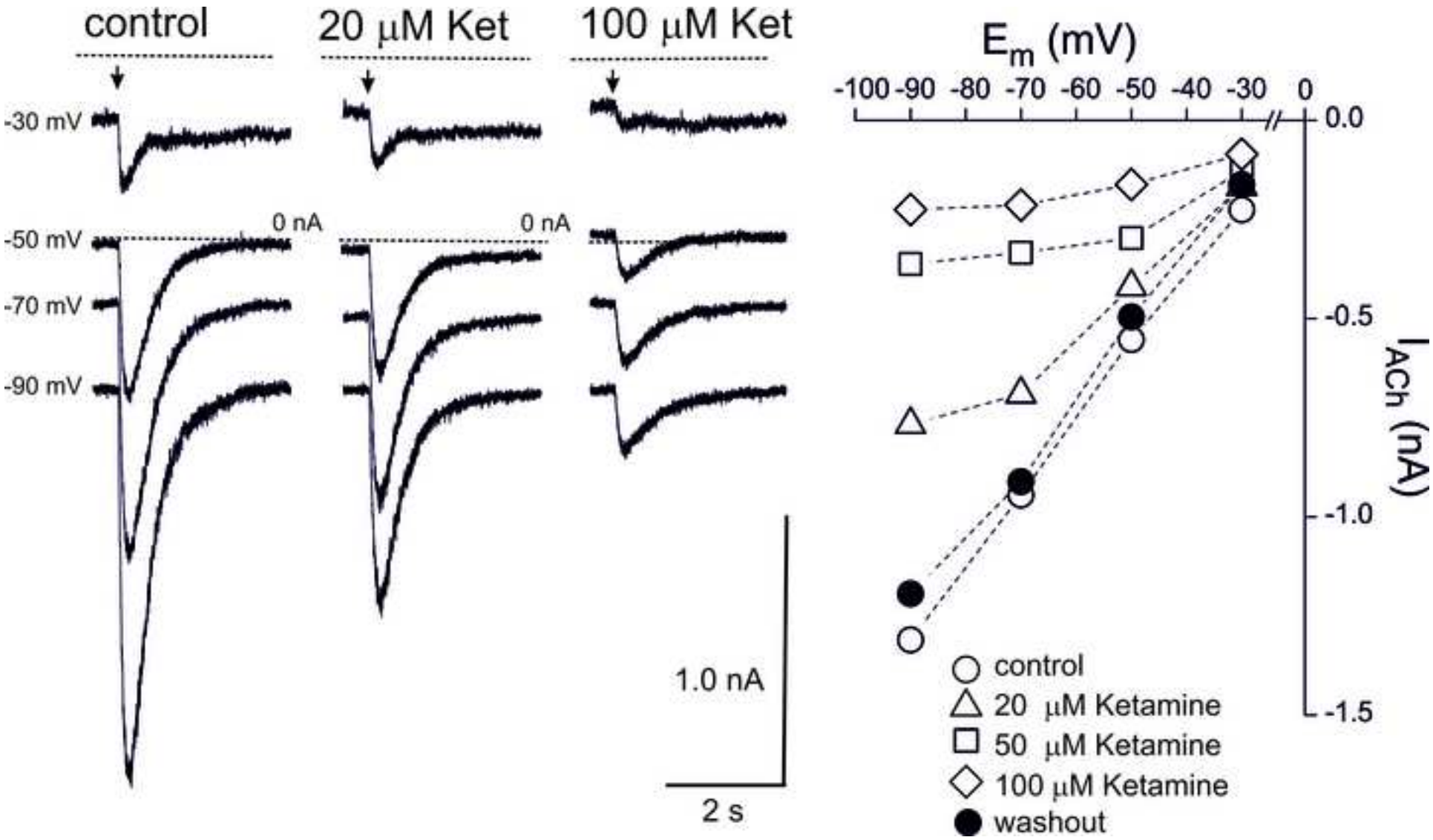

Authors contributions

Performed experiments and analysed the data: A.A.H., K.R., J.R.D., J.R.M., D.J.A.

Conceived and designed the studies, supervised the project and wrote the manuscript: A.A.H., D.J.A.