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The depressor response to intracerebroventricular hypotonic saline is sensitive to TRPV4 antagonist RN1734

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Abstract

Several reports have shown the periventricular region of the brain, including the paraventricular nucleus (PVN) is critical to sensing and responding to changes in plasma osmolality. Further studies also implicate the transient receptor potential ion channel, type V4 (TRPV4) channel in this homeostatic behaviour. In previous work we show that TRPV4 ion channels couple to calcium activated potassium channels in the PVN to decrease action potential firing frequency in response to hypotonicity. In the present study we investigated whether, similarly, intracerebroventricular (ICV) application of hypotonic solutions modulated cardiovascular parameters, and if so whether this was dependent on the TRPV4 channel.

We found that ICV injection of 270mOsmol artificial cerebrospinal fluid (ACSF) decreased mean blood pressure, but not heart rate compared either to naïve mice or mice injected with 300mOsmol ACSF. This effect was abolished by treatment with the TRPV4 inhibitor RN1734.

This data shows that periventricular targets within the brain are capable of generating depressor action in response to TRPV4 ion channel activation and raises the TRPV4 channel, or the TRPV4–KCa coupling mechanism as a potential therapeutic target for treatment of cardiovascular disease.

Keywords: Ion Channel, TRPV4, blood pressure, heart rate, omolality, cell volume.

Introduction

Body fluid osmolality is usually regulated within an extremely narrow range (~290-300 mosmol) (Bourque, 2008). This is largely maintained through regulation of renal function, but control areas exist both within and outside of the central nervous system (CNS). One important reason why animals have evolved to control osmolarity within the CNS is that osmoregulation is a complex process and needs to be integrated with other homeostatic elements. Body systems defend electrolyte composition and osmolarity in parallel with blood pressure and blood volume (Share and Claybaugh, 1972). In laboratory experiments each of these may be differentially regulated, but a healthy functioning animal each must be controlled as part of the overall pattern of homeostasis. Within the brain, the area surrounding the third ventricle within the hypothalamus is particularly important for osmoregulation. Key areas identified to date include the subfornical (SFO), organum vasculosum lamina terminalis (OVLT), circumventricular organs (CVO), medial preoptic (MPO) area and paraventricular nucleus (PVN) of the hypothalamus (Stocker *et al.*, 2007; Toney *et al.*, 2003). Our particular focus has been the PVN since this is an established autonomic control centre exerting influence over heart rate (HR) and blood pressure (BP) in response to a number of homeostatic challenges including temperature (Cham and Badoer, 2008), day-night cycle (Feetham and Barrett-Jolley, 2014), volume load (Lovick *et al.*, 1993) and osmolarity (Stocker *et al.*, 2004a). The PVN is conveniently subdivided into two major areas; the parvocellular and magnocellular “sub-nuclei” (Swanson and Sawchenko, 1983).

The magnocellular region would be a logical site of osmosensation since it contains a high density of neurones that secrete vasopressin (also known as anti-diuretic hormone, ADH) (Swanson and Sawchenko, 1983) from the neurohypophysis (posterior pituitary) which then exert profound effects on vascular contractility and on kidney water reabsorption as each of its two alternative names imply (Share, 1988). The parvocellular region of the PVN is named after the smaller "parvocellular" neurones within. These neurones sub-serve diverse functions. Many release CRF into the hypophyseal portal circulation, which, in turn, evokes release of ACTH from the adenohypophysis (anterior pituitary) and is a key part of the ACTH-adrenal-cortisol axis (Antoni, 1993). Additionally, the parvocellular region of the PVN also contains a number of neurones with autonomic control actions. These latter neurones project to areas such as the intermediolateralis (IML) of the spinal cord and synapse with preganglionic sympathetic neurones (Barrett-Jolley *et al.*, 2000; Motawei *et al.*, 1999; Pyner and Coote, 2000). When activated, these "pre-autonomic" neuroneselevate heart rate, blood pressure and sympathetic nerve activity (SNA) including renal SNA (rSNA) (Womack *et al.*, 2007). Some authors have alternatively concluded that the spinally projecting pre-autonomic PVN neurones are neither parvocellular, nor magnocellular, but a family of intermediately sized neurones they named mediocellular neurones with some merit (Kiss *et al.*, 1991). Under resting conditions the pre-autonomic parvocellular neurones exist under a state of partially tonic inhibition by GABAergic input. Furthermore, application of the $GABA_A$ antagonist bicuculline evokes increases in rSNA, HR and BP (Chen *et al.*, 2003). This tonic inhibition is not absolute, however, since paraventricular application of the $GABA_A$ agonist muscamol produces

powerful inhibition of SNA with associated decreases of HR and BP (Zhang *et al.*, 2002), even in control rats. Toney and colleagues, however, report that this response is more apparent in chronically dehydrated rats where the tonic inhibition of the PVN is reduced (Holbein *et al.*, 2014; Stocker *et al.*, 2004b). This reduced tonic inactivity is due to an additional excitatory input from the MPO, rather than a demonstrable alleviation of the tonic GABA-ergic inhibition (Stocker and Toney, 2005). Thus, dehydration and hypertonicity (direct to hypothalamus or intra-carotid) lead to elevated c-fos expression in pre-autonomic PVN neurones (Arnhold *et al.*, 2007; Gottlieb *et al.*, 2006; Stocker *et al.*, 2004a) and increased spiking activity of hypothalamic neurones (Cross and Green, 1959), glutaminergic activity in medulla-projecting neurones (Stocker *et al.*, 2006) and activity of vasopressin-ergic spinal neurones (Antunes *et al.*, 2006).

The true complexity of the whole-animal osmoregulation begins to emerge, however, when one also considers the cardiovascular responses to consumption of water. Initially this would be expected to increase plasma volume and decrease plasma osmolality. The response to water consumption in people is variable, however, depending on age and health status. In people with autonomic failure, consumption of moderate quantities of water evokes a substantial rise in blood pressure of up to 100mHg (Cariga and Mathias, 2001; Jordan *et al.*, 1999; Lipp *et al.*, 2005). This is absent in young healthy human subjects (Jordan *et al.*, 2000). The detailed pattern of cardiovascular response to water consumption also includes increases in total peripheral resistance, presumably reflecting sympathetic vasomotor activity and decrease in heart rate despite very little overall change in blood pressure (Brown *et al.*, 2005). This decrease in heart rate results from an

increase in cardiac vagal drive (Routledge *et al.*, 2002). Since these effects are seen with water, rather than oral consumption of isotonic saline (Lipp *et al.*, 2005), plasma osmolality is clearly key. However, physiologically this may be an adaptation to rapidly redistribute plasma to the capacitance apparatus (Greenway and Lister, 1974), rather than simply initiating diuresis. From an evolutionary context this would make sense since animals tend to preserve water and ions where possible (Share and Claybaugh, 1972). It appears that in healthy animals, blood volume increase is opposed by increasing (sympathetically driven) vascular tone and blood pressure elevation is then limited by a vagal decrease in heart rate, this is more complex than a simple implementation of the baroreceptor reflex and whilst the site of such integrative control is not known, the hypothalamic PVN is well placed to contribute since it contains both sympathetic and vagal pre-autonomic neurones (Li *et al.*, 2003). The PVN has been shown to be critical to the sympathetic nerve response to isotonic volume expansion (Haselton *et al.*, 1994), but not the baroceptor reflex, which is centred in the medulla (Spyer, 1994). There have been far fewer studies of hypotonic response than those to hypertonic exposure. Intra-carotid application of hypotonic solution shows decrease in SNA, and BP with HR increased (Brooks *et al.*, 2005). However, these responses were seen only in water-deprived animals and little response was seen in euhydrated rats. The early studies of Cross and Green (Cross and Green, 1959) showed a correspondingly limited suppression of action potential activity in the hypothalamus following intra-carotid hypotonic saline. In our own ex vivo “brain-slice” work, we found that a proportion of parvocellular neurones did respond to direct application of hypotonic solutions (Feetham *et al.*, 2014). This would certainly have been expected for magnocellular

neurones, which would be expected to switch off vasopressin release and thus increase diuresis, but was unexpected in parvocellular neurones. In our ex vivo work we established that TRPV4 was a critical element in this osmosensing and this is very much what would be expected from the observations that TRPV4 is expressed in the PVN (Carreno *et al.*, 2009) and that TRPV4$^{-/-}$knock-out leads to animals unable to detect hypo-osmolarity and respond with diuresis (Liedtke and Friedman, 2003; Mizuno *et al.*, 2003).

In the current work therefore, we investigated simply whether direct intra-cerebroventricular application of hypotonic saline to normal rats modulated their cardiovascular system, and if so whether this response was sensitive to the TRPV4 antagonist RN1734.

Methods

Ethics approval

All animal work (CD1 mice) was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. All use of animals and animal tissue was authorised by UK Home Office licence. All surgery was performed under anaesthesia as described in detail below.

Immunohistochemistry

Immunohistochemistry was performed using a rabbit primary antibody for TRPV4 (1:300; Abcam, UK), and goat secondary antibody anti rabbit CY3 (1:300; Abcam, UK). Blue DAPI dye was applied as a nuclear counter-stain, using VECTASHIELD mounting medium with DAPI (Vector laboratories, UK). Negative controls involved primary antibody omission.

Cannulation

Adult CD1 wild-type male mice (30-40g) and were anaesthetised with a combination of urethane and α-chloralose (Sigma-Aldrich, UK), administered at an appropriate dose IP in saline. Urethane was used to minimise the effects on the cardiovascular system (Carruba *et al.*, 1987). Following injection of the anaesthetic, the mice were returned to their cage for several minutes until they lost consciousness. Body temperature was recorded immediately and continuously by rectal probe and maintained at 37±0.5 °C by use of a heat lamp. Once loss of paw-withdrawal and

eye-blink reflexes was achieved the trachea was intubated in order to maintain respiration. The carotid artery was cannulated with stretched PE25 tubing filled with heparinised saline. Blood pressure was recorded by a pressure transducer attached to the tubing and connected to a Neurolog (Digitimer Ltd, Herefordshire, UK) blood pressure amplifier. BP signals were digitised to PC with a CED Micro1401 (Cambridge Electronic Design, Cambridge, UK) using WinEDR at 5 kHz.

Heart rate measurement

Heartbeats were annotated to the amplified AC coupled blood pressure signal using Wabp from the PhysioNet suite of programs to give heart rate of the animals (Goldberger *et al.*, 2000). Briefly, the signal was analysed at 1/10th sampling frequency (ie. 500 Hz), and resampled to 125 Hz for optimal beat detection by Wabp. Annotated beats were then reverted to 10 times speed to give the actual heart rate.

Intracerebroventricular injections

For intracerebroventricular (ICV) injections the anaesthetised mice were placed in a stereotaxic frame adapted for mice (Kopf instruments). Their heads were clamped gently and held in a level position and the skull was exposed. Bregma was located at coordinates 3.8 ± 0.3mm rostral and 5.8± 0.5mm dorsal to intraural line taken from the Paxinos and Franklin adult mouse stereotaxic atlas (Paxinos and Franklin, 2001); a 2mm craniotomy, 1mm lateral and 0.2mm caudal to Bregma allowed for drug or vehicle to be applied via 10µL Hamilton syringe. All drugs were given in ACSF as the vehicle, and injected in a 1µl volume gradually over a 30 second period. All

injections were given into the lateral ventricle at the following coordinates; 0.2mm caudal, 1mm lateral, 3.2mm vertical. The syringe was left at the injection site for 2 minutes and elevated to just above the injection site after this time, where it was kept in place for the duration of the recording. At the end of the procedure all animals were injected with 1% pontamine blue dye (Sigma-Aldrich, UK) at the same injection site using the same volume in order to confirm the correct location for the injection site. Post mortem, the brain removed and sliced to 300µm on a Campden Instruments Ltd 752 M Vibroslice to locate the injection site.

Drug injections

Drugs/ vehicle controls used were; 1µL isotonic/isotonic+DMSO ACSF, hypotonic ACSF (~280 mosmol) and RN1734 (Tocris, UK) in vehicle (ACSF) (100nmol/kg).

Results

It has been suggested TRPV4 may be responsible for volume control centrally (Bourque, 2008).Therefore we began by verifying the presence of the TRPV4 ion channel within the PVN with immunohistochemistry. We detected clear immunoreactivity to the TRPV4 channel within the parvocellular subnucleus of the PVN (Figure 1). Next, we investigate whether simple ICV injection had a confounding effect on cardiovascular parameters. In mice injected ICV with isotonic ACSF plus DMSO 0.01% (~300 mosmol) neither BP nor HR were significantly changed (Figure 2 and Figure 5 n=6; $p>0.05$ by ANOVA using Tukey's post hoc comparison). Next, ICV injections of 1µl ~270 mosmol ACSF were given to investigate the central effects of hypo-osmolality on cardiovascular parameters. Hypotonic ACSF administered centrally resulted in a significant decrease in mean arterial pressure of -9 ± 2 mmHg (n=6; $p<0.01$ by ANOVA using Tukey's post hoc comparison). No change in heart rate was observed (Figure 2 and Figure 5; n=6;

p>0.05 by ANOVA; Figure 3). Reduction in BP upon hypotonic injection was also significant compared to control (isotonic) ICV ACSF injection (Figure 5; -9 ± 2 mmHg vs -2 ± 1 mmHg; n=6; p<0.01 by ANOVA with Tukey's post hoc comparison).

Finally, we investigated whether the depressor action of hypotonic solution was dependent on TRPV4 channels. We tested this using 100 nM/kg of the selective TRPV4 inhibitor RN1734 (Vincent *et al.*, 2009) along with hypotonic ASCF was injected ICV into anaesthetised mice. Addition of the TRPV4 inhibitor prevented the reduction in mean arterial pressure observed with injection of hypotonic ACSF alone (Figure 4 and Figure 5; n=6; p>0.05 by ANOVA using Tukey's post hoc comparison). Again, no difference in heart rate was observed (Figure 4 and Figure 5; n=6; p>0.05 by ANOVA using Tukey's post hoc comparison).

Discussion

In this study we identify a clear depressor action of euhydrated CD1 mice challenged with ICV hypotonic solution. This effect was prevented by treatment with the TRPV4 antagonist RN1734.

Several studies have indicated that the periventricular region of the brain is key to detecting and responding to osmotic challenge (Stocker *et al.*, 2007; Toney *et al.*, 2003), and our own work has demonstrated that, in vitro, PVN neurons can detect and respond to hypotonic solutions with a decrease in action potential firing (Feetham *et al.*, 2014). *In vivo* the complex homeostatic response to PVN challenge with osmotic stimuli is likely to include both vasopressin release and activity of spinally projecting neurones; including interactions between these two pathways via dendritic-dendritic signalling (Stern, 2014).

Our previous report analysed this coupling in fine detail and found that TRPV4 channels initially allow Ca^{2+} entry which in turn activates potassium channels and subsequently inhibits the firing of action potentials (Feetham *et al.*, 2014). This effect is consistent with previous reports that the TRPV4 channel is known to be activated by osmolality changes (Liedtke *et al.*, 2000) and has a role in volume control in other tissues (Becker *et al.*, 2005; Benfenati *et al.*, 2011; Guilak *et al.*, 2010). Our combination of isolated neuron and brain slice experiments suggested that this is a direct effect within PVN parvocellular neurons rather than an indirect modulation of PVN projecting neurons. The latter, however, remains a possibility since spinally projecting PVN neurons receive functional inputs from a number of other hypothalamic nuclei (Cui *et al.*, 2001; Stocker and Toney, 2005; Womack and Barrett-Jolley, 2007). This region of the PVN includes spinally projecting neurons which positively modulate the cardiovascular system (Coote, 2007) and so we hypothesised that such inhibition may result in a depressor action and reduction in HR. In the present study we find that whilst ICV hypotonic solutions do reduce BP, they have little effect on heart rate. Furthermore, the nature of this injection site means that it is not possible to know if the target neurons are in the PVN or some other peri-ventricular site. It is clear however that this effect involves TRPV4 channels, since the selective inhibitor RN1734 (Vincent *et al.*, 2009) abolished the effect. It is also important to note that it was necessary to conduct this study in anaesthetised animals, which may have affected the response to hypotonicity. Anaesthetics do affect the cardiovascular system; for example, by reducing resting BP which is quite low in this study compared to what one may expect in a conscious

animal study, but in line to that recorded previously for wildtype mice (Nunn *et al.*, 2013).

These data support previous reports that changes in central osmolality results in the modulation of blood pressure (Brooks *et al.*, 2005; Scrogin *et al.*, 1999). In previous studies altered heart rate has also been recorded upon osmotic change (Chen and Toney, 2001); however, our results show no statistically significant differences. This is not completely unexpected due to the baroreceptor reflex, which would be working to counteract the reduction in blood pressure (Spyer, 1994). Together, this and our previous data strongly support the notion that there is a role for central TRPV4 channels in sensing osmolality changes and initiating changes in blood pressure. Although the exact position of these channels centrally is unknown, they could be within the PVN itself. Further, perhaps identified spinally projecting neurone work, will be required to establish this.

Potentially, pharmacological modulation of blood pressure via TRPV4 channels could be useful in the treatment of cardiovascular disease; however, the widespread distribution of these ion channels could limit their practical usefulness. Therefore future studies will be aimed at identifying the receptor and neurotransmitter profile of PVN osmosensing neurones to determine if we can identify more specific therapeutic targets.

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Abbreviations

ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
Ang II	angiotensin II
CRF	corticrotrophin releasing factor
DMSO	dimethyl sulfoxide
GABA	γ-aminobutyric acid
ICV	intracerebroventricular
IML	intermediolateralis
IP	intraperitoneal
K_{Ca}	Ca^{2+} activated K^+ channel
KO	knock out

MPO	Medial Preoptic Area
NMDA	N-methyl-D-aspartate receptor
NO	nitric oxide
NOS	nitric oxide synthase
PVN	paraventricular nucleus
RSNA	renal sympathetic nervous activity
SCN	suprachiastmatic nucleus
SFO	subfornical organ
SNA	sympathetic nervous activity
SNS	sympathetic nervous system
TRP	transient receptor potential
TRPV	transient receptor potential vanilloid

Figure Legends

Figure 1. Immunofluorescent identification of TRPV4 in the paraventricular nucleus.

Rat PVN coronal slice labelled with antibody to TRPV4**. (A)** Negative control showing DAPI blue, with the absence of red TRPV4 staining **(B)** Red staining is positive for the TRPV4 channel, blue is DAPI nuclei staining. Scale bar 100µm and 3V indicates the 3rd ventricle. **(C)** High magnification images of a section seen in (B). Red staining is TRPV4 and blue is DAPI nuclei staining; arrows indicate where overlap can be seen. Scale bar is 25 µm.

Figure 2. Intracerebroventricular injection of isotonic ASCF has no effect on cardiovascular parameters.

Adult male CD1 mice were anaesthetised with urethane-chloralose, and blood pressure was recorded by cannulation of the carotid artery. **(A)** Raw blood pressure trace with annotated beats (purple lines), before (i) and after (ii) injection of 300mOsm ACSF/DMSO vehicle. Annotated beats are used to derive R-R interval and heart rate. **(B)** Example R-R interval trace shows no difference before (i) and after (ii) ICV injection of isotonic ACSF. **(C)** Example heart rate trace shows no difference before (i) and after (ii) injection of isotonic ACSF. **(D)** Average blood pressure and **(E)** heart rate do not change with injection of isotonic vehicle (n=6; $p>0.05$).

Figure 3. Intracerebroventricular injection of hypotonic ASCF decreases blood pressure but has no effect on heart rate.

Blood pressure significantly decreases after injection of hypotonic ACSF. **(A)** Raw blood pressure trace with annotated beats (purple lines), before (i) and after (ii) injection. Annotated beats are used to derive R-R interval and heart rate. **(B)** Example R-R interval trace shows no difference before (i) and after (ii) ICV injection of hypotonic ACSF. **(C)** Example heart rate trace shows no difference before (i) and after (ii) injection of hypotonic ACSF. **(D)** Average blood pressure is significantly reduced with injection of hypotonic ASCF (n=6; *$p<0.01$), but heart rate **(E)** remains unchanged ($p>0.05$).

Figure 4. Intracerebroventricular injection of the TRPV4 inhibitor RN1734 prevents the effect of hypotonic ACSF on blood pressure.

(A) Raw blood pressure trace with annotated beats (purple lines), before (i) and after (ii) injection. Annotated beats are used to derive R-R interval and heart rate. **(B)** Example R-R interval trace shows no difference before (i) and after (ii) ICV injection. **(C)** Example heart rate trace shows no difference before (i) and after (ii) ICV injection. **(D)** Average blood pressure response to hypotonic ASCF is prevented by injection of RN1734 (n=6; $p>0.05$). **(E)** Average heart rate remains unchanged (n=6; $p>0.05$).

Figure 5. Summary average changes in cardiovascular parameters from ICV injections.

(A) Average change in blood pressure compared to control of several ICV injection treatments. No significant change was seen with vehicle (isotonic ACSF) or the TRPV4 inhibitor, RN1734 (100 nM/kg) alone *vs* control (n=6; $p>0.05$). Blood pressure is significantly reduced in animals injected with hypotonic ACSF compared to those injected with vehicle (n=6; $*p<0.01$). ICV injections with RN1743 + hypotonic ACSF did not produce a significant blood pressure change compared to vehicle injections (n=6; $p>0.05$), but blood pressure change was significantly reduced compared to hypotonic injections (n=6; $\#p<0.01$). **(B)** Average heart rate did not change significantly between any of the conditions stated (n=6; $p>0.05$ by ANOVA).

Figure 1.TIF

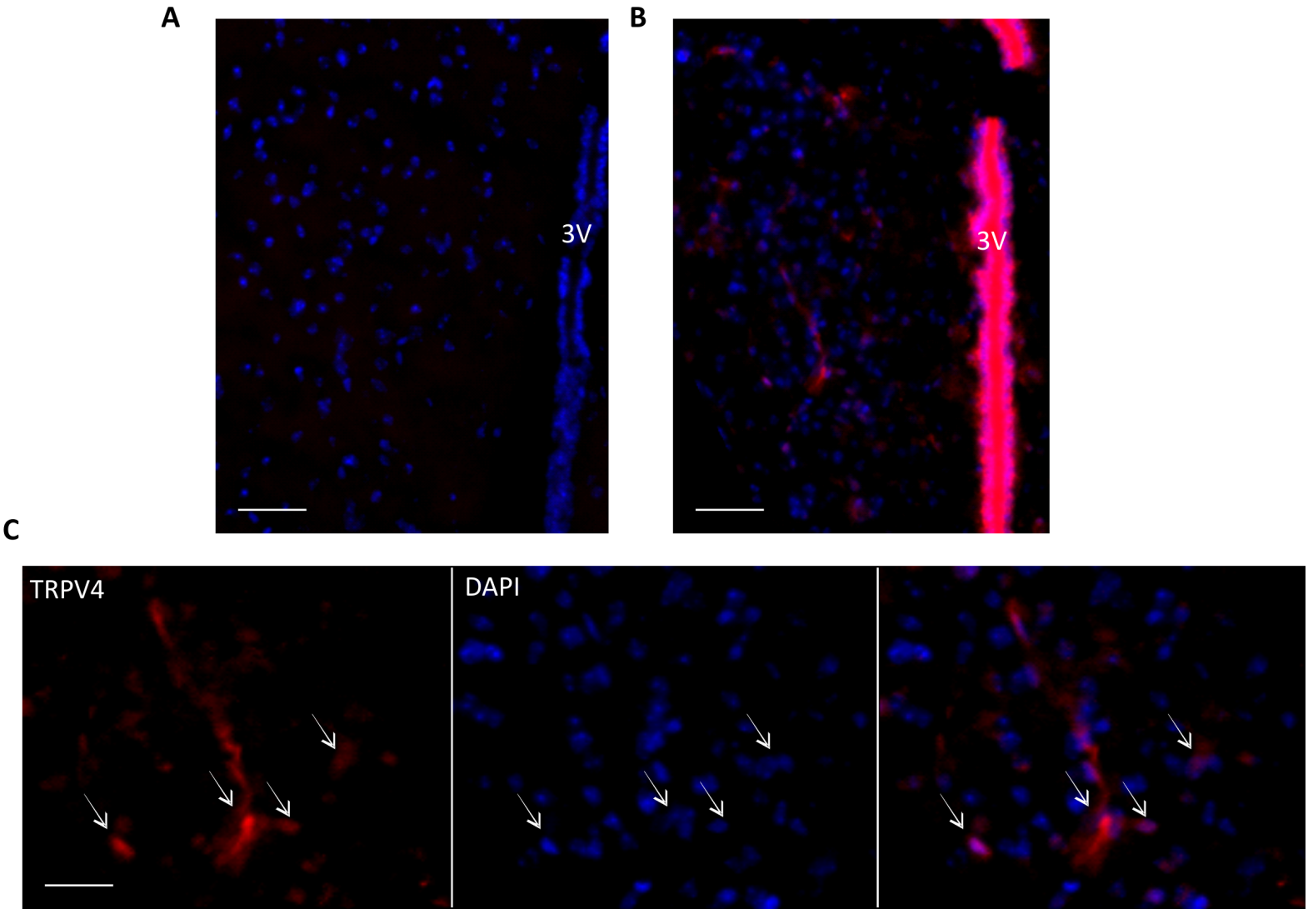


Figure 2.TIF

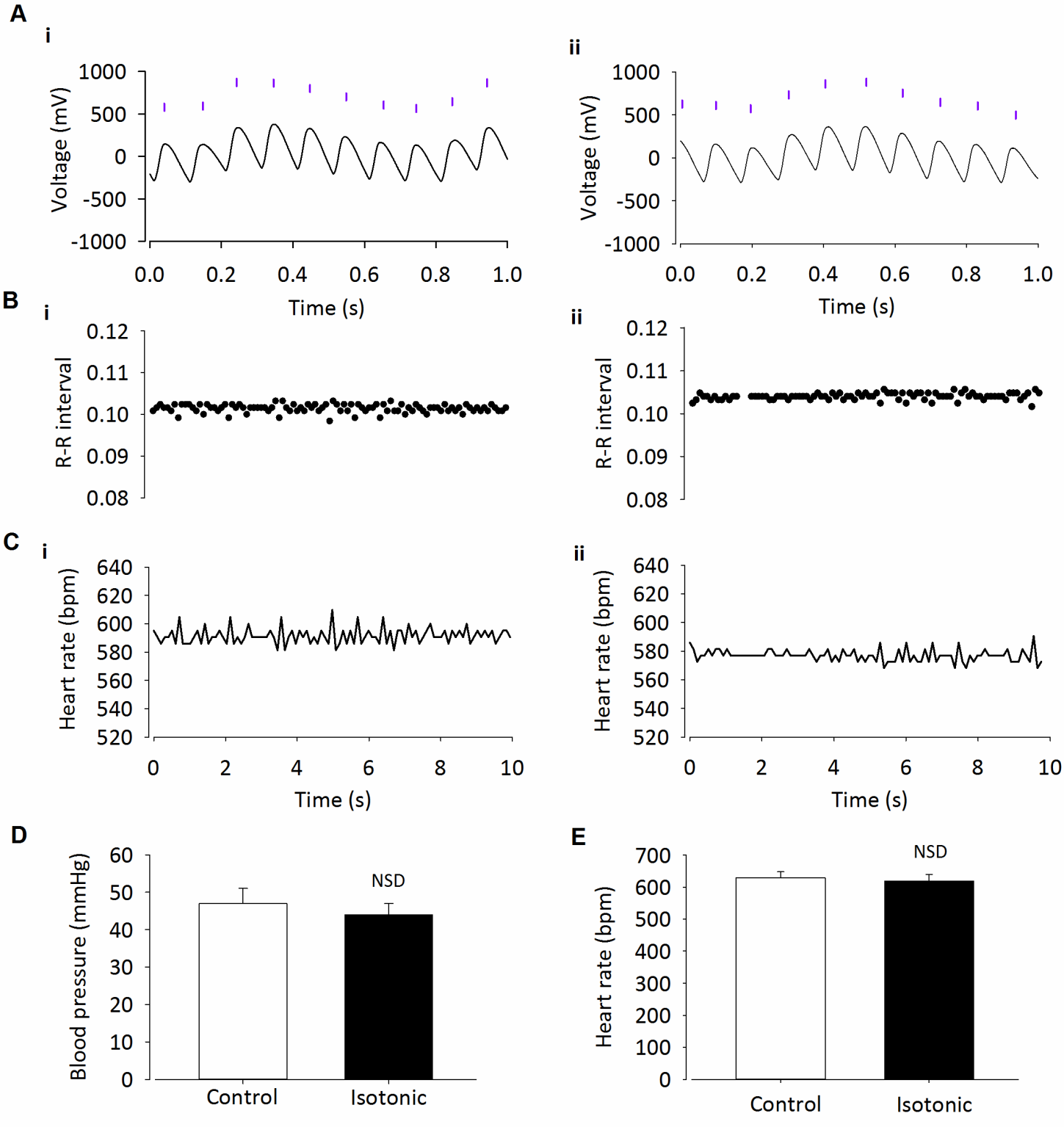

A
i
ii
Voltage (mV)
1000
500
0
-500
-1000
0.0
0.2
0.4
0.6
0.8
1.0
Time (s)
B
R-R interval
0.12
0.11
0.10
0.09
0.08
C
Heart rate (bpm)
640
620
600
580
560
540
520
0
2
4
6
8
10
D
Blood pressure (mmHg)
60
50
40
30
20
10
0
NSD
Control
Isotonic
E
Heart rate (bpm)
700
600
500
400
300
200
100
0
NSD
Control
Isotonic

Figure 3.TIF

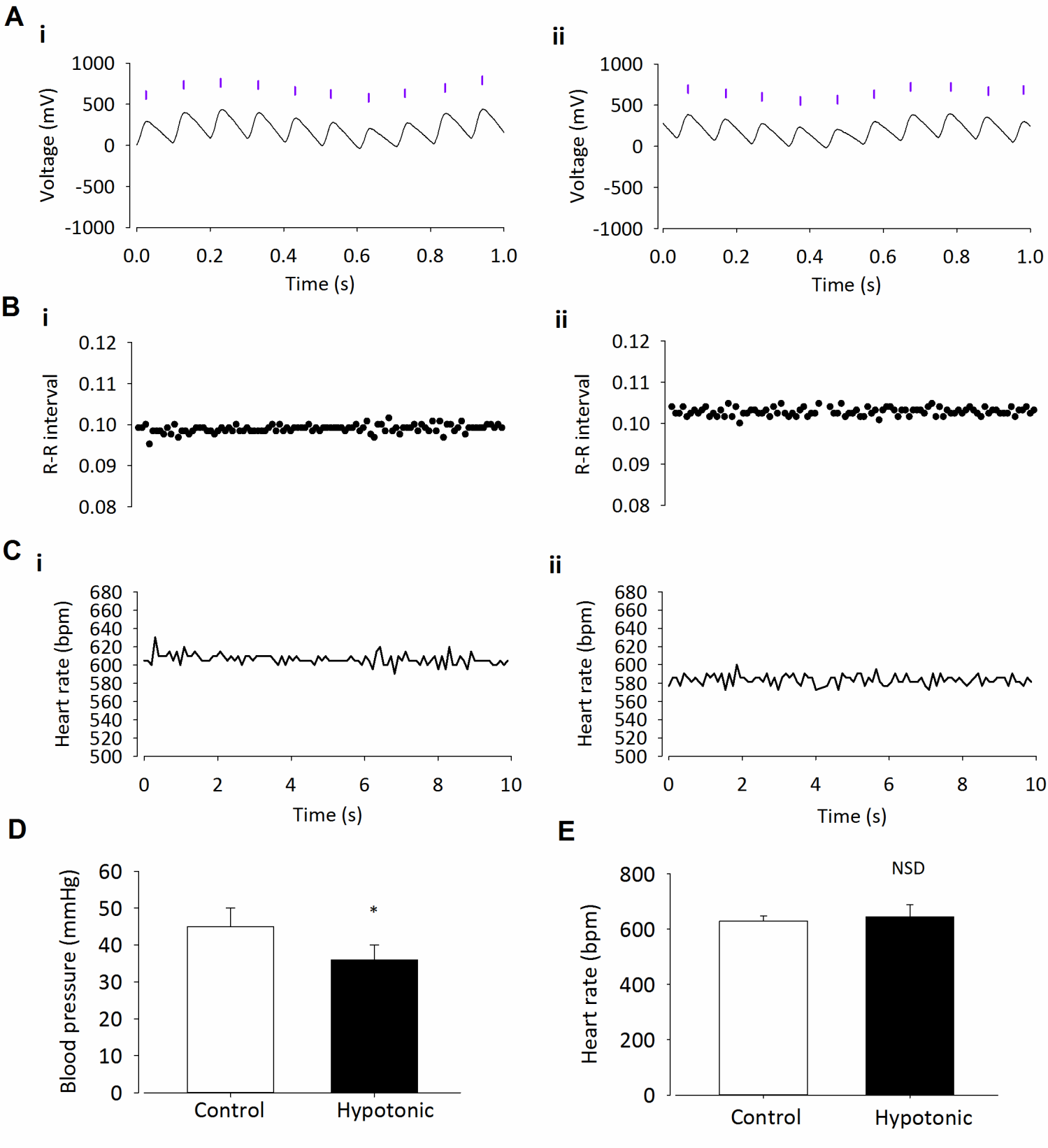

A
i
ii
Voltage (mV)
1000
500
0
-500
-1000
0.0
0.2
0.4
0.6
0.8
1.0
Time (s)
B
R-R interval
0.12
0.11
0.10
0.09
0.08
C
Heart rate (bpm)
680
660
640
620
600
580
560
540
520
500
0
2
4
6
8
10
D
Blood pressure (mmHg)
60
50
40
30
20
10
0
*
Control
Hypotonic
E
NSD
800
600
400
200
0

Figure 4.TIF

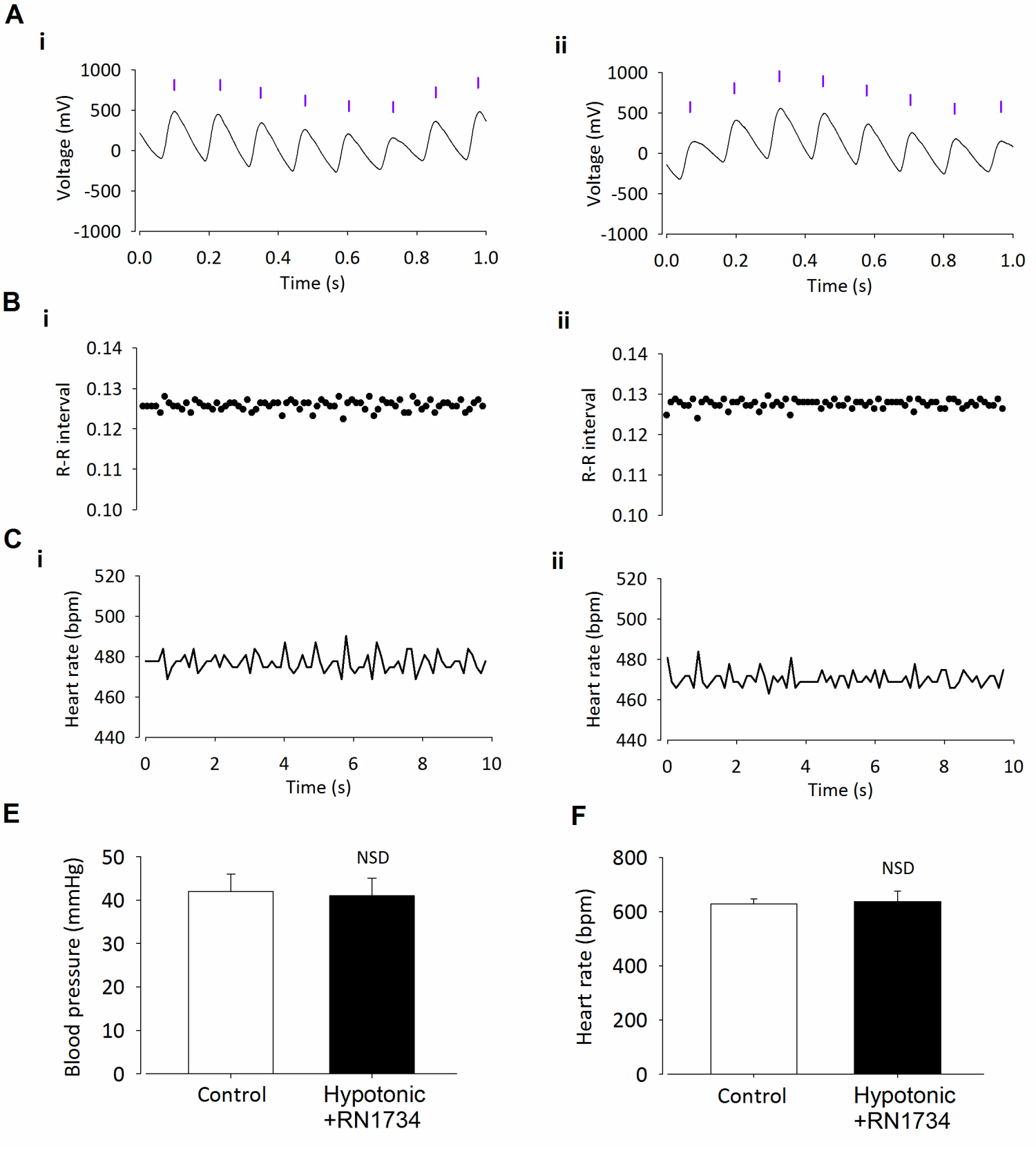
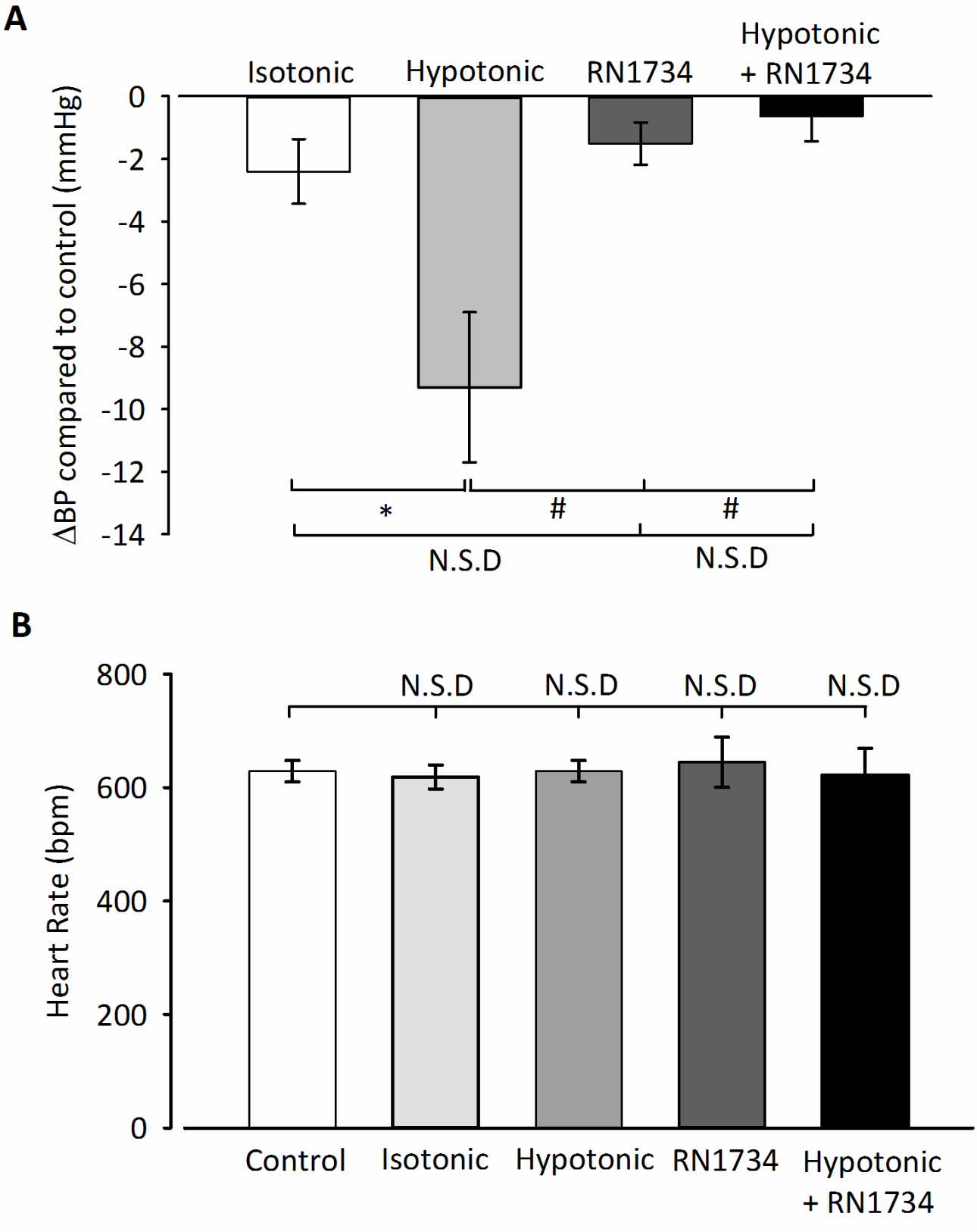

A
i
ii
Voltage (mV)
Time (s)
B
R-R interval
C
Heart rate (bpm)
E
Blood pressure (mmHg)
NSD
Control
Hypotonic
+RN1734
F
Heart rate (bpm)
NSD
Control
Hypotonic
+RN1734

Figure 5.TIF


A
Isotonic
Hypotonic
RN1734
Hypotonic + RN1734
0
-2
-4
-6
-8
-10
-12
-14
ΔBP compared to control (mmHg)
*
#
#
N.S.D
N.S.D
B
800
600
400
200
0
Heart Rate (bpm)
N.S.D
N.S.D
N.S.D
N.S.D
Control
Isotonic
Hypotonic
RN1734
Hypotonic + RN1734