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Stepan Kortus, Chinnapaiyan Srinivasan, Oksana Forostyak, Martin Zapotocky, Yoichi Ueta, et al.. Sodium-calcium exchanger and R-type  $\text{Ca}^{2+}$  channels mediate spontaneous  $[\text{Ca}^{2+}]_i$  oscillations in magnocellular neurones of the rat supraoptic nucleus. *Cell Calcium*, 2016, 59 (6), pp.289-298. 10.1016/j.ceca.2016.03.010 . hal-02000181

**HAL Id: hal-02000181**

**<https://hal.umontpellier.fr/hal-02000181>**

Submitted on 21 Jun 2022

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# Sodium-calcium exchanger and R-type Ca<sup>2+</sup> channels mediate spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations in magnocellular neurones of the rat supraoptic nucleus

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## ARTICLE INFO

### Article history:

Received 1 March 2016

Received in revised form 30 March 2016

Accepted 30 March 2016

Available online 31 March 2016

### Keywords:

Ca<sup>2+</sup> signalling

Voltage-gated Ca<sup>2+</sup> channels

Ca<sup>2+</sup> homeostasis

Ca<sup>2+</sup> imaging

Ca<sup>2+</sup> oscillations

Ca<sup>2+</sup> clearance

Transgenic rats

Vasopressin

Oxytocin

## ABSTRACT

Isolated supraoptic neurones generate spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations in isolated conditions. Here we report in depth analysis of the contribution of plasmalemmal ion channels (Ca<sup>2+</sup>, Na<sup>+</sup>), Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX), intracellular Ca<sup>2+</sup> release channels (InsP<sub>3</sub>Rs and RyRs), Ca<sup>2+</sup> storage organelles, plasma membrane Ca<sup>2+</sup> pump and intracellular signal transduction cascades into spontaneous Ca<sup>2+</sup> activity. While removal of extracellular Ca<sup>2+</sup> or incubation with non-specific voltage-gated Ca<sup>2+</sup> channel (VGCC) blocker Cd<sup>2+</sup> suppressed the oscillations, neither Ni<sup>2+</sup> nor TTA-P2, the T-type VGCC blockers, had an effect. Inhibitors of VGCC nifedipine, ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA (for L-, N-, P and P/Q-type channels, respectively) did not affect [Ca<sup>2+</sup>]<sub>i</sub> oscillations. In contrast, a specific R-type VGCC blocker SNX-482 attenuated [Ca<sup>2+</sup>]<sub>i</sub> oscillations. Incubation with TTX had no effect, whereas removal of the extracellular Na<sup>+</sup> or application of an inhibitor of the reverse operation mode of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger KB-R7943 blocked the oscillations. The mitochondrial uncoupler CCCP irreversibly blocked spontaneous [Ca<sup>2+</sup>]<sub>i</sub> activity. Exposure of neurones to Ca<sup>2+</sup> mobilisers (thapsigargin, cyclopiazonic acid, caffeine and ryanodine); 4-aminopyridine (A-type K<sup>+</sup> current blocker); phospholipase C and adenylyl cyclase pathways blockers U-73122, Rp-cAMP, SQ-22536 and H-89 had no effect. Oscillations were blocked by GABA, but not by glutamate, apamin or dynorphin. In conclusion, spontaneous oscillations in magnocellular neurones

**Abbreviations:** AVP, arginine vasopressin; OT, oxytocin; SON, supraoptic nucleus; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; SERCA, sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase; VGCC, voltage-gated Ca<sup>2+</sup> channels; EGTA, ethylene glycol (bis-aminoethyl ether)-N,N,N',N'-tetra acetic acid; TTX, tetrodotoxin; PMCA, plasmatic-Ca<sup>2+</sup>-ATPase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; 4-AP, 4-aminopyridine; PACAP, pituitary adenylyl cyclase activating polypeptide; NCX, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; GABA, gamma-aminobutyric acid; AHP, after hyperpolarisation; DAP, depolarizing after potential; InsP<sub>3</sub>, 1,4,5-trisphosphate; DMSO, dimethyl sulfoxide; eGFP, enhanced green fluorescent protein; mRFP, monomeric red fluorescent protein; NMDG-Cl, N-methyl-d-glutamine; CPA, cyclopiazonic acid; ER, endoplasmic reticulum.

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Hypothalamus  
 Supraoptic nucleus  
 Na<sup>+</sup>/Ca<sup>2+</sup> exchanger  
 Mitochondria  
 Plasma membrane calcium pump  
 Sarcoplasmic reticulum  
 Ca<sup>2+</sup>-ATPase  
 GABA  
 Glutamate  
 1,4,5-Trisphosphate  
 Tetrodotoxin  
 Ca<sup>2+</sup> channel toxins

are mediated by a concerted action of R-type Ca<sup>2+</sup> channels and the NCX fluctuating between forward and reverse modes.

## 1. Introduction

Neurosecretory oxytocin (OT) and arginine-vasopressin (AVP) magnocellular neurones in the rat supraoptic nucleus (SON) project their axons to neurohypophysis where they secrete AVP and OT into the bloodstream in response to physiological stimulation: this secretion is also modulated by central mechanisms within the SON [1]. Circulating AVP regulates water reabsorption in kidney and instigates vasoconstriction to increase blood pressure. OT is released as a part of the milk-ejection reflex and during parturition when it induces uterine contraction to facilitate labour [2,3]. Apart from their peripheral actions, both OT and AVP are also secreted within the SON from dendrites [4,5]; they stimulate OT and AVP receptors expressed in magnocellular neurones activating the intracellular signalling pathway that causes the increase of cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) [6]. In OT neurones, this increase in [Ca<sup>2+</sup>]<sub>i</sub> resulted from Ca<sup>2+</sup> release from intracellular stores [7], whereas in AVP neurones, the [Ca<sup>2+</sup>]<sub>i</sub> rise reflects both the Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels (VGCCs) and Ca<sup>2+</sup> mobilisation from intracellular stores [1,8]. These responses are mediated via the activation of specific AVP and OT receptors [9,10] and multiple intracellular transduction signals [11].

The release of OT and AVP from terminals in neurohypophysis and from dendrites in the hypothalamus occurs strictly by Ca<sup>2+</sup>-regulated exocytosis [12]. In terminals the [Ca<sup>2+</sup>]<sub>i</sub> elevation is triggered by action potential arriving from the SON neurones. In the SON, any agent that increases [Ca<sup>2+</sup>]<sub>i</sub> (by intracellular Ca<sup>2+</sup> release or plasmalemmal Ca<sup>2+</sup> entry) evokes the release of hormone from dendrites without increasing the electrical activity of the cell body, and without inducing secretion from the nerve terminals [4,13]. Both AVP and OT neurones exhibit specific intrinsic electrical activities. AVP neurones exhibit 'phasic' firing activity with intervals, whereas OT neurones fire high frequency synchronized bursts of action potentials without intervals; these bursts are associated with suckling-induced milk ejection [2]. These characteristic electrical patterns are crucial for the efficient release of AVP and OT at the neurohypophysis [14]. Both cell types exhibit Ca<sup>2+</sup>-dependent after-hyperpolarisations (AHPs), including an apamin-sensitive, medium duration AHP and a slower, apamin-insensitive AHP (sAHP) [2]. AVP neuronal excitability is also influenced by slow (sDAP) and fast (fDAP) depolarising after potentials that underlie phasic bursting activity [15].

Freshly isolated SON neurones exhibited spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations [7,16]. Experiments on transgenic animals showed that these [Ca<sup>2+</sup>]<sub>i</sub> oscillations exist in both AVP and OT neurones [1,17] yet mechanisms underlying these oscillations remain unknown; similarly unknown is the effect of [Ca<sup>2+</sup>]<sub>i</sub> oscillations on the release of AVP and OT [17]. Spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations, similar to those exhibited in freshly isolated SON neurones may be the key element in the regulation of hormone release. In general, two types of [Ca<sup>2+</sup>]<sub>i</sub> oscillations can be recognised, depending on the main source of Ca<sup>2+</sup>, i.e., release from intracel-

lular stores such as endoplasmic reticulum (ER) or plasmalemmal Ca<sup>2+</sup> entry [18]. The ER-driven [Ca<sup>2+</sup>]<sub>i</sub> oscillations result from the periodical release of Ca<sup>2+</sup> mediated by the inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) receptor or the ryanodine receptor/Ca<sup>2+</sup> release channel, whereas the extracellular Ca<sup>2+</sup> driven oscillations require Ca<sup>2+</sup> entry through plasmalemmal channels or exchangers. Cellular Ca<sup>2+</sup> and electrical properties of the SON neurones are closely and mutually associated although they can operate independently. In this study, we analysed spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations using a battery of specific pharmacological tools and propose the possible mechanism driving these oscillations.

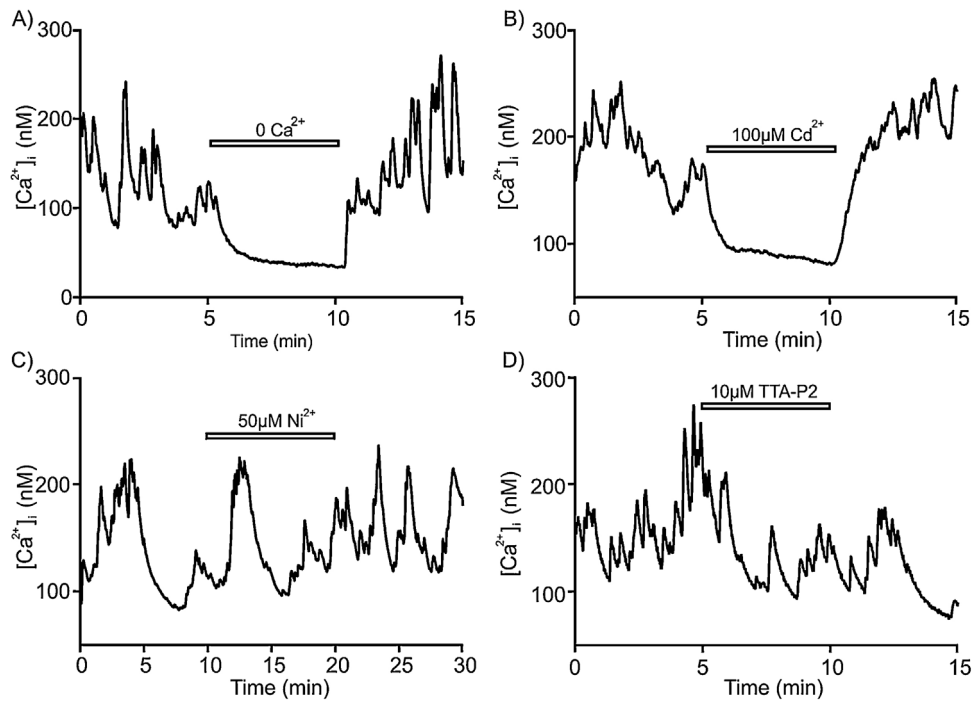
## 2. Materials and methods

### 2.1. Animals and experimental procedures

Adult male Wistar rats (wild type) and homozygous transgenic male rats carrying a fluorescent reporter for AVP (AVP-eGFP), or OT (OT-mRFP), or double transgenic rats simultaneously expressing reporters for both AVP-eGFP and OT-mRFP, were used as described in detail in earlier work [17,19,20]. All animals (weighting 150–300 g; 4–8 weeks old) were bred and housed at 22–23°C, 12:12 h light/dark cycle (lights on 07:00–19:00 h), with food and drinking water available *ad libitum*. The animals were sacrificed by decapitation after anaesthesia with 5% isoflurane for 5 min, the brain was rapidly removed and the SON was dissected. All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethics Committee of the Institute of Experimental Medicine, AS CR, Prague, Czech Republic (license #CZ 205/2010-Revised in 2013).

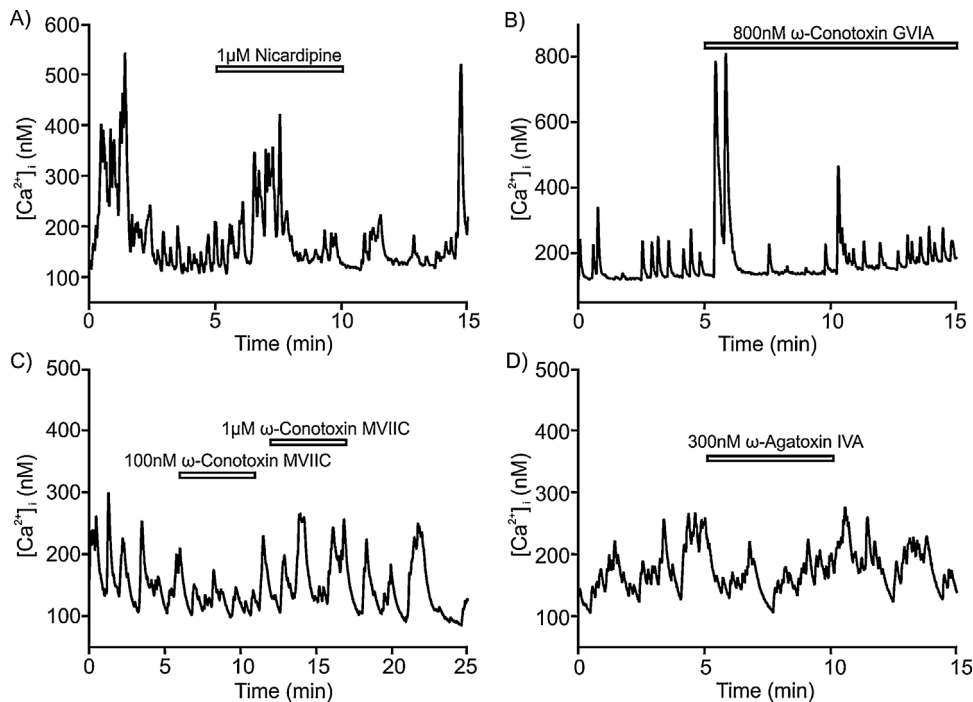
### 2.2. Isolation of supraoptic neurones

SON neurones were acutely dissociated by enzymatic and mechanical treatments of the tissues as described previously [6,7] with modifications [12,21]. In brief, the blocks (1 mm × 0.5 mm × 0.5 mm) of SON tissues were dissected and enzymatically dissociated by incubation for 30 min in oxygenated HEPES-buffered normal Locke's solution (NL; in mM: 140 NaCl, 5 KCl, 2CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, 10HEPES, pH was adjusted to 7.25 with tris; the osmolarity was 298–300 mosm l<sup>-1</sup>; temperature 37°C) supplemented with 1 mg/ml deoxyribonuclease I, 0.5 mg/ml proteases X, and 0.5 mg/ml protease XIV. After incubation, tissues were washed with NL and triturated gently using a Gilson-Pipetman (1 ml) with a polypropylene white pipette-tip to isolate the SON cells. The cells were plated onto 22 mm glass Petri dishes (WillCo Wells-Amsterdam, The Netherlands). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, USA) or from Alomone labs (Jerusalem, Israel). Fura-



**Fig. 1.** Effect of external  $\text{Ca}^{2+}$  removal and VGCC blockers on  $[\text{Ca}^{2+}]_i$  oscillations.

A: A typical  $[\text{Ca}^{2+}]_i$  oscillation observed in a neurone before and after removal of external  $\text{Ca}^{2+}$ . B: Inhibition of spontaneous  $[\text{Ca}^{2+}]_i$  oscillations in the presence of  $\text{Cd}^{2+}$ . C,D: Exposure of SON neurones to T-type VGCCs inhibitors  $\text{Ni}^{2+}$  and TTA-P2 fail to affect spontaneous  $[\text{Ca}^{2+}]_i$  oscillations.



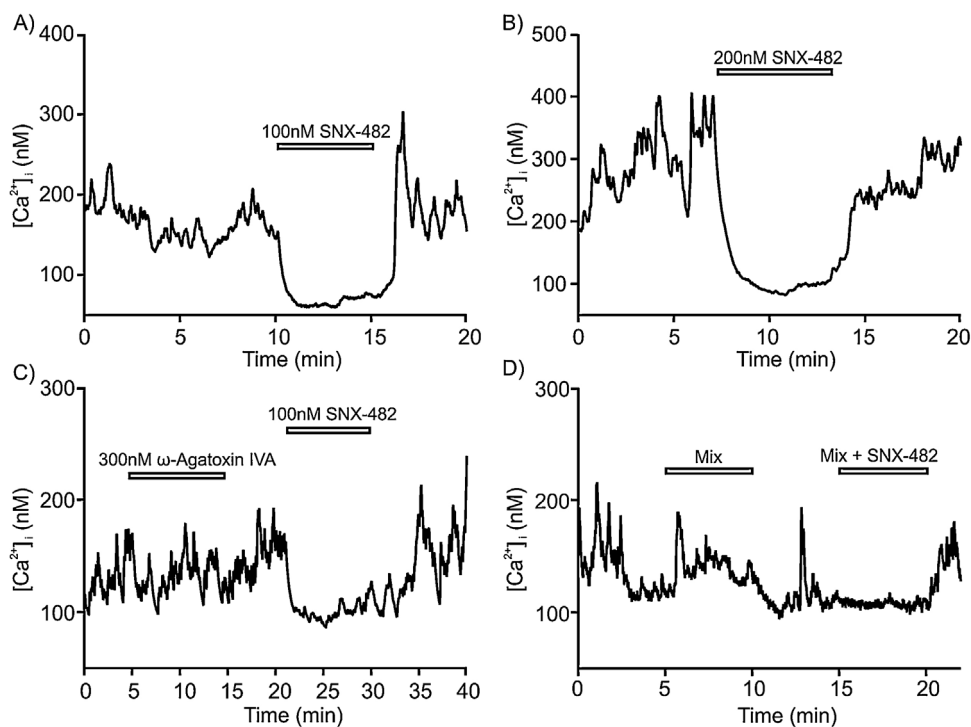
**Fig. 2.** Effect of specific HVGCC blockers on  $[\text{Ca}^{2+}]_i$  oscillations.

The representative traces showing the oscillations from individual neurones in the presence of different HVGCC blockers: nicardipine ( $1 \mu\text{M}$ ) for L-type (A);  $\omega$ -conotoxin GVIA ( $800 \text{ nM}$ ) for N-type (B); two different concentrations of  $\omega$ -conotoxin MVIIC for P-type (C) and  $\omega$ -agatoxin ( $300 \text{ nM}$ ) for P/Q-type (D). Note that none of the blockers affected the oscillations.

2-AM, TTA-P2, nicardipine, KB-R7943, thapsigargin, CPA, CCCP, ryanodine and 4AP were dissolved in DMSO. TTX and apamin were dissolved in acetic acid as suggested by the suppliers. Other drugs were dissolved in total ion-free distilled  $\text{H}_2\text{O}$  (EMD Millipore Corporation, Germany) and all stock solutions and buffers were prepared using this total-ion-free  $\text{H}_2\text{O}$ .

### 2.3. $[\text{Ca}^{2+}]_i$ measurements and drug applications

The  $[\text{Ca}^{2+}]_i$  measurements using a fluorescent probe Fura-2 AM were performed on isolated single neurones from WT [7,21,22] or from transgenic (AVP-eGFP-positive and OT-mRFP-positive) rats as described previously [17,19,21,23]. Video imaging of  $[\text{Ca}^{2+}]_i$  was



**Fig. 3.** Effect of R-type VGCC blocker on  $[Ca^{2+}]_i$  oscillations.

Traces A and B show  $[Ca^{2+}]_i$  oscillations from individual neurones recorded in the presence of 100 nM and 200 nM SNX-482, a specific blocker of R-type channels, respectively. The trace C shows the specific block of oscillations by SNX-482 in comparison to no effect of  $\omega$ -agatoxin IVA. Trace D shows  $[Ca^{2+}]_i$  oscillations from a neurone that was first exposed to the mixture of blockers of  $Ca^{2+}$  channels (L-, N-, P/Q-type) followed by the addition of SNX-482.

performed using an inverted microscope AxioObserver D1 (Zeiss) equipped with a CCD camera and Lambda-DG4 fast rotating wheel illumination system (Sutter Instrument, Novato, USA) for double excitation at 340 and 380 nm. The fluorescence intensity of the Fura-2 emission was measured at 510 nm as a ratio of signals obtained after excitation at 340 and 380 nm. The microscope was also equipped with GFP and RFP filters for observing cells bearing fluorescent markers. To estimate the range of change in absolute  $[Ca^{2+}]_i$  in nM a calibration following the Grynkiewicz method [24] was performed on a few neurones. The calibration produced  $R_{\min} = 0.2$ ,  $R_{\max} = 7.2$ ,  $f_{340\max}/f_{380\min} = 7.7$ , dissociation constant for Fura-2 at  $37^\circ C K_d = 224$  nM. An estimation of  $[Ca^{2+}]_i$  was then determined from the  $f_{340}/f_{380}$  ratio using the Grynkiewicz equation [24].

Cells were continuously perfused with NL solution at  $37^\circ C$ . In some experiments the  $CaCl_2$  concentration in the NL was 200 nM or 500 nM. In experiments where extracellular  $Ca^{2+}$  was removed, EGTA (100 nM) was added to NL with no added  $CaCl_2$ . To obtain  $Na^+$  free solutions the NaCl was replaced with NMDG-Cl and osmolarity adjusted to 295–300 mOsmol/l. Solutions were exchanged using a multiple capillary perfusion system, as described previously [25,26], with appropriate modifications [12,21,27] using a computer controlled multichannel peristaltic pump (REGLO ICC, Ismatec, Germany). Neurones with minimal diameter size of 10  $\mu m$  and persisted dendrites were randomly chosen for  $[Ca^{2+}]_i$  measurements.

#### 2.4. Data analysis

Data are presented as mean values  $\pm$  SEM ( $n$  = the number of tested neurones). All experiments were performed in at least two independent sessions.

### 3. Results

#### 3.1. Removal of external $Ca^{2+}$ inhibits $[Ca^{2+}]_i$ oscillations

Removal of extracellular  $Ca^{2+}$  led to an immediate cessation of spontaneous  $[Ca^{2+}]_i$  oscillations in all tested neurones ( $n = 9$ ; Fig. 1A). After extracellular  $Ca^{2+}$  removal the basal  $[Ca^{2+}]_i$  of  $133 \pm 14$  nM started to decline until it reached a low steady state level of  $32 \pm 6$  nM in about 3 min. Inhibition of  $[Ca^{2+}]_i$  oscillations by removal of external  $Ca^{2+}$  was reversible and the oscillations (as well as basal  $[Ca^{2+}]_i$ ) were restored rapidly after switching perfusion back to the normal extracellular solution (Fig. 1A). The  $[Ca^{2+}]_i$  oscillations still persisted when external  $Ca^{2+}$  was lowered from 2 mM to 500 nM ( $n = 6$ ) or to 200 nM ( $n = 4$ ), but no oscillations were observed at external  $Ca^{2+}$  concentration of 100 nM ( $n = 5$ ).

#### 3.2. Voltage dependent $Ca^{2+}$ channels and $[Ca^{2+}]_i$ oscillations

A non-specific  $Ca^{2+}$  channel blocker  $Cd^{2+}$  at 50  $\mu M$  or 100  $\mu M$  led to a complete inhibition of  $[Ca^{2+}]_i$  oscillations in all tested neurones ( $n = 17$ , Fig. 1B). In contrast, incubation with  $Ni^{2+}$  (50  $\mu M$ ,  $n = 10$ , Fig. 1C) or TTA-P2 (10 mM,  $n = 9$ , Fig. 1D), both being blockers of T-type VGCCs [28], did not affect  $[Ca^{2+}]_i$  oscillations. We further probed more specific antagonists of high-threshold VGCCs, the effects of which on spontaneous  $[Ca^{2+}]_i$  oscillations are summarised in Fig. 2. Neither L-type channel blocker nifedipine (1  $\mu M$ ,  $n = 10$ , Fig. 2A), nor a specific blocker of the N-type channels  $\omega$ -conotoxin GVIA (800 nM,  $n = 12$ , Fig. 2B), nor a specific blocker of the Q-type,  $\omega$ -conotoxin MVIIC (100 nM,  $n = 6$ , 1  $\mu M$ ,  $n = 2$ , Fig. 2C), nor a specific blocker of the P/Q-type channels  $\omega$ -agatoxin IVA (300 nM,  $n = 5$ , Fig. 2D) blocked the  $[Ca^{2+}]_i$  oscillations. In contrast, a specific inhibitor of R-type VGCCs SNX-482 [29–31] affected  $[Ca^{2+}]_i$  oscillations when applied at various concentrations. At 40 nM SNX-482 only partially inhibited  $[Ca^{2+}]_i$  oscillations in 1 out of 6 neurones



(data not shown). At 100 nM of SNX-482  $[Ca^{2+}]_i$  oscillations were completely blocked in 9 out of 19 neurones, partially inhibited in 5 cells and had no effect in the remaining 5 neurones. In cells in which SNX-482 completely inhibited  $[Ca^{2+}]_i$  oscillations, the basal  $[Ca^{2+}]_i$  decreased from  $153 \pm 17$  nM to  $87 \pm 6$  nM ( $n=9$ ; Fig. 3A). In neurones in which oscillations were only partially blocked the basal  $[Ca^{2+}]_i$  also dropped from  $143 \pm 15$  to  $102 \pm 12$  nM (see Fig. 3C). In the remaining 5 cells which were insensitive to the drug, the basal  $[Ca^{2+}]_i$  did not change significantly. When applied at 200 nM SNX-482  $[Ca^{2+}]_i$  oscillations were completely blocked in 4 out of 6 neurones (Fig. 3B). To further corroborate the contribution of R-type VGCCs, the neurones were exposed to a mixture of specific blockers for L-, N-, P/Q- and T-type channels (1  $\mu$ M nifedipine, 800 nM  $\omega$ -agatoxin IVA, 300 nM  $\omega$ -conotoxin GVIA and 50  $\mu$ M  $Ni^{2+}$ ), which failed to inhibit  $[Ca^{2+}]_i$  oscillations. The addition of 100 nM of SNX-482 to this cocktail, however inhibited  $[Ca^{2+}]_i$  oscillations ( $n=3$ , Fig. 3D) again indicating the role of R-type channels.

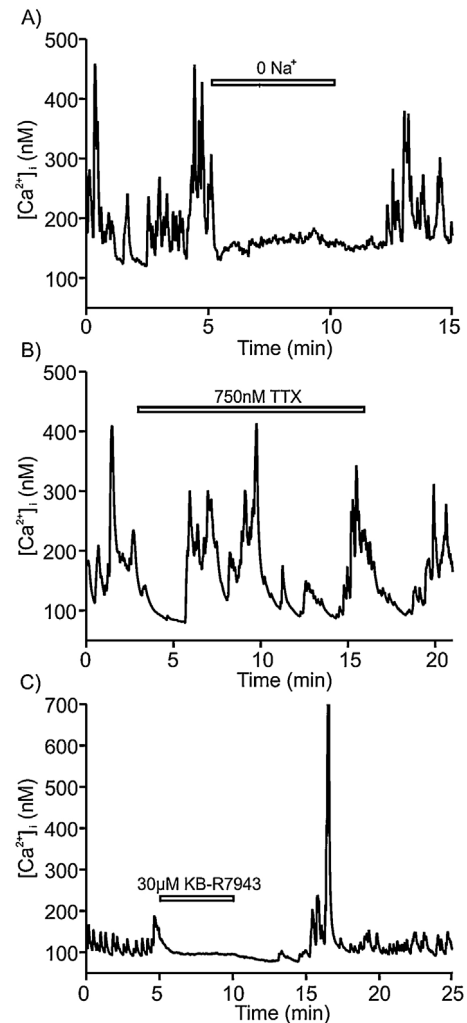
### 3.3. Role of $Na^+$ channels and $Na^+$ transport

Removal of extracellular  $Na^+$  caused a complete, rapid and reversible block of  $[Ca^{2+}]_i$  oscillations in 7 neurones and significantly inhibited oscillations in 2 cells (Fig. 4A). At the same time, treatment with the specific blocker of voltage-gated  $Na^+$  channels TTX at concentrations between 750 nM to 5  $\mu$ M (Fig. 4B) did not affect  $[Ca^{2+}]_i$  oscillations in all tested cells ( $n=26$ ). On the contrary, treatment of oscillating neurones with KB-R7943, a selective inhibitor of the reverse mode of  $Na^+/Ca^{2+}$  exchanger, NCX [32,33] caused a complete block of oscillations and lowered basal  $[Ca^{2+}]_i$  to  $95 \pm 5$  nM in 26 out of 33 neurones (Fig. 4C). In the remaining 7 neurones, the oscillations were inhibited only partially and the basal  $[Ca^{2+}]_i$  also dropped to  $102 \pm 12$  nM.

### 3.4. Role of intracellular $Ca^{2+}$ stores and $Ca^{2+}$ clearance mechanisms

The treatment of SON neurones with TG (irreversible blocker of SERCA) did not affect  $[Ca^{2+}]_i$  oscillations in all tested neurones ( $n=8$ ), except for producing a short transient effect when oscillations were inhibited for 1–2 min after exposure to TG but fully recovered in the continuing presence of TG. This transient effect of TG was observed in 6 out of 8 neurones (Fig. 5A). The treatment of SON neurones ( $n=4$ ) with a reversible blocker of SERCA pumps CPA (10  $\mu$ M) also had no visible effect on  $[Ca^{2+}]_i$  oscillations in 3 out of 4 neurones; in 1 cell, similarly to TG, CPA produced transient inhibition (Fig. 5B). In addition, we tested the CPA effect in the absence of external  $Ca^{2+}$  by using  $0Ca^{2+}$  buffer and then exposing the neurones to 10  $\mu$ M CPA. This resulted in a transient elevation of  $[Ca^{2+}]_i$ . When  $0Ca^{2+}$  buffer was switched back to the 2 mM  $Ca^{2+}$  NL, we observed an immediate transient increase in  $[Ca^{2+}]_i$  and the oscillations were restored. Subsequent exposure to CPA failed to affect the ongoing oscillations. This protocol was tested with the same result in 7 neurones (Fig. 5C).

Ryanodine and caffeine are known to mobilize  $Ca^{2+}$  from ER in SON neurones [12] through activation of RyRs; in addition caffeine is a potent inhibitor of  $InsP_3$  receptors [34,35]. Both drugs induced a transient elevation of  $[Ca^{2+}]_i$  in non-oscillating SON neurones which lasted between 1–3 min (data not shown). However, application of ryanodine (10  $\mu$ M,  $n=6$ ) or caffeine (10 mM,  $n=4$ ) to neurones exhibiting  $[Ca^{2+}]_i$  oscillations did not affect the latter, except (similar to TG and CPA), inducing a short (1–2 min) transient inhibition that was observed in 2 out of 6 neurones treated with ryanodine and in 2 out of 4 neurones treated with caffeine (Fig. 5D, E). Subsequently, contribution of the PMCA was tested by applying 100  $\mu$ M of Lanthanum ( $La^{3+}$ ), which is known to effectively block plasmalemmal  $Ca^{2+}$  pump [12,17,36]. Exposure to 100  $\mu$ M of  $La^{3+}$



**Fig. 4.** Role of extracellular  $Na^+$ , voltage-gated  $Na^+$  channels and NCX. A: Removal of external  $Na^+$  completely inhibits  $[Ca^{2+}]_i$  oscillations; B: Inhibition of voltage-gated  $Na^+$  channels with TTX does not affect spontaneous  $[Ca^{2+}]_i$  oscillations. C: Exposure of magnocellular neurone to KB-R7943, a specific inhibitor of NCX reverse mode, completely inhibits spontaneous  $[Ca^{2+}]_i$  oscillations.

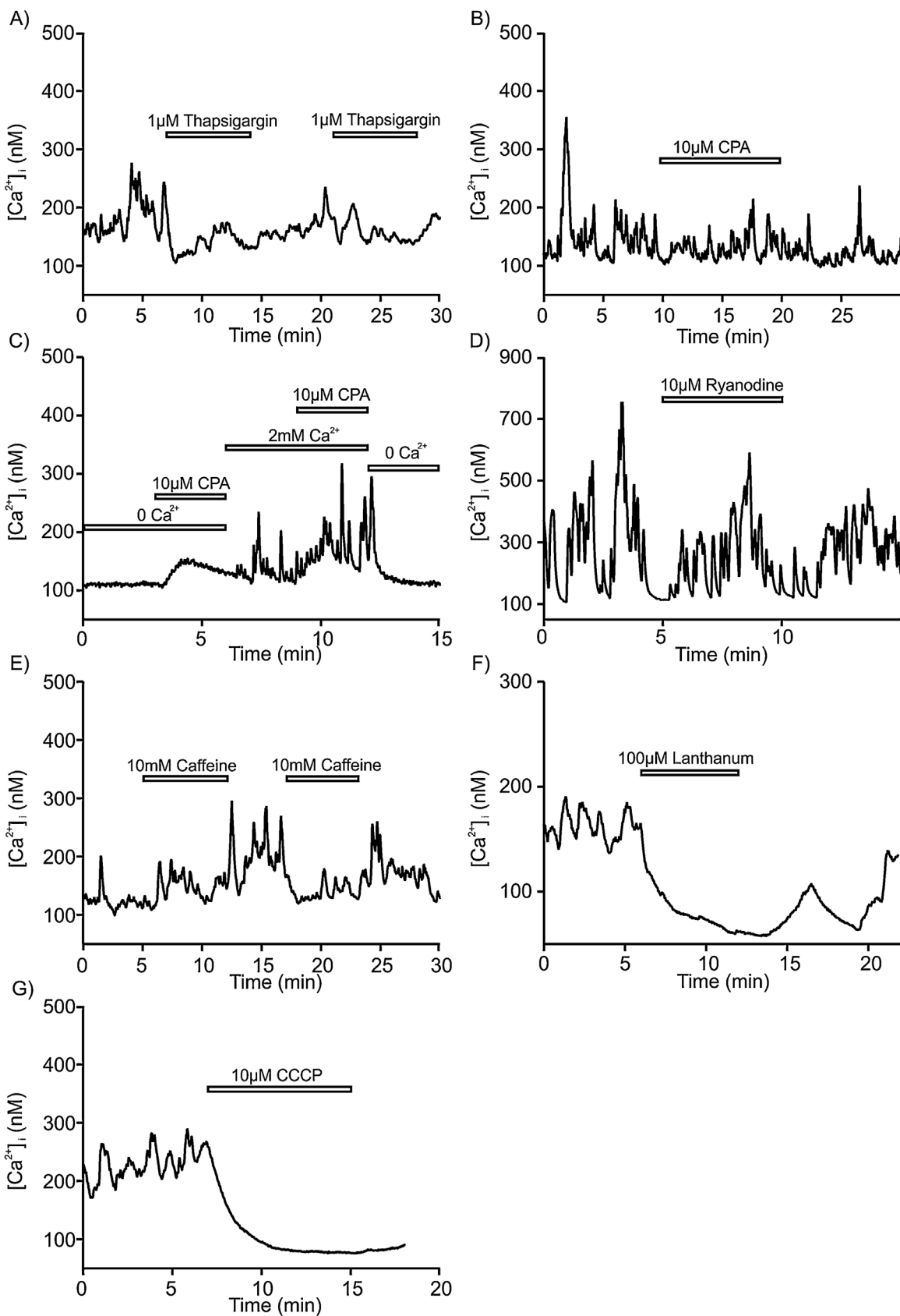
inhibited  $[Ca^{2+}]_i$  oscillations in 8 out of 9 neurones (Fig. 5F) with rather poor recovery.

To test the role of mitochondria in spontaneous oscillations, treatment of the oscillating SONs with CCCP, an  $H^+$  ionophore and uncoupler of oxidative phosphorylation in mitochondria, caused a complete and irreversible block of  $[Ca^{2+}]_i$  oscillations in 6 out of 7 neurones (Fig. 5G).

### 3.5. Effect of neurotransmitters and blockers of $K^+$ currents on $[Ca^{2+}]_i$ oscillations

Exposure of magnocellular neurones to various concentrations of GABA (3  $\mu$ M,  $n=12$ ; 20  $\mu$ M,  $n=4$ ; and 50  $\mu$ M,  $n=4$ ) completely inhibited  $[Ca^{2+}]_i$  oscillations in all cells tested; this inhibition was fully reversible (examples: Fig. 6A, for 3  $\mu$ M). The inhibitory effect of GABA was completely blocked by GABA<sub>A</sub> antagonist gabazine (at 10  $\mu$ M,  $n=8$ ; Fig. 6B). Similar (albeit slightly weaker) inhibition was observed using taurine (500  $\mu$ M, in 6 cells blocked, in 1 partially blocked, in 2 no effect; Fig. 6C) and using glycine (20  $\mu$ M, in 4 cells blocked, in 1 partially blocked, in 3 no effect; Fig. 6D).

Other neurotransmitters such as glutamate or NMDA were shown to have an excitatory effect on SON neurones [37,38]. In our



**Fig. 5.** Effect of intracellular  $Ca^{2+}$  mobilisers and inhibitors of  $Ca^{2+}$  clearance mechanisms.

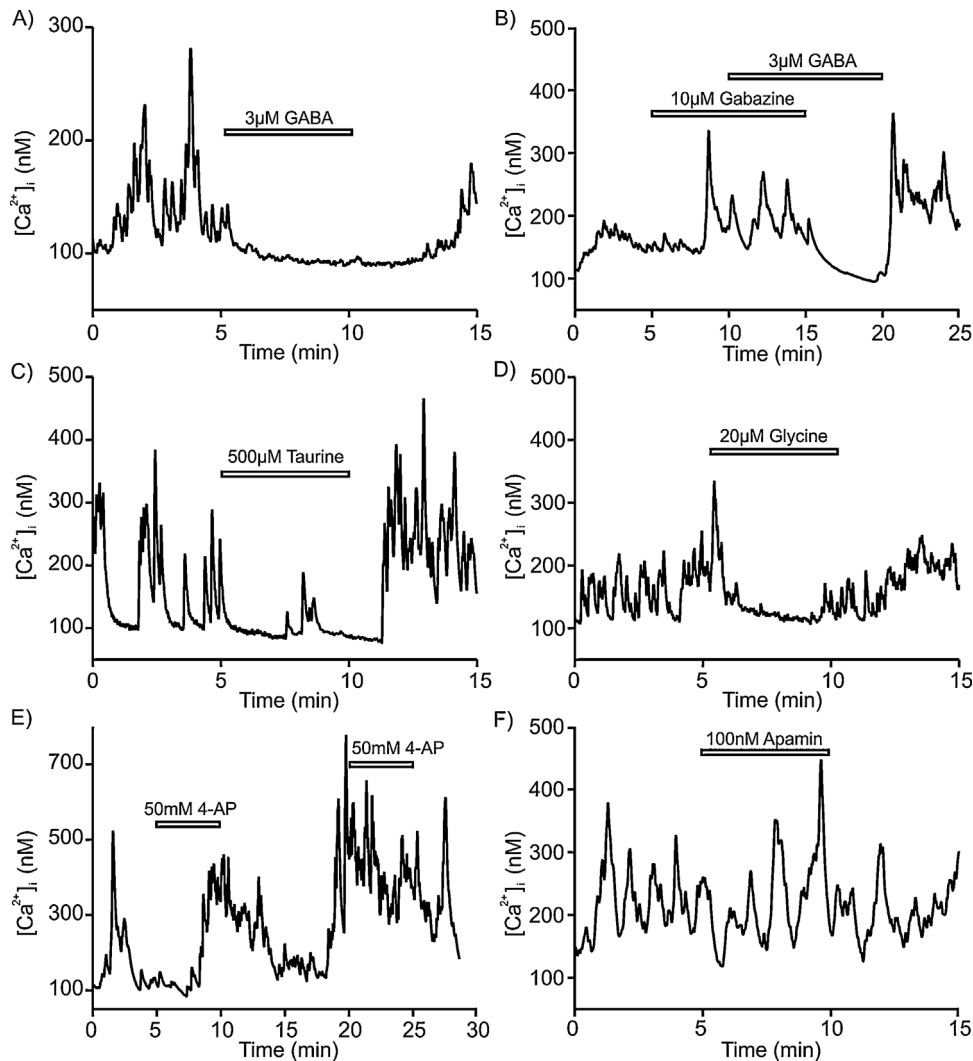
A, B: Inhibition of endoplasmic reticulum  $Ca^{2+}$  pumps (of SERCA type) by thapsigargin (A) and CPA (B) does not affect spontaneous  $[Ca^{2+}]_i$  oscillations.

C: Shows the effect of CPA on  $[Ca^{2+}]_i$  in the presence and absence of external  $Ca^{2+}$ . The CPA-induced  $[Ca^{2+}]_i$  rebound after readmission of extracellular  $Ca^{2+}$  reflects store-operated  $Ca^{2+}$  entry (SOCE) activation.

D, E: Mobilisation of ER  $Ca^{2+}$  by  $10 \mu M$  ryanodine (D) or  $10 mM$  caffeine (E) does not influence spontaneous  $[Ca^{2+}]_i$  oscillations.

F: Exposure of magnocellular neurone to  $La^{3+}$  (a blocker of plasma membrane  $Ca^{2+}$ -ATPase) inhibits spontaneous  $Ca^{2+}$  oscillations.

G: Mitochondrial uncoupler CCCP completely and irreversibly inhibits spontaneous  $[Ca^{2+}]_i$  oscillations.



**Fig. 6.** Effect of neurotransmitters and blockers of  $K^+$  currents on  $[Ca^{2+}]_i$  oscillations.

A: Trace showing the blocking effect of GABA (3  $\mu$ M) on spontaneous  $[Ca^{2+}]_i$  oscillations.

B: Trace showing the reversibility of GABA effect by its antagonist, gabazine.

C, D: Traces showing the blocking effect of taurine and glycine on spontaneous  $[Ca^{2+}]_i$  oscillations.

Exposure of magnocellular neurones to 4-AP (E), an inhibitor of A-type  $K^+$  current or to apamin (F), an inhibitor of  $Ca^{2+}$ -activated  $K^+$ -current, did not affect spontaneous  $Ca^{2+}$  oscillations.

hands, neither glutamate (in concentrations up to 100  $\mu$ M;  $n=4$ ) nor NMDA (100  $\mu$ M;  $n=5$ ) influenced spontaneous  $[Ca^{2+}]_i$  oscillations (data not shown). Exposure of SON neurones to 10–50 mM of 4-AP (inhibitor of  $I_A$   $K^+$  channels) did not affect spontaneous  $[Ca^{2+}]_i$  oscillations ( $n=5$ ); the inhibitor of SK  $Ca^{2+}$ -dependent  $K^+$  channels apamin (100 nM,  $n=5$ , Fig. 6E, F) was also similarly impotent.

### 3.6. Second messengers and spontaneous $[Ca^{2+}]_i$ oscillations

We used specific blockers of PLC (5  $\mu$ M U-73211; Fig. 7A); a competitive antagonist of cAMP dependent PKA (50  $\mu$ M Rp-cAMP; Fig. 7B), an AC inhibitor (10  $\mu$ M SQ-22536; Fig. 7C) and an inhibitor of PKA (10  $\mu$ M H-89; Fig. 7D). While none of these inhibitors affected the oscillations in all tested cells ( $n=5$ –8 neurones for each drug), H-89 partially blocked the oscillations only in a few neurones (3 out of 8). Finally, dynorphin, known to modulate the bursting properties of AVP neurones [39] did not affect spontaneous  $[Ca^{2+}]_i$  oscillations (at 100 nM;  $n=10$ , Fig. 7E).

## 4. Discussion

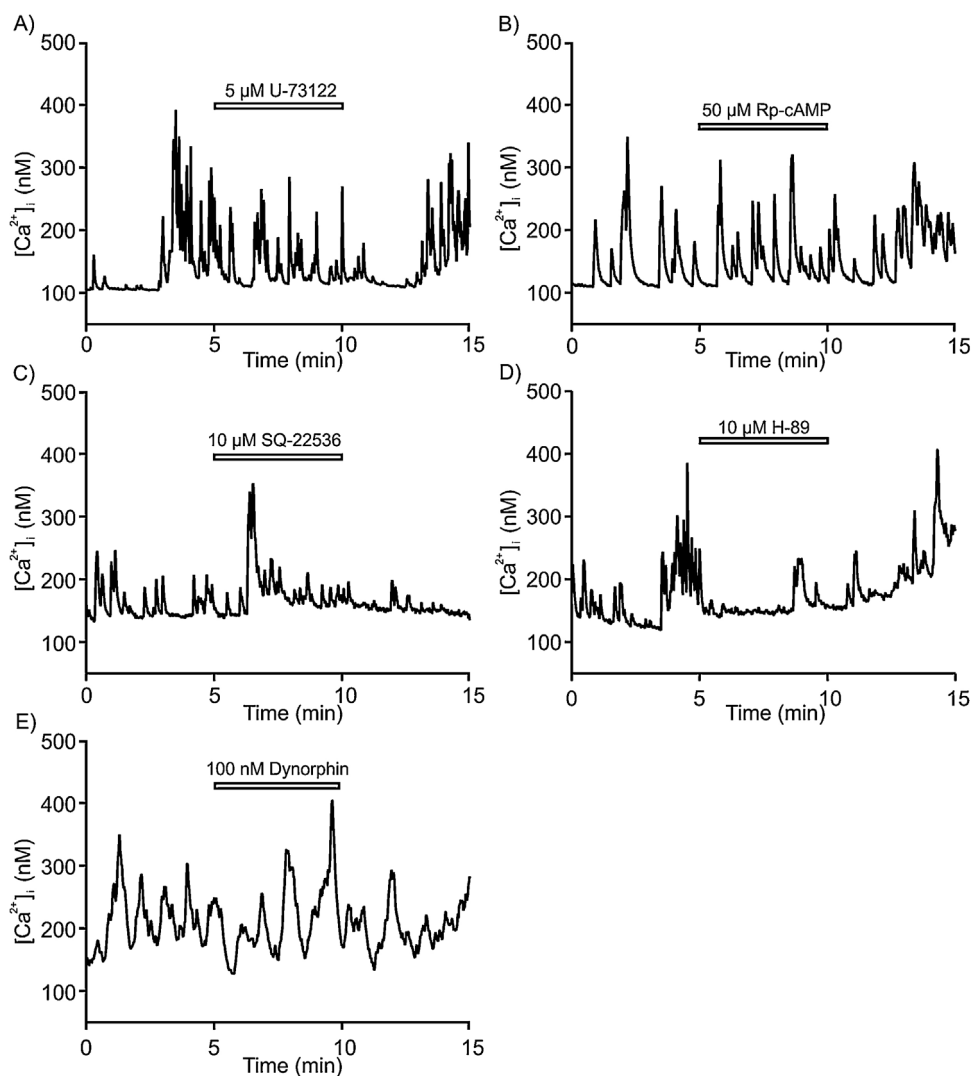
### 4.1. Spontaneous oscillations are not linked to electrical activity

Action potentials fired by SON neurones activate  $Ca^{2+}$  entry [15], in our experiments, however, inhibition of electrical excitability with TTX did not affect  $[Ca^{2+}]_i$  oscillations, indicating that action potentials are not involved in the spontaneous  $[Ca^{2+}]_i$  oscillations. Of note, the pituitary adenylyl cyclase-activating peptide-induced  $[Ca^{2+}]_i$  oscillations in SON neurones were sensitive to TTX [16], which probably reflects their peculiar nature. Complete dissociation of action potentials and spontaneous  $[Ca^{2+}]_i$  dynamics reflects the operation of a specific pacemaker.

### 4.2. Spontaneous oscillations are $Na^+$ dependent and result from transmembrane $Ca^{2+}$ entry

Removal of extracellular  $Na^+$  effectively inhibited spontaneous  $[Ca^{2+}]_i$  oscillations in SON neurones. The very same inhibition was achieved by KB-R7943, a specific inhibitor of the reverse mode of NCX. The reversal potential of the exchanger in SON neurones (at





**Fig. 7.** The effect of blockers of phospholipase C and adenylyl cyclase intracellular transduction pathways on oscillations.

A-D: Inhibition of signalling cascades by PLC blocker (U-73122; A), competitive blocker of cAMP-dependent PKA (Rp-cAMP; B), an AC blocker (SQ-22536; C) and PKA blocker (H-89; D) does not affect spontaneous  $[Ca^{2+}]_i$  oscillations. E: Trace showing the absence of effect of dynorphin on spontaneous  $[Ca^{2+}]_i$  oscillations.

$[Ca^{2+}]_i = 130$  nM,  $[Na^+]_i = 10$  mM,  $[Na^+]_o = 140$  mM) is about  $-45$  to  $-50$  mV, and thus a moderate depolarisation will reverse the NCX operation. In the reverse mode NCX provides for  $Ca^{2+}$  entry, which logically, underlies observed  $Ca^{2+}$  oscillations. This is in contrast to numerous examples of spontaneous  $[Ca^{2+}]_i$  dynamics originating from  $Ca^{2+}$  release from the ER [32,40,41]. In SON neurones this seems not to be the case. Targeting the ER using TG, CPA, ryanodine or caffeine failed to affect  $[Ca^{2+}]_i$  oscillations. Caffeine has a double action on the ER activating RyRs and effectively inhibiting  $InsP_3$  receptors [34,35]; absence of any effect on spontaneous  $[Ca^{2+}]_i$  oscillations argued against the role of ER  $Ca^{2+}$  release. At the same time removal of extracellular  $Ca^{2+}$  effectively blocked spontaneous  $[Ca^{2+}]_i$  dynamics. It's of interest to note that the dependence of oscillations on extracellular  $Ca^{2+}$  has been observed in OT-sensitive SON neurones [7] and in AVP-sensitive neurones [6]. The NCX, however, is not the only mechanism for  $Ca^{2+}$  delivery associated with spontaneous  $[Ca^{2+}]_i$  activity of SON neurones. Of interest, in nerve terminal preparations, the release of AVP was unexpectedly modulated by extracellular  $Na^+$ : removal of  $Na^+$  inhibited secretion, whereas increases of extracellular  $Na^+$  (in  $Na^+$ -depleted media) increased AVP secretion suggesting that the  $Na^+/Ca^{2+}$  exchanger has a particular functional role in the regulation of secretion—both somatic as well as at the level of the releasing nerve terminals in the

neurohypophysis [42]. The  $[Ca^{2+}]_i$  oscillations appear to be specifically associated with R-type VGCCs: treatment with a selective blocker of these channels, SNX-482, inhibited  $[Ca^{2+}]_i$  oscillations in a concentration-dependent manner. The pharmacological inhibition of other VGCCs (T-, L-, N- and P/Q-types) was without effect. Similarly, inhibition of intercellular second messenger cascades and plasmalemmal  $K^+$  channels did not affect spontaneous  $[Ca^{2+}]_i$  activity. The role for NCX and R-type  $Ca^{2+}$  channels are also supported (albeit indirectly) by the inhibitory action of GABA that completely blocked spontaneous  $[Ca^{2+}]_i$  dynamics. This action was mediated through  $GABA_A$  receptors (as revealed by gabazine sensitivity) and most likely resulted from cell hyperpolarisation. The latter, arguably, prevented reversal of the NCX and activation of R-type  $Ca^{2+}$  channels.

#### 4.3. The need for ATP-dependent $Ca^{2+}$ extrusion

Efficient  $Ca^{2+}$  clearance from the cytosol is another necessary component for maintaining spontaneous  $[Ca^{2+}]_i$  oscillations. Inhibition of PMCA by  $La^{3+}$  effectively suppressed spontaneous  $Ca^{2+}$  dynamics; the same effect was achieved by mitochondrial uncoupling. Thus we may conclude that energy dependent  $Ca^{2+}$  extrusion

joins NCX and R-type VGCCs in creating a pacemaker mechanism driving  $\text{Ca}^{2+}$  oscillatory activity.

## 5. Conclusions

Taken together, these results unveil, for the first time, the idiosyncrasies of  $\text{Ca}^{2+}$  signalling in AVP and OT neurones and demonstrate that the spontaneous  $[\text{Ca}^{2+}]_i$  oscillations in the SON neurones are driven by plasmalemmal R-type  $\text{Ca}^{2+}$  channels and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger oscillation between forward and reverse modes; maintenance of oscillations also requires the activity of the plasmalemmal  $\text{Ca}^{2+}$  pump.

## Conflict of interest

The authors state that they have no conflict of interest pertaining to this manuscript.

## Authors' contribution

SK, CS, GD: performed experiments.

YU, OF: prepared and maintained the heterozygous and homozygous transgenic rats for both AVP and OT and developed double transgenic rats for AVP and OT; performed genotyping, manuscript writing.

SK, MZ, AV, GD: prepared the concept of the project, manuscript writing, data and statistical analysis.

AC, ES, GD: project management and logistics, manuscript writing.

## Acknowledgements

This work was supported by the grants 14-34077S and GACR P304/12/G069 from the Grant Agency of the Czech Republic and GAUK 22214 from the Grant Agency of Charles University in Prague, and by institutional support RVO#67985823. CS and GD were supported in part by the FP7 Initial Training Network EduGLIA program (PITN-GA-2009-237956). This publication is partly a result of the "Advanced Bioimaging of Living Tissues" project, registration number #CZ.2.16/3.1.00/21527, which was financed from the budget of the European Regional Development Fund and public budgets of the Czech Republic through the Operational Programme Prague-Competitiveness. AV was supported by the Wellcome Trust, by the Alzheimer's research foundation (UK) and by a grant (agreement from August 27 2013 № 02.B.49.21.0003) between The Ministry of Education and Science of the Russian Federation and Lobachevsky State University of Nizhny Novgorod, by a grant of the Russian Scientific Foundation № 14-15-00633 and by the Ministry of Education of the Russian Federation, unique identity number RFMEFI57814X0079. Govindan Dayanithi belongs to the "Centre National de la Recherche Scientifique-The French Ministry of Research and Higher Education-Paris", France. We are grateful to Kip Allan Bauersfeld, IEM ASCR, for critical reading and helpful comments on the manuscript.

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