Sodium-calcium exchanger and R-type Ca2+ channels mediate spontaneous [Ca2+]i oscillations in magnocellular neurones of the rat supraoptic nucleus

Stepan Kortus, Chinnapaiyan Srinivasan, Oksana Forostyak, Martin Zapotocky, Yoichi Ueta, Eva Sykova, Alexandr Chvatal, Alexei Verkhratsky, G. Dayanithi

► To cite this version:

Stepan Kortus, Chinnapaiyan Srinivasan, Oksana Forostyak, Martin Zapotocky, Yoichi Ueta, et al.. Sodium-calcium exchanger and R-type Ca2+ channels mediate spontaneous [Ca2+]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

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Sodium-calcium exchanger and R-type Ca^{2+} channels mediate spontaneous $[Ca^{2+}]_i$ oscillations in magnocellular neurones of the rat supraoptic nucleus

Stepan Kortus[a,b,c], Chinnapaiyan Srinivasan[a], Oksana Forostyak[a,d], Martin Zapotocky[b,c], Yoichi Ueta[e], Eva Sykova[d,f], Alexandr Chvatal[d,g], Alexei Verkhratsky[h,i,j,k,**], Govindan Dayanithi[a,l,m,*]

[a] Department of Molecular Neurophysiology, Institute of Experimental Medicine, Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic
[b] Institute of Physiology, Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic
[c] Institute of Biophysics and Informatics, First Medical Faculty, Charles University in Prague, Salmovska 1, 12000 Prague, Czech Republic
[d] Department of Neuroscience, Charles University, Second Medical Faculty, V Uvalu 84, 15006 Prague, Czech Republic
[e] Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan
[f] Department of Neuroscience, Institute of Experimental Medicine, Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic
[g] Department of Cellular Neurophysiology, Institute of Experimental Medicine, Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic
[h] University of Manchester, School of Biological Sciences, D.4417 Michael Smith Building, Oxford Road, M13 9PT Manchester, United Kingdom
[i] Achucarro Center for Neuroscience, IKERBASQUE, Basque Foundation for Science, 48011 Bilbao, Spain
[j] Department of Neurosciences, University of the Basque Country UPV/EHU and CIBERNED, Leioa, Spain
[k] University of Nizhny Novgorod, Nizhny Novgorod 603022, Russia
[l] Institut National de la Santé et de la Recherche Médicale, Unité de recherche U1198, Université Montpellier, 34095 Montpellier, France
[m] Ecole Practique des Hautes Etudes, Sorbonne, 75014 Paris, France

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^{2+} signalling
Voltage-gated Ca^{2+} channels
Ca^{2+} homeostasis
Ca^{2+} imaging
Ca^{2+} oscillations
Ca^{2+} clearance
Transgenic rats
Vasopressin
Oxytocin

ABSTRACT

Isolated supraoptic neurones generate spontaneous $[Ca^{2+}]_i$ oscillations in isolated conditions. Here we report in depth analysis of the contribution of plasmalemmal ion channels (Ca^{2+}, Na^+), Na^+/Ca^{2+} exchanger (NCX), intracellular Ca^{2+} release channels ($InsP_3$Rs and RyRs), Ca^{2+} storage organelles, plasma membrane Ca^{2+} pump and intracellular signal transduction cascades into spontaneous Ca^{2+} activity. While removal of extracellular Ca^{2+} or incubation with non-specific voltage-gated Ca^{2+} channel (VGCC) blocker Cd^{2+} suppressed the oscillations, neither Ni^{2+} nor TTA-P2, the T-type VGCC blockers, had an effect. Inhibitors of VGCC nicardipine, ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA (for L-, N-, P and P/Q-type channels, respectively) did not affect $[Ca^{2+}]_i$ oscillations. In contrast, a specific R-type VGCC blocker SNX-482 attenuated $[Ca^{2+}]_i$ oscillations. Incubation with TTX had no effect, whereas removal of the extracellular Na^+ or application of an inhibitor of the reverse operation mode of Na^+/Ca^{2+} exchanger KB-R7943 blocked the oscillations. The mitochondrial uncoupler CCCP irreversibly blocked spontaneous $[Ca^{2+}]_i$ activity. Exposure of neurones to Ca^{2+} mobilisers (thapsigargin, cyclopiazonic acid, caffeine and ryanodine); 4-aminopyridine (A-type K^+ 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; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; VGCC, voltage-gated Ca^{2+} channels; EGTA, ethylene glycol (bis-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetra acetic acid; TTX, tetrodotoxin; PMCA, plasmatic-Ca^{2+}-ATPase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; 4-AP, 4-aminopirydine; PACAP, pituitary adenylate cyclase activating polypeptide; NCX, Na^+/Ca^{2+} exchanger; GABA, gamma-aminobutyric acid; AHP, after hyperpolarisation; DAP, depolarizing after potential; $InsP_3$, 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.

* Corresponding author at: Department of Molecular Neurophysiology, Institute of Experimental Medicine, Czech Academy of Sciences, Videnska 1083, CZ-142 20 Prague 4, Czech Republic.
** Corresponding author at: University of Manchester, School of Biological Sciences, D.4417 Michael Smith Building, Oxford Road, M13 9PT Manchester, United Kingdom.
E-mail addresses: Alexej.Verkhratsky@manchester.ac.uk (A. Verkhratsky), gdaya@univ-montp2.fr, gdaya@biomed.cas.cz (G. Dayanithi).

Hypothalamus
Supraoptic nucleus
Na^+/Ca^{2+} exchanger
Mitochondria
Plasma membrane calcium pump
Sarcoendoplasmic reticulum
Ca^{2+}-ATPase
GABA
Glutamate
1,4,5-Trisphosphate
Tetrodotoxin
Ca^{2+} channel toxins

are mediated by a concerted action of R-type Ca^{2+} 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^{2+} concentration ($[Ca^{2+}]_i$) [6]. In OT neurones, this increase in $[Ca^{2+}]_i$ resulted from Ca^{2+} release from intracellular stores [7], whereas in AVP neurones, the $[Ca^{2+}]_i$ rise reflects both the Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) and Ca^{2+} 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^{2+}-regulated exocytosis [12]. In terminals the $[Ca^{2+}]_i$ elevation is triggered by action potential arriving from the SON neurones. In the SON, any agent that increases $[Ca^{2+}]_i$ (by intracellular Ca^{2+} release or plasmalemmal Ca^{2+} 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^{2+}-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 $[Ca^{2+}]_i$ oscillations [7,16]. Experiments on transgenic animals showed that these $[Ca^{2+}]_i$ oscillations exist in both AVP and OT neurones [1,17] yet mechanisms underlying these oscillations remain unknown; similarly unknown is the effect of $[Ca^{2+}]_i$ oscillations on the release of AVP and OT [17]. Spontaneous $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ oscillations can be recognised, depending on the main source of Ca^{2+}, i.e., release from intracellular stores such as endoplasmic reticulum (ER) or plasmalemmal Ca^{2+} entry [18]. The ER-driven $[Ca^{2+}]_i$ oscillations result from the periodical release of Ca^{2+} mediated by the inositol 1,4,5-trisphosphate ($InsP_3$) receptor or the ryanodine receptor/Ca^{2+} release channel, whereas the extracellular Ca^{2+} driven oscillations require Ca^{2+} entry through plasmalemmal channels or exchangers. Cellular Ca^{2+} and electrical properties of the SON neurones are closely and mutually associated although they can operate independently. In this study, we analysed spontaneous $[Ca^{2+}]_i$ 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, $2CaCl_2$, 1 $MgCl_2$, 10 glucose, 10HEPES, pH was adjusted to 7.25 with tris; the osmolarity was 298–300mosm l^{-1}; 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-

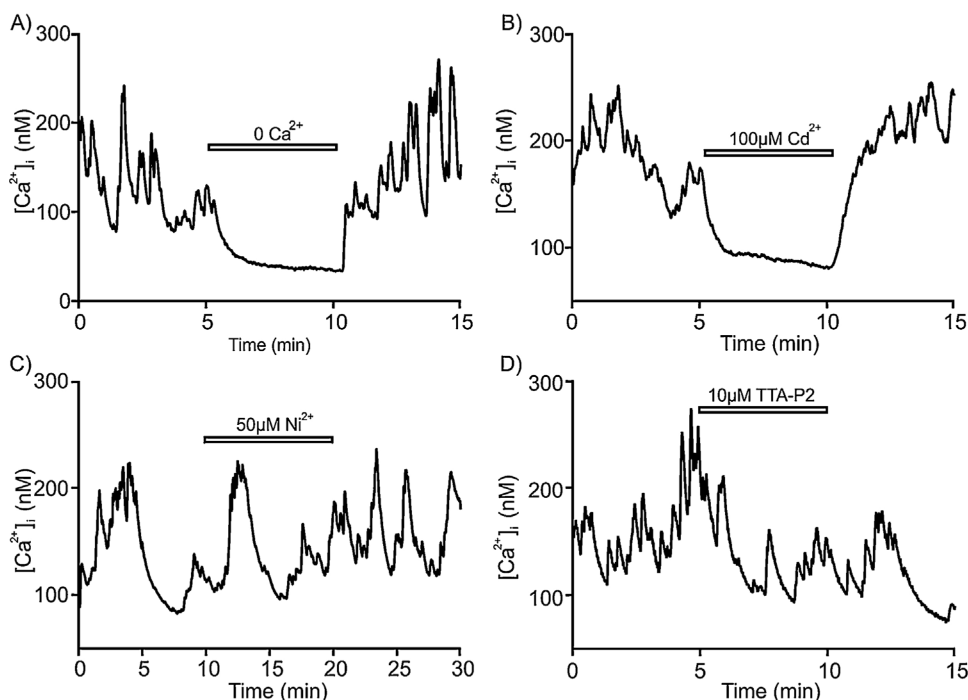

Fig. 1. Effect of external Ca^{2+} removal and VGCC blockers on $[Ca^{2+}]_i$ oscillations.
A: A typical $[Ca^{2+}]_i$ oscillation observed in a neurone before and after removal of external Ca^{2+}. B: Inhibition of spontaneous $[Ca^{2+}]_i$ oscillations in the presence of Cd^{2+}. C,D: Exposure of SON neurones to T-type VGCCs inhibitors Ni^{2+} and TTA-P2 fail to affect spontaneous $[Ca^{2+}]_i$ oscillations.

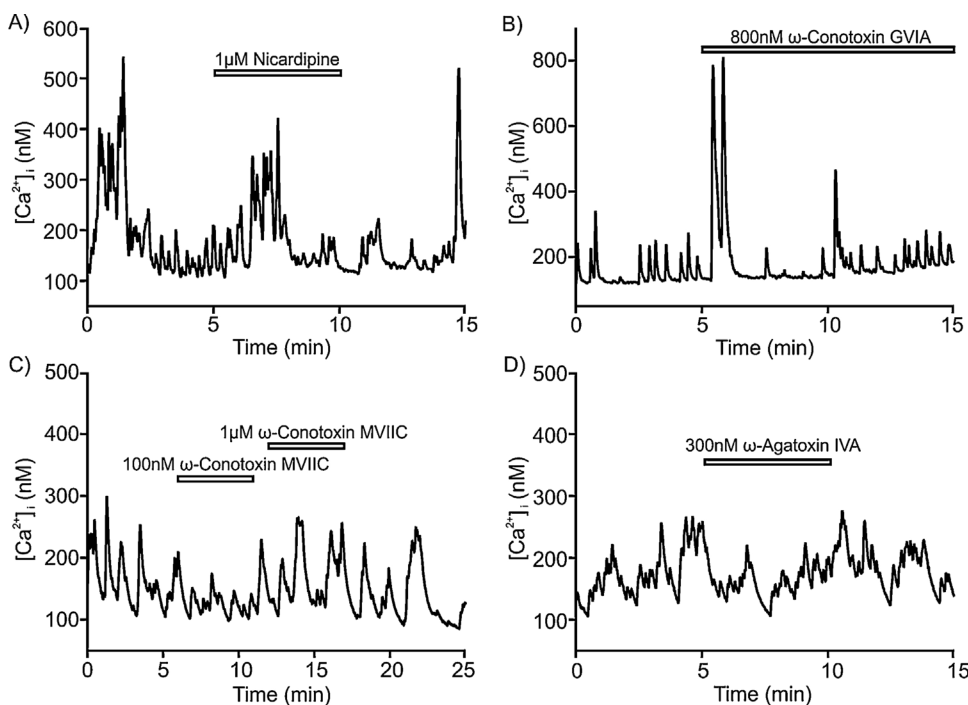

Fig. 2. Effect of specific HVGCC blockers on $[Ca^{2+}]_i$ oscillations.
The representative traces showing the oscillations from individual neurones in the presence of different HVGCC blockers: nicardipine (1 μM) for L-type (A); ω-conotoxin GVIA (800 nM) for N-type (B); two different concentrations of ω-conotoxin MVIIC for P-type (C) and ω-agatoxin (300 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 H_2O (EMD Millipore Corporation, Germany) and all stock solutions and buffers were prepared using this total-ion-free H_2O.

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

The $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ was

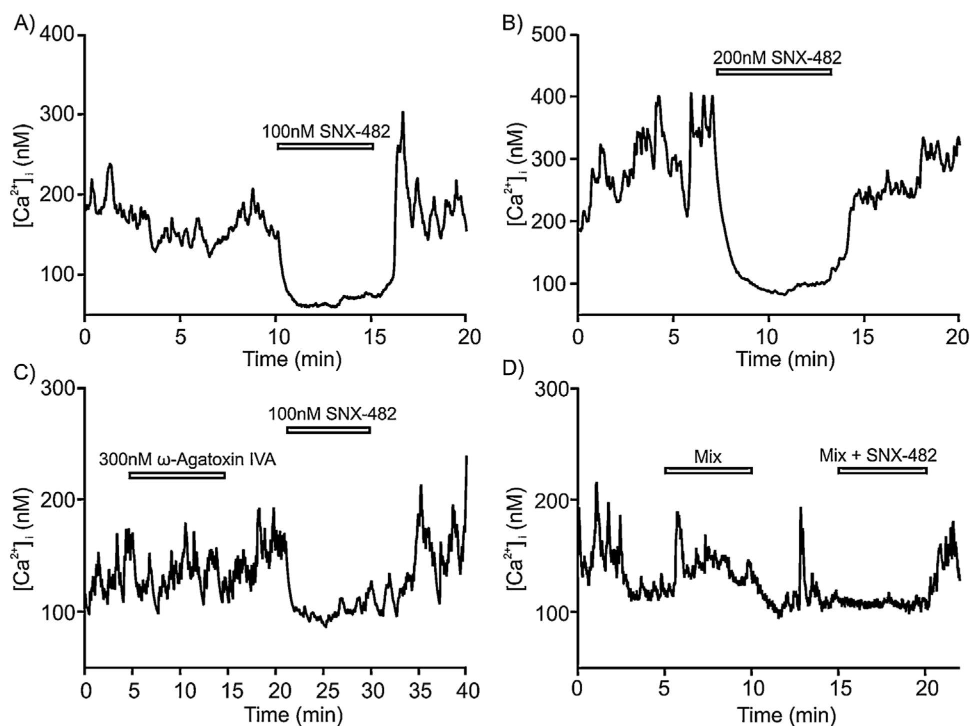

Fig. 3. Effect of R-type VGCC blocker on $[Ca^{2+}]_i$ oscillations.
Traces A and B show $[Ca^{2+}]_i$ oscillations from individual neurones recorded in the presence of 100 nM and 200 nM SNX-482, a specific blocker of R-type channels, respectively. The trace C shows the specific block of oscillations by SNX-482 in comparison to no effect of ω-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, $f340_{max}/f380_{min}$ = 7.7, dissociation constant for Fura-2 at 37 °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° 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 μm and persisted dendrites were randomly chosen for $[Ca^{2+}]_i$ measurements.

2.4. Data analysis

Data are presented as mean values ± 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 ± 14 nM started to decline until it reached a low steady state level of 32 ± 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 μM or 100 μM led to a complete inhibition of $[Ca^{2+}]_i$ oscillations in all tested neurones (n = 17, Fig. 1 B). In contrast, incubation with Ni^{2+} (50 μM, 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 nicardipine (1 μM, n = 10, Fig. 2A), nor a specific blocker of the N-type channels ω-conotoxin GVIA (800 nM, n = 12, Fig. 2B), nor a specific blocker of the Q-type, ω-conotoxin MVIIC (100 nM, n = 6, 1 μM, n = 2, Fig. 2C), nor a specific blocker of the P/Q-type channels ω-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 ± 17 nM to 87 ± 6 nM (n = 9; Fig. 3A). In neurones in which oscillations were only partially blocked the basal $[Ca^{2+}]_i$ also dropped from 143 ± 15 to 102 ± 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 μM nicardipine, 800 nM ω-agatoxin IVA, 300 nM ω-conotoxin GVIA and 50 μM 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 μM (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 ± 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 ± 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 μM) 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 μM 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 μM, 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 μM of Lanthanum (La^{3+}), which is known to effectively block plasmalemmal Ca^{2+} pump [12,17,36]. Exposure to 100 μM of La^{3+} inhibited $[Ca^{2+}]_i$ oscillations in 8 out of 9 neurones (Fig. 5F) with rather poor recovery.

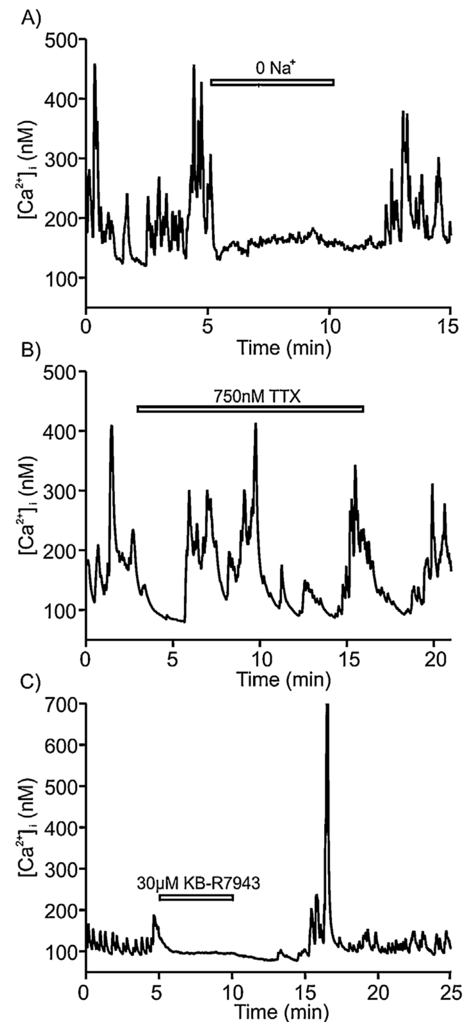

Fig. 4. Role of extracellular Na^+, voltage-gated Na^+ channels and NCX.
A: Removal of external Na^+ completely inhibits $[Ca^{2+}]_i$ oscillations;
B: Inhibition of voltage-gated Na^+ channels with TTX does not affect spontaneous $[Ca^{2+}]_i$ oscillations.
C: Exposure of magnocellular neurone to KB-R734, a specific inhibitor of NCX reverse mode, completely inhibits spontaneous $[Ca^{2+}]_i$ oscillations.

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

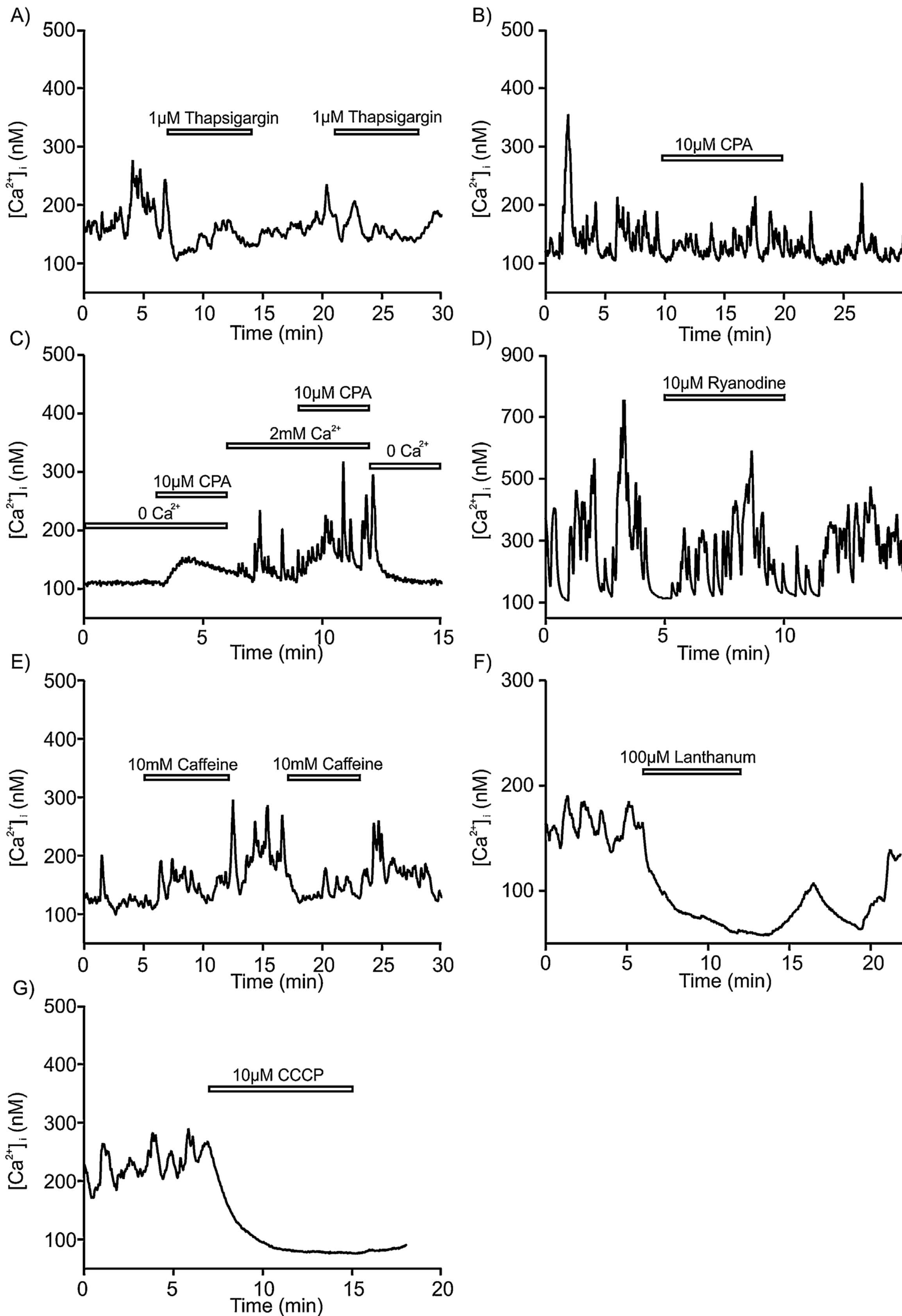

Fig. 5. Effect of intracellular Ca^{2+} mobilisers and inhibitors of Ca^{2+} clearance mechanisms.
A, B: Inhibition of endoplasmic reticulum Ca^{2+} pumps (of SERCA type) by thapsigargin (A) and CPA (B) does not affect spontaneous $[Ca^{2+}]_i$ oscillations.
C: Shows the effect of CPA on $[Ca^{2+}]_i$ 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 μM 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.

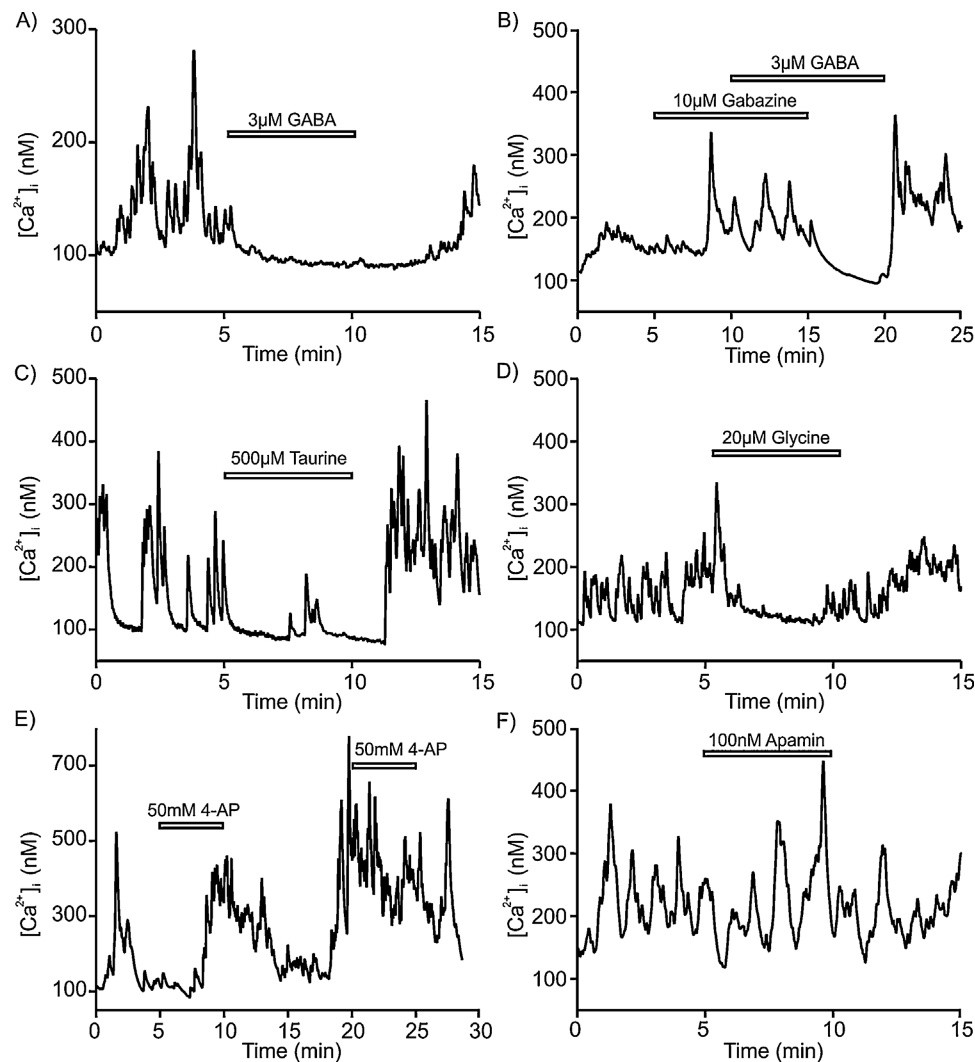

Fig. 6. Effect of neurotransmitters and blockers of K^+ currents on $[Ca^{2+}]_i$ oscillations.
A: Trace showing the blocking effect of GABA (3 μM) on spontaneous $[Ca^{2+}]_i$ oscillations.
B: Trace showing the reversibility of GABA effect by its antagonist, gabazine.
C, D: Traces showing the blocking 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 μM; n = 4) nor NMDA (100 μM; 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 μM U-73211; Fig. 7A): a competitive antagonist of cAMP dependent PKA (50 μM Rp-cAMP; Fig. 7B), an AC inhibitor (10 μM SQ-22536; Fig. 7C) and an inhibitor of PKA (10 μM 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

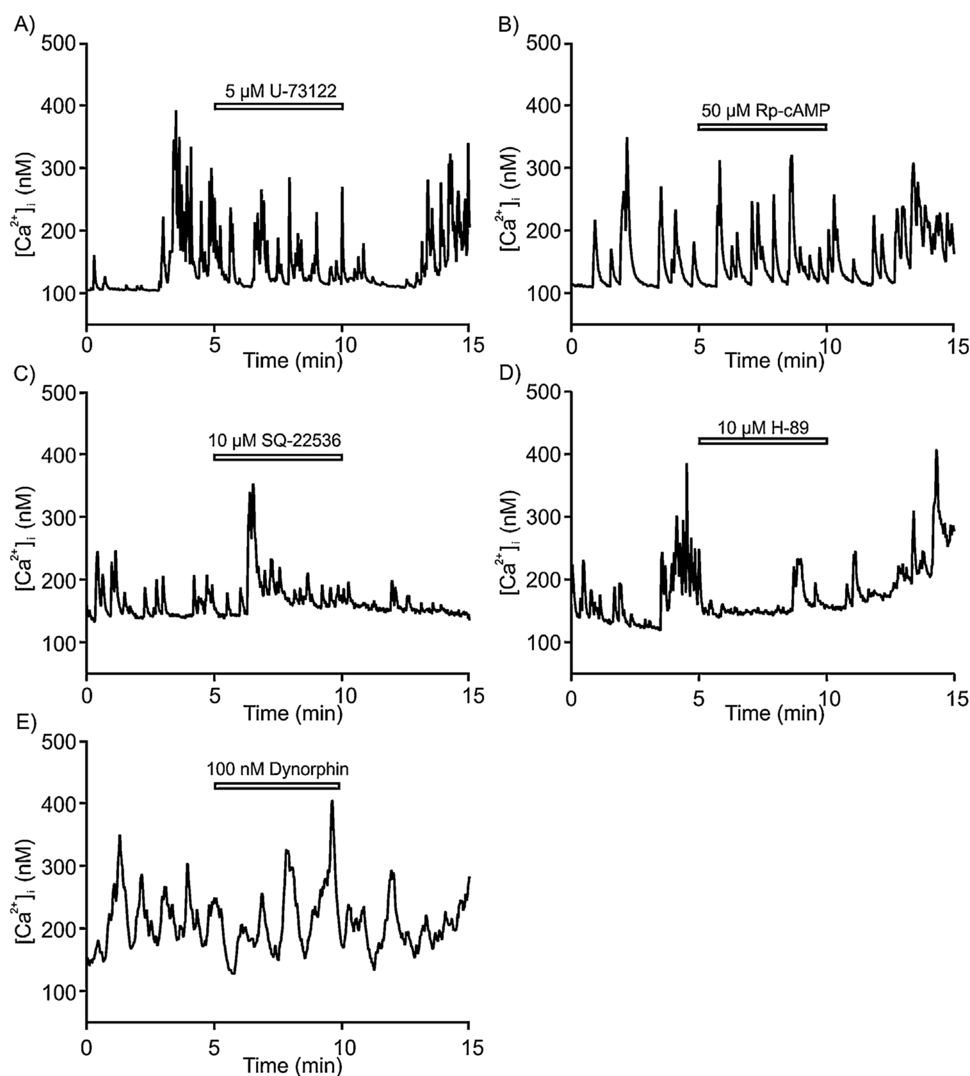

Fig. 7. The effect of blockers of phospholipase C and adenylyl cyclase intracellular transduction pathways on oscillations.
A-D: Inhibition of signalling cascades by PLC blocker (U-73122; A), competitive blocker of cAMP-dependent PKA (Rp-cAMP; B), an AC blocker (SQ-22536; C) and PKA blocker (H-89; D) 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 Ca^{2+} oscillatory activity.

5. Conclusions

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

References

[1] G. Dayanithi, N. Sabatier, H. Widmer, Intracellular calcium signalling in magnocellular neurones of the rat supraoptic nucleus: understanding the autoregulatory mechanisms, Exp. Physiol. 85 (2000) 75S–84S (Spec No).

[2] W.E. Armstrong, Morphological and electrophysiological classification of hypothalamic supraoptic neurons, Prog. Neurobiol. 47 (1995) 291–339.

[3] C.H. Brown, J.S. Bains, M. Ludwig, J.E. Stern, Physiological regulation of magnocellular neurosecretory cell activity: integration of intrinsic, local and afferent mechanisms, J. Neuroendocrinol. 25 (2013) 678–710.

[4] M. Ludwig, N. Sabatier, P.M. Bull, R. Landgraf, G. Dayanithi, G. Leng, Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites, Nature 418 (2002) 85–89.

[5] P. Richard, F. Moos, G. Dayanithi, L. Gouzenes, N. Sabatier, Rhythmic activities of hypothalamic magnocellular neurons: autocontrol mechanisms, Biol. Cell 89 (1997) 555–560.

[6] G. Dayanithi, H. Widmer, P. Richard, Vasopressin-induced intracellular Ca^{2+} increase in isolated rat supraoptic cells, J. Physiol. 490 (Pt 3) (1996) 713–727.

[7] R.C. Lambert, G. Dayanithi, F.C. Moos, P. Richard, A rise in the intracellular Ca^{2+} concentration of isolated rat supraoptic cells in response to oxytocin, J. Physiol. 478 (Pt 2) (1994) 275–287.

[8] N. Sabatier, P. Richard, G. Dayanithi, L-, N- and T- but neither P- nor Q-type Ca^{2+} channels control vasopressin-induced Ca^{2+} influx in magnocellular vasopressin neurones isolated from the rat supraoptic nucleus, J. Physiol. 503 (Pt 2) (1997) 253–268.

[9] N. Sabatier, P. Richard, G. Dayanithi, Activation of multiple intracellular transduction signals by vasopressin in vasopressin-sensitive neurones of the rat supraoptic nucleus, J. Physiol. 513 (Pt 3) (1998) 699–710.

[10] L. Gouzenes, N. Sabatier, P. Richard, F.C. Moos, G. Dayanithi, V1a- and V2-type vasopressin receptors mediate vasopressin-induced Ca^{2+} responses in isolated rat supraoptic neurones, J. Physiol. 517 (Pt 3) (1999) 771–779.

[11] N. Sabatier, I. Shibuya, G. Dayanithi, Intracellular calcium increase and somatodendritic vasopressin release by vasopressin receptor agonists in the rat supraoptic nucleus: involvement of multiple intracellular transduction signals, J. Neuroendocrinol. 16 (2004) 221–236.

[12] Y. Komori, M. Tanaka, M. Kuba, M. Ishii, M. Abe, N. Kitamura, A. Verkhratsky, I. Shibuya, G. Dayanithi, Ca(2+) homeostasis, Ca(2+) signalling and somatodendritic vasopressin release in adult rat supraoptic nucleus neurones, Cell Calcium 48 (2010) 324–332.

[13] M. Ludwig, P.M. Bull, V.A. Tobin, N. Sabatier, R. Landgraf, G. Dayanithi, G. Leng, Regulation of activity-dependent dendritic vasopressin release from rat supraoptic neurones, J. Physiol. 564 (2005) 515–522.

[14] M. Cazalis, G. Dayanithi, J.J. Nordmann, The role of patterned burst and interburst interval on the excitation-coupling mechanism in the isolated rat neural lobe, J. Physiol. 369 (1985) 45–60.

[15] P. Roper, J. Callaway, T. Shevchenko, R. Teruyama, W. Armstrong, AHP's, HAP's and DAP's: how potassium currents regulate the excitability of rat supraoptic neurones, J. Comput. Neurosci. 15 (2003) 367–389.

[16] I. Shibuya, J. Noguchi, K. Tanaka, N. Harayama, U. Inoue, N. Kabashima, Y. Ueta, Y. Hattori, H. Yamashita, PACAP increases the cytosolic Ca^{2+} concentration and stimulates somatodendritic vasopressin release in rat supraoptic neurons, J. Neuroendocrinol. 10 (1998) 31–42.

[17] G. Dayanithi, O. Forostyak, Y. Ueta, A. Verkhratsky, E.C. Toescu, Segregation of calcium signalling mechanisms in magnocellular neurones and terminals, Cell Calcium 51 (2012) 293–299.

[18] J. Sneyd, J. Keizer, M.J. Sanderson, Mechanisms of calcium oscillations and waves: a quantitative analysis, FASEB J. 9 (1995) 1463–1472.

[19] Y. Ueta, H. Fujihara, R. Serino, G. Dayanithi, H. Ozawa, K. Matsuda, M. Kawata, J. Yamada, S. Ueno, A. Fukuda, D. Murphy, Transgenic expression of enhanced green fluorescent protein enables direct visualization for physiological studies of vasopressin neurons and isolated nerve terminals of the rat, Endocrinology 146 (2005) 406–413.

[20] H. Fujihara, K. Sasaki, E. Mishiro-Sato, T. Ohbuchi, G. Dayanithi, M. Yamasaki, Y. Ueta, N. Minamino, Molecular characterization and biological function of neuroendocrine regulatory peptide-3 in the rat, Endocrinology 153 (2012) 1377–1386.

[21] T. Moriya, R. Shibasaki, T. Kayano, N. Takebuchi, M. Ichimura, N. Kitamura, A. Asano, Y.Z. Hosaka, O. Forostyak, A. Verkhratsky, G. Dayanithi, I. Shibuya, Full-length transient receptor potential vanilloid 1 channels mediate calcium signals and possibly contribute to osmoreception in vasopressin neurones in the rat supraoptic nucleus, Cell Calcium 57 (2015) 25–37.

[22] H. Fujihara, Y. Ueta, H. Suzuki, A. Katoh, T. Ohbuchi, H. Otsubo, G. Dayanithi, D. Murphy, Robust up-regulation of nuclear red fluorescent-tagged fos marks neuronal activation in green fluorescent vasopressin neurons after osmotic stimulation in a double transgenic rat, Endocrinology 150 (9) (2009) 5633–5638.

[23] C. Viero, I. Shibuya, N. Kitamura, A. Verkhratsky, H. Fujihara, A. Katoh, Y. Ueta, H.H. Zingg, A. Chvatal, E. Sykova, G. Dayanithi, Oxytocin: crossing the bridge between basic science and pharmacotherapy, CNS Neurosci. Ther. 16 (2010) e138–e156.

[24] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.

[25] G. Dayanithi, I. Mechaly, C. Viero, H. Aptel, S. Alphandery, S. Puech, F. Bancel, J. Valmier, Intracellular Ca^{2+} regulation in rat motoneurons during development, Cell Calcium 39 (2006) 237–246.

[26] C. Viero, I. Mechaly, H. Aptel, S. Puech, J. Valmier, F. Bancel, G. Dayanithi, Rapid inhibition of Ca^{2+} influx by neurosteroids in murine embryonic sensory neurones, Cell Calcium 40 (2006) 383–391.

[27] N. Sasaki, G. Dayanithi, I. Shibuya, Ca^{2+} clearance mechanisms in neurohypophysial terminals of the rat, Cell Calcium 37 (2005) 45–56.

[28] F.M. Dreyfus, A. Tscherter, A.C. Errington, J.J. Renger, H.S. Shin, V.N. Uebele, V. Crunelli, R.C. Lambert, N. Leresche, Selective T-type calcium channel block in thalamic neurons reveals channel redundancy and physiological impact of I(T)window, J. Neurosci. 30 (2010) 99–109.

[29] E. Bourinet, S.C. Stotz, R.L. Spaetgens, G. Dayanithi, J. Lemos, J. Nargeot, G.W. Zamponi, Interaction of SNX482 with domains III and IV inhibits activation gating of alpha(1E) (Ca(V)2.3) calcium channels, Biophys. J. 81 (2001) 79–88.

[30] G. Wang, G. Dayanithi, R. Newcomb, J.R. Lemos, An R-type Ca(2+) current in neurohypophysial terminals preferentially regulates oxytocin secretion, J. Neurosci. 19 (1999) 9235–9241.
[31] S. Ortiz-Miranda, G. Dayanithi, E. Custer, S.N. Treistman, J.R. Lemos, Micro-opioid receptor preferentially inhibits oxytocin release from neurohypophysial terminals by blocking R-type Ca^{2+} channels, J. Neuroendocrinol. 17 (2005) 583–590.
[32] S. Kawano, K. Otsu, S. Shoji, K. Yamagata, M. Hiraoka, Ca(2+) oscillations regulated by Na(+)-Ca(2+) exchanger and plasma membrane Ca(2+) pump induce fluctuations of membrane currents and potentials in human mesenchymal stem cells, Cell Calcium 34 (2003) 145–156.
[33] T. Brustovetsky, M.K. Brittain, P.L. Sheets, T.R. Cummins, V. Pinelis, N. Brustovetsky, KB-R7943 an inhibitor of the reverse Na^{+}/Ca^{2+} exchanger, blocks *N*-methyl-D-aspartate receptor and inhibits mitochondrial complex I, Br. J. Pharmacol. 162 (2011) 255–270.
[34] M. Wakui, Y.V. Osipchuk, O.H. Petersen, Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca2(+)-induced Ca^{2+} release, Cell 63 (1990) 1025–1032.
[35] I. Bezprozvanny, S. Bezprozvannaya, B.E. Ehrlich, Caffeine-induced inhibition of inositol(1,4,5)-trisphosphate-gated calcium channels from cerebellum, Mol, Biol. Cell 5 (1994) 97–103.
[36] N. Wanaverbecq, S.J. Marsh, M. Al-Qatari, D.A. Brown, The plasma membrane calcium-ATPase as a major mechanism for intracellular calcium regulation in neurones from the rat superior cervical ganglion, J. Physiol. 550 (2003) 83–101.
[37] Y. Hattori, I. Shibuya, K. Tanaka, N. Kabashima, Y. Ueta, H. Yamashita, Ionotropic and metabotropic glutamate receptor agonist-induced $[Ca^{2+}]i$ increase in isolated rat supraoptic neurons, J. Neuroendocrinol. 10 (1998) 383–389.
[38] I. Shibuya, N. Kabashima, N. Ibrahim, S.V. Setiadji, Y. Ueta, H. Yamashita, Pre- and postsynaptic modulation of the electrical activity of rat supraoptic neurones, Exp. Physiol. 85 (2000) 145s–151s (Sec No).
[39] C.H. Brown, G. Leng, In vivo modulation of post-spike excitability in vasopressin cells by kappa-opioid receptor activation, J. Neuroendocrinol. 12 (2000) 711–714.
[40] J. Sneyd, K. Tsaneva-Atanasova, D.I. Yule, J.L. Thompson, T.J. Shuttleworth, Control of calcium oscillations by membrane fluxes, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1392–1396.
[41] A. Verkhratsky, Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons, Physiol. Rev. 85 (2005) 201–279.
[42] E.C. Toescu, J.J. Nordmann, Effect of sodium and calcium on basal secretory activity of rat neurohypophysial peptidergic nerve terminals, J. Physiol. 433 (1991) 127–144.