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In vitro differentiation between oxytocin- and vasopressin-secreting magnocellular neurons requires more than one experimental criterion

M.P. da Silva [a,1], R.M. Merino [a,1], A.S. Mecawi [b,2], D.J. Moraes [a], W.A. Varanda [a,*]

[a] Department of Physiology, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, SP, Brazil
[b] Department of Physiology, Faculty of Medicine, University of Malaysia, Malaysia

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ABSTRACT

The phenotypic differentiation between oxytocin (OT)- and vasopressin (VP)-secreting magnocellular neurosecretory cells (MNCs) from the supraoptic nucleus is relevant to understanding how several physiological and pharmacological challenges affect their electrical activity. Although the firing patterns of OT and VP neurons, both *in vivo* and *in vitro*, may appear different from each other, much is assumed about their characteristics. These assumptions make it practically impossible to obtain a confident phenotypic differentiation based exclusively on the firing patterns. The presence of a sustained outward rectifying potassium current (SOR) and/or an inward rectifying hyperpolarization-activated current (IR), which are presumably present in OT neurons and absent in VP neurons, has been used to distinguish between the two types of MNCs in the past. In this study, we aimed to analyze the accuracy of the phenotypic discrimination of MNCs based on the presence of rectifying currents using comparisons with the molecular phenotype of the cells, as determined by single-cell RT-qPCR and immunohistochemistry. Our results demonstrated that the phenotypes classified according to the electrophysiological protocol in brain slices do not match their molecular counterparts because vasopressinergic and intermediate neurons also exhibit both outward and inward rectifying currents. In addition, we also show that MNCs can change the relative proportion of each cell phenotype when the system is challenged by chronic hypertonicity (70% water restriction for 7 days). We conclude that for *in vitro* preparations, the combination of mRNA detection and immunohistochemistry seems to be preferable when trying to characterize a single MNC phenotype.

1. Introduction

Magnocellular neurosecretory cells (MNCs) located in the hypothalamic supraoptic nuclei are responsible for the synthesis and secretion of vasopressin (VP) and oxytocin (OT). Once released by the neurohypophysis to the circulation, these neuropeptides have different effects. Because they can be released independently (Antunes-Rodrigues et al., 2004), studies have been performed to identify parameters that would allow a quick and reliable phenotypic differentiation between MNCs.

From the 1960s until the end of the 1980s, the classical view of hormone production by the MNCs of the supraoptic nucleus relied on the premise that OT and VP are produced by mutually exclusive types of neurons (Mohr et al., 1988a; Sokol and Valtin, 1967). Later, it was shown that a third phenotype, which was able to produce both hormones, was also present in the supraoptic nucleus (Kiyama and Emson, 1990; Mezey and Kiss, 1991). Currently, evidence shows that any MNC can produce the mRNA for both VP and OT, albeit in different quantities. Thus, a given phenotype may be defined based on the predominantly expressed peptide, i.e., vasopressinergic neurons would produce circa two orders of magnitude more vasopressin than oxytocin and *vice versa*. The third, "intermediate" phenotype, from now on referred to as intermediate cells, would produce both peptides in equivalent amounts (Custer et al., 2007; Glasgow et al., 1999; Xi et al., 1999).

Distinguishing phenotypes among MNCs becomes relevant in experiments studying the modulatory effects of drugs on electrical activity because different results can be obtained depending on the phenotype of each cell (Hirasawa et al., 2003; Hoyda et al., 2007; Zampronio et al., 2010). In this regard, it has been suggested that OT and VP neurons can show different electrophysiological properties with a coupling between the firing rate pattern and the release of a given neuropeptide (Poulain and Wakerley, 1982). From these types of parameters, cells were identified by associating

* Corresponding author. Department of Physiology, Ribeirão Preto Medical School, University of São Paulo, 14049-900, Ribeirão Preto, SP, Brazil. Tel.: +55 16 3602 3024; fax: +55 16 3630 0017.
E-mail address: wvaranda@fmrp.usp.br (W.A. Varanda).
[1] da Silva MP and Merino RM contributed equally to this paper.
[2] Present address: Department of Physiological Sciences, Institute of Biology, Federal Rural University of Rio de Janeiro, Brazil.

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phenotypes to specific action potential firing characteristics: a firing pattern in which action potentials are intercalated with silent periods was considered typical of VP neurons, whereas a pattern showing bursts of high frequency firing would characterize OT neurons (Brimble and Dyball, 1977; Poulain et al., 1977; Yamashita et al., 1983). However, Moos and Ingram (1995), Armstrong (1995) and Armstrong et al. (2010) observed that *in vivo* and *in vitro*, both VP and OT neurons may fire action potentials at high or low frequency, phasically or in bursts, or they may even be silent. These findings suggest that the action potential firing pattern should not be used as the single criterion to distinguish between OT and VP phenotypes.

Another procedure to electrophysiologically distinguish between these two neuronal types was suggested by Stern and Armstrong (1995, 1997) based on their observation that for negative current injections, the current–voltage relationship of VP neurons is linear, while that of OT neurons is not. The authors report that this difference occurs because OT neurons exhibit the so-called sustained outward rectifying potassium current (SOR), which is activated upon the application of negative current steps (Armstrong and Stern, 1998; Stern and Armstrong, 1995). In addition, experiments using immunolabeled neurons or neurons expressing eGFP have suggested that OT neurons have not only SOR but also a hyperpolarization-activated inward rectifying current (IR); this property could also be used to distinguish them from VP neurons (Hirasawa et al., 2003; Zampronio et al., 2010). Although these protocols have been used, they clearly disregard the existence of intermediate neurons. Studies using single-cell RT-qPCR demonstrated that all MNCs express both OT and VP mRNA, albeit in different quantities (Glasgow et al., 1999; Xi et al., 1999).

In this paper, we combined real-time single-cell RT-qPCR, immunofluorescence and patch-clamp recordings of MNCs in slices of the supraoptic nucleus of male rats to determine the reliability of the different methods for detecting a particular type of MNC. In addition, we also used water restriction and salt-loading rat models to investigate changes in the proportion of phenotypically different cells. Our results show that the *whole cell* voltage clamp electrophysiology protocol is not sensitive enough to discriminate between MNC phenotypes. This inadequacy mainly occurs because vasopressinergic and intermediate neurons can both exhibit non-linear current–voltage relationships, and oxytocin neurons exhibit a linear relationship. Furthermore, we also demonstrated that in rats subjected to a water restriction protocol, the proportion of OT and VP neurons changes, suggesting that MNCs are, in principle, able to synthesize and modulate the quantities of both peptides.

2. Material and methods

2.1. Ethics

The experimental protocols used in the present study were approved by the Ethical Committee for Animal Experimentation of the Ribeirão Preto Medical School, University of São Paulo (023/2012) in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health (USA).

2.2. Hypothalamic slices preparation

Hypothalamic brain slices from male *Wistar* rats (25–30 days old) were prepared as described previously (da Silva et al., 2013). In short, following decapitation, the brain was rapidly removed from the cranial vault and submerged in cooled (0–4 °C), oxygenated (95% O_2–5% CO2) artificial cerebro-spinal fluid solution (ACSF) containing (in mM) 121 NaCl, 2.5 KCl, 26 $NaHCO_3$, 25 glucose, 2 sodium lactate, 0.4 sodium ascorbate, 2 $CaCl_2$ and 1 $MgCl_2$ that was saturated with carbogen (pH = 7.4 and osmolality = 310 mosm/kg H_2O (Fiske Mark 3 Osmometer, Norwood, USA)). Next, 230 μm thick coronal slices containing the supraoptic nucleus were obtained using a Leica vibratome (model VT 1200 S, Heidelberg, Germany). They were incubated for at least 1 h at 36 °C in a modified ACSF containing (in mM) 121 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 glucose and 1.25 NaH_2PO_4 (constantly gassed with carbogen, pH = 7.4 and osmolality = 295 mosm/kgH_2O) for metabolic reestablishment.

2.3. Electrophysiology

Magnocellular neurons of the supraoptic nucleus were visualized using infrared differential contrast optics with images captured by a CCD digital camera (SensiCam qe, The Cooke Corporation, Romulus, MI, USA). Anatomical and morphological characteristics were used for their identification (Paxinos and Watson, 2007). Electrophysiological recordings were made using the *voltage clamp* mode in the *whole cell* configuration of the patch clamp technique. Micropipettes were made from borosilicate glass capillaries and had resistance ranging from 4 to 6 MΩ when filled with the following pipette solution (in mM): 140 potassium gluconate, 10 KCl, 0.75 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Na-ATP and 0.25 Na- GTP (pH = 7.3, and osmolality ranging from 285 to 295 mosm/KgH_2O). The "Milli Q" water used to prepare pipette solutions was treated overnight with Diethylpyrocarbonate (DEPC) (Sigma Chemical Corporation, St. Louis, MO, USA). Recordings were performed using an Axon 200B patch clamp amplifier (Molecular Devices, Foster City, CA, USA), and signals were low-pass filtered at 2 KHz and digitized at 10 KHz using a Digidata 1440A (Molecular Devices) controlled by Clampex (Molecular Devices). Recordings started 5 min after achieving the *whole cell* configuration, and the membrane capacitance and series resistance were compensated (40–60%) using the amplifier settings. The liquid junction potential between the pipette and bath solutions was calculated (–15 mV) using Clampex, and the values presented here were corrected accordingly. All data were analyzed using Clampfit (Molecular Devices) and OriginPro 8 (OriginLab Corporation, Northampton, MA, USA).

2.3.1. Electrophysiological phenotypic differentiation protocol

The electrophysiological protocol used to differentiate OT from VP cells was based on that described by others (Armstrong and Stern, 1998; Hirasawa et al., 2003). Briefly, the discrimination is based on the presence or absence of sustained outward and/or hyperpolarized-activated rectifying potassium currents. Under *voltage clamp*, hyperpolarizing voltage pulses from –40 to –130 mV from a holding potential of –40 mV and lasting 250 ms were applied, and steady-state current–voltage (I–V) relationships were constructed. In this context, linear relationships defined VP neurons, and non-linear relationships defined OT neurons.

2.4. Molecular identification of phenotypes

Subsequent to the electrophysiological recordings, the cytoplasm of each cell was collected into the recording pipette and transferred to a reaction tube containing reverse transcription master mix in DEPC-treated water (High Capacity cDNA Reverse Transcription Kit, Life Technologies Corporation, Carlsbad, CA, USA). The reverse transcription was performed immediately after the recording in a thermocycler (Master Cycler, Eppendorf, Hamburg, Germany) using the protocol recommended by the reverse transcription kit manufacturer.

2.4.1. cDNA pre-amplification reaction

Because the amount of cDNA obtained from a single-cell is very small, a pre-amplification step was performed using the TaqMan PreAmp Master Mix Kit (Life Technologies Corporation) with the following hydrolysis probes: Rn00564446_g1 (oxytocin) and