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## Store-operated Ca<sup>2+</sup> entry and Ca<sup>2+</sup> responses to hypothalamic releasing hormones in anterior pituitary cells from *Orai1*<sup>-/-</sup> and heptaTRPC knockout mice<sup>★</sup>

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### Abstract

Store operated Ca<sup>2+</sup> entry (SOCE) is the most important Ca<sup>2+</sup> entry pathway in non-excitabile cells. However, SOCE can also play a pivotal role in excitable cells such as anterior pituitary (AP) cells. The AP gland contains five different cell types that release six major AP hormones controlling most of the entire endocrine system. AP hormone release is modulated by Ca<sup>2+</sup> signals induced by different hypothalamic releasing hormones (HRHs) acting on specific receptors in AP cells. TRH and LHRH both induce Ca<sup>2+</sup> release and Ca<sup>2+</sup> entry in responsive cells while GHRH and CRH only induce Ca<sup>2+</sup> entry. SOCE has been shown to contribute to Ca<sup>2+</sup> responses induced by TRH and LHRH but no molecular evidence has been provided. Accordingly, we used AP cells isolated from mice devoid of *Orai1* channels (noted as *Orai1*<sup>-/-</sup> or *Orai1* KO mice) and mice lacking expression of all seven canonical TRP channels (TRPC) from TRPC1 to TRPC7 (noted as heptaTRPC KO mice) to investigate contribution of these putative channel proteins to SOCE and intracellular Ca<sup>2+</sup> responses induced by HRHs. We found that thapsigargin-evoked SOCE is lost in AP cells from *Orai1*<sup>-/-</sup> mice but unaffected in cells from heptaTRPC KO mice. Conversely, while spontaneous intracellular Ca<sup>2+</sup>-oscillations related to electrical activity were not affected in the *Orai1*<sup>-/-</sup> mice, these responses were significantly reduced in heptaTRPC KO mice. We also found that Ca<sup>2+</sup> entry induced by TRH and LHRH is decreased in AP cells isolated from *Orai1*<sup>-/-</sup>. In addition, Ca<sup>2+</sup> responses to several HRHs, particularly TRH and GHRH, are decreased in the heptaTRPC KO mice. These results indicate that expression of *Orai1*, and not TRPC channel proteins, is necessary for thapsigargin-evoked SOCE and is required to support Ca<sup>2+</sup> entry induced by TRH and LHRH in mouse AP cells. In contrast, TRPC channel proteins appear to contribute to spontaneous Ca<sup>2+</sup>-oscillations and Ca<sup>2+</sup> responses induced by TRH and GHRH. We conclude that expression of *Orai1* and TRPC channels proteins may play differential and significant roles in AP physiology and endocrine control.

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## Keywords

Anterior pituitary cells; Orai1; TRPC channels; Store-operated calcium entry; Hypothalamic releasing factors

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## 1. Introduction

The anterior pituitary (AP) gland is a major endocrine modulator that regulates multiple endocrine glands and tissues by the release of different AP hormones including prolactin (PRL), growth hormone (GH), thyrotropin (TSH), adrenocorticotropin (ACTH), and the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The secretory activity of the gland is under control of chemical signals derived from the hypothalamus in the form of hypothalamic-releasing hormones (HRHs) that reach the AP through the portal system vasculature. HRHs include thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), corticotropin-releasing hormone (CRH), and gonadotropin-releasing hormone (LHRH). AP hormone secretion is limited by hypothalamic inhibitory factors such as dopamine and somatostatin as well as by negative feedback loops of peripheral hormones as well as AP hormones [1].

The effect of HRHs on AP hormone secretion is mediated by intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}_i^{2+}$ ) mobilization in target cells. In many AP cells, this  $\text{Ca}^{2+}$  signal is in the form of spontaneous intracellular  $\text{Ca}^{2+}$ -oscillations that are generated by spontaneous electrical activity in the form of  $\text{Ca}^{2+}$  action potentials in which the depolarizing current is carried by  $\text{Ca}^{2+}$  instead of  $\text{Na}^+$  [2]. Inhibition of electrical activity by selected  $\text{Ca}^{2+}$  channel antagonists and/or physiological inhibitors like dopamine or somatostatin prevents the occurrence of  $\text{Ca}^{2+}$  oscillations and inhibits AP hormone secretion [2,3]. Several HRHs induce a rise in  $\text{Ca}_i^{2+}$  in target cells and elicit AP hormone secretion, but the mechanisms underlying these  $\text{Ca}_i^{2+}$  signals are not completely understood [4]. For example, TRH activates TRH receptors in thyrotropes storing TSH as well as in PRL producing cells (mammotropes), and induce a biphasic  $\text{Ca}_i^{2+}$  response. This biphasic  $\text{Ca}_i^{2+}$  response is composed of a first phase due to  $\text{IP}_3$  dependent-release of  $\text{Ca}^{2+}$  from intracellular stores. The second phase is complex and is associated with an increased firing of action potentials which leads to increased  $\text{Ca}_i^{2+}$  oscillations and enhanced entry of  $\text{Ca}^{2+}$  through L-type voltage-gated  $\text{Ca}^{2+}$  channels [3,5]. However, we reported that store-operated  $\text{Ca}^{2+}$  entry (SOCE) also contributed to  $\text{Ca}^{2+}$  entry induced by TRH [3]. At that time, the molecular players involved in SOCE were unknown and no molecular evidence for the involvement of SOCE in  $\text{Ca}^{2+}$  responses to TRH and other HRHs has been provided so far. Similarly, LHRH promotes gonadotropin secretion mediated by  $\text{Ca}^{2+}$  release in gonadotropes followed by a second phase associated to a series of intracellular  $\text{Ca}_i^{2+}$  oscillations. However, the occurrence and contribution of SOCE to LHRH-induced  $\text{Ca}_i^{2+}$  responses remain controversial. GHRH increases  $\text{Ca}_i^{2+}$  in somatotropes and promotes GH secretion [2,6].  $\text{Ca}^{2+}$  responses elicited by GHRH are mediated by cAMP-dependent activation of protein kinase A (PKA) and a  $\text{Na}^+$  conductance that increases electrical activity of the somatotrope [7]. However, the nature of the  $\text{Na}^+$

conductance remains unknown. Finally, CRH also increases  $Ca_i^{2+}$  in a fraction of rat and mouse AP cells by a PKA-dependent mechanism and enhances electrical activity. In this case the mechanism is believed to be mediated by the closure of TREK-1  $K^+$  channels involved in resting electrical activity in the corticotrope [8,9].

It is worth noting that these hormone-specific actions are not restricted/partitioned into separate, specialized cells with a single receptor type. In fact, many rat and mouse AP cells express more than one HRH receptor type [10]. These cells, termed multiresponsive cells, may contribute to AP cell plasticity and secrete AP hormones in response to several HRHs [4]. AP cells may also be polyhormonal cells storing and releasing more than one AP hormones [11]. Multiresponsive and polyhormonal AP cells are multifunctional cells present in each of the five “classic” AP cell types including mammotropes, somatotropes and gonadotropes [12] and thyrotropes [13]. Multifunctional cells are also present in human pituitary tumors [14,15] and their relative abundance is dictated by the gender and physiological status of the donor [16] as well as external influences such as stress [17]. Accordingly, signal transduction pathways are probably not partitioned into specific cell types and may contribute to hormone secretion in a complex manner, probably depending on the gender, age and physiological status.

Since TRH and LHRH induce release of  $Ca^{2+}$  from intracellular stores, it is plausible that SOCE contributed to evoke  $Ca_i^{2+}$  signals. In 1995, it was reported that SOCE contributed to  $Ca_i^{2+}$  entry induced by TRH in prolactin producing cells [3,18]. It was the first evidence on the occurrence and physiological role of SOCE in excitable cells. It was observed that blocking L-type, voltage-gated  $Ca^{2+}$  entry with dihydropyridines, PRL secretion and hormone gene expression was only partially inhibited. This data suggested the presence of another channel responsible for  $Ca^{2+}$  entry and hormone secretion in AP cells. In testing for the presence of SOCE in gonadotropes, the authors could find no evidence of calcium release activated calcium currents (CRAC), which underlie SOCE in non-excitabile cells [19]. In addition, observations on ACTH release by rat corticotropes could find no role for SOCE [20]. As stated above, no molecular evidence for contribution of SOCE to AP cell function has been reported to date.

At the molecular level, SOCE is mediated by interactions between stromal interaction molecule 1 (STIM1) [21], a sensor of the  $Ca^{2+}$  concentration inside the endoplasmic reticulum (ER), with Orai1, a  $Ca^{2+}$  channel at the plasma membrane [22]. Related protein STIM2, as well as Orai2 and Orai3, may also contribute in some cases to SOCE but their contribution is considered minor [23]. Unfortunately, there is no information on expression of Orai and STIM proteins in AP cells.

Some transient receptor potential (TRP) channels may contribute to SOCE as well in some cell types [24,25]. TRP channels were initially considered candidates for the channels involved in SOCE [26]. However, the controversy remains as to whether some canonical TRPs (TRPC) channels contribute to SOCE. The present view is that TRPC channels may rather modulate SOCE after their recruitment to the plasma membrane by Orai1-dependent  $Ca^{2+}$  influx [27]. For instance, contribution of TRPC channels to SOCE may depend on

the phenotypic transformation of cells [28]. Expression of TRPCs has been assessed by qRT-PCR in rat AP cells. Results show expression of mRNA transcripts for TRPC1  $\gg$  TRPC6 > TRPC4 > TRPC5 > TRPC3 in rat and mouse AP cells [20,29]. In addition, in AP cells, constitutively active cation channels are stimulated further by PKA and contribute to calcium signaling indirectly by controlling the pacemaking depolarization in a sodium-dependent manner and directly by conducting calcium [29]. TRPC channel function in the different axes has not been addressed except for the case of the reproductive axis. Thus, TRPC5 has been functionally characterized in female murine gonadotropes [30]. It has been also demonstrated that TRPC5 in gonadotropes contributes to depolarization of the plasma membrane upon GnRH stimulation and increases the intracellular  $\text{Ca}^{2+}$  concentration via its own  $\text{Ca}^{2+}$  permeability and subsequent activation of voltage-gated  $\text{Ca}^{2+}$  channels [31]. However, conditional gene targeting experiments are required to unambiguously dissect the physiological role of TRPC channels in the different cell types of the reproductive axis in vivo.

The detection and measurement of SOCE is technically challenging in AP cells. The observed  $\text{Ca}^{2+}$  entry signals associated with SOCE are small in excitable cells including neurons [32] and AP cells [3] compared with the magnitude of this pathway in non-excitabile cells or tumor cells [28]. In addition,  $\text{Ca}^{2+}$  entry signal is often contaminated with the large voltage-gated  $\text{Ca}^{2+}$  channels that may be active even in resting conditions when cells have not been treated with hormones. A major challenge in these studies is due to the large heterogeneity of AP cell population, containing up to five different AP cell types, which may also be further complicated by differential expression of up to seven different types of TRPC channel proteins in addition to Orai1, all of which might affect SOCE.

Here we have addressed the role of Orai1 and all seven TRPCs in SOCE and  $\text{Ca}_i^{2+}$  responses to all four HRHs in mouse AP cells. This was achieved by performing calcium imaging measurements on primary AP cells isolated from Orai1 $^{-/-}$  and heptaTRPC KO mice. Results indicate that Orai1 channels, but not TRPC channels, contribute to SOCE in mouse AP cells. In addition, we show for the first time that Orai1 channels contribute to  $\text{Ca}^{2+}$  entry signal induced by TRH and LHRH in mouse AP cells. Conversely, TRPC channels, but not Orai1 channels, are involved in spontaneous  $\text{Ca}_i^{2+}$  oscillations. Finally, we also show that TRPC channels could be involved in the  $\text{Ca}^{2+}$  responses induced by TRH and GHRH.

## 2. Methods

### 2.1. Reagents

Hypothalamic releasing factors including thyrotropin-releasing hormone (TRH), growth-hormone releasing hormone (GHRH), gonadotropin-releasing hormone (LHRH) and corticotropin-releasing hormone (CRH) were purchased from Sigma.-Aldrich and Fura5f/AM from Setereh Biotech (now Thermo Fisher).

## 2.2. Animals

Mice carrying *Orai1* null alleles (*Orai1*<sup>-/-</sup>, also noted as *Orai1* KO in the text) were kindly provided by Jean-Pierre Kinet (Harvard Medical School, Boston, MA) and were generated using gene trap mutagenesis [33]. These *Orai1* KO mice had a high incidence of perinatal lethality, which was improved by further outbreeding this line (C57/DBA/129 background) with Institute of Cancer Research (ICR) mice [Harlan Laboratories Inc., strain Hsd:ICR (CD-1)] and by delaying weaning in potential KO animals. Genotyping was performed on tail DNA by PCR. The following primers and reaction conditions were used to distinguish *Orai1*<sup>+/+</sup> (488 bp), *Orai1*<sup>+/-</sup> and *Orai1*<sup>-/-</sup> (300 bp): 5'-TCA CGC TTG CTC TCC TCA TC-3', 5'-TAA GGG CGA CAC GGA AAT G-3' and 5'-AGG TTG TGG ACG TTG CTC AC-3'; cycling for 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 10 min. For these studies we used five *Orai1* KO mice and five WT mice (4–6 months old, all male).

Mice lacking all seven canonical TRP proteins (heptaTRPC KO mice) were originally created by Dr. Lutz Birnbaumer and maintained by the Comparative Medicine Branch at the NIEHS, Research Triangle Park, North Carolina, USA. Initially, TRPC1, TRPC3, TRPC5 and TRPC6 knockout animals (QuadKO) were generated on a mixed C57BL/6J:129SvEv background by combinatorial breeding of single knockout alleles, TRPC1, TRPC3, TRPC5, TRPC6 as reported previously [34]. Quad KO mice exhibited generally good health and TRPC3/6 double knockout mice were crossed with C57BL/6 mice to generate wildtype (WT) control animals. For these studies we used five HeptaTRPC KO mice (3 months old, 3 male and 2 female) and five WT mice (3 male and 2 female).

## 2.3. Primary anterior pituitary cell culture

Mouse AP cells were obtained as reported previously [11]. Mice were euthanized by cervical dislocation, and the AP glands were rapidly dissected. After isolating the neurointermediate lobe, the glands were chopped into small pieces with dissecting scissors. A cell suspension was then generated by digestion with 1 mg/ml trypsin (Sigma-Aldrich) in Hanks' balanced salt solution (Life Technologies, Inc., Gaithersburg, MD) at 37 °C with gentle shaking for 30 min. Every 15 min, tissue pieces were triturated to release single cells by passing them repeatedly through a fire-polished siliconized Pasteur pipette. Isolated cells were then sedimented by centrifugation at 200g for 7 min, washed twice with Hanks' balanced salt solution. This process generated about  $1 \times 10^6$  cells per pituitary gland. Assessing viability by Trypan blue exclusion, dead cells were < 5%. In preparation for calcium measurements, monodispersed cells were plated onto round, 30 mm coverslips (#1.5) previously coated with 0.01 mg/ml poly-L-lysine. Plated cells were bathed in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics, and incubated in a humidified 95% air, 5% CO<sub>2</sub> at 37 °C and 95% CO<sub>2</sub>/O<sub>2</sub> until used. Cells were used within 2–4 h following isolation for calcium imaging experiments.

## 2.4. Single cell calcium measurements

Fluorescence measurements were made in AP cells loaded with the calcium sensitive dye fura-5F ( $K_D$  400 nm) as described in detail previously [35]. Coverslips with attached cells were mounted in a Teflon chamber and incubated in DMEM with 1 μM of the acetoxymethyl

ester form of fura-5F (fura-5F AM, Molecular Probes, USA) for 25 min in a humidified 95% air–5% CO<sub>2</sub> incubator at 37 °C. Before Ca<sub>i</sub><sup>2+</sup> measurements were made, cells were washed 3 times and incubated for 30 min at room temperature (25 °C) in a Hepes-buffered salt solution (HBSS (mM): NaCl 120; KCl 5.4; MgCl<sub>2</sub> 0.8; Hepes 20; CaCl<sub>2</sub> 1.8 and glucose 10 mM, with pH 7.4 adjusted by NaOH). In these experiments, nominally Ca<sup>2+</sup>-free solutions were HBSS with no added CaCl<sub>2</sub>.

Fluorescence was monitored by placing the Teflon chamber with fura-5F-loaded cells onto the stage of a Nikon TS-100 inverted microscope equipped with a 20× fluor objective (0.75 NA). Fluorescence images of the cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH, USA) equipped with a light-sensitive CCD camera (Cooke PixelFly, ASI, Eugene, OR, USA). Fura-5F fluorescence was monitored by alternately exciting the dye at 340 and 380nm, and collecting the emission wavelength at 510nm. Changes in Ca<sub>i</sub><sup>2+</sup> are represented as the ratio of fura-5F fluorescence due to excitation at 340nm to that due to excitation at 380nm (ratio= $F_{340}/F_{380}$ ). The ratio changes in fields of fura-5F-loaded cells were collected from a multiple regions of interest (ROI), with each ROI representing an individual cell. Typically, about 100 ROIs were monitored per experiment. In all cases, ratio values have been corrected for contributions by autofluorescence, which is measured after treating cells with 10 μM ionomycin and 20 mM MnCl<sub>2</sub>. Peak ratio values typically were less than 2.0 while  $R_{max}$ , determined in situ, averaged 6.5, indicating that the ratio values were less than half-saturation and thus essentially proportional to Ca<sup>2+</sup> concentration.

To assess SOCE, AP cells were initially bathed in HBSS lacking extracellular calcium and containing 200 μM of the calcium chelator BAPTA and 5 μM of nisoldipine to prevent Ca<sup>2+</sup> entry and activation of L-type voltage-gated Ca<sup>2+</sup> channels. The cells were then treated with the sarcoplasmic and endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) pump blocker thapsigargin (TG, 1 μM). This treatment results in a transient rise in intracellular Ca<sub>i</sub><sup>2+</sup> and the complete depletion the ER calcium stores. After 15 min, the external HBSS bathing cells exchanged to include 5 mM Ca<sup>2+</sup> in the presence of nisoldipine. This condition will permit the observation of any rise in intracellular Ca<sub>i</sub><sup>2+</sup> due to TG-induced, store-operated calcium entry (SOCE). The magnitude of the TG-induced calcium release and calcium entry (SOCE) was expressed as a Ratio, and represents the peak Ca<sub>i</sub><sup>2+</sup> Ratio with the baseline Ca<sub>i</sub><sup>2+</sup> Ratio subtracted.

To estimate the size of the releasable Ca<sup>2+</sup> store, we monitored the magnitude of the Ca<sub>i</sub><sup>2+</sup> signal evoked by the Ca<sup>2+</sup> ionophore ionomycin (1 μM), which was added to AP cells in the absence of extracellular Ca<sup>2+</sup> [3]. A Ratio for the resulting transient Ca<sub>i</sub><sup>2+</sup> response was calculated as described above. Observations of spontaneous Ca<sub>i</sub><sup>2+</sup> oscillations were performed over a period of at least 10 min. Cells displaying significant changes in Ca<sub>i</sub><sup>2+</sup> during the recording period were pooled as a fraction of oscillating cells. When measuring the Ca<sub>i</sub><sup>2+</sup> responses of AP cells to HRHs, a protocol was followed whereby cells were

stimulated sequentially with mouse TRH, GHRH, LHRH and CRH. All HRHs were tested at concentrations of 10 nM or 1  $\mu$ M. For analysis of  $\text{Ca}^{2+}$  responses in WT and *Orai1*<sup>-/-</sup> mice we performed 57 independent experiments and analyzed about 5.000 individual cells. For analysis of  $\text{Ca}^{2+}$  responses in WT and heptaTRPC KO mice we performed 45 independent experiments and analyzed more than 4.000 individual cells.

## 2.5. Analysis of intracellular calcium signals

An R package developed by Elena Hernando-Pérez and Enrique Pérez-Riesgo in our lab and named CimageD4 v1.10.1 is available at <https://github.com/emodoro/CimageD4>. This software permits the analysis of many parameters elicited from calcium imaging experiments. These include measuring the peak and areas of  $\text{Ca}_i^{2+}$  responses within each individual AP cell in a particular experiment, and the identification and removal of ‘outliers’ within each experimental under a multivariate criterion. The software package summarizes the peak and areas values for each experiment. As spontaneous  $\text{Ca}_i^{2+}$  oscillations in AP cells are quite variable, it is not simple to measure amplitude and/or frequency of oscillations. We have developed previously a parameter termed oscillations index [3,36]. This parameter is computed as the average of all changes in  $\text{Ca}_i^{2+}$  (in absolute values) during a period of time. OI, therefore, is a parameter influenced by both the amplitude and the frequency of  $\text{Ca}_i^{2+}$  oscillations. Cells showing no oscillations had OI values lower than 0.05.

## 2.6. Statistics

Data are presented as mean  $\pm$  SEM. When only two means were compared, Student’s *t*-test was used. For more than two groups, statistical significance of the data was assessed by one-way ANOVA. Differences were considered significant at  $p < 0.05$ . To compare the effects of channel removal on  $\text{Ca}^{2+}$  responses to HRHs, a Completely Randomized Design (CRD) has been set up. Regarding the assignment of treatments to experimental units, in order to control the experimental error as much as possible, all experiments were carried out on a pairwise basis, such that each stimulus is applied to both WT and KO samples at the same time, and experimental day is used as blocking factor (Complete Block Designs). Error rates were controlled using the Bonferroni method which controls the Family-Wise Error Rate, or the Benjamini-Hochberg method which controls the False Discovery Rate. The signification level from which we reject the null hypothesis is 0.05. Outlier detection was carried out by Multivariate Criteria employing Mahalanobis distance which takes into account the correlation structure between different variables, which are the height and areas of all different response variables measured from each experiment (hypothalamic factors, or calcium stimulus), in addition to basal fluorescence. After outlier elimination, the median response of all measurement units within the experimental unit is employed as summary response for each experimental unit.

### 3. Results

#### 3.1. Contribution of Orai1 to store-operated $\text{Ca}^{2+}$ entry and spontaneous $\text{Ca}^{2+}$ oscillations in mouse anterior pituitary cells

The presence of SOCE was tested in AP cells isolated from WT and Orai1 KO mice by use of the SERCA blocker thapsigargin (TG). As described in the Methods section, AP cells isolated from WT mice were treated with TG in the absence of extracellular  $\text{Ca}^{2+}$ , resulting in a transient rise in  $\text{Ca}_i^{2+}$  due to passive leak of  $\text{Ca}^{2+}$  from the ER (Fig. 1A). After 15 min, extracellular  $\text{Ca}^{2+}$  (5 mM) was restored to the extracellular medium and resulted in a sustained increase in  $\text{Ca}_i^{2+}$ , which was inhibited by 2  $\mu\text{M}$   $\text{Gd}^{3+}$  (Fig. 1A). The sustained  $\text{Ca}_i^{2+}$  signal induced by TG, and blocked by  $\text{Gd}^{3+}$ , is indicative of SOCE. We repeated the same experiment in AP cells isolated from Orai1KO mice. In these experiments, while TG induced a similar (Fig. 1A & B), transient  $\text{Ca}_i^{2+}$  rise in the absence of extracellular calcium, the rise in  $\text{Ca}_i^{2+}$  observed in the presence of extracellular  $\text{Ca}^{2+}$  was lost (Fig. 1A & C). Indeed, the residual  $\text{Ca}_i^{2+}$  entry signal observed was similar to that observed in control experiments with Orai1 KO cells not treated with TG.

The transient  $\text{Ca}_i^{2+}$  response induced by TG was similar in AP cells from WT and Orai1KO mice (Fig. 1C) suggesting that  $\text{Ca}^{2+}$  store content is similar in AP cells derived from these mice. To confirm this data, we measured the effects of the  $\text{Ca}^{2+}$  ionophore ionomycin in  $\text{Ca}^{2+}$  free medium, the transient nature of which is indicative of  $\text{Ca}^{2+}$  store (Fig. 1D). Consistently, we found that release of  $\text{Ca}^{2+}$  from intracellular stores evoked by ionomycin was similar in AP cells from WT and Orai1KO cells (Fig. 1E). These results indicate that while the expression of Orai1 is required for SOCE in mouse AP cells, the lack of Orai1 does not reduce  $\text{Ca}^{2+}$  store content.

Mouse AP cells, just like rat and human AP cells, display spontaneous  $\text{Ca}^{2+}$ -oscillations that are caused by spontaneous firing of  $\text{Ca}^{2+}$  action potentials and opening of voltage-gated  $\text{Ca}^{2+}$  channels (VOCCs), particularly L-type VOCCs. We tested whether the expression of Orai1 channels could be involved in the firing of spontaneous  $\text{Ca}^{2+}$  oscillations in mouse AP cells. Fig. 2A shows representative recordings of spontaneous  $\text{Ca}^{2+}$  oscillations in single AP cells from WT and Orai1KO mice, respectively. We found that a fraction of mouse AP cells display no spontaneous  $\text{Ca}_i^{2+}$  oscillations in WT and Orai1 $^{-/-}$  animals. The remaining cells displayed different types of oscillations with variable frequency and amplitude (Fig. 2A). Both oscillating and non-oscillating cells are observed in the same cell cultures. The fraction of cells showing spontaneous  $\text{Ca}_i^{2+}$  oscillations was similar in cell cultures from WT and Orai1KO animals (Fig. 2B). To quantify oscillations, we calculated the oscillations index (OI) for oscillating cells as reported previously [3,36]. This parameter reflects the amplitude and/or frequency of oscillations. We found that OI was similar for oscillating AP cells from WT and Orai1 $^{-/-}$  mice suggesting that lack of Orai1 channels does not contribute to spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells.



### 3.2. Contribution of TRPC channels to store-operated $\text{Ca}^{2+}$ entry and spontaneous $\text{Ca}^{2+}$ oscillations in mouse anterior pituitary cells

TRPC channels have been suggested to be involved in SOCE in different cell types. Most data have been derived from studies in which a single TRPC channel is silenced or knocked-down. However, since multiple TRPC channels are often expressed in the same cell, interpretation of such results is complex. In this study, we have utilized the heptaTRPC KO mice in which all seven TRPC channels (from TRPC1 to TRPC7) were knocked-down. This would allow us to unambiguously investigate the contribution of TRPC channels to SOCE in mouse AP cells.

As described for the experiments in Fig. 1, we used fura-5F loaded AP cells isolated from WT and heptaTRPC KO mice to observe the effects of TG in the absence and presence of extracellular  $\text{Ca}^{2+}$  (Fig. 3). As can be seen in Fig. 3A, the pattern of  $\text{Ca}_i^{2+}$  responses induced by TG-treatment was indistinguishable between AP cells isolated from WT or heptaTRPC KO mice.

Analysis of the transient TG-induced  $\text{Ca}_i^{2+}$  response in the absence of extracellular  $\text{Ca}^{2+}$  is summarized in Fig. 3B, and the TG-induced  $\text{Ca}^{2+}$  entry response is summarized in Fig. 3C. The  $\text{Ca}^{2+}$  entry signal observed in the absence of TG was also similar in both AP cell populations (Fig. 3C). Moreover,  $\text{Ca}^{2+}$  store content assessed by ionomycin-induced  $\text{Ca}_i^{2+}$  transients (Fig. 3D) was also similar in AP cells from WT and heptaTRPC KO mice (summarized in Fig. 3E). These results suggest that expression of TRPC proteins do not contribute neither to SOCE nor the  $\text{Ca}^{2+}$  store content in mouse AP cells.

We tested next whether the presence or absence of TRPC channel proteins could be involved in the firing of spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells. As in the case of *Orai1*<sup>-/-</sup> mice, a fraction of WT and heptaTRPC KO cells display no spontaneous  $\text{Ca}_i^{2+}$  oscillations while the remaining cells displayed different types of oscillations with variable frequency and amplitude (Fig. 4A). However, the fraction of AP cells showing no spontaneous oscillations was significantly lower in the heptaTRPC KO mice than in the WT mice (Fig. 4B). In addition, average OI values for oscillating cells are significantly lower in AP cells from the heptaTRPC KO mice than in AP cells from WT animals. These data suggest that, in contrast to *Orai1* channels, TRPC channels could contribute to spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells.

### 3.3. Effect of *Orai1* expression on $\text{Ca}_i^{2+}$ responses induced by hypothalamic releasing hormones in mouse anterior pituitary cells

We assessed the effects of hypothalamic releasing hormones (HRHs) on  $\text{Ca}_i^{2+}$  signals in AP cells isolated from WT and *Orai1*<sup>-/-</sup> mice. Fura-5f-loaded AP cells were stimulated sequentially with the four HRHs in standard medium devoid of dihydropyridines. Each HRH increases  $\text{Ca}_i^{2+}$  in a fraction of responsive cells that may depend on age, sex and physiological status of the AP donor as reported previously [11,17]. Fig. 5A and B show

representative single-cell recordings of  $\text{Ca}_i^{2+}$  responses to individual HRHs in mouse AP cells from WT and *Orai1*<sup>-/-</sup> mice, respectively. The fraction of cells responsive to each HRH is shown in Fig. 5C. The data indicate that the fraction of AP cells responsive to TRH, CRH and LHRH was similar in AP cells from WT and *Orai1*<sup>-/-</sup> mice. However, the percent of mouse AP cells responsive to GHRH was slightly but significantly lower in the *Orai1*<sup>-/-</sup> mice compared to WT mice.

To compare the magnitude of  $\text{Ca}_i^{2+}$  responses to the four HRHs in AP cells from WT and *Orai1*KO, we calculated the peak  $\text{Ca}_i^{2+}$  response induced by each HRH as a Ratio (Fig. 5D). Indeed, the  $\text{Ca}_i^{2+}$  response for all HRHs was similar for WT and *Orai1*KO cells.

We have previously reported that  $\text{Ca}_i^{2+}$  responses to TRH and LHRH are biphasic corresponding to  $\text{Ca}^{2+}$  release (first phase) followed by enhanced  $\text{Ca}^{2+}$  entry (second phase) [3,11]. We tested and compared the biphasic effects of TRH and LHRH in AP cells isolated from WT and *Orai1*<sup>-/-</sup> mice (Fig. 6). The peak  $\text{Ca}_i^{2+}$  response of each agonist primarily reflects intracellular  $\text{Ca}^{2+}$  release, and was similar in WT and *Orai1*KO cells as shown above. However,  $\text{Ca}_i^{2+}$  responses after 1 to 2 min of stimulation depend on  $\text{Ca}^{2+}$  entry, and differences could be observed between WT and *Orai1*KO AP cells. In summary, for both TRH and LHRH, the number of AP cells exhibiting a sustained  $\text{Ca}^{2+}$  entry signal was reduced in *Orai1*KO cells (Fig. 6C), and the magnitude of the observed  $\text{Ca}^{2+}$  entry signal was significantly reduced in *Orai1*KO cells compared to WT cells (Fig. 6D). These data indicate that expression of *Orai1* channels contributes to sustained  $\text{Ca}^{2+}$  entry induced by TRH and LHRH in mouse AP cells. In addition, it is known that second phase in response to TRH and LHRH are also associated to increased  $\text{Ca}_i^{2+}$  oscillations. This effect is best appreciated at low agonist concentrations. Consistently, we found that, at low concentrations, TRH induced or increased  $\text{Ca}_i^{2+}$  oscillations and this effect is similar in AP cells from wt and *Orai*<sup>-/-</sup> mice (data not shown).

#### 3.4. Effect of TRPC expression on $\text{Ca}_i^{2+}$ responses induced by hypothalamic releasing hormones in mouse anterior pituitary cells

We also assessed the contribution of TRPC channels to  $\text{Ca}_i^{2+}$  responses induced by HRHs in mouse AP cells. Using the protocols described for Fig. 6, we assessed the effects of HRHs in AP cells isolated from WT and heptaTRPC KO mice. As before, AP cells were stimulated sequentially with the four HRHs and representative recordings are shown in Fig. 7A and B. The fraction of AP cells responsive to CRH and LHRH was similar in WT and heptaTRPC KO mice. However, the percent of cells responsive to TRH and GHRH were reduced significantly in heptaTRPC KO mice (Fig. 7C). The magnitude (Ratio) of agonist-induced  $\text{Ca}_i^{2+}$  responses was also assessed (Fig. 7D). The  $\text{Ca}_i^{2+}$  responses induced by TRH and GHRH were similar for WT and TRPC KO cells. The  $\text{Ca}_i^{2+}$  increase induced by LHRH was

slightly, but significantly lower in heptaTRPC KO cells. However, surprisingly, the  $\text{Ca}_i^{2+}$  response induced by CRH was significantly larger in heptaTRPC KO cells compared to WT.

Finally, we also compared the effects of TRH and LHRH on  $\text{Ca}_i^{2+}$  responses in AP cells from WT and heptaTRPC KO mice (Fig. 8A and B, respectively). As can be seen in representative experiments, the peak  $\text{Ca}_i^{2+}$  response to each agonist was similar in AP cells from WT and heptaTRPC KO cells. However, the second phase response to each agonist was differentially affected by lack of TRPC channels. Specifically,  $\text{Ca}^{2+}$  entry induced by TRH was reduced in the heptaTRPC KO cells while the entry of  $\text{Ca}^{2+}$  induced by LHRH was unaffected (Fig. 8A and B). Nevertheless, as illustrated in Fig. 8C, the fraction of cells showing a sustained  $\text{Ca}^{2+}$  entry in response to LHRH was slightly but significantly reduced in AP cells isolated from heptaTRPC KO cells compared to WT. In contrast, the fraction of cells showing a sustained  $\text{Ca}^{2+}$  entry in response to TRH was similar in WT and heptaTRPC KO cells.

To ascertain contribution of TRPC channels to  $\text{Ca}^{2+}$  entry induced by each agonist, we have computed the magnitude of the  $\text{Ca}^{2+}$  entry response induced by TRH and LHRH in all cells responsive to each agonist (Fig. 8D). The data illustrate that TRH-induced  $\text{Ca}^{2+}$ -entry is significantly smaller in TRPC KO mice compared to WT. In contrast, no difference is observed for LHRH-induced  $\text{Ca}^{2+}$  entry between WT and heptaTRPC KO cells. These results suggest that expression of TRPC channels may play a role in supporting sustained  $\text{Ca}^{2+}$  entry induced by TRH. Consistently, oscillatory activity induced by low concentrations of TRH is decreased in mouse AP cells from TRPCKO animals relative to wt mice (data not shown). In contrast, contribution of TRPC channels to  $\text{Ca}^{2+}$  entry induced by LHRH is minor or negligible.

#### 4. Discussion

The AP gland plays a pivotal role in control of the entire endocrine system by the release of important AP hormones, a process primarily dictated by hypothalamic factors, most particularly HRHs that reach the AP via the hypophyseal portal system [1]. These peptides activate specific receptors in target cells leading to  $\text{Ca}^{2+}$  responses and release of specific AP hormones [3,5–8]. It has been previously suggested that store-operated channels (SOCs) and non-selective channels may contribute to  $\text{Ca}^{2+}$  responses to HRHs but the molecular evidence has remained elusive. Here we show that Orai1 channels, but not TRPC channels, are essential for thapsigargin-evoked SOCE in mouse AP cells. This does not mean necessarily that TRPC channels play no role in SOCE in AP cells. In fact, TRPC channels are believed to modulate SOCE in some cell types [27]. In addition, we do not know whether removal of TRPC channels might influence expression of other ion channels and regulatory proteins involved in SOCE. Further research is required to address whether this is also the case in AP cells. However, our results clearly indicate that Orai1 channels are mandatory for SOCE in mouse AP cells while TRPC channels are not.

One of the physiological roles of SOCE is to provide a  $\text{Ca}^{2+}$  influx pathway for store refilling after  $\text{Ca}^{2+}$  release. However,  $\text{Ca}^{2+}$  store content is similar in AP cells from Orai1-/-

– and WT mice suggesting that other  $\text{Ca}^{2+}$  entry pathways contribute to store refilling in AP cells. We have no information on whether other Orai channels (Orai2, Orai3) are expressed differentially in mouse AP cells from Orai1  $-/-$  mice. However, the single removal of Orai1 is enough to abolish thapsigargin-evoked SOCE without affecting  $\text{Ca}^{2+}$  store content. Nevertheless, removal of Orai1 could influence the kinetics of  $\text{Ca}^{2+}$  refilling after stimulation.

About 50 to 60% of all mouse AP cells display spontaneous  $\text{Ca}_i^{2+}$  oscillations that are related to electric activity and VOCCs, thus providing alternative means for store refilling. However, our results show that inhibition of VOCCs with nisoldipine does not affect  $\text{Ca}^{2+}$  store content, thus suggesting that VOCCs are also dispensable for  $\text{Ca}^{2+}$  store refilling.

Our results indicate a role for TRPC channels, but not Orai1 channels, in spontaneous  $\text{Ca}_i^{2+}$  oscillations in AP cells. Thus, loss of Orai1 channels has no influence on spontaneous oscillations in mouse AP cells or on  $\text{Ca}^{2+}$  oscillations induced by TRH. Consistently, we have reported previously that thapsigargin-evoked SOCE does not increase  $\text{Ca}_i^{2+}$  oscillations in GH<sub>3</sub> pituitary cells [3]. In contrast, lack of TRPC channels decreases both the percent of AP cells showing spontaneous oscillations and also the frequency and/or amplitude of oscillations without decreasing  $\text{Ca}^{2+}$  store content. These data suggest that, in contrast to Orai1 channels, TRPC channels may contribute to AP cell excitability. Whether lack of TRPC channels promotes overexpression of VOCCs or other ion channels involved in AP cell excitability remains to be established. In summary, our results indicate that Orai1 channels are critical for SOCE but do not contribute to spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells. Conversely, TRPC channels are dispensable for SOCE but may contribute to spontaneous  $\text{Ca}^{2+}$  oscillations in the same cells.

We have investigated also whether Orai1 and TRPC channels contribute to  $\text{Ca}^{2+}$  responses elicited by HRHs in mouse AP cells. There are four main HRHs including TRH, LHRH, GHRH and CRH. It is well known that TRH and LHRH evoked a biphasic  $\text{Ca}^{2+}$  response. The first phase corresponds to agonist-evoked synthesis of  $\text{IP}_3$  and the ensuing release of intracellular  $\text{Ca}^{2+}$  stores. The second phase corresponds to enhanced  $\text{Ca}^{2+}$  entry associated to enhanced electric activity. As TRH and LHRH induce  $\text{Ca}^{2+}$  release, it has been proposed that SOCE may contribute as well to the second phase of  $\text{Ca}^{2+}$  entry in response to these stimuli. In contrast, GHRH and CRH do not induce  $\text{Ca}^{2+}$  release and SOCE should not play a role in  $\text{Ca}^{2+}$  responses to these agonists.

TRH is involved in control of TSH and PRL secretion from thyrotropes and mammotropes, respectively. As stated above, TRH induces  $\text{Ca}^{2+}$  release followed by enhanced electric activity and  $\text{Ca}^{2+}$  entry (second phase) in mammotropes [5]. We proposed long ago that TRH activated not only VOCCs but also SOCE in mammotropes [3]. Here, we provide molecular evidence that both Orai1 and TRPC channels contribute also to  $\text{Ca}^{2+}$  entry (second phase) induced by TRH in mouse AP cells. Specifically, removal of Orai1 channels prevents partially  $\text{Ca}^{2+}$  entry induced by TRH without influencing the fraction of AP cells responsive to TRH or the release of  $\text{Ca}^{2+}$  induced by TRH (first phase). In turn, lack of TRPC channels also decreases  $\text{Ca}^{2+}$  entry induced by TRH without influencing  $\text{Ca}^{2+}$  release. Therefore, both

Orai1 channels and TRPC channels are involved in  $\text{Ca}^{2+}$  entry and  $\text{Ca}^{2+}$  responses to TRH, thus contributing together with VOCCs to  $\text{Ca}^{2+}$  entry induced by TRH. Since Orai1 channels are activated by store-depletion, and TRPC channels also contribute to TRH-induced  $\text{Ca}^{2+}$  entry, it is possible that TRPC channels will modulate SOCE evoked by TRH-induced  $\text{Ca}^{2+}$  release in mouse AP cells.

Our results invite speculation on the role of Orai1 and TRPC channels in TSH and PRL secretion evoked by TRH. It has been previously reported that non-selective blockers of SOCs inhibit resting electric activity of AP cells and PRL release [37]. In addition, Orai1 channel has been previously involved in lactation and breast cancer [38,39]. During lactation, milk is ejected by way of pulsatile contractions of alveolar units. Interestingly, in mammary glands of Orai1<sup>-/-</sup> mice, these contractions are infrequent and poorly coordinated and this effect has been attributed to defects of SOCE in the mammary epithelial cells [40]. Whether defective TRH signaling in Orai1 KO mice may influence TSH and PRL secretion as well deserves further consideration.

Hypothalamic LHRH activates LHRH receptors in a fraction of AP cells (gonadotropes) promoting gonadotropin release for control of the reproductive axis. LHRH induces  $\text{Ca}^{2+}$  release (first phase) followed by  $\text{Ca}_i^{2+}$  oscillations associated to entry of  $\text{Ca}^{2+}$  (second phase) [41].  $\text{Ca}^{2+}$  entry during LHRH-induced oscillations involves several ion channels. However, whether Orai1 and TRPC channels contribute to this entry remains unknown. We show here that lack of Orai1 channels decreases partially  $\text{Ca}^{2+}$  entry induced by LHRH, but not  $\text{Ca}^{2+}$  release or the fraction of AP cells responsive to LHRH. In striking contrast, removal of TRPC channels has no effect on  $\text{Ca}^{2+}$  entry induced by LHRH (second phase) but it reduces slightly the fraction of AP cells responsive to LHRH and the fraction of cells showing LHRH-induced second phase. These results indicate that Orai1 channels are involved in  $\text{Ca}^{2+}$  entry induced by LHRH in mouse gonadotropes. However, the role of TRPC channels in  $\text{Ca}^{2+}$  entry induced by LHRH is minor if any.

There is presently no information on whether Orai1 is involved in gonadotropin release. However, it has been also recently reported that male (but not female) mice lacking Orai1 channels show defective fertility [40]. Specifically, Orai1<sup>-/-</sup> male mice are sterile and have severe defects in spermatogenesis, with prominent deficiencies in mid- to late-stage elongating spermatid development. These data suggest an essential *in vivo* role for Orai1 channels in male reproductive function and identify Orai1 channels as potential non-steroidal regulators of male fertility [40]. The suggested mechanism includes SOCE downregulation in mouse testis cells and lack of proper sperm development or spermatogenesis. Whether defective  $\text{Ca}^{2+}$  signaling to LHRH in mouse gonadotropes contributes to male fertility defects warrants also further research.

Hypothalamic GHRH is involved in GH secretion by somatotropes. In contrast to TRH and LHRH, activation of GHRH receptors does not induce release of  $\text{Ca}^{2+}$  from intracellular stores. Instead, GHRH enhances electrical activity and  $\text{Ca}^{2+}$  entry by a not fully understood mechanism [2,7]. Here we show that lack of Orai1 channels does not influence  $\text{Ca}^{2+}$  entry induced by GHRH in mouse AP cells. However, it decreased slightly, but significantly, the fraction of AP cells responsive to GHRH. It has been previously reported that Orai1<sup>-/-</sup> mice

show short stature that has been attributed to dysfunction of skeletal muscle development [42]. Whether decreased responsiveness to GHRH contributes to growth deficits in the *Orai1*<sup>-/-</sup> mice remains to be established. More importantly, lack of TRPC channels decreased largely the fraction of AP cells responsive to GHRH without changing the rise in  $\text{Ca}_i^{2+}$  induced by this agonist in the responsive population. These data suggest an important role of TRPC channels in control of GHRH receptor expression and/or signaling in mouse somatotropes.

The secretion of ACTH from corticotropes is a key component in the endocrine response to stress. The resting potential of corticotropes is set by the basal activities of TWIK-related  $\text{K}^+$  (TREK)-1 channels. CRH, the major ACTH secretagogue, closes background TREK-1 channels via the cAMP-dependent pathway, resulting in depolarization and a sustained rise in  $\text{Ca}_i^{2+}$  [9]. We show here that removal of *Orai1* channels does not influence either the fraction of CRH responsive cells or the rise in  $\text{Ca}_i^{2+}$  induced by CRH, thus excluding any role for *Orai1* channels in ACTH secretion induced by CRH. Consistently, blockers of SOCs do not affect ACTH release induced by CRH [20]. However, these results do not exclude that *Orai1* channels may contribute to ACTH secretion induced by  $\text{Ca}^{2+}$ -releasing agonists like arginine vasopressin [20]. Nevertheless, in contrast to previous reports [20], our results indicate that SOCE in corticotropes involves *Orai1* channels but not TRPC channels. Surprisingly, we found that lack of TRPC channels increased slightly but significantly the rise in  $\text{Ca}_i^{2+}$  induced by CRH without affecting the fraction of cells responsive to this agonist. Further research is required to address this intriguing point.

In summary, these results indicate the critical role of *Orai1* channels in SOCE and  $\text{Ca}^{2+}$  entry induced by TRH and LHRH in mouse AP cells. In addition, our results also suggest a role for TRPC channels in spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells and  $\text{Ca}^{2+}$  responses to several HRHs, particularly TRH and GHRH. The present study focuses solely on AP cells, and therefore we cannot extrapolate it to the more general question of the relative importance of TRPC and *Orai* channels in  $\text{Ca}^{2+}$  entry mechanisms elsewhere. Additional work will be necessary to establish the roles of these two channel types on store-operated and receptor-operated channels in other tissues. Hopefully, the availability of these and other mouse models will facilitate the important identification of channels underlying  $\text{Ca}^{2+}$  signaling in a variety of important physiological systems.

### Transparency document

The Transparency document associated with this article can be found, in online version.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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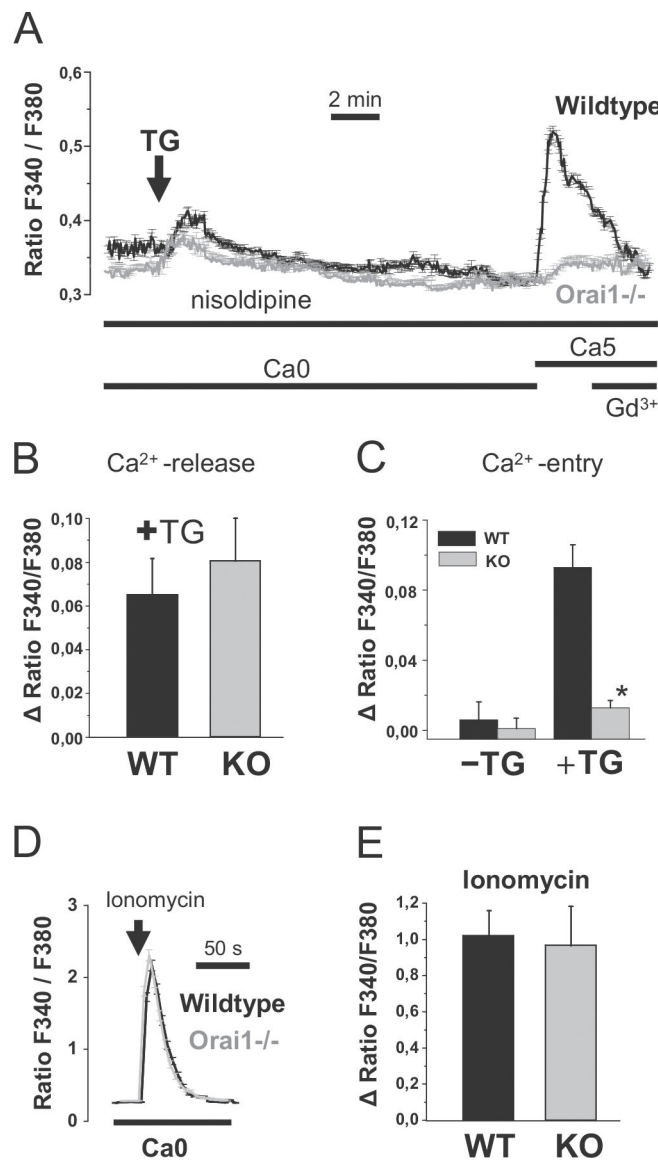
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**Fig. 1.** Contribution of Orai1 channels to store-operated Ca<sup>2+</sup> entry and Ca<sup>2+</sup> store content in mouse AP cells.

Isolated AP cells from male WT and Orai1<sup>-/-</sup> mice were subjected to fluorescence imaging for monitoring SOCE and calcium store content. A. Cells were treated with thapsigargin 1 μM (TG) in Ca<sup>2+</sup> free medium containing 1 μM nisoldipine. Then, medium containing 5 mM Ca<sup>2+</sup> (Ca5) was added to estimate SOCE. Finally, medium containing 2 μM Gd<sup>3+</sup> was added to prevent SOCE. Shown are average (mean ± SEM) recordings of Ca<sub>i</sub><sup>2+</sup> (Ratio F340/F380) (black trace, 100 cells wt; gray trace 95 cells Orai1<sup>-/-</sup>) from two representative experiments. B. Bars show average (mean ± SEM) values of Ratio increases induced by addition of thapsigargin in Ca<sup>2+</sup> free medium. C. Bars show average (mean ± SEM) values of Ratio increases induced by addition of extracellular Ca<sup>2+</sup> to cells treated with thapsigargin (TG) or untreated cells (-TG). Data in B and C are from n = 5 experiments in

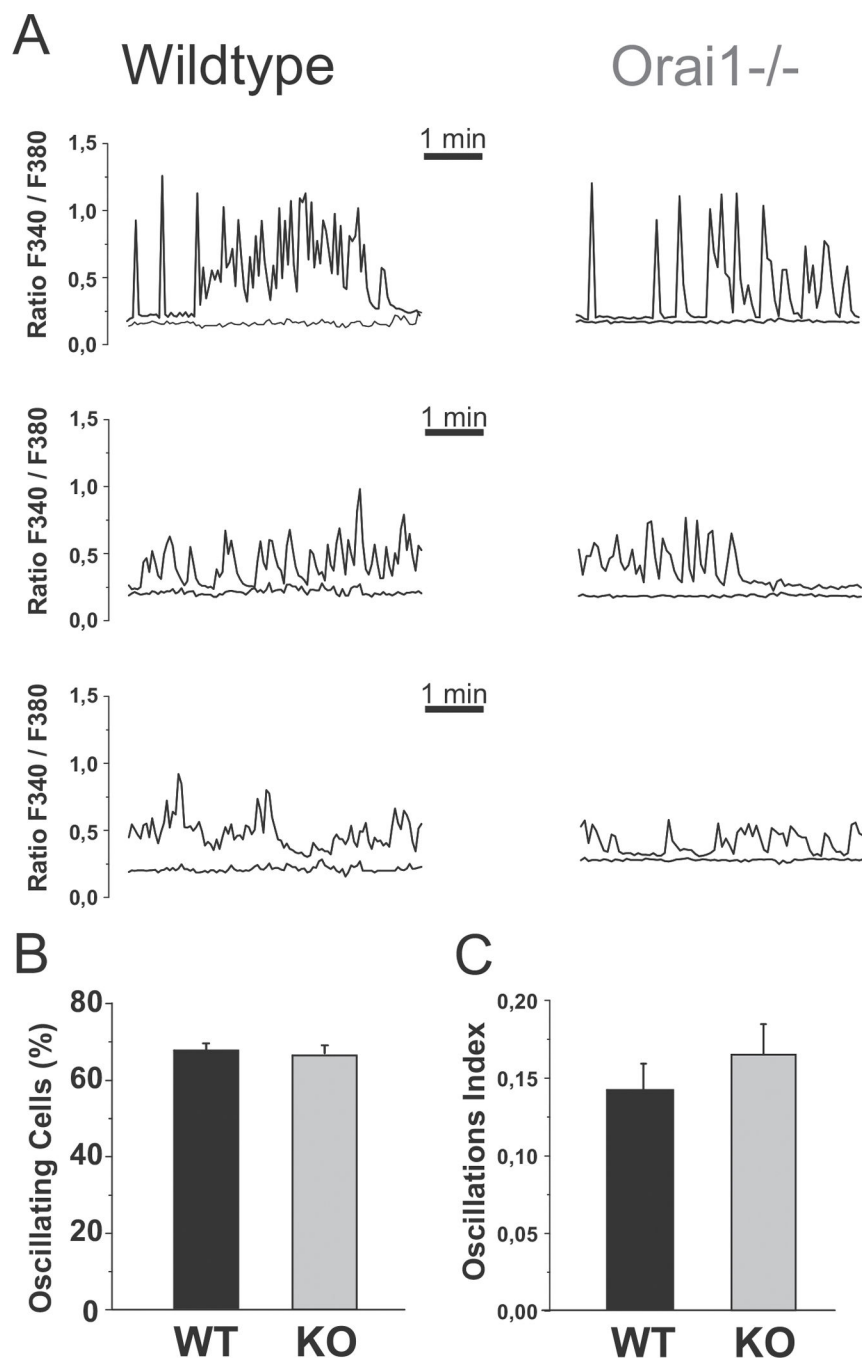
WT mice (237 cells) and n = 6 experiments in KO mice (332 cells). D. Cells were treated with 1  $\mu$ M ionomycin (TG) in  $\text{Ca}^{2+}$  free medium to assess  $\text{Ca}^{2+}$  store content. Average recordings (mean  $\pm$  SEM) of  $\text{Ca}_i^{2+}$  (Ratio F340/F380) of AP cells from WT (black trace, 90 cells) and *Orai1*<sup>-/-</sup> (gray trace, 81 cells) mice in two representative experiments. E. Average (mean  $\pm$  SEM) values of Ratio increases induced by ionomycin in  $\text{Ca}^{2+}$  free medium in AP cells from wt and *Orai1*<sup>-/-</sup> (KO) mice. n = 6 experiments (wt, 331 cells) and n = 6 experiments (*Orai1*<sup>-/-</sup>, 313 cells). \*p < 0.05.

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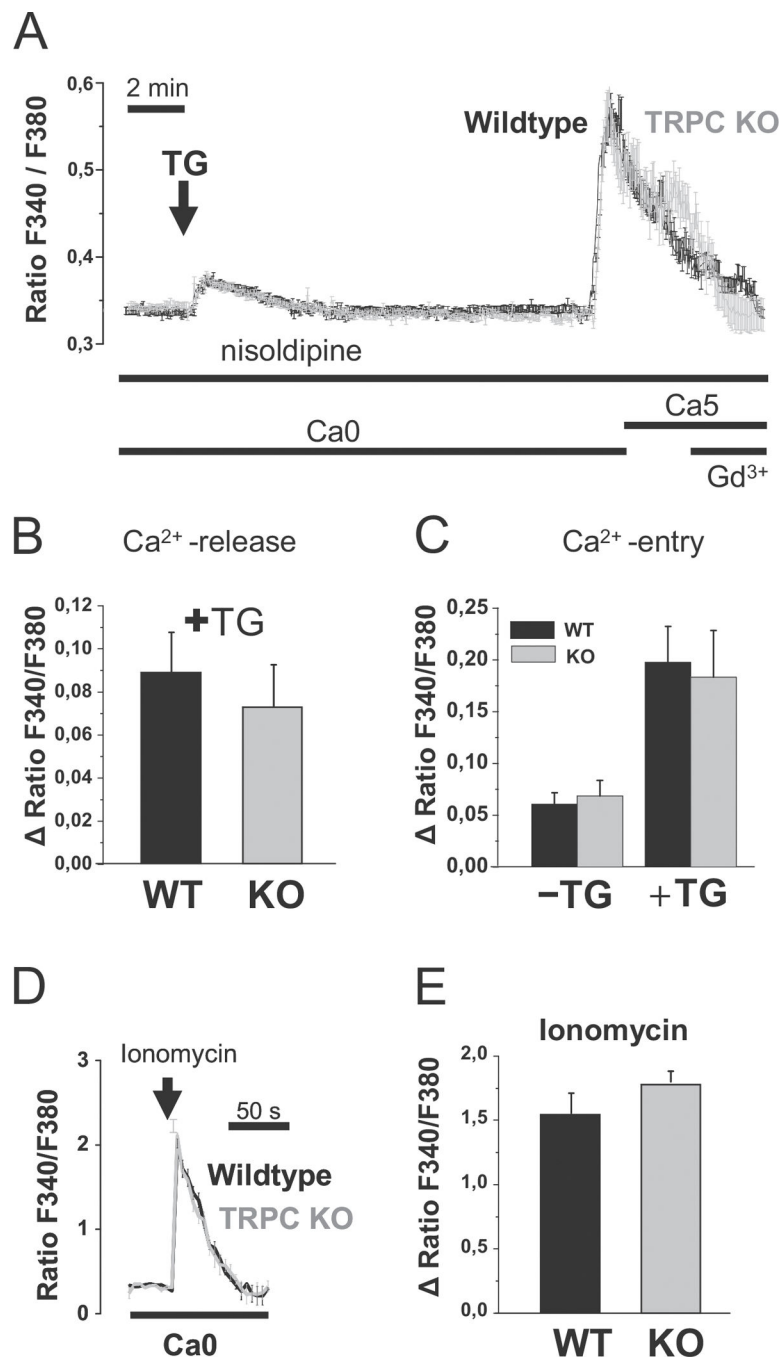


**Fig. 2.**

Contribution of Orai1 channels to spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells.

Isolated AP cells from wt and Orai1<sup>-/-</sup> mice were subjected to fluorescence imaging for monitoring spontaneous  $\text{Ca}_i^{2+}$  oscillations. A. Representative recordings of resting Ratio F340/F380 in 6 individual AP cells from WT (left) and 6 individual AP cells from Orai1<sup>-/-</sup> (right) mice. Paired recordings are representative examples of individual cells present in the same microscopic field showing no oscillations (flat recordings) and different oscillations.

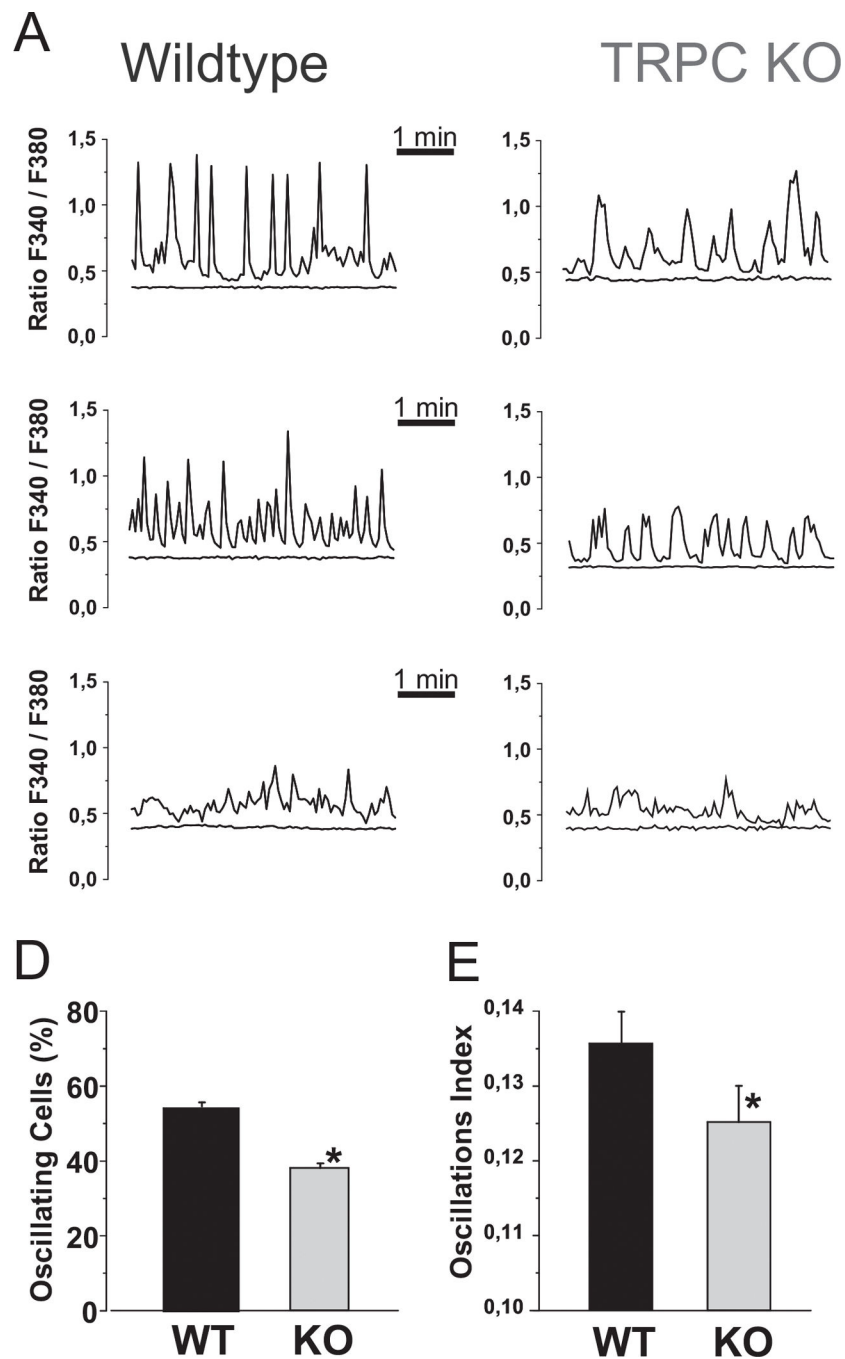
B. Bars show average (mean  $\pm$  SEM) fraction of AP cells (%) displaying spontaneous  $\text{Ca}_i^{2+}$  oscillations. C. Bars show average values (mean  $\pm$  SEM) of oscillations index (average changes in  $\text{Ca}_i^{2+}$  in absolute values during the recording period) in the fraction of oscillating AP cells from wt and KO mice. Data in B and C are from n = 9 experiments in WT mice (711 cells) and n = 10 experiments in *Orai1*<sup>-/-</sup> mice (932 cells).



**Fig. 3.** Contribution of TRPC channels to store-operated Ca<sup>2+</sup> entry and Ca<sup>2+</sup> store content in mouse AP cells.

Isolated AP cells from male WT and heptaTRPC KO mice were subjected to fluorescence imaging for monitoring SOCE and calcium store content. A. Cells were treated with thapsigargin 1 μM (TG) in Ca<sup>2+</sup> free medium containing 1 μM nisoldipine. Then, medium containing 5 mM Ca<sup>2+</sup> (Ca5) was added to estimate SOCE. Finally, medium containing 2 μM Gd<sup>3+</sup> was added to prevent SOCE. Shown are average (mean ± SEM) recordings

of  $\text{Ca}_i^{2+}$  (Ratio F340/F380) (black trace, 90 cells wt; gray trace 89 cells heptaTRPC KO) from two representative experiments. B. Bars show average (mean  $\pm$  SEM) values of Ratio increases induced by addition of thapsigargin in  $\text{Ca}^{2+}$  free medium. C. Bars show average (mean  $\pm$  SEM) values of Ratio increases induced by addition of extracellular  $\text{Ca}^{2+}$  to cells treated with thapsigargin (TG) or untreated cells (-TG). Data in B and C are from n = 6 experiments in WT mice (343 cells) and n = 6 experiments in KO mice (501 cells). D. Cells were treated with 1  $\mu\text{M}$  ionomycin (TG) in  $\text{Ca}^{2+}$  free medium to assess  $\text{Ca}^{2+}$  store content. Average recordings (mean  $\pm$  SEM) of  $\text{Ca}_i^{2+}$  (Ratio F340/F380) of AP cells from WT (black trace, 62 cells) and heptaTRPC KO (gray trace, 80 cells) mice in two representative experiments. E. Average (mean  $\pm$  SEM) values of Ratio increases induced by ionomycin in  $\text{Ca}^{2+}$  free medium in AP cells from wt and heptaTRPC KO mice. n = 4 experiments (wt, 147 cells) and n = 4 experiments (heptaTRPC KO, 262 cells). \*p < 0.05.

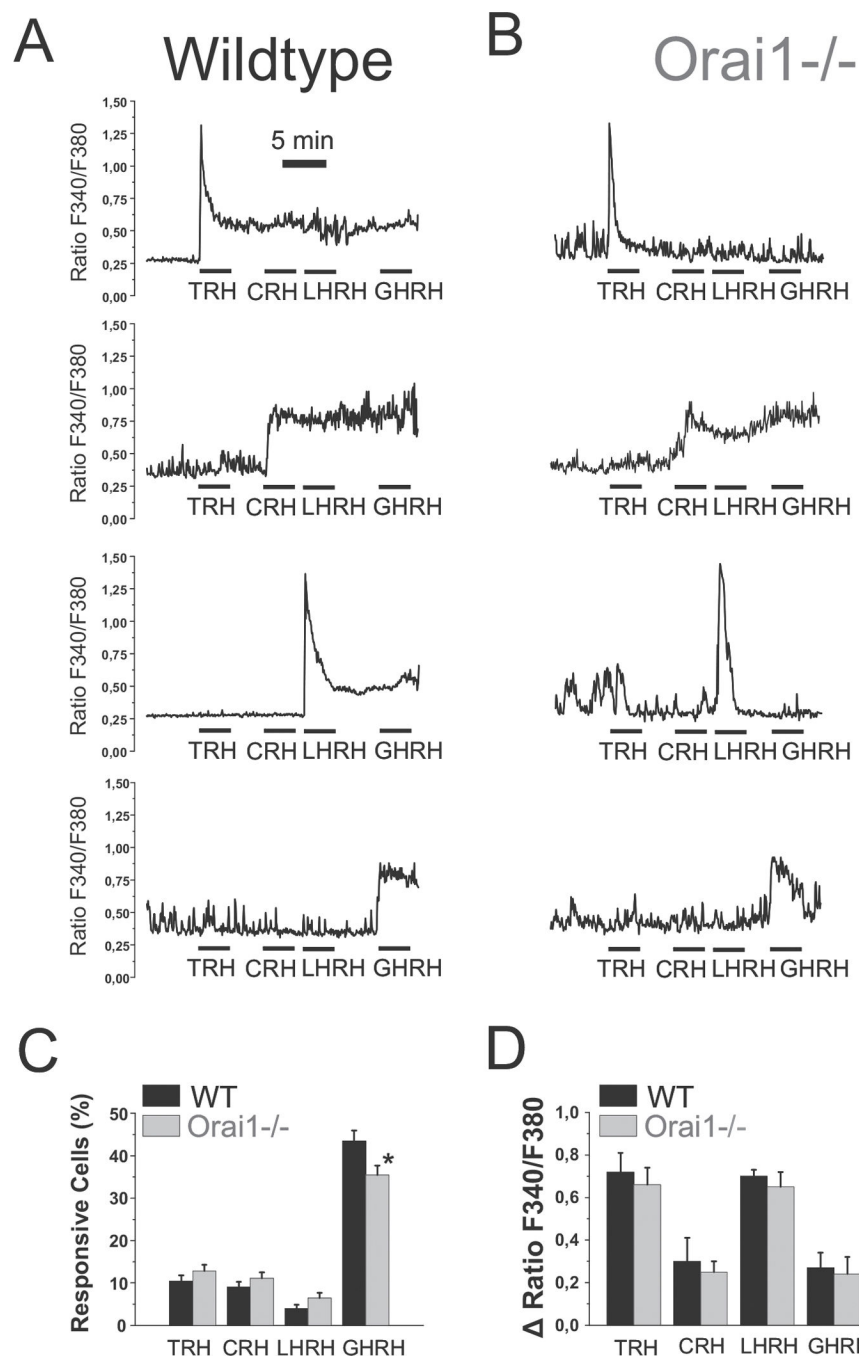
**Fig. 4.**

Contribution of TRPC channels to spontaneous  $\text{Ca}_i^{2+}$  oscillations in mouse AP cells.

Isolated AP cells from wt and heptaTRPC KO mice were subjected to fluorescence imaging for monitoring spontaneous  $\text{Ca}_i^{2+}$  oscillations. A. Representative recordings of resting Ratio F340/F380 in 6 individual AP cells from WT mice (left panels) and 6 AP cells from KO mice (right panels). Paired recordings are representative examples of individual cells present in the same microscopic field showing no oscillations (flat recordings) and different oscillations. B. Bars show average (mean  $\pm$  SEM) fraction of AP cells (%) displaying



spontaneous  $\text{Ca}_i^{2+}$  oscillations. C. Bars show average values (mean  $\pm$  SEM) of oscillations index (average changes in  $\text{Ca}_i^{2+}$  in absolute values during the recording period) in the fraction of oscillating AP cells from wt and KO mice. Data in B and C are from n = 23 experiments in WT mice (2275 cells) and n = 22 experiments in KO mice (1338 cells). \*p < 0.05.



**Fig. 5.** Contribution of Orai1 channels to  $\text{Ca}^{2+}$  responses induced by hypothalamic releasing hormones in mouse AP cells. Isolated AP cells from wt and Orai1<sup>-/-</sup> mice were subjected to fluorescence imaging for monitoring  $\text{Ca}^{2+}$  responses to HRHs. Shown are representative recordings of individual AP cells from WT (A) and Orai1<sup>-/-</sup> (B) mice responsive to TRH, CRH, LHRH and GHRH. C. Average (mean  $\pm$  SEM) fraction of AP cells (%) showing clear cut responses to each HRH for WT and Orai1<sup>-/-</sup> mice. Data are from n = 6 experiments (WT, 564 cells) and n = 6

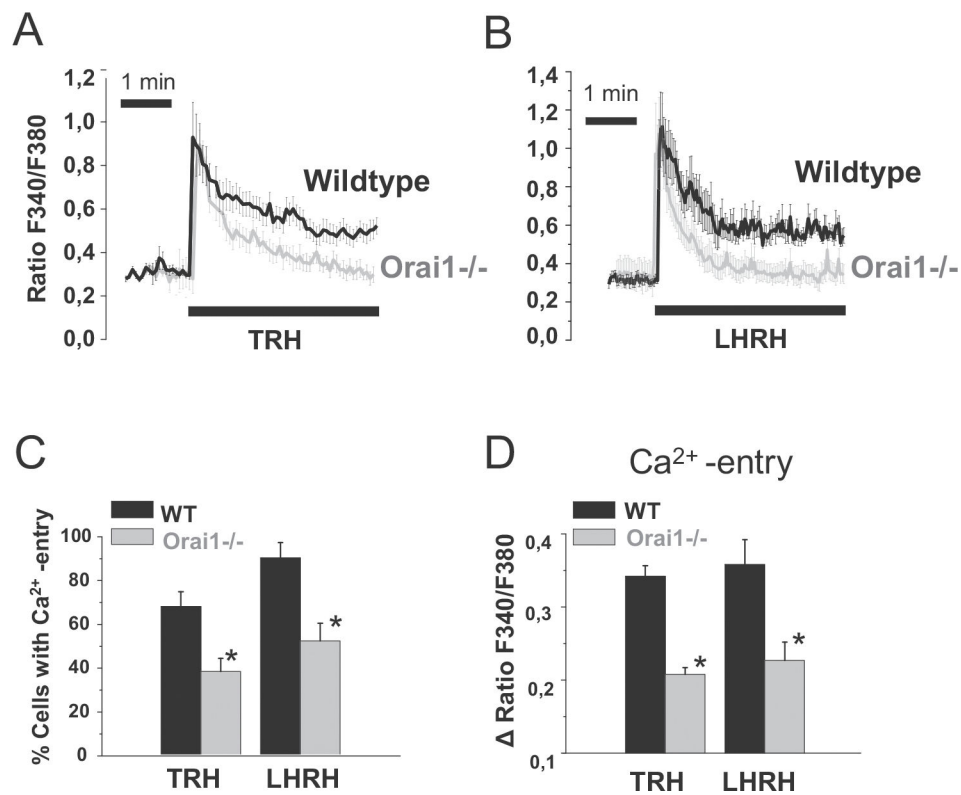
experiments (KO, 562 cells). D. Average (mean  $\pm$  SEM) values of the increases in  $\text{Ca}_i^{2+}$  ( Ratio) induced by each HRH in responsive cells from wt and *Orai1*<sup>-/-</sup> mice. Data are from n = 6 experiments (wt) and n = 6 experiments (*Orai1*<sup>-/-</sup>). \*p < 0.05.

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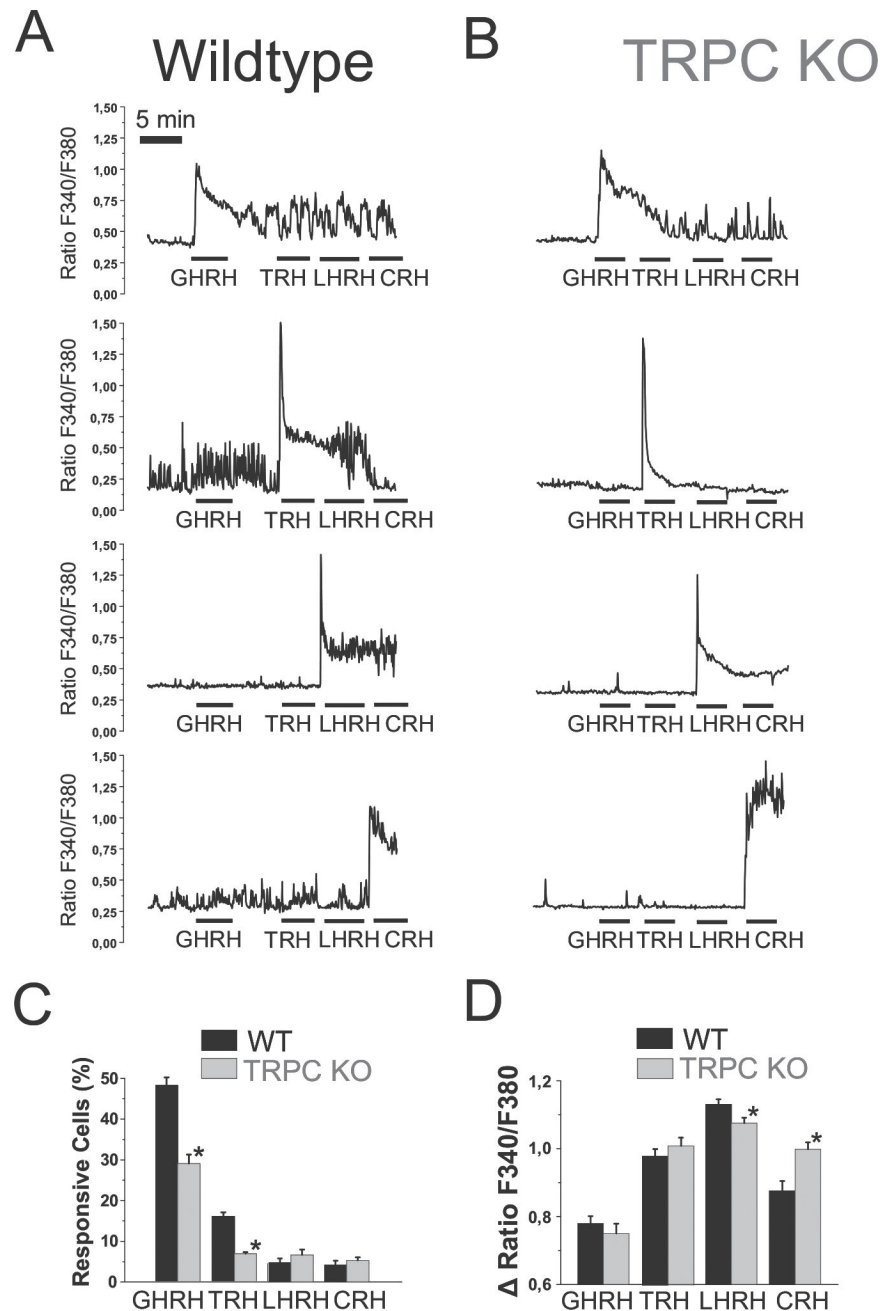
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**Fig. 6.** Contribution of Orai1 channels to Ca<sup>2+</sup> entry induced by TRH and LHRH in mouse AP cells. Isolated AP cells from WT and Orai1<sup>-/-</sup> mice were subjected to fluorescence imaging for monitoring Ca<sup>2+</sup> entry induced by TRH and LHRH. Shown are average (mean ± SEM) recordings of Ratio in AP cells responsive to TRH (A, 15 cells WT, 12 cells Orai1<sup>-/-</sup>) and LHRH (B, 7 cells WT and 10 cells Orai1<sup>-/-</sup>), respectively, from two representative experiments. C. Average (mean ± SEM) fraction of HRH responsive AP cells (%) showing second phase in response to TRH and LHRH. Data are from n = 6 experiments in WT mice (55 cells TRH; 19 cells LHRH) and n = 6 experiments in Orai1<sup>-/-</sup> mice (68 cells TRH; 31 cells LHRH). D. Average (mean ± SEM) values of the increases in Ca<sub>i</sub><sup>2+</sup> (Ratio) induced by TRH and LHRH during the second phase of Ca<sup>2+</sup> entry in responsive cells from WT and Orai1<sup>-/-</sup> mice. Data are from the same experiments as in C. \*p < 0.05.



**Fig. 7.** Contribution of TRPC channels to  $\text{Ca}^{2+}$  responses induced by hypothalamic releasing hormones in mouse AP cells. Isolated AP cells from WT and heptaTRPC KO mice were subjected to fluorescence imaging for monitoring  $\text{Ca}^{2+}$  responses to HRHs. Shown are representative recordings of individual AP cells from WT (A) and heptaTRPC KO (B) mice responsive to GHRH, TRH, LHRH and CRH. C. Average (mean  $\pm$  SEM) fraction of AP cells (%) showing clear cut responses to each HRH. Data are from  $n = 11$  experiments (WT, 949 cells) and  $n = 11$  experiments (KO, 914 cells). D. Average (mean  $\pm$  SEM) values of the increases in  $\text{Ca}_i^{2+}$

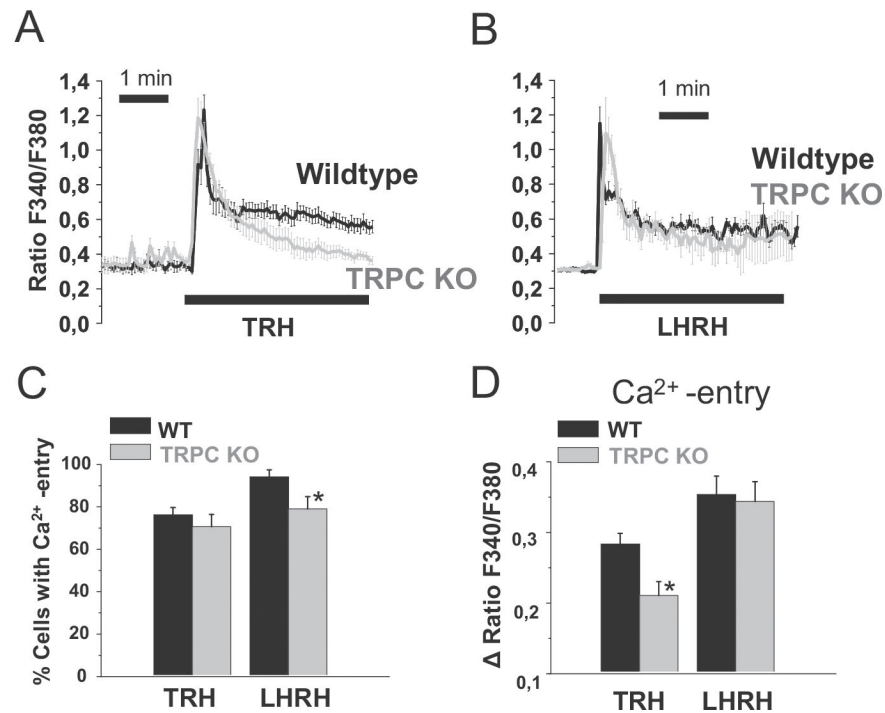
( Ratio) induced by each HRH in responsive cells from WT and heptaTRPC KO mice. Data are from n = 11 experiments (WT) and n = 11 experiments (heptaTRPC KO). \*p < 0.05.

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**Fig. 8.** Contribution of TRPC channels to  $\text{Ca}^{2+}$  entry induced by TRH and LHRH in mouse AP cells. Isolated AP cells from wildtype (WT) and heptaTRPC KO mice were subjected to fluorescence imaging for monitoring of  $\text{Ca}^{2+}$  entry induced by TRH and LHRH. Shown are average (mean  $\pm$  SEM) recordings of Ratio increases in AP cells responsive to TRH (A, 10 cells WT, 6 cells heptaTRPC KO) and LHRH (B, 6 cells WT and 5 cells heptaTRPC KO), respectively, from two representative experiments. C. Average (mean  $\pm$  SEM) fraction of HRH responsive AP cells (%) showing second phase in response to TRH and LHRH. Data in WT mice are from  $n = 11$  experiments for TRH (150 cells) and  $n = 10$  experiments for LHRH (45 cells). Data in heptaTRPC KO mice are from  $n = 11$  experiments for TRH (63 cells) and  $n = 8$  experiments of LHRH (55 cells). D. Average (mean  $\pm$  SEM) values of the increases in  $\text{Ca}_i^{2+}$  (Ratio) induced by TRH and LHRH during the second phase of  $\text{Ca}^{2+}$  entry in responsive cells from WT and *Orai1*<sup>-/-</sup> mice. Data are from the same experiments as in C. \* $p < 0.05$ .