ACTIVATION OF ERK1/2 AND AXOGENESIS INDUCED BY ESTRADIOL DEPEND ON DIFFERENT CALCIUM POOLS IN MALE RAT HYPOTHALAMIC NEURONS *IN VITRO*

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INTRODUCTION

- Estrogens generate a wide diversity of rapid "non-classical" effects which occur in a range from some seconds to few minutes (Arevalo et al., 2012), including the triggering of Ca^{2+} signals (Wong et al., 2012) and the activation of several signaling pathways such as the protein kinase C (PKC) and the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascades (Wu et al., 2005).
- Previous studies from our laboratory have shown that 17β-estradiol (E2) induces axonal growth through ERK1/2 activation in hypothalamic neurons of male embryos of 16 days of gestation (E16; Gorosito & Cambiasso, 2008).
- Both axogenesis and ERK1/2 activation mediated by the hormone depend on Ca^{2+} and Ca^{2+}-dependent PKC isoforms (Gorosito & Cambiasso, 2008).

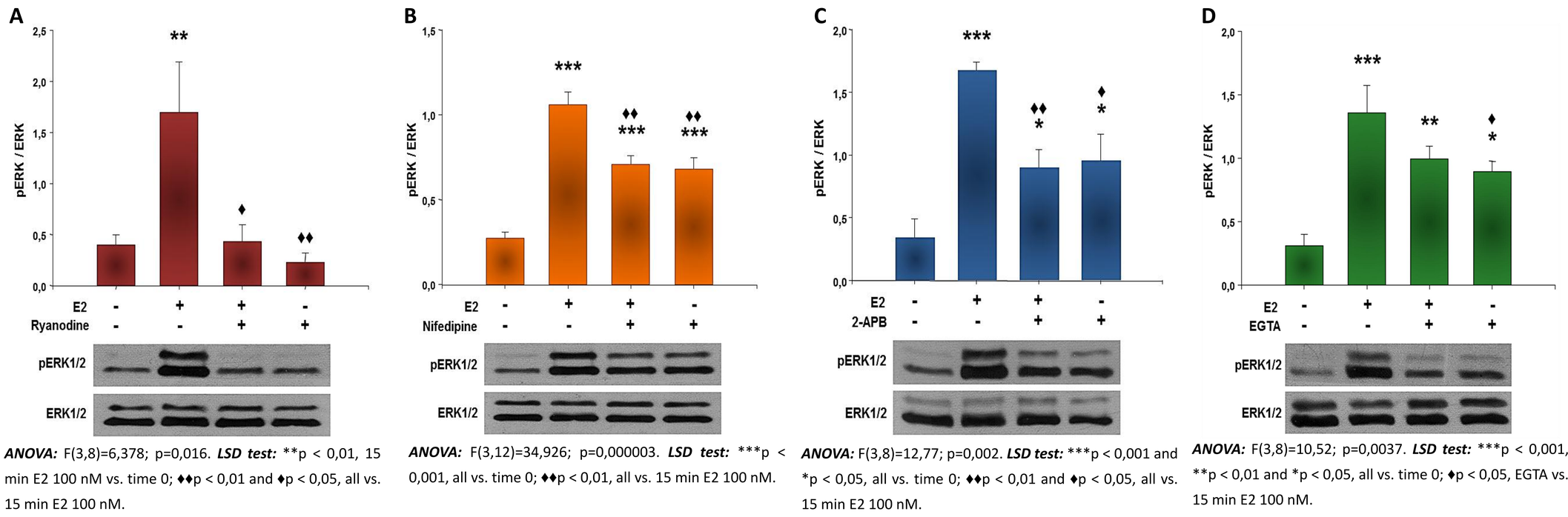
In the present study we investigate the Ca^{2+} pools that participate in the activation of ERK1/2 and axogenesis by E2.

MATERIALS and METHODS

Primary hypothalamic neuron cultures were established from E16 male rat embryos and maintain during 48 h *in vitro*. Neurons were pre-treated for 1 h with an extracellular Ca2+ chelator (EGTA, 1 mM), an L-type voltage-gated Ca2+ channel (L-VGCC) blocker (nifedipine, 2 µM), a ryanodine receptor (RyR) inhibitor (ryanodine, 50 µM), or an inositol-1,4,5-trisphosphate receptor (IP3R) inhibitor (2-APB, 100 µM) and then stimulated with E2 to analyze: 1) ERK1/2 phosphorylation levels by Western blot, 2) morphometric parameters by immunocytochemical staining and computational measurements and 3) intracellular calcium concentration ($[Ca^{2+}]_i$) dynamics by Ca^{2+} imaging using Cal-520 AM.

RESULTS

Figure 1. A 15 min pulse of 100 nM E2 induced ERK1/2 phosphorylation. This effect was completely abolished by 50 µM ryanodine **(A)** and partially attenuated by 2 µM nifedipine **(B)** and 100 µM 2-APB **(C)**. 1 mM EGTA **(D)** slightly decreased E2-induced ERK1/2 activation but this effect was not statistically significant.



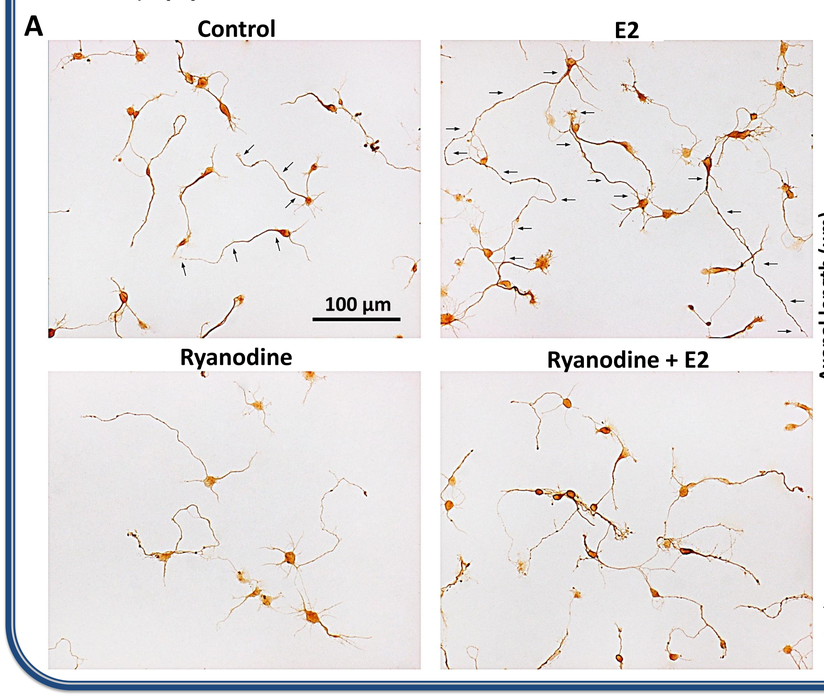
ANOVA: F(3,8)=6,378; p=0,016. ***LSD test:*** **p < 0,01, 15 min E2 100 nM vs. time 0; ♦♦p < 0,01 and ♦p < 0,05, all vs. 15 min E2 100 nM.

ANOVA: F(3,12)=34,926; p=0,000003. ***LSD test:*** ***p < 0,001, all vs. time 0; ♦♦p < 0,01, all vs. 15 min E2 100 nM.

ANOVA: F(3,8)=12,77; p=0,002. ***LSD test:*** ***p < 0,001 and *p < 0,05, all vs. time 0; ♦♦p < 0,01 and ♦p < 0,05, all vs. 15 min E2 100 nM.

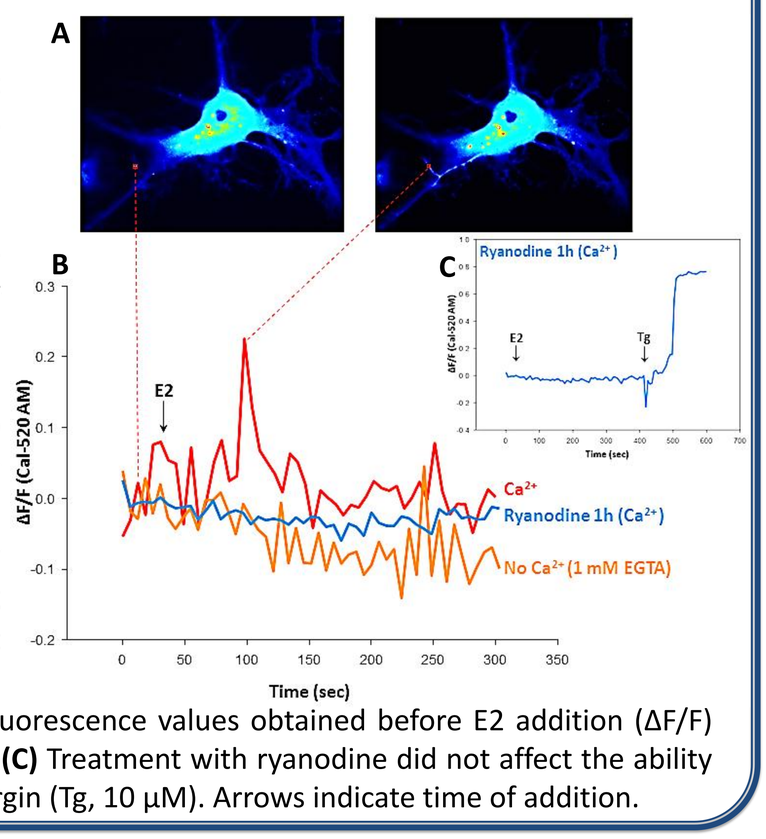
ANOVA: F(3,8)=10,52; p=0,0037. ***LSD test:*** ***p < 0,001, **p < 0,01 and *p < 0,05, all vs. time 0; ♦p < 0,05, EGTA vs. 15 min E2 100 nM.

Figure 2. (A) Male hypothalamic neurons cultured for 48 h. 10 nM E2 induced axogenesis, which was prevented by 1 h incubation with 50 µM ryanodine (arrows indicate the axon of some neurons). **(B)** Quantitation of the effects of the different treatments on axonal length.



ANOVA: F(3,12)=4,515; p=0,024. ***LSD test:*** *p < 0,05, all vs. control; ♦♦p < 0,01, all vs. E2 10 nM.

Figure 3. Neurons were loaded with Cal-520 AM for 30 min at 37°C and changes in $[Ca^{2+}]_i$ were measured with confocal microscopy. 100 nM E2 generated Ca^{2+} oscillations, which were dependent on the presence of Ca^{2+} in the extracellular medium and were abolished by 1 h 50 µM ryanodine pre-incubation. **(A)** Confocal images in pseudo-colour showing Ca^{2+} oscillations induced by E2. **(B)** Fluorescence intensity values were obtained by selecting a 2x2 pixels area from subsequent images captured during recording. Values were normalized against fluorescence values obtained before E2 addition (ΔF/F) and plotted as a function of time. **(C)** Treatment with ryanodine did not affect the ability of neurons to respond to thapsigargin (Tg, 10 µM). Arrows indicate time of addition.



CONCLUSIONS

- E2 treatment rapidly induced ERK1/2 activation, which was completely abolished by ryanodine and partially attenuated by nifedipine and 2-APB.
- E2-induced axonal growth was completely inhibited by ryanodine.
- Preliminary results from Ca^{2+} imaging recording suggest that E2 induces Ca^{2+} oscillations, which depend on Ca^{2+} influx and RyRs-regulated Ca^{2+} pools.

In summary, results suggest that Ca^{2+} mobilization from extracellular space as well as from endoplasmic reticulum are necessary to E2-induced ERK1/2 activation and axogenesis. Whereas L-VGCCs and IP_3Rs might participate in the Ca^{2+} signaling evoked by E2, the predominant role is mediated by RyRs.

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