The Role of Ryanodine Receptors in Neuronal Outgrowth under Physiologic and Oxygen Glucose Deprived Conditions in Culture
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Ryanodine receptor (RyR) is a calcium permeable homotetrameric channel that resides on the membrane of the endoplasmic reticulum and mediates calcium-induced calcium release. Calcium transients serve as a key messenger to control a myriad of intracellular processes and are therefore tightly regulated. Calcium can enter the cytosol from the extracellular space or intracellular stores like the endoplasmic reticulum. In this study, we investigated the contributions of RyRs to early neuronal development in mouse primary cortical neurons under physiologic, oxygen glucose deprived and caffeine stressed conditions. Neurite lengths and development were evaluated using immunocytochemistry under pharmacological inhibition, caffeine treatment and oxygen-glucose deprivation treatment. Confocal laser scanning microscope was used for image acquisition and images were analyzed using SynD, semi-automatic immune-fluorescence image analysis routine. RyRs were demonstrated to be a contributing factor to neurite outgrowth under physiological conditions. Pharmacologically antagonizing the channel with dantrolene significantly shortened neurites. Under caffeine-induced stress, inhibition of RyRs was found to partially rescue the neurite length shortening by caffeine. Dantrolene inhibition of RyRs was also found to both rescue immature neurons affected by oxygen glucose deprivation and lengthen mature neurons not shortened by oxygen glucose deprivation. Our data suggests that the inhibition of RyRs significantly shortened neurite length under physiologic conditions but promoted neurite elongation under oxygen glucose deprived and caffeine stressed conditions.

Introduction

Calcium serves as a key secondary messenger in many important pathways in the neuron including growth cone motility. Intracellular calcium levels are tightly regulated in a spatiotemporal manner. Calcium can enter the cytosol from the extracellular space or intracellular stores. One of the main intracellular stores is the endoplasmic reticulum (ER), controlled by ryanodine and inositol triphosphate receptor channels. In mammalian species there are 3 isoforms of the ryanodine receptor (RyR): RyR1, predominantly expressed in skeletal muscle cells, RyR2 in cardiac muscle cells, and RyR3 in neurons. Specifically in the embryonic mouse brain, RyR2 is highly expressed in purkinje cells of the cerebellum and the cerebral cortex while RyR3 is found in hippocampal neurons, purkinje cells and the corpus striatum. These homotetrameric calcium release channels are allosterically regulated by calcium ions, ryanodine, caffeine and dantrolene. Ca\(^{2+}\) entry through voltage-gated calcium channels induces calcium release via RyR from the ER, raising cytoplasmic Ca\(^{2+}\).

The presence or absence of this calcium-induced calcium release, calcium gradient steepness and cytosolic calcium concentration are especially critical in growth cone motility, as they dictate whether a guidance cue or adhesion molecule initiates an attractive or repulsive growth cone turning response. Ca\(^{2+}\) gradients and Ca\(^{2+}\)-dependent enzymes can, directly or thru Rho family GTPases, create an imbalance of tubule, microtubule associated protein phosphorylation and actin filament reorganization, thus steering the growth cone. RyRs are highly involved in steering the growth cone but it is unclear the role it plays under un-stimulated conditions.

Neuronal development is also affected by environmental stresses including caffeine, hypoxia and ischemia. Caffeine is a widely used stimulant, toxic to neurons at high concentrations and also a RyR agonist. Caffeine has a significantly longer half-life in pregnant humans and is able to cross the placental barrier into the fetus. Acute exposure to high dosages of caffeine, 100µm, have been shown to induce disturbances in early neurogenesis, disrupt closing of neural tube and induce premature evagination of the telencephalic vesicles in whole cultured embryos. Current studies focus on the more evident defects in the fetus and use high dosages that are unlikely to be consumed by the average person. It would be noteworthy to explore the effects of chronic exposure to lower doses of caffeine at a cellular level and whether or not RyRs play a role.

Under oxygen and glucose-deprived conditions, ion homeostasis is disrupted, causing degeneration and neuronal cell death. Oxygen and glucose deprivation (OGD) causes elevations of Ca\(^{2+}\) and Na\(^{+}\) ions in the soma mainly through NMDA glutamate receptors, sodium calcium exchangers and voltage-gated sodium channels resulting in compromised neurite outgrowth and cell death. The role of RyR under OGD conditions in developing neurons is not clear. An in vitro study using mouse hippocampal cell cultures finds dantrolene to be ineffective in neuroprotection against OGD. On the contrary, blocking RyRs with dantrolene proves to be protective in C6 glial and human neuronal cell cultures subjected to OGD insult and in neonatal rat models of hypoxic-ischemia. This project will attempt to characterize the role RyRs in axon elongation and neuron maturation by pharmacologically inhibiting under physiologic, caffeine-stressed and hypoxic glucose-deprived conditions. We hypothesize that RyRs differentially regulate early development of neurons under physiologic and stressed conditions. This project will attempt to determine whether activation of RyRs is required for neurite outgrowth, the role of RyRs in neurite outgrowth under caffeine treatment stress and oxygen and glucose deprivation (OGD) stress in early development. This project is in collaboration with a graduate student in Feng Lab.
Cell Culture, Dantrolene and Caffeine Treatment

This project used primary mouse cortical neuron culture harvested from E16 embryos. The dissected cortices were digested with trypsin, incubated at 37°C and centrifuged at 1000RPM for 5 minutes. The cells were resuspended in plating media (Neurobasal media with B27, GlutaMAX and Penicillin Streptomycin) and plated on Poly-D-Lysine coated dishes at a density of 100,000 cells neurons per ml. Dantrolene powder was dissolved in DMSO to a concentration of 50mM and added to media resulting in a final concentration of 50µM dantrolene and 0.032% DMSO. 5µM Caffeine or dantrolene is added and left in media for 3 days.

Oxygen Glucose Deprivation

On day 2, plating media was replaced with a glucose free culture media and bubbled with N₂ gas for 30 minutes to sequester all dissolved O₂. The plate was then transferred to a hypoxia chamber containing 5% CO₂ and 95% N₂ for 90 minutes. Finally, the media was changed back to original plating media and dantrolene was added.

Immunocytochemistry

Cells were fixed on day 3 with 4% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 20 minutes and incubated with the primary antibody, mouse monoclonal anti-β-Tubulin antibody (1:1000, Sigma, no. T0198 diluted in PBS, 2% bovine serum albumin, 2% fetal bovine serum) for 1 hour at room temperature. Cells were washed with PBS and incubated with goat polyclonal anti-Mouse IgG secondary antibody (1:500, Life Technologies, Alexa Fluor A31553) and Rhodamine Phalloidin (f-actin marker, 1:500, R415) for 2 hours at room temperature. A brief timeline of the study is available in the appendix (fig. 1B)

Image Acquisition, Neurite Analysis and Statistical analysis

Maximally projected confocal images were captured with Leica TCS LS confocal laser-scanning microscope (Heidelberg, Germany) equipped with ×40 oil immersion lens and 405- and 540-nm lasers. All neurons were traced and analyzed with SynD, a semi-automatic immune-fluorescence image analysis routine.

Microsoft Excel was used to compute the average neurite and dendrite length for each neuron and all basic statistical parameters. GraphPad Prism software was used for statistical analysis. One-way ANOVA and Bonferroni post hoc test was used to assess for statistically significant differences between treatment groups. Fisher’s exact non-parametric test was used to compare the proportion of neurons at each stage in each group. Values of p<0.05 are taken as statistically significant. Data is presented as mean ± standard error of the mean.

Results

Classification of neuronal developmental stages in culture

Neurite data was categorized into 1 control, 5 treatment groups and by stage of neuronal development. The stage of development was determined by observing the morphology and using the length difference formula (fig. 1B). As a neuron matures in culture it goes through 5 main stages:

1. At approximately 6 hours, lamellipodia from the soma explore the extracellular space then
2. At 24 hours neurite processes will start to project at the same pace and
3. At about 36-48 hours, polarization begins. One neurite will elongate faster than the others and the cell will start to express tau-1 and MAP 2 preferentially in the axon and the dendrites, respectively. (4) Branching and development of the neurite will continue on the 4th day until finally (5) on approximately the 14th day the arbors will mature and synapses may form. Figure 1A shows representative images of neurons in each stage.

In this report, outgrowth properties of cortical neurons in stage 2 & 3 were studied. To further differentiate between stage 2 and stage 3 neurons, the length different formula (fig. 1B) was used. The length difference formula was derived in an earlier paper publish by our lab. The neuron is designated as a stage 2 neuron if its length difference falls below 0.67. Representative images of stage 2 and stage 3 neurons from each treatment group are shown in figure 2.

Inhibition of RyR by dantrolene shortens neurite lengths

Inhibition of RyR by dantrolene had no effect on the proportion of neurons at stage 2 & 3 as compared to untreated control neurons (fig. 3A). Under dantrolene treatment neurite lengths of stage 2 neurons were significantly shortened (P<0.01) (fig.3B). Inhibition of RyR had negligible effects on axon and dendritic lengths of stage 3 neurons (fig. 3C,D). Dantrolene is an
inhibitor of RyR isoforms 1 and 3\(^8\), isoforms highly expressed in our cortical cell cultures\(^4\). Dantrolene has no other known targets in the neuron thus effect of dantrolene treatment can be fully attributed to the inactivity of RyR. Therefore basal activities of RyR isoforms 1 and/or 3 are a contributing factor to neuronal outgrowth at the second developmental stage.

**Caffeine treatment induces delay of neuron maturation and shortening of stage 2 neurites**

Caffeine treatment significantly reduced the percentage of neurons in stage 3, (fig. 3E, P<0.01), reduced neurite lengths of stage 2 neurons (fig. 3F, P<0.05) and had little effect on stage 3 neurons (fig 3G,H). Caffeine is the classical agonist for RyR receptor at the concentration of mM range\(^28\), and antagonist of adenosine A2 receptors (A2AR) at low concentrations (µM). To determine the effect of caffeine on neurite outgrowth, a low concentration of caffeine was used to determine the functional range of caffeine. Caffeine constrained neurons in the earlier developmental stage and shortened the neurite lengths of those constrained in the earlier developmental stage. The effect of caffeine appears to be stage-dependent, as it decreases neurite lengths but only in stage 2. Caffeine has other effectors in the cell; it inhibits inositol triphosphate receptors\(^29\), adenosine 2A receptors\(^30\), GABA-A receptors and phosphodiesterases\(^31\). Dantrolene and caffeine were administered together to determine if RyR mediates the effects of caffeine. Dantrolene’s effect on caffeine stressed neurons is unclear. The average neurite length of stage 2 neurons under caffeine and dantrolene treatment is not significantly different from the control group or caffeine treatment group (fig. 3F). Stage 3 neurons treated with both dantrolene and caffeine showed no difference in neurite length compared to control and caffeine treatment group (fig. 3G,H). Dantrolene partially countered caffeine’s shortening of stage 2 neurons but not full. It is likely caffeine acted via pathways including RyR-dependent pathways to cause neurite retraction of stage 2 neurons.

**OGD suppressed of neurite outgrowth via RyR-dependent mechanisms**

The proportion of neurons under OGD condition is lower in the stage 3 (fig. 3I), but higher in the stage 2 although not significant. OGD insult reduced neurite lengths of stage 2 neurons (fig. 3J, P<0.05). OGD insult had negligible effect on axon and dendrite lengths of stage 3 neurons (fig. 3K, L). This is consistent with current literature suggesting that more immature neurons are more affected by OGD\(^32\). It is possible that more neurons constrained in the early developmental stage under hypoxic condition and those constrained to the earlier developmental stage have shortened neurites. To determine if OGD shortened neurite lengths through RyR-dependent pathways, dantrolene was applied under OGD stress. Dantrolene significantly increased neurite lengths of stage 2 neurons, the resulting neurite length is comparable to control (fig. 3J, P<0.01). Interestingly, although OGD did not affect neurite lengths, dantrolene applied under OGD conditions significantly increased both axonal (P<0.001) and dendritic (P<0.05) lengths in both developmental stages (fig. 3K,L). Dantrolene applied under OGD condition also appeared to decrease the proportion of neurons constrained in stage 2, although no significant. OGD significantly shortened neurite lengths of stage 2 neurons through a RyR-mediated pathway and dantrolene applied under OGD condition promoted neurite elongation.

**Discussion**

**Early stage neurons are more susceptible to intracellular calcium homeostasis disruption**

We observed that dantrolene inhibition of RyR had detrimental effects to neurite outgrowth but only in stage 2 neurons. Similarly, caffeine and OGD-induced neurite shortening was only observed in stage 2 neurons. Under the same conditions, later stage neurons are less affected by the disruption of calcium homeostasis.

Calcium homeostasis regulation may be more robust in more mature neurons. It is possible that in the early stage of development calcium regulatory elements are not fully functional. Other calcium channels and calcium buffers may not be expressed at their full capacity at early developmental stages. For example, IP\(_3\)R and calbindin-1 are not expressed at high levels in the embryonic mouse brain until E18.5\(^33\). Additionally, feedback mechanisms may not be fully functional at early stages. For example, calmodulin binding transcription activator (CAMTA) 2, normally interacting with calcium bound calmodulin is not expressed at high levels in the embryonic mouse brain until E18.5\(^35\). CAMTA 2 may be involved in regulating the transcription of calcium regulatory mechanisms in response to raised cytosolic calcium levels. This delayed expression and/or functionality of intracellular calcium regulators causes early stage neurons to be more susceptible to calcium imbalance.

Early stage neurons may have a narrower optimal intracellular calcium concentration range for growth. Neurons have a range of calcium concentration that is optimal for neurite outgrowth\(^34\) and it may be wider in more mature neurons. A2A receptors, NMDA receptor expression levels in the mouse brain do not differ significantly between E15.5 and E18.5\(^33\). Therefore we assume that caffeine and OGD stress should directly affect calcium levels to a similar magnitude in both stage 2 and 3 neurons. However, we observe similar shifts in intracellular calcium levels to have more pronounced effects on more immature neurons. This suggests that stage 2 neurons may be subjected to a narrower optimal calcium range that is promoting of neurite outgrowth.
RyR1 & 3 activity may be less important in later stages of neuronal development. Our data shows that under normal conditions, RyR activity contributes to neurite outgrowth in stage 2 neurons. RyR inhibition in stage 3 neurons shows a negligible effect on neurite length. It is possible that RyR activity does not play a big role in later development, the receptor channel could be down regulated as the neurite transitions into stage 3 (or expression of the protein may have decreased). The expression of RyR1 and 3 in the cortex of embryonic mice peak at E15 but RyR2 expression is just beginning and on the rise. Dantrolene is an effective inhibitor of RyR1 and 3 and had insignificant effects on stage 3 neurons. It is possible that RyR2 activity becomes the main contributor in this later stage and therefore dantrolene was unsuccessful in affecting neurite outgrowth.

**Micromolar levels of caffeine can act through RyR to shorten neurite lengths**

At a dosage comparable to the effect of 1 cup of coffee in the blood and not believed to stimulate RyRs, caffeine was able to activate RyRs to shorten neurite lengths and also delay neuron maturation. This is conflicting with current literature that suggests the effective dose of caffeine to activate RyR mediated calcium release to be at least 2mM, which was 400 times greater than the amount that we used in this study. However the effects under high concentrations of caffeine (mM) were observed following a few hours of treatment because of its extreme toxicity. Our findings of 5µM caffeine treatment with 3 days suggest that long-term treatment of low concentration caffeine may also activate RyR.

When channel antagonist dantrolene was applied in conjunction, there is not a full recovery of neurite lengths. The residual effects of caffeine after dantrolene application can be attributed to possible RyR2 activation or RyR-independent pathways. Caffeine is also an A2AR antagonist. A2ARs act through phospholipase C and phosphoinositide3-kinase to promote axonal elongation, therefore caffeine antagonization of A2AR could explain some of the shortening we observe.

**Dantrolene has an elongating effect on OGD stresses neurons**

OGD stress induced shortening of stage 2 neurites and dantrolene rescued neurite lengths. OGD causes calcium influx from extracellular space into the cytosol primarily through NMDA and voltage gated calcium channels, this influx may then turn activate RyRs to cause release of calcium from the intracellular store raising calcium concentrations to a level not suitable for neurite growth. Therefore, dantrolene inhibition of RyRs under OGD stress could be preventing the concentration to reach that magnitude, rescuing neurite outgrowth.

A similar but more interesting effect from dantrolene is observed in stage 3 neurons under OGD stress. OGD did not significantly shorten neurite lengths of stage 3 neurons but dantrolene administration still produced significantly longer axons and dendrites.

Alluding to an earlier hypothesis, OGD stress may not have affected stage 3 neurons because more mature neurons may have more robust calcium regulatory mechanisms and therefore able to combat high intracellular calcium levels and detrimental effects presented by OGD stress. Additionally, the population of neurons surviving OGD insult maybe inherently or conditioned by the OGD to be more energetically efficient. When glucose containing media is returned to the culture, neurons subjected to OGD are more efficiently bioenergetically but held back by the higher than optimal cytosolic calcium level. Therefore when dantrolene is applied to return calcium levels to control, OGD conditioned neurons has a bioenergetic advantage and elongate at a faster rate than control neurons under the same conditions.

OGD may be altering the expression of RyRs. We saw that under control conditions, dantrolene application had no significant effect on neurite outgrowth but under OGD condition inhibition of RyRs significantly increase neurite lengths. Perhaps, OGD stress altered the expression or localized of RyRs. It is not unlikely that under OGD condition, neurons will have altered gene expression and protein function to help moderate the effects of OGD. For example, OGD lowers the expression and function of gap junctions in slices of spinal cord white matter, which will help to preserve axonal function and nerve regrowth following ischemic injury. An increased expression or preferential localization of RyRs in the axons and dendrites could cause dantrolene’s effects under OGD condition to be more pronounced.

Calcium imbalance may have been masking elongating effects of hypoxia. In stage 3 neurons, the calcium regulatory elements are strong enough to combat the detrimental effects of OGD conditioning but are faced with lowered glucose levels. Hypoxia conditioning has been shown to enhance neurite outgrowth but when combined with glucose deprivation, neurite outgrowth is compromised even when calcium containing media is returned after 1 hour. Hypoxia enhanced outgrowth may have been masked by calcium imbalance and therefore dantrolene returning calcium levels removed the only hurdle preventing this enhanced outgrowth.

**Future Directions**

An important next step to take is increasing sample size to strengthen the power of the study. Additionally, incorporating the use of axonal and dendritic markers (e.g. tau-1 and MAP2 antibodies) would create more accurate axon dendrite data. To study the change in RyR’s role through development would require exploring cells fixed on earlier and later dates. Neuron death and
neurite branching are two areas that have no been explored yet and would aid in clarifying whether the observed shorter and more immature neurons under OGD and caffeine treatments is due to retraction or cell death. This can be achieved by analyzing PI staining of dead cells and Sholl analysis or manual recording of neurite branching. Specific inhibitors of each isoform (e.g. heparin to block RyR1) would help to determine the contributions of each isoform to neuron development. Studying the subcellular localization and functionality of the RyR between stages of neuron development could help to explain the stage dependent effects of RyR antagonist. Similar studies under OGD conditions would help to explain dantrolene’s elongating effects in later stage neurons.

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References

Appendix

Fig. 1A Representative images of development stages of cortical neurons. Antibodies and stains used: Primary Mouse monoclonal anti-β-Tubulin antibody (1:1000, Sigma, no. T0198), goat polyclonal anti-Mouse IgG secondary antibody (1:500, Life Technologies, Alexa Fluor A31553) and RhodaminePhalloidin (f-actin marker, 1:500, R415.) Scale bars are 20μm.

B Timeline of study

C Length difference formula

If Length Difference < 0.67 then Stage 2 Neuron
≥ 0.67 then Stage 3 Neuron
Fig. 2A Representative images of neurons in stage 2 (left) and stage 3 (right) in different treatment groups. Scale bars are 20µm.
Fig. 3 A,E,I Proportion of neurons in stages 2 and 3 in different treatment groups  
B,F,J Average neurite length of DIV 3 stage 2 neurons 2 (control n=9 , dantrolene n=18, caffeine n=23, caffeine and dantrolene n=6, OGD n=17 and OGD dantrolene n=12 )  
C,G,K Average axonal and D,H,L dendritic lengths of DIV 3 stage 3 neurons 2 (control n=16 , dantrolene n=22, caffeine n=19, caffeine and dantrolene n=26, OGD n=21 and OGD dantrolene n=20 )