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# High-frequency stimulus and high-$K^+$ solution cause kiss-and-run fusion at the crayfish neuromuscular junction

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## ABSTRACT

The conventional method of exocytosis in synaptic cells, full collapse fusion, occurs when the vesicles containing neurotransmitters collapse entirely into the presynaptic membrane. Kiss-and-run fusion (KR), an alternative to full collapse fusion, suggests that instead of fully collapsing into the membrane, vesicles briefly open a pore into the cleft and retain their spherical shape so they can be quickly reused by the cell. KR has been discovered at some synapses, but the existence of KR at the neuromuscular junction (NMJ) is not definitive, and the effects of variables such as stimulus frequency on KR fusion are subject to further research. We contribute to these areas of interest by determining the rate of KR fusion at the NMJ and exploring the effects of two kinds of nerve stimulation (100Hz, and high-potassium solution). We studied KR by loading the fluorescent dye FM1-43, which allows us to see and measure fluorescence intensity, along with the quencher bromophenol blue (BPB), which partially suppresses fluorescence, into NMJ vesicles and recorded the fluorescence by taking photographs and comparing fluorescence levels using the program MetaMorph. Our experiment was based on the premise that the rate of KR would manifest in an increase of fluorescence (signaling that the quencher BPB, which has more hydrophilic properties than FM1-43, had left the vesicle through the vesicle pore). We hypothesized that KR would occur in baseline conditions and would occur at a lower rate during depolarization caused by electrical stimulation and high-potassium. We found that KR is not present in the NMJ under normal conditions but can be caused by both electrical stimulation and high-potassium solutions. We speculate that residual calcium from these depolarizing influences is causally responsible for the increased rate of KR.

## INTRODUCTION

Kiss-and-run fusion (KR), in which neurotransmitter vesicles open a pore into the presynaptic membrane and release contents while retaining their spherical structure, is a relatively new proposal in neuroscience. It is unconventional compared to full-collapse fusion (FCF), in which the vesicle collapses entirely into the membrane and will be recycled but cannot be immediately reused. If KR proves to be a viable method of fusion, it may explain vesicle reuse and energy conservation in cell junctions.

KR has been discovered at junctions of hippocampal cells (Alabi and Tsein 2013), but its existence elsewhere is highly contested. Fitzpatrick et al (2010) and Hunter et al (2008) suggested that KR may exist at the crayfish neuromuscular junction (NMJ). Hunter et al. (2008) used the fluorescent dyes FM1-43 and BPB to determine the rate of KR. In KR, only a small pore will be opened into the cleft. FM1-43, the less hydrophilic of the two, will remain inside the vesicle while BPB will be drawn into the synaptic cleft since it is more hydrophilic. When BPB diffuses out of the vesicle, it will stop quenching FM1-43's fluorescence and we will observe an increase in fluorescence ( et al. 2006). During FCF, however, the two dyes will disperse into the cleft evenly, since the synaptic vesicles collapse entirely into the membrane, regardless of any difference in hydrophilicity.

We expect tetanic stimulation to create a build-up of residual calcium in the NMJ. Calcium binds to calcium receptor calmodulin and may alter the mechanism by which vesicles release their contents. We expect low calcium concentrations would hinder vesicles' ability to fuse fully with the membrane, causing the cell to resort to KR, so high stimulation (or depolarization caused by a high-potassium solution) should have the opposite effect. Conversely, Ales et al. (1999) showed that high calcium levels increase the rate of KR, but the reason for this is unknown.

Our experiment sought to identify whether the crayfish NMJ relies primarily on KR or FCF, and how the rate of KR is influenced by tetanic stimulation and high-potassium solutions. We found that both electrical and chemical stimulation led to an increase in fluorescence, but saw no such increase in our control data, leading us to conclude that while the NMJ does not normally use KR, the depolarization caused by stimulation leads the NMJ to use KR. We speculate that this is caused by residual calcium

accumulating in the presynaptic terminal following repeated depolarization.

## MATERIALS AND METHODS

*Dye function*

FM1-43 dye is an amphipathic fluorescent molecule which partially lodges into the cell's membrane without fully permeating it. During endocytosis, FM1-43 molecules on the outer cell membrane get pulled into the cell and trapped inside vesicles, allowing us to track fluorescence (Amaral et al., 2011). FM1-43 can be used as a membrane marker to indicate the loss of fluorescence by dispersion. Additionally, bromophenol blue (BPB) can be used as a fluorescence quencher (Harata et al 2006). When it is loaded with FM1-43, it will partially quench FM1-43's fluorescence. If KR is occurring, BPB will leave the vesicle before FM1-43, causing the fluorescence to increase.

*Dye loading*

We created stock solutions of FM1-43 and BPB from Invitrogen at 8mM. After neutralizing the crayfish for 5 minutes with standard Ringer's solution, we immersed the crayfish in high-K+ solution to induce depolarization and added the dyes, diluting them each to 8μM in the high-K+ solution. We set the crayfish in the dark for 20 minutes to allow the dyes to undergo endocytosis and load into the vesicles. Lastly, we rinsed the crayfish to remove any remaining extracellular dye molecules and immersed it in standard Ringer's solution.

*Crayfish preparation*

We anesthetized our crayfish in ice water to humanly anesthetize it and preserve nerve function before we removed its tail. We cut the ventral part of the tail off and removed the mass of muscle in the dorsal part, exposing the superficial muscle extensor. We cut the remainder of the tail in half lengthwise and pinned it in a small dissection bowl immersed with crayfish saline to preserve nerve function for stimulation. The saline composition was 5.4mM KCl, 19.6 mM NaCl, 2.6mM $MgCl_2 \cdot 6H2O$, 10mM Na HEPES buffer and 13.5 mM $CaCl_2 \cdot 2H_2O$ (pH=7.4).

*Electrical and chemical stimulation*

We located a nerve using an Olympus SZ61 microscope and hooked a wire around the nerve to provide stimulation. We tetanically stimulated it for trials of five and ten minutes at 100 Hz. We also stimulated the NMJ chemically by replacing the standard Ringer's solution with 54mM high-potassium Ringer's solution.

*Microscope and camera*

We carefully moved the preparation from the Olympus microscope to the Nikon DS-Ri1 fluorescence microscope with camera. We used a 40x water-immersible objective and a GFP filter. We identified regions of interest at which clusters of vesicles (boutons) could be observed fluorescing (we looked for a line of small bright circular spots). We photographed these regions at 15 second intervals using the computer program NIS-Elements.

*Data Analysis*

We used the computer program MetaMorph to measure the fluorescence intensity of our photos' regions of interest. We entered the data into Excel and normalized it (calculated the percent change of the boutons opposed to the background). We graphed the fluorescence against time and noted when we had applied electrical stimulation or the high-potassium solution.

## RESULTS

Our experiment sought to determine whether or not kiss-and-run fusion occurs at the crayfish neuromuscular junction, and if so, what factors influence the rate at which it occurs. Following the methodology of Hunter et al. (2008) and Harata et al. (2006), we applied the fluorescent dye FM1-43 and quencher BPB to determine the rate of KR. By loading these dyes into the cell's synaptic vesicles, we were able to see clusters of synaptic vesicles under a fluorescence microscope, track dye dispersion and ultimately measure fluorescence intensity over time (Figure 1). FM1-43 provided "baseline" fluorescence, and BPB partially quenched it, so that we could tell BPB had left the vesicle when fluorescence increased. If KR is occurring, the small pores on the vesicles' exterior will allow BPB, but not FM1-43, to leave. This means that when we see significant increases in fluorescence intensity—indicating that BPB has left—KR is occurring at that time. We hypothesized that both electrical and chemical stimulation would decrease the rate of KR, as signified by increases in fluorescence intensity.

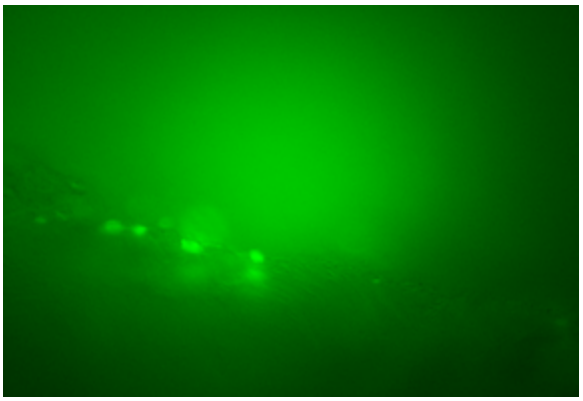

Figure 1. Photograph of dyed boutons. The bright spots in the lower left region of this photograph are boutons (clusters of vesicles) in the crayfish neuromuscular junction. The FM1-43 provides fluorescence and is quenched (though not entirely) by BPB. We measured fluorescence by selecting one to four small, bright, circular regions of interest on the photographs and matching them with one to four background regions. We calculated the difference in maximum fluorescence between the bright areas (boutons) and background areas.

Using Metamorph, we analyzed 11 series of photographs by graphing the relative fluorescence of selected regions against time. Regions of interest were selected by identifying outstanding fluorescence intensity and a morphology appropriate to clusters of vesicles (boutons)—i.e., small, bright circular spots (Figure 1). In all four baseline sets of data, and three of the five high-potassium series, the final fluorescence was either lower or unchanged from the initial level. Two out of five applications of the high-potassium Ringer's solution resulted in an immediate spike in fluorescence, which returned to a steady rate afterwards (Figure 2). Both instances of 100Hz stimulation gradually increased fluorescence in the duration of five minutes then decreased back to its starting rate (Figure 3).

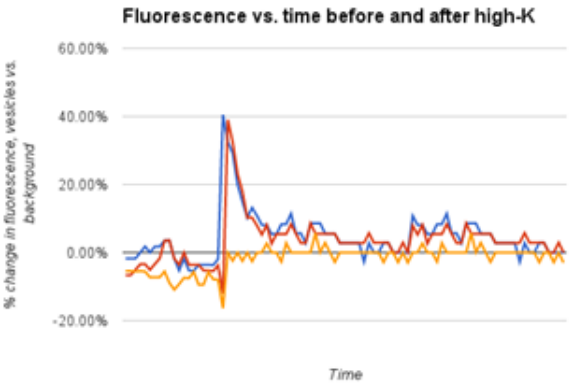


Figure 2. Fluorescence vs. time in minutes. Spike in fluorescence (at t=5 minutes) resulted directly after we replaced the standard Ringer's solution with the high-K+ version. Each line represents one region, or bouton, which we took data from in this particular series. Blue, for example, would correspond to a distinct vesicle cluster, which was analyzed in such a way that it was isolated from the rest of the picture in Metamorph; likewise for red and yellow.

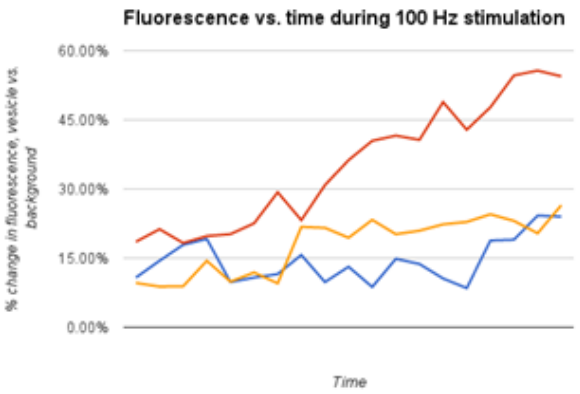


Figure 3. Fluorescence vs. time in minutes. We began 100Hz stimulation at the beginning of this series and continued it for 5 minutes. Each line represents one region, or bouton, which we took data from in this particular series. We hypothesize that if we had continued stimulation in this series, fluorescence would have gradually decreased. As in Figure 2, colors correspond to distinct vesicle clusters that were isolated in the process of data analysis.

## DISCUSSION

Our hypothesis that kiss-and-run fusion would occur at lower rates during electrical and chemical stimulation was not supported. We found no evidence of KR when the crayfish was at rest in the standard Ringer's solution, but the increase in fluorescence during electrical and chemical stimulation led us to conclude that KR occurs under those conditions, possibly due to the increase in residual calcium.

As shown in our results, we did not observe an increase in fluorescence during the resting state in standard Ringer's solution and we observed an increase in fluorescence twice during 100Hz stimulation and twice during the high-K+ solution. From this we concluded that KR fusion does not normally occur in the crayfish NMJ, but can be induced by electrical or chemical depolarization.

These results align with Ales et al. (1999), who determined that increased levels of calcium cause cells to resort to KR. The mechanics behind this change are unknown and should be studied further. However, there are others ways, besides calcium, of explaining our results. Alabi and Tsien suggest, for example, that increased electrical frequency may somehow augment either the size or amount of pores on a synaptic vesicle—a condition clearly conducive to KR. In general, the study of the specific mechanisms through which KR occurs is a relatively undeveloped area of research; hence we propose only a few possibilities here.

Our experiment was subject to some opportunities for error. Firstly, all of our data fluctuated greatly from photo to photo over the series (as seen in Figures 2 and 3). Our baseline

measurements were not wholly stable. Secondly, due to technical issues with our microscope, we had trouble keeping the selected region in focus or consistent, so it was sometimes difficult to compare photographs within a data set. Due to limited space in which to conduct the experiment, we were unable to use a recording electrode when stimulating the specimen, which made us blind to whether the wire was actually connected to the nerve and whether stimulation was occurring properly.

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## REFERENCES

Alabi, Abdul Rasheed A. and Richard W. Tsein. 2013. Perspectives on Kiss-and-Run: Role in Exocytosis, Endocytosis, and Neurotransmission. *Annual Review of Physiology* 75: 393–422.

Ales E., Tabares L., Poyato J., Valero V., Lindau M., and Guillermo A. 1999. High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. *Nature Cell Biology* 1: 40–44.

Amaral E, Guatimosim S, Guatimosim C. 2011. Using the fluorescent styryl dye FM1-43 to visualize synaptic vesicles exocytosis and endocytosis in motor nerve terminals. *Methods Mol Biol* 689: 137-48.

Arvanis A., Pyle J., and Tsien R. 2003. Single synaptic vesicles fusing transiently and successively without loss of identity. *Nature* 423: 643-647.

Fitzpatrick, Megan, Cassie Koid, and David Satzer. 2010. The Calcium Dependence of Kiss-and-Run Exocytosis at the Crayfish. *Pioneering Neuroscience* 10: 51–54.

Hunter, Lindsay, Jessica Jackson, and Natalia Maurer. 2008. Kiss-and-Run Fusion can be Detected at the Crayfish Neuromuscular Junction. *Pioneering Neuroscience* 9: 1–4.

Harata NC, Choi S, Pyle JL, Aravanis AM, Tsien RW. 2006. Frequency-dependent kinetics and prevalence of kiss-and-run and reuse at hippocampal synapses studied with novel quenching methods. *Neuron* 49.2: 243-53.