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Alkalinization of the Synaptic Cleft during Excitatory Neurotransmission

Peter J. Niesman[1] and Valeria Silva[2]

[1]Interdepartmental Neuroscience Program, Yale University, New Haven, Connecticut 06510, and [2]Centro Interdisciplinario de Neurociencia de Valparaíso, Universidad de Valparaíso, Valparaíso 2360102, Chile

Review of Stawarski et al.

Synaptic transmission has been studied for decades to understand the fundamental properties underlying neural communication, yet some basic questions about synaptic transmission remain unanswered. Historically, ribbon synapses have been a particularly important model because of the massive amounts of vesicles released during neurotransmission (Von Gersdorff and Mathews, 1994). Preparations of ribbon synapses from various organisms revealed the importance of calcium influx on vesicle release exocytosis and also showed acidification of the synaptic cleft because of joint release of neurotransmitters with protons (H^+; DeVries, 2001; Palmer et al., 2003; Hirasawa et al., 2012; Wang et al., 2014; Vincent et al., 2018). Although the necessity of calcium for vesicle release has been confirmed across synapse types, whether cleft acidification occurs at all synapses remains unclear. The current belief that transmitter release acidifies the cleft is mostly based on ribbon synapses, which release 10 to 100 times more vesicles than a typical mammalian central synapse.

Whether central excitatory synapses undergo acidification or alkalinization during synaptic transmission is less clear because of mixed results from different groups. A previous study using rat hippocampal slices reported acidification in the cleft during neurotransmitter release (Krishtal et al., 1987), as observed at ribbon synapses, while a different group showed alkalinization after transmission in the same preparation (Gottfried and Chesler, 1996; reviewed in Sinning and Hübner, 2013). These variations may have resulted from technical limitations, since in both studies the authors measured pH changes with pH sensitive dyes filling the entire extrasynaptic space, instead of directly examining the pH shifts in the synaptic cleft. Additionally, the pH shifts at ribbon synapses are larger and easier to detect than the pH shifts at central synapses.

A recent study (Stawarski et al., 2020) published in *The Journal of Neuroscience* addresses some of these weaknesses and strengthens the support for alkalinization of glutamatergic synaptic clefts by neurotransmitter release. The authors used a pH fluorescent sensor called "pHusion-Ex" to measure pH oscillations in the synaptic cleft. pHusion-Ex was created by fusing a pH-sensitive green fluorescent protein-based sensor (pHluorin; Sankaranarayanan et al., 2000) with a low-toxicity red fluorescent protein (FusionRed; Shemiakina et al., 2012). This fusion protein was then expressed at the cleft of the *Drosophila* larva neuromuscular junction (NMJ). Ratiometric imaging *in vivo* in intact larvae revealed alkalinization of the cleft in response to locomotion. The authors then measured pH transients in response to single action potentials at the larval NMJ in *ex vivo* preparations. Again, alkalinization was measured at the cleft, albeit at a much smaller magnitude than during locomotion *in vivo*. The authors switched to using a chemical indicator to detect pH transients at the NMJ to strengthen the support for alkalinization, and this also indicated alkalinization of the cleft.

To address the possibility that rapid cleft acidification went undetected, high-frequency (560 Hz) imaging was performed at the cleft. No evidence of acidification was found using the pHusion-Ex sensor. Yet, it is possible that acidification occurs only immediately proximal to active zones of vesicle release. To test this, SE-pHluorin, another pH sensor, was fused to voltage-gated calcium channels to restrict its expression to active zones in the presynaptic neuron. Again, no evidence for any acidification was seen: only alkalinization of the cleft was observed.

Regulation of pH at the synapse is complex (Chesler, 2003), but evidence points to calcium transporters such as Ca^{2+}-ATPase (PMCA) as key components in synaptic pH homeostasis. These transporters can rapidly exchange cytosolic Ca^{2+} for external H^+ (Makani and Chesler, 2010). Therefore, Stawarski and colleagues hypothesized that calcium, through the PMCA transporter, drives cleft alkalinization. Simultaneous

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Correspondence should be addressed to Peter J. Niesman at peter.niesman@yale.edu or Valeria Silva at valeria.silva@cinv.cl.

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imaging with SE-pHluorin in the cleft and jRGECO1a (intracellular calcium indicator) in the postsynaptic neuron shows cross-correlation between these transients. However, these measurements are indirect and alone do not support a causal role for the PMCA transporter. An informative extension of this work would be to block the PMCA transporter using PMCA inhibitors such as carboxyeosin (Makani and Chesler, 2010), and measure if cleft alkalinization still occurs.

To extend these conclusions past the *Drosophila* NMJ, Stawarski et al. (2020) examined the Calyx of Held synapse in acute brain slices from mice. Extracellular-facing pHusion-Ex revealed alkalinization of the cleft during an action potential train. In contrast, acidification of the presynaptic cytosol was seen using cytosolic facing pHusion-Ex. The magnitude of the acidification appeared larger and more persistent than the cleft alkalinization. As expected, the cleft alkalinization was reduced by increasing extracellular buffering capacity, whereas cytosolic acidification was unaffected. Together these results provide the strongest evidence to date for cleft alkalinization of excitatory synapses using the *Drosophila* NMJ and the mouse Calyx of Held synapse.

One likely effect of alkalinization of the cleft at glutamatergic synapses is altering the action of pH-sensitive potassium channels in neurons. Since most two-pore acid sensitive K^+ (TASK) channels are impaired by cleft acidification and enhanced by cleft alkalinization (Ma et al., 2012), repeated neuronal firing would increase outward K^+ currents, lowering neuronal excitability. Although these ion channels are not broadly expressed, both TASK-1 and TASK-3 are present in the rodent spinal cord (García et al., 2019). These TASK channels play an antinociceptive role by reducing overall firing rate induced by formalin injection. These results fit together nicely, suggesting that *in vivo* firing of a glutamatergic spinal afferent may lead to cleft alkalinization and increase outward K^+ currents, homeostatically lowering excitability.

It is well accepted that pH homeostasis is critical for neural communication, although no direct role has been identified for physiological pH changes during synaptic transmission (Chesler, 2003). The most frequently hypothesized role of physiological pH changes at the synaptic cleft is to affect short term synaptic plasticity by altering the sensitivity of neurotransmitter receptors. While numerous studies have examined the pH-dependence of various neurotransmitter receptors, including the ionotropic glutamate receptors AMPA and NMDA, the magnitude of the differences are often small at physiological pH ranges (Traynelis and Cull-Candy, 1991). Also, many short-term plasticity mechanisms are well accounted for by calcium signaling pathways affecting presynaptic release (Jackman and Regehr, 2017), suggesting pH may not be involved in short-term plasticity. While it is possible that pH may play a complementary role in short-term synaptic plasticity, it may play a key role in other cellular processes involved in neurotransmitter release, such as regulating endocytosis and vesicle refilling.

Acidification of glutamatergic presynaptic terminals and alkalinization of the cleft may play a role in the regulation of the synaptic vesicle cycle. The rate of various steps of the synaptic vesicle cycle is regulated by neurotransmitter transporters, the H^+ electrochemical gradient, and the actin cytoskeleton. Previous work has demonstrated how acidic pH facilitates glutamate uptake into vesicles through an allosteric mechanism (Eriksen et al., 2016). The recently published structure of VGLUT2 identified pH sensitive residues that are critical for transporter function (F. Li et al., 2020), further supporting the regulation of glutamate loading by pH. Recently, the regulation of the H^+ gradient by cation/H^+ exchangers (Na^+/H^+ and K^+/H^+), which facilitate the vesicle refill process of glutamatergic synapses (Goh et al., 2011), was shown to be very important for high-frequency spiking neurons (D. Li et al., 2020). These exchangers may contribute to acidification of the presynaptic terminal, which can enhance the rate of glutamate loading into vesicles. Additionally, actin dynamics have been shown to be important for mediating endocytosis (Boulant et al., 2011), and actin polymerization has been shown to be enhanced by acidic conditions (Crevenna et al., 2013). Therefore, acidification of the presynaptic cytosol (Stawarski et al., 2020) could link vesicle release to vesicle endocytosis, and the magnitude of acidification of the presynaptic terminal could determine the rate of endocytosis.

The recently published study by Stawarski and colleagues provides strong support for the alkalinization of the synaptic cleft at excitatory glutamatergic synapses. While the mechanism underlying the alkalinization remains incompletely understood, it likely involves the PMCA transporter. We think these findings will prove important not for short-term synaptic plasticity, but for regulating key steps of the synaptic vesicle cycle such as vesicle loading and endocytosis.

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