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Slow neuromodulation mediated by ATP P2X receptors

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Abstract

ATP-gated P2X receptors are widely expressed in the nervous system, but their physiological roles are not fully understood. New insights in this issue of *Neuron* show that postsynaptic P2X receptors may be activated by ATP released from astrocytes and function to down regulate synaptic AMPA receptors in hippocampal neurons (Pougnet et al., 2014).

Over 40 years ago Geoffrey Burnstock proposed the existence of purinergic nerves that released ATP (Burnstock, 1972). Although initially met with considerable skepticism, there is now overwhelming evidence that ATP is widely used as a signaling molecule in the body, including in the brain (Khakh and Burnstock, 2009). ATP functions as a neurotransmitter and as a neuromodulator by activating plasma membrane ionotropic P2X receptors and metabotropic P2Y receptors. The P2X receptor family belongs to the superfamily of neurotransmitter-gated ion channels that includes ionotropic glutamate and cys-loop receptor families, but P2X receptors are structurally (Kawate et al., 2009) and functionally distinct in several important ways (Khakh and North, 2012). Cationic P2X receptors are assembled from three subunits with just two transmembrane domains per subunit, and the family comprises seven subunits (P2X1 to P2X7), six homomeric receptors and several heteromeric assemblies. Of these P2X2 and P2X4 are widely expressed in the brain (Collo et al., 1996), but in relation to other neurotransmitter-gated ion channels we still know very little about their physiological roles. From this perspective, the work by Pougnet *et al.* markedly extends our understanding of the diversity of mechanisms utilized by P2X receptors to regulate neurons by revealing slow neuromodulatory functions of ATP rather than fast actions that are usually assumed and frequently ascribed to neurotransmitter-gated ion channels.

Early work showed that P2X receptors mediate sparse fast synaptic transmission in some brain nuclei (Edwards et al., 1992), but subsequent studies over several years failed to

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identify widespread fast ATP synaptic transmission at brain neuro-neuronal synapses (Khakh and North, 2012). P2X receptors are also present in presynaptic nerve terminals, where their activation increases neurotransmitter release probability (Gu and MacDermott, 1997; Khakh and Henderson, 1998), but the physiological settings under which this occurs in the brain are still unclear. Other pharmacological studies have suggested that P2X receptors may be involved in synaptic plasticity (Pankratov et al., 2009), but definitive evidence has been lacking because of the paucity of selective antagonists with which to explore roles for distinct P2X receptors. In addition to these findings, it was recognized that P2X2 and P2X4 receptors were localized to dendrites, where they were situated at the edges of the postsynaptic density (PSD) and thus distanced from synaptically activated AMPA receptors located within PSDs (Rubio and Soto, 2001). These high resolution immunogold electron microscopy studies were extended using single P2X2 receptor imaging on dendrites using quantum dots. Here, P2X2 channels sampled the dendritic surface at ~0.01 $\mu m^2/s$, but failed to enter the PSD and did not mediate fast synaptic transmission (Richler et al., 2011). The current study of Pougnet *et al*. starts with these findings as a back story and simply asks: what happens to excitatory synaptic transmission when postsynaptic P2X receptors are activated on hippocampal neurons? The experiments utilize several complementary approaches, including hippocampal cultures, *Xenopus* oocytes, acute hippocampal slices, biochemistry and high resolution imaging to address this question. The new insights are summarized below.

The core observations are reported in Figures 1 and 7 of Pougnet *et al*. The authors started their studies by applying ATP for ~1 min to hippocampal neurons in culture and analyzed miniature excitatory postsynaptic currents mediated by AMPA receptors (mEPSCs) over the next ~30 min. They found that ATP reliably decreased the amplitude of mEPCSs in the majority of neurons over a time course of ~20–30 min. However, ATP did not change the frequency of mEPSCs, which argues against a presynaptic effect. In these settings, the authors argued against a potential role for postsynaptic metabotropic ATP receptors, and argued for the presence of P2X receptors in hippocampal neurons. The key observation is that brief applications of ATP caused enduring slow decreases in mEPSC amplitudes over tens of minutes. Interestingly, a pioneering study by the Bains lab had previously reported a related response to ATP mediated by P2X receptors in magnocellular neurosecretory cells of the paraventricular nucleus (Gordon et al., 2005). In that study, responses mediated by exogenous ATP were mimicked by ATP released endogenously from astrocytes in response to norepinephrine (NE) applications. In accord, Pougnet *et al* found that astrocytes in hippocampal cultures expressed α1 adrenoceptors and that noradrenaline NE mimicked the effects of exogenous ATP, resulting in mEPSCs with reduced amplitude. The effects of both NE and ATP were blocked by PPADS, an antagonist that blocks P2X receptors, and the effects of NE were also blocked by fluoroacetate (FAC), a metabolic toxin that severely impairs (perhaps even destroys) astrocyte functions. The basic observations made with hippocampal cultures and mEPSCs were reproduced when field excitatory postsynaptic potentials (fEPSPs) were measured in the CA1 region of hippocampal slices; ATP and NE decreased fEPSP slopes in a manner that was blocked PPADS and FAC. Taken together, these data suggest that NE activation of adrenoceptors on astrocytes released substantial

amounts of ATP that in turn activated postsynaptic P2X receptors leading to a decrease in mEPSC amplitudes (in culture) and decreased fEPSP slope (in slices).

Next, the authors performed a series of functional and biochemical experiments with heterologously expressed P2X2 or P2X4 receptors together with GluA1/A2 heteromeric and GluA1 homomeric AMPA receptors. These data show that P2X receptor activation caused Ca^{2+}-dependent reduction in AMPA receptor mediated responses even within highly reduced systems such as the *Xenopus* oocytes. However, in oocytes the effect of ATP occurred within minutes and recovered slowly over tens of minutes. The reduction of AMPA receptor mediated electrophysiological responses was mirrored by a reduction in GluA1 and GluA2 subunits on the cell surface of oocytes. The mechanism proposed involves loss of GluA1 and GluA2 receptors from the surface with a presumed concomitant increase in their clathrin-mediated endocytosis. In this scenario, the initial trigger for endocytosis is ATP-evoked Ca^{2+} entry via P2X receptors.

The next section of the paper explored the aforementioned mechanisms within cultured hippocampal neurons expressing fluorescent protein tagged GluA1 and GluA2 receptors with and without P2X2 receptors. The use of pH-sensitive pHluorin tags on the AMPA receptor subunits allowed the authors to monitor their expression on the surface of neuronal dendrites; they found that ATP activation of P2X2 receptors caused the dynamin-dependent loss of cell surface dendritic AMPA receptors, which was in accord with their expectations from oocyte studies. These data were bolstered with experiments that imaged natively expressed AMPA receptor subunits within hippocampal neurons before and after ATP applications. Here, the use of direct Stochastic Optical Reconstruction Microscopy (dSTORM) showed that ATP applications caused the loss of GluA2 receptors from synapses. The final section of the paper explored if ATP can cause synaptic depression of AMPA receptor mediated fEPSPs in acute slices through a mechanism involving P2X receptors. The author's data suggest that this did occur, and that the mechanisms underlying the ATP effect were distinct from those which underlie long-term depression (LTD), because the ATP effect was not occluded by LTD. The authors provided pharmacological evidence to show that the ATP-evoked reduction in fEPSP slope involved phosphatase or Ca^{2+}/calmodulin dependent protein kinase, and that the same mechanisms are involved in the reduction of AMPA receptor currents in *Xenopus* oocytes following activation of P2X2 receptors.

In summary, the paper by Pougnet *et al.*, shows that applications of ATP and NE (to evoke endogenous ATP release) reduced mEPSC amplitudes in hippocampal cultures, that activation of P2X2 receptors coexpressed with AMPA receptor subunits resulted in the reduction of AMPA responses in *Xenopus* oocytes, and that ATP applications caused a reduction in fEPSPs within hippocampal slices. As such the paper raises the intriguing possibility that endogenous signaling by P2X receptors may be triggered by ATP release from astrocytes, and perhaps other cells such as microglia, rather than ATP release from neurons. If so, this may explain the lack of evidence for fast ATP synaptic transmission, and would be in accord with the location of P2X receptors outside the PSD (Rubio and Soto, 2001). In this view, ATP P2X receptors would serve neuromodulatory roles and would perhaps only be engaged under circumstances when astrocytes release ATP.

The new findings by Pougnet *et al* set the stage for additional studies to fully elucidate the modulation of glutamatergic transmission by P2X signaling. It needs to be demonstrated directly that ATP is released from astrocytes, and under what physiological stimuli this occurs. What precisely is the mechanistic link between adrenoceptor activation and ATP release? Does NE application to astrocytes trigger P2X receptor mediated currents in hippocampal neurons? Which type of adrenoceptor mediates these NE responses? Do mEPSCs decrease in amplitude in slices following ATP and NE applications? What is the timescale, magnitude and relevance of the resultant modulation? To that end, the biophysical and pharmacological properties of these P2X receptors *in situ* should be further investigated. For instance, a role for Ca^{2+} entry through P2X receptors in the mediation of this response could be explored with whole-cell voltage-clamp recordings, where neurons could be dialyzed with known concentrations of fast and slow organic Ca^{2+} buffers with known Ca^{2+} affinities. Exploiting the availability of the well characterized P2X2 and P2X4 knockout mice would strengthen the case that this form of neuromodulation relies exclusively on the ionotropic signaling cascade the authors propose. More generally, NE-evoked ATP release from astrocytes results in reduced fEPSPs in the hippocampus, but increased synaptic efficacy in the paraventricular nucleus (Gordon et al., 2005). Are these differences due to the differential expression of post synaptic P2X receptors, downstream signaling or the spatiotemporal profile of the Ca^{2+} signal?

The intriguing implication of the current work by Pougnet *et al* is that it may offer a clue for the neuronal microcircuit effects that begin to occur several seconds after startle and arousal responses *in vivo*, which recent beautiful studies show are accompanied by widespread NE-mediated Ca^{2+} signaling in cortical astrocytes (Ding et al., 2013; Paukert et al., 2014). If so, neuronal P2X receptors may contribute to norepinephrine regulation of startle, arousal and related behaviors. All together, the current study by Pougnet *et al.* raises many questions for future exploration.

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