NEUROSCIENCE

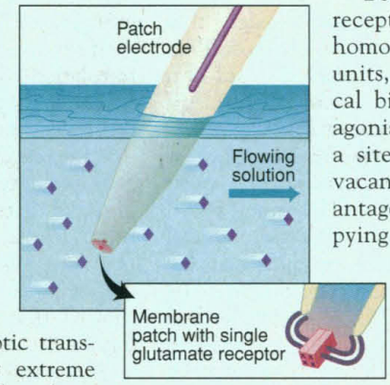
Glutamate Receptor Activation: A Four-Step Program

Christopher Miller

We run on electricity. All of our physical movements, all sensations, all emotion, all thought, and even (in the view of reductionists) human consciousness itself are constructed from electrical signals coursing along the billions of neurons that make up the nervous system. Central to the operation of this symphonically coordinated machine are the synapses—those tiny way stations where the nerve action potential, dying on the presynaptic membrane at the terminus of one neuron, is resurrected at the postsynaptic membrane of the next cell in the circuit. Synaptic transmission is brought about by neurotransmitters squirted out of the presynaptic cell onto the postsynaptic membrane, where receptor proteins await to trigger the newborn electrical signal.

In the vertebrate brain, glutamate is the predominant excitatory neurotransmitter, and the ionotropic glutamate receptors make up a molecular family of postsynaptic ion channels that mediate synaptic transmission. Thus burdened by extreme neurobiological relevance, the glutamate receptors have been furiously poked and probed over the last decade. Yet despite this scrutiny, one of the most fundamental molecular properties of this ion channel remains in doubt—its subunit stoichiometry. Many other neurotransmitter-activated ion channels are known to be constructed with five identical (or similar) subunits surrounding a central transmembrane pore, like the staves of a barrel, and so it has been generally assumed that glutamate receptors are pentamers too. But now, by clever use of single-channel recording, Rosenmund *et al.* (*1*) on page 1596 propose that these channels are instead tetramers.

The experiment at the core of the study is simple and elegant—the ohmigod-why-didn't-I-think-of-that kind. A high-resolution electrical recording is established on a patch of cell membrane carrying a single homo-oligomeric glutamate receptor channel (see inset). Initially, the patch is bathed in a solution with a high concentration of antagonist, a molecule that occupies the glutamate-binding site but prevents channel opening (X in the figure). The patch is suddenly switched into a flowing stream containing a high concentration of agonist, a glutamate analog that opens the channel by causing a conformational change upon binding (♦ in the figure). The authors then follow the pattern of channel opening after this sudden exposure to agonist.

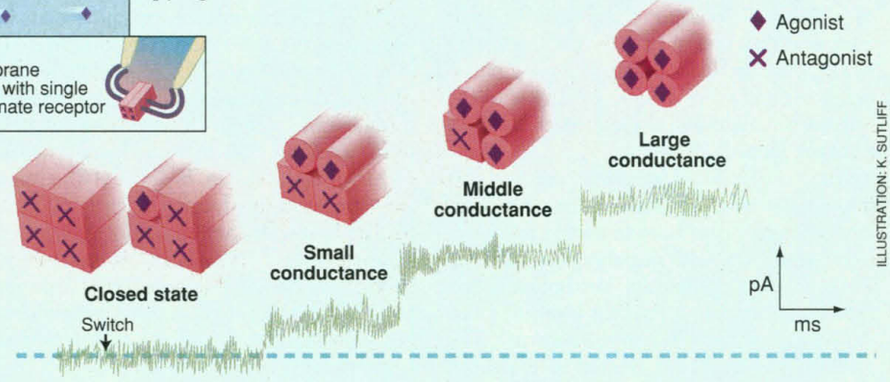


Because the glutamate receptor used here is a homo-oligomer of N subunits, there are N identical binding sites. For an agonist molecule to bind, a site must first become vacant: It must lose the antagonist that was occupying it before the solution was switched. This, it turns out, takes a few hundred milliseconds, time enough to watch each individual antagonist molecule fall off the site it had originally occupied. Immediately upon being vacated, the site becomes occupied by an agonist molecule waiting in the bathing solution. So the question becomes: What is actually observed as the N sites switch from occupancy by antagonists to occupancy by agonists, and can this tell us the value of N?

The results are delightful, both for themselves and for the mechanistic information they provide. As agonists replace antagonists, the channel opens in three discrete steps, seen as "substate" conductances smaller than that associated with the fully agonist-occupied channel (see lower part of the figure). From a closed state (C), the channel first opens to a small conductance (S), then to a middle state (M), and finally to the fully open large conductance (L). After many repetitions of the experiment, statistical distributions are built up of the dwell times in all the states on the way to full opening, and these furnish information about the pathway of activation. The $S \rightarrow M$ and $M \rightarrow L$ transitions behave statistically as if each occurs with the dissociation of a single antagonist molecule (and prompt replacement by a single agonist). The fully closed state, however, is different; it pauses before entering the S state, as if *two* agonists must replace antagonists before S can be established. Counting up the agonists that bind on the way to full channel opening, we find two to get from C to S, one to get from S to M, one to get from M to L—four sites in all. Ergo, the glutamate receptor is a tetramer, not a pentamer.

This picture is buttressed by a quantitative analysis of the waiting times between the various substate transitions. The authors find that these times vary in exactly the 7/6/12 ratio predicted by the simplest picture—that is, the dissociation of antagonist from each subunit is completely oblivious to the state of the other subunits. The precise confirmation of this waiting-time ratio is truly remarkable, as it was predicted without any adjustable parameters, once a tetrameric channel with independent subunits is assumed.

The experiment is aesthetically win-



Traces of a tetramer. (Inset) A patch of cell membrane with a single glutamate receptor channel in a flowing solution containing an agonist. (Bottom) Upon sudden switch to a channel-activating agonist, full opening of the channel proceeds via partially open "substrates."

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some, but is its conclusion correct? The authors, recognizing that conflicting studies have recently deduced both pentameric (*2*) and tetrameric (*3*) architecture for glutamate receptors, are careful to point out that their conclusion follows only under an extremely simple picture of ligand binding. This is a problem because multisubunit ligand-binding proteins typically show lots of cross-talk between subunits; this is ugly and messy, but it is real, as if Rube Goldberg rather than Isaac Newton were the Great Designer of proteins. So at this point we are left with a devil's choice between a tetrameric glutamate receptor channel with a mechanism pristine—and unprecedented—in its simplicity, or a pentameric channel that behaves in a kinetically lumpy but wholly familiar way.

References

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2. L. S. Premkumar and A. Auerbach, *J. Gen. Physiol.* **110**, 485 (1997).
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CIRCADIAN RHYTHMS

An End in the Beginning

Jay Dunlap

Circadian rhythms and the cellular oscillators that underlie them are ubiquitous—and for good reason. For most organisms, dawn means food (either fixing carbon or hunting prey), predation, and changes in all the geophysical variables that accompany the sun—warmth, winds, and so on. It's a big deal when the sun comes up, and most living things time their days with an internal clock that is synchronized by external cues. Given this common and ancient evolutionary pressure, circadian clocks probably evolved early, and common elements are likely to be present up and down the evolutionary tree. A series of papers appearing in this week's *Science* (*1*, *2*) on pages 1564 and 1599, *Cell* (*3*, *4*), and the *Proceedings of the National Academy of Sciences* (*5*) reveals an appealingly similar pattern in the assembly of circadian oscillators ranging from fungi to mammals and gives us a close-up view of the way the gears within a clock drive its circadian feedback loop.

For some years evidence has been building in support of a model for a core circadian oscillator comprising, at least in part, a transcription/translation-based negative feedback loop wherein clock genes are rhythmically expressed, giving rise to cycling levels of clock RNAs and proteins (negative elements). The proteins then feed back, after a lag, to depress the level of their own transcripts, perhaps by interfering with positive elements that increase transcription of the clock genes. Although individual negative elements (canonical clock genes like *Drosophila per* and *Neurospora frq*) and positive elements (*CLOCK* in mammals, *white collar–1* and *white collar–2* in *Neurospora*) had been identified, yielding clues as to the general layout of the loop, a clear picture had not yet emerged. Another clue appeared last spring, when the the mouse gene *CLOCK* and the *Neurospora* genes *wc-1* and *wc-2* were found to contain PAS domains (*6*, *7*), regions also found in PER that interact with other PAS domain–containing proteins (*8*). Now several groups working independently have brought us to the next chapter in this story.

It is a truth universally acknowledged, that a single protein in possession of a good PAS dimerization domain, must be in want of a partner (with apologies to Jane Austen). Applying this maxim, Weitz and colleagues (*2*) used the recently identified mouse *CLOCK* gene sequence (*7*) in a yeast two-hybrid screen of hamster hypothalamic cDNAs and pulled up several likely candidates. Decades of careful analyses had pinpointed the mammalian pacemakers in the suprachiasmatic nuclei of the brain (where indeed *CLOCK* and mammalian *per* gene are expressed) and in the eye (*9*), so candidates were sifted by virtue of where they were expressed and all were found wanting except one, an orphan, BMAL1 (*10*). A similar screen, executed independently by Bradfield and his colleagues to catalog interactions among bHLH-PAS proteins, also turned up a strong interaction between a BMAL1 isoform (MOP3) and CLOCK. A third independent, but simultaneous, investigation in *Drosophila* began in Kay's lab with the careful application of low-stringency hybridizations with the mouse *CLOCK* gene to identify the *Drosophila* homolog, d*CLOCK* (d*CLK*). A collaboration between the Weitz and Kay labs ported the analysis of the CLOCK partner to the tractable fly genetic system. And finally (good news for those of us who still find comfort in informative phenotypes) classical forward genetic screens for rhythms mutations in the laboratories of Rosbash and Hall had identified and mapped two new *Drosophila* clock genes, *cyc* and *Jrk*, mutations in both of which eliminate expression of *per* and *tim*. This phenotype is enticingly similar to that accompanying mutations in *wc-1* and *wc-2* in *Neurospora*, which eliminate expression of the clock gene *frq* and are required for sustained rhythmicity, and to some extent to that of the phenotype of mice with a mutation in *CLOCK* (*6*). As expressed sequence tags corresponding to bHLH-PAS proteins, including dCLK (identified genetically as *Jrk*) and dBMAL1 (*cyc*) were deposited in the databases, it became clear that these mutations had yielded the phenotypes that were essential to anchor the emerging molecular biology to the organism's overt rhythms. The next step was to obtain a concrete description of what these proteins really do—and happily that is just what materialized, in a satisfying tale of great science flawlessly executed.

If the oscillator includes a transcription/translation-based negative feedback loop in which PAS protein partners are positive regulators, the right experiment is to show that the proteins bind to the pertinent clock gene promoters to activate their transcription and that the proposed negative regulators block this activation. This is just what was done. Weitz and colleagues, Kåy and colleagues, and Bradfield and colleagues all showed that the CLOCK-BMAL1 dimer binds DNA via a promoter sequence termed an E-box and activates transcription in vivo (*1*, *2*, *5*). Careful work by Hardin and colleagues (*11*) had already shown that a small enhancer region of the *per* promoter containing an E-box was sufficient to confer circadian regulation on *per* transcription in whole flies; the transcription part of the clock loop closed at least in part through an E-box. These studies were sufficient to suggest a model that has now been elegantly tested in intact cells. Weitz and colleagues in a collaboration with Takahashi have found the E-boxes in the mammalian *per1* promoter, showed them sufficient to activate *per1* transcription, and confirmed that the dominant negative phenotype of the original *CLOCK* allele (*7*) is due to the mutant protein's inability to activate transcription, although it retained the ability to form heterodimers with BMAL1. Kay and colleagues have used the E-box element in the promoter of the other *Drosophila* negative clock element *tim*, shown it sufficient to confer dCLK responsiveness to a reporter in a naïve cell line and, in the *coup de grace*,

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