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Developmental Neurotransmitters?

Previous studies support an early role for neurotransmitter signaling before synaptogenesis, but puzzlingly, a neurological phenotype is absent in embryonic mice that lack vesicular release. Demarque et al. (in this issue of *Neuron*) now report that early release of transmitter is unconventional in not requiring action potentials, Ca^{2+} entry, or vesicle fusion, thus potentially reconciling the discrepancy.

The role of neuronal activity (i.e., action potentials and synaptic transmission) in the refinement of developing neural circuits has been a dominant theme in developmental neurobiology (Cohen-Cory, 2002). Neurotransmitter signaling, however, may also act to influence earlier developmental events, some of which occur prior to synapse formation, such as proliferation, migration, and differentiation (Nguyen et al., 2001). The idea that neurotransmitters can serve as chemical signals during development in both nervous and non-nervous tissue is not new (McMahon, 1974). However, in order for these influences to be physiologically relevant, functional neurotransmitter systems must be present early in the developing nervous system. For example, in the developing cortex, precociously expressed GABA and glutamate receptors appear to be activated by endogenously released ligand as early as stages of cellular proliferation (LoTurco et al., 1995). From these studies, questions have arisen as to the exact mode of early neurotransmitter action. Is transmitter released by conventional mechanisms, as occurs at mature synapses, or is there an alternate form of nonsynaptic release? Once released, how do transmitters activate their nonsynaptic receptors? Is there point-to-point signaling, as at traditional synapses, or does a paracrine or endocrine form of action exist?

In this issue of *Neuron*, Demarque and colleagues examine early transmitter release in immature hippocampal neurons (Demarque et al., 2002). The authors focus on perinatal CA1 hippocampal neurons that are synaptically silent. Evidence is obtained for two forms of receptor activation: a tonic current mediated by activation of $GABA_A$ receptors, and an evoked slow current mediated by both $GABA_A$ and NMDA-type glutamate receptors. The latter slow current, lasting 10–30 s, is termed the "early slow current" (ESC). A current similar to the evoked ESC was found to occur spontaneously at low frequencies (approximately 0.001 Hz). The authors explore the mechanism of transmitter release mediating both types of currents and demonstrate that, for the most part, neither current depends on action potential firing or activation of voltage-gated calcium channels (VGCCs). Additionally, the authors were able to rule out conventional vesicular release of transmitter, since the tonic current and the ESC persisted in the presence of Botulinum toxin, an agent known to prevent vesicular exocytosis. Furthermore, the authors made use of the Munc18-1 knockout mouse that has a complete absence of neurotransmitter secretion from synaptic vesicles due to deficient SNARE complex formation (Verhage et al., 2000). These mutant mice die at birth, but the authors recorded from hippocampal neurons of embryonic mice and found that, despite deficient vesicular release, these mice had spontaneous and evoked ESCs and tonic GABA currents. The authors conclude that synaptically silent neurons exhibit tonic and episodic GABA and glutamate receptor activation mediated by nonvesicular (or SNARE-independent) transmitter release.

This work extends previous findings of early, nonsynaptic transmitter signaling by providing additional details concerning the mechanism of transmitter release (see also Flint et al., 1998). These results may also reconcile previous findings of a developmental role for neurotransmitter signaling with a surprising lack of a developmental phenotype in the Munc18-1 mice. If transmitters are having developmental effects via nonvesicular modes of release, then one might not expect to see developmental abnormalities in mice with deficient vesicular release mechanisms. What remains to be established is whether transmitter released by either of the two forms (tonic or episodic) identified by Demarque et al. actually influences the development of the activated cells. In this regard, it may be worth considering the possible actions of early released transmitters along with the possible source of transmitter and the molecular machinery of release.

The basis of any signaling system must include a source and a target, and in the case of early nonsynaptic transmitter signaling, the source remains unknown. An obvious source for both GABA and glutamate release would be neurons that synthesize these amino acids; however, it is also possible that glial cells including astrocytes or perhaps even radial glial cells may secrete these transmitters. Future studies aimed at identifying the actual cellular source of transmitter will be important in order to understand the significance and potential range of action of these early transmitter signals.

The concept that neurotransmitters may provide diffuse signals in addition to localized synaptic actions has been suggested for both the developing and adult brain. This form of nonsynaptic transmission has been variously termed crosstalk, spill over, volume transmission, and paracrine signaling. While it is likely that such forms of transmitter action may be informationally rich, it remains to be determined if these nonsynaptic signals convey specificity in the developing brain. The extracellular environment in the immature brain is quite permissive to passive diffusion, a favorable condition for long-distance signaling. Depending on the source of ligand and the location and properties of the receptors, it is possible that instructive transmitter gradients are present in the developing brain. As a cell either moves or extends processes into a region of high concentration, for example, a specific signal may be transduced by receptor activation, thereby imparting positional infor-

mation. In this sense, transmitters may be acting in a way similar but not identical to graded morphogens, a common theme in developmental patterning. While this idea is speculative, immunohistochemical localization of various transmitter-producing cells and processes in the developing brain does show regional variation. Therefore, an understanding of the distribution of extracellular ligand would help determine whether such a mechanism could provide positional signaling.

Alternatively, a constant concentration of transmitter may simply provide a permissive environment in which other signaling events proceed more effectively. It is unlikely that signaling specificity can result if cells are in a uniform concentration of transmitter. Additionally, chronic receptor activation could lead to receptor desensitization and limit the temporal signaling potential of ambient transmitters. In this regard, the episodic spontaneous ESCs described by Demarque et al. would provide phasic transmitter action and overcome this limitation of chronic release.

As to the mode of secretion, a number of mechanisms other than vesicular release have been identified and are potential candidates. These include reverse transport and exchanger-mediated release, although both these modes were viewed as improbable by Demarque et al. Reverse transport of GABA was suggested not to occur since inhibition of the GAT-1 GABA transporter was found to be without effect on extracellular GABA clearance. But since other GABA transporters exist, it may be premature to completely rule out reverse transport. Additionally, since only one exchanger, the cysteine glutamate exchanger, was suggested to be absent in the hippocampus, release mediated by other exchangers remains a possibility. The more novel concept that the transmitter may be released by gap junction hemichannels, as has been suggested for ATP release from glial cells, is also mentioned. Alternatively, the authors suggest that transmitter may be leaked from source cells, as has been shown for acetylcholine (Ach) release at the neuromuscular junction, by an as yet unidentified mechanism. It is worth noting that in an analysis of Ach secretion in the 1970s, Katz and Miledi concluded that about 99% of the total Ach released under resting conditions was due to leakage rather than quantal secretion (Katz and Miledi, 1977). Therefore, significant amounts of transmitter can be released into the extracellular space in the absence of vesicular release; however, the membrane potential change induced by tonic transmitter release was small (approximately 40μV). Is it plausible then that the early released transmitters can have a significant physiological effect? In small immature cells that display high membrane resistances, much less charge transfer is needed to appreciably change the membrane potential. This applies to the cells under investigation in the present study. From Ohms law, for example, in cells with a membrane resistance ranging from 1 to 3 GΩ, just 10 pA of current could potentially produce a significant 10mV to 30mV of depolarization. The ESC could provide even more significant levels of membrane depolarization. Furthermore, slower channel kinetics, reduced rates of desensitization, and higher ligand affinity, all properties of immature receptors, may further increase the likelihood of effective nonsynaptic activation. Future studies may further define the source and mode of release of these early transmitter signals.

Combined with work from other groups, the present study by Demarque et al. suggests that in the developing brain, the majority of early transmitter signaling is mediated by the GABA system (Owens and Kriegstein, 2002). However, mice with null mutations of genes encoding the two primary GABA biosynthetic enzymes, GAD65 and GAD67, show relatively few developmental brain abnormalities despite having only 0.02% of the normal GABA content (Ji et al., 1999). In fact, nearly all the genetic experiments to date that perturb the GABA system, whether at the level of ligand, receptor, or transporter, have demonstrated little, if any, developmental defects. Therefore from a genetic point of view, early GABA signaling appears to be dispensable, although a more rigorous analysis of the various GABA mutants may reveal subtle developmental defects. Additionally, since all knockout experiments conducted so far involve germline mutations, they may be subject to developmental compensation that could reduce the severity of a brain phenotype. As other ligands, such as β-alanine and taurine, can also activate GABA receptors, it would be interesting to see if the tonic and slow currents reported by Demarque et al. are present in the GAD65/67 knockout mice. Furthermore, Ach, glutamate, and glycine can all depolarize embryonic cortical neurons as does GABA at early ages, so that pathways involving one or more of these transmitters could potentially reduce the severity of the GABA loss-of-function mutations. In the future, it will be important to analyze similarities in downstream signaling induced by receptor activation in a variety of transmitter systems that are present in the early developing brain. Such studies could reveal overlapping roles for early-appearing transmitter signaling during brain development.

It appears then that transmitters, particularly GABA and glutamate, are released by nonvesicular means in the early differentiating cortical environment and activate their cogent ionotropic receptors. These signals possibly mediate a wide range of development effects, from proliferation to differentiation and synapse formation. Connecting these early transmitter-dependent currents to specific downstream consequences remains a problem for future research. However, in view of the relatively mild phenotypes observed after mutation of components of a number of individual transmitter systems, it is likely that early developmental neurotransmitters act more as modulators than directors of developmental events. While development can proceed in its absence, neurotransmitter signaling may potentially shape specific downstream developmental events. For example, neurons can differentiate and form synapses without transmitter action, but signaling may be required for the remodeling of dendritic processes or the maturation of synaptic specializations. In this regard, a recent study of the mouse neuromuscular junction (NMJ) development in the absence of Ach has demonstrated that although nerve terminal differentiation does occur, a number of features both within and adjacent to the synapse are abnormal when transmitter is missing. The authors conclude, "neurotransmission is better viewed as coordinating rather than promoting synaptic maturation" (Misgeld et al., 2002). However, it is still unclear if

any of these early transmitter effects seen at the NMJ are mediated by synaptic or nonsynaptic release mechanisms or a combination of both. A role for nonvesicular glutamate release in the regulation of synaptic field size during NMJ development in the fly has recently been demonstrated (Featherstone et al., 2002). Therefore, evidence continues to be provided suggesting that transmitters do indeed act as developmental signals. The exact role of these early signals in the mammalian brain, however, remains to be defined.

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PKG and the Neural Basis for Behavioral Phenotypes

Cyclic GMP-dependent protein kinase (PKG) has been implicated in the regulation of diverse aspects of vertebrate and insect behavior, yet the mechanisms underlying these effects are poorly understood. In this issue of *Neuron*, Fujiwara et al. and L'Etoile et al. address the neural basis for PKG function in *C. elegans* and demonstrate the power of behavioral genetic analysis in simple systems in the elucidation of neuronal signaling mechanisms in vivo.

Cyclic GMP-dependent protein kinase, or PKG, is a relatively site-specific serine/threonine kinase that is widely expressed in the nervous systems of vertebrates and other animals. Although cGMP is an important second messenger in neurons as well as other cell types, other signaling kinases, such as PKA, PKC, and CaM kinase, have made considerably greater impressions on the consciousness of neurobiologists. It is true that PKG has been shown to act downstream of an NO-sensitive guanylyl cyclase to trigger the relaxation of capillary smooth muscle, a pathway that has provided gainful employment to Bob Dole and uncounted internet spammers. However, the functions of PGK in neurons have been much less clearly defined.

In addition to its vasorelaxive functions, NO has also been implicated as a retrograde messenger in some forms of synaptic plasticity; thus, significant attention has been devoted to the possibility that PKG might be an effector for NO in the establishment of cellular memory. Indeed, some studies using PKG inhibitors have suggested that NO may act through PKG in cerebellar LTP (Zhuo et al., 1994) and hippocampal LTD (Lev-Ram et al., 1997). However, studies of PKG knockout mice have failed to demonstrate a link between PKG and LTP, suggesting that other effectors (such as ADP-ribose) may be more important for these processes (Kleppisch et al., 1999). Moreover, links between PKG gene knockouts and behavioral deficits in learning or memory have not been reported.

However, recent genetic studies have revealed unexpected roles for PKG in the modulation of complex behaviors. In particular, elegant genetic studies in *Drosophila* have shown that the *for* locus, which encodes an isoform of PKG, dramatically influences heritable patterns of larval foraging behavior (Osborne et al., 1997). Animals carrying a "rover" allele (for^R) move large distances while feeding, while animals homozygous for the "sitter" allele (for^S), a naturally occurring variant that produces less PKG enzyme, are relatively inactive in the presence of food. Since the *for* locus has no effect on locomotion rate in the absence of food, PKG appears to specifically affect the animal's propensity to forage during feeding. Remarkably, a more recent study found that in honeybee colonies, the age-related transition from hive workers to foragers is associated with and dependent on an increase in the expression of *Amfor*, the honeybee *for* homolog (Ben-Shahar et al., 2002). Although these results suggest the possibility of a conserved role for PKG in insect foraging behavior, the neural mechanism for these effects is not well understood, since the sites of action for PKG's foraging-related activities in the *Drosophila* and honeybee brains have not been identified.

Two new studies (in this issue of *Neuron*) in the nematode *C. elegans* not only define the behavioral consequences of mutations in PKG genes but also elegantly address the neural and molecular basis for these phenotypes. In the first study, McIntire and colleagues define a role for PKG in mediating the effects of sensory information on development and the modulation of behavioral states (Fujiwara et al., 2002). This study originated from the authors' observation that mutant strains defective in the structure of chemosensory cilia exhibited two, seemingly unrelated abnormalities: they were unusually