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Non-Cell-Autonomous Mechanism of Activity-Dependent Neurotransmitter Switching

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SUMMARY

Activity-dependent neurotransmitter switching engages genetic programs regulating transmitter synthesis, but the mechanism by which activity is transduced is unknown. We suppressed activity in single neurons in the embryonic spinal cord to determine whether glutamate-gamma-aminobutyric acid (GABA) switching is cell autonomous. Transmitter respecification did not occur, suggesting that it is homeostatically regulated by the level of activity in surrounding neurons. Graded increase in the number of silenced neurons in cultures led to graded decrease in the number of neurons expressing GABA, supporting non-cell-autonomous transmitter switching. We found that brain-derived neurotrophic factor (BDNF) is expressed in the spinal cord during the period of transmitter respecification and that spike activity causes release of BDNF. Activation of TrkB receptors triggers a signaling cascade involving JNK-mediated activation of cJun that regulates *tlx3*, a glutamate/GABA selector gene, accounting for calcium-spike BDNF-dependent transmitter switching. Our findings identify a molecular mechanism for activity-dependent respecification of neurotransmitter phenotype in developing spinal neurons.

INTRODUCTION

Specification of neurotransmitters is a fundamental aspect of neuronal development, allowing establishment of functional connections at synapses and enabling normal operation of the nervous system. Distinct expression patterns of transmitters are initially determined by transcription factors via genetic programs (Cheng et al., 2004; Mizuguchi et al., 2006; Pillai et al., 2007) and subsequently respecified by electrical activity (Dulcis and Spitzer, 2008; Demarque and Spitzer, 2010; Marek et al., 2010). Activity-dependent transmitter respecification is a recently discovered form of brain plasticity, distinct from changes in synaptic strength and number of synapses, in which neurons acquire an additional transmitter, lose a transmitter, or switch between one transmitter and another (Spitzer, 2012). It can be driven by target-derived factors, experimental manipulations of spontaneous activity, or by natural fluctuations in sensory stimuli in both developing and mature neurons (Furshpan et al., 1976; Landis, 1976; Walicke et al., 1977; Schotzinger and Landis, 1988; Gutiérrez, 2002; Borodinsky et al., 2004; Dulcis and Spitzer, 2008; Demarque and Spitzer, 2010; Marek et al., 2010; Dulcis et al., 2013). Spontaneous Ca^{2+} spikes regulate inhibitory and excitatory transmitter phenotypes homeostatically in embryonic *Xenopus* spinal neurons. When Ca^{2+} spikes are suppressed, more neurons express the excitatory neurotransmitters glutamate and acetylcholine. In contrast, when Ca^{2+} spiking is increased, more neurons express the inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and glycine (Borodinsky et al., 2004). Here we identify the signal transduction cascade linking activity to changes in gene expression that lead to transmitter switching.

Electrical activity leads to a wide range of elevations of intracellular Ca^{2+} that could regulate expression of genes determining excitatory or inhibitory phenotype in a cell-autonomous manner. However, these transient elevations of intracellular Ca^{2+} could also regulate cellular secretion enabling inductive interactions among cells to specify neurotransmitter via a non-cell-autonomous mechanism (Spitzer, 2006). The role of cell-autonomous versus non-cell-autonomous mechanisms is often examined in purified and sparsely plated cultures (Tonge and Andrews, 2010), but is more challenging to address in vivo (Lee and Luo, 1999; Zong et al., 2005). We address this issue by developing a single-neuron targeting method in vivo. No transmitter switch was observed in single neurons in which Ca^{2+} spikes had been suppressed, indicating that a non-cell-autonomous mechanism is involved.

Brain-derived neurotrophic factor (BDNF) then became an attractive candidate to regulate activity-dependent transmitter respecification because its expression and release are regulated by neuronal activity (Balkowiec and Katz, 2002; Gärtner and Staiger, 2002; Tabuchi et al., 2000), and it has been implicated in mechanisms that optimize neuronal differentiation and neuronal plasticity (Vicario-Abejón et al., 2002; Park and Poo, 2013). Application or overexpression of BDNF promotes development of inhibition (Ohba et al., 2005), whereas decreased expression or disruption of the function of BDNF impairs development of inhibitory synapses (Hong et al., 2008; Shinoda et al., 2011). On the other hand, application of BDNF leads to suppression of excitatory synaptic transmission (Yang et al., 2002). BDNF exerts its action by binding preferentially to its tyrosine receptor kinase (TrkB) but also through its low-affinity receptor p75. Upon ligand binding, TrkB

receptor dimerization leads to *trans*-autophosphorylation and activation of intracellular signaling cascades, including MAP kinase (MAPK), PI 3-kinase (PI3K), and phospholipase-Cγ (PLCγ) pathways (Huang and Reichardt, 2003).

We demonstrate Ca^{2+} spike-dependent BDNF release, identify critical components of the molecular signaling pathway downstream of BDNF, and show that BDNF regulates glutamate/GABA switching. Thus, genetic programs and collective electrical activity that drives transmitter induction via BDNF determine the mature transmitter phenotype. Understanding the molecular basis of transmitter respecification identifies potential points of intervention for therapeutically enhancing or restoring synaptic transmission that is impaired in neurological or psychiatric disorders.

RESULTS

Suppression of Ca^{2+} Spike Activity in Single Neurons In Vivo

Ca^{2+}-dependent electrical activity in embryonic *Xenopus* spinal neurons homeostatically regulates respecification of the neurotransmitters that neurons express without affecting cell identities both in vivo and in vitro (Borodinsky et al., 2004). Misexpression of human inward rectifier K^+ channels (hKir2.1) by injection of hKir2.1 mRNA causes more neurons to express the excitatory transmitters glutamate and acetylcholine, while fewer neurons express the inhibitory transmitters GABA and glycine in the spinal cord. We developed a single-neuron targeting system to determine whether activity-dependent neurotransmitter respecification is cell autonomous in vivo. Expression from DNA constructs is mosaic and only a few cells express large amounts of transcript whereas most cells express none (Kroll and Amaya, 1996). Accordingly, blastomeres were injected with hKir2.1 DNA instead of mRNA. Although mosaic expression from DNA constructs has been regarded as a nuisance, here it becomes an asset. In addition, neuronal lineages have been determined at the 16-cell stage (Moody, 1989), enabling more specific manipulation of Ca^{2+} spike activity in particular classes of spinal neurons. We thus performed injections at this stage to further limit the number of cells expressing the DNA of interest.

We injected hKir2.1-mCherry DNA into the D1.1 or D1.2 blastomeres of the 16-cell blastula (Figure S1A available online), both of which make a major contribution to neurons in the ventral spinal cord (http://www.xenbase.org). When embryos reached the late tailbud stage (stage 41), mCherry was typically observed in several neurons separated along the spinal cord (Figure S1B), indicating the success of targeting single neurons by this method. Eighty percent were located on the ventral side of the neural tube and only 20% were located on the dorsal side of the neural tube (Figure S1C). Because V1.2 blastomeres make a major contribution to the dorsal spinal cord (http://www.xenbase.org), DNA injection of these cells predominantly targeted neurons on the dorsal side (data not shown).

Activity Blockade in Single Neurons Does Not Switch Neurotransmitters

hKir2.1 has been used to suppress neuronal excitability both in vitro (Burrone et al., 2002) and in vivo (Borodinsky et al., 2004; Mizuno et al., 2007). To determine whether misexpression of hKir2.1-mCherry in single neurons suppresses Ca^{2+} spikes, we assessed Ca^{2+} activity in these mCherry-labeled neurons by confocal imaging of Fluo-4 AM. Although neurons located on both dorsal and ventral surfaces spike in situ in *X. laevis*, those positioned on the dorsal surface spike at lower frequencies at early stages of development (Belgacem and Borodinsky, 2011; Borodinsky et al., 2004; Gu et al., 1994; Root et al., 2008). We thus imaged the intact ventral spinal cord at stage 23-25 when most classes of neurons exhibit a higher incidence and frequency of Ca^{2+} spikes (Borodinsky et al., 2004). Neurons without hKir2.1 expression in the same embryos served as internal controls (Figure 1A). Misexpression of hKir2.1-mCherry decreased both spike incidence and frequency in single neurons (Figures 1B and 1C; Figure S1D). The incidence and frequency of spiking in single neurons expressing mCherry alone were similar to those in internal controls, suggesting that mCherry has no effect on Ca^{2+} spike activity and allows hKir2.1 to effectively exert its hyperpolarizing function (Figure 1C; Figure S1D).

We then determined whether homeostatic transmitter switching occurred in the single neurons in which Ca^{2+} spike activity had been suppressed. The primary nervous system in *Xenopus* embryos is composed of ~1,000 neurons (Hartenstein, 1993). Neurons on the ventral side of the neural tube include cholinergic motor neurons, GABAergic ascending interneurons, and cholinergic/glutamatergic descending interneurons (Li et al., 2004; Roberts et al., 1987). Neurons on the dorsal side of the neural tube comprise the glutamatergic Rohon-Beard sensory neurons and GABAergic/glycinergic dorsolateral ascending interneurons (Roberts et al., 1987; Sillar and Roberts, 1988). If Ca^{2+} spikes acted cell autonomously, we expected that suppressing activity in single neurons on the ventral side of the spinal cord would cause neurons not normally glutamatergic to acquire a glutamatergic phenotype, identified by expression of vesicular glutamate transporter (vGluT1). VGluT1-, but not vGluT2- or vGluT3-immunoreactivity, colocalizes with glutamate immunoreactivity (Glu-IR), making vGluT1 a useful glutamatergic marker (Borodinsky et al., 2004). In parallel, we expected that suppressing activity in single neurons on the dorsal side of the spinal cord would cause them to lose the GABAergic phenotype, assessed by GABA-immunoreactivity (GABA-IR). Neurons were identified by position and morphology. Strikingly, the incidence of vGluT1-IR neurons among hKir2.1-mCherry-labeled ventral neurons and among mCherry-alone-labeled ventral neurons did not differ from each other. This result indicates that the glutamatergic phenotype was not acquired in single neurons in which Ca^{2+} spikes had been suppressed (Figure 1D). Moreover, no difference was observed in the incidence of GABA-IR between hKir2.1-mCherry-labeled neurons and mCherry-alone-labeled dorsal neurons, indicating that there is no decrease in the incidence of GABA-IR cells among the single neurons in which Ca^{2+} spikes have been suppressed (Figure 1E). These data suggest that the mechanism for Ca^{2+} spike activity-dependent transmitter respecification in the embryonic spinal cord is non-cell autonomous.

To further analyze network-dependent regulation of transmitter switching, we changed the ratio of silenced to unsilenced neurons in a graded manner. Because most chemical synapses