2-5-2014

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Recommended Citation
Chancey, Jessica H.; Poulsen, David J.; Wadiche, Jacques I.; and Overstreet-Wadiche, Linda, "Hilar Mossy Cells Provide the First Glutamatergic Synapses to Adult-Born Dentate Granule Cells" (2014). Biomedical and Pharmaceutical Sciences Faculty Publications. Paper 16.
http://scholarworks.umt.edu/biopharm_pubs/16

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Brief Communications

Hilar Mossy Cells Provide the First Glutamatergic Synapses to Adult-Born Dentate Granule Cells

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Adult-generated granule cells (GCs) in the dentate gyrus must establish synapses with preexisting neurons to participate in network activity. To determine the source of early glutamatergic synapses on newborn GCs in adult mice, we examined synaptic currents at the developmental stage when glutamatergic synapses are first established. We show that hilar mossy cells provide initial glutamatergic synapses as well as disynaptic GABAergic input to adult-generated dentate GCs.

Key words: adult neurogenesis; circuit; dentate gyrus; GABA; glutamate; synaptic

Introduction

Synaptic integration of adult-generated granule cells (GCs) has been well characterized as a multistage process that occurs over many weeks. Newborn GCs first receive synapses from local GABAergic interneurons in the first week after mitosis followed by glutamatergic synapses during the second and third postmitotic week (Espósito et al., 2005; Ge et al., 2006; Kumamoto et al., 2012). GABAergic innervation by interneurons that generate slow postsynaptic currents, like Ivy/Neurogliaform cells (Markwardt et al., 2011), is followed by innervation by perisomatic-projecting interneurons around the time when glutamatergic innervation is strengthening and outputs onto hilar neurons and CA3 pyramidal cells is established (Espósito et al., 2005; Faullker et al., 2008; Gu et al., 2012). Synaptic integration continues over subsequent weeks, eventually resulting in functional connectivity that is indistinguishable from GCs generated from the postnatal period (Laplagne et al., 2006). Due to the role of the earliest synaptic connections in activity-dependent maturation and survival, recent attention has focused on the cellular identity of the initial synaptic partners (Markwardt et al., 2011; Song et al., 2012; Vivar et al., 2012; Deshpande et al., 2013).

Here we identify the source of early glutamatergic synapses on adult-generated neurons. Pro-opiomelanocortin-enhanced green fluorescent protein (POMC-EGFP) reporter mice label newborn GCs at an early developmental stage when the first NMDA receptor (NMDAR)-only silent synapses are established (Chancey et al., 2013). POMC-EGFP newborn GCs are ~11–12 d postmitotic (Overstreet-Wadiche et al., 2006a), consistent with retroviral labeling studies demonstrating that glutamatergic synapse formation initiates in the second week after retroviral infection (Ge et al., 2006; Kumamoto et al., 2012). POMC-EGFP newborn GCs have dendrites mainly restricted to the inner molecular layer (IML; Overstreet-Wadiche et al., 2006a,b); thus, glutamatergic terminals could arise from (1) hilar mossy cells (MCs) that form the associational/commissural pathway located in the IML, (2) projection neurons in the medial entorhinal cortex (MEC) that comprise the medial perforant path (MPP) located in the middle molecular layer (MML) that may also impinge into the IML, or (3) mossy fibers (MFs) from mature GCs (Vivar et al., 2012). We use pharmacology and optogenetics to distinguish between these possibilities.

Materials and Methods

Adult (>8 weeks old) female hemizygous POMC-EGFP transgenic mice (Cowley et al., 2001; Overstreet et al., 2004) were maintained on a C57BL/6J background. Animal procedures followed the Guide for the Care and Use of Laboratory Animals, U.S. Public Health Service, and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Mice were housed in an enriched environment.

The pAAV-hSyn-hChR2(H134)-mCherry construct from K. Deisseroth (Stanford University, Stanford, CA) was purchased from Addgene (plasmid 26976) and packaged into recombinant adenovirus-associated virus (AAV8) with a titer of $6 \times 10^{13}$ genomic particles/ml. Approximately $1.5 \times 10^7$ HEK 293T cells were seeded in complete DMEM (Cellgro) with 10% fetal bovine serum, 1 mM MEM sodium pyruvate, 0.1 mM MEM nonessential amino acids solution, and 0.05% Penicillin-Streptomycin (5000 U/ml). After 24 h, media was changed to culture media containing 5% fetal bovine serum, and cells were transfected with three plasmids: Adeno helper plasmid (pF). AAV2 inverted terminal repeats. Plasmids were transfected using Polyfect (Qiagen). Cultures were incubated at 37°C, 5% CO2, for 72 h, and then pelleted by centrifugation and resuspended in 10 mM Tris,
Results

Pharmacological identification of IML- and MPP-evoked EPSCs

To confirm the pharmacological signature of glutamate release from MCs and MPP terminals, we first recorded EPSCs from mature GCs while stimulating in the IML or MML, respectively. MPP terminals contain Group II mGluRs that decrease release probability (Macek et al., 1996; Kilbride et al., 2001; Chiu and Castillo, 2008). Thus, the Group II mGluR agonist (2S,20R,30R)-2-[20,30-dicarboxycyclopropyl]glycine (DCG-IV; 1 μM) reduced MML-evoked EPSCs and increased the paired-pulse ratio (PPR; Fig. 1A; 69 ± 4% reduction; PPR: 0.87 ± 0.07 to 1.35 ± 0.10, p < 0.01, paired t test). IML-evoked EPSCs, however, were minimally affected by DCG-IV (21 ± 11% reduction, p < 0.001 compared with MML EPSCs, ANOVA with post hoc Bonferroni; PPR: 0.92 ± 0.11 to 0.94 ± 0.14, p = 0.45). Conversely, MC terminals contain presynaptic type I cannabinoid (CB1) receptors (Chiu and Castillo, 2008). The CB1 agonist WIN 55,212–2 (WIN; 5 μM) selectively reduced IML-evoked EPSCs compared with MML-evoked EPSCs (Fig. 1B; 61 ± 10% reduction compared with 20 ± 9%, respectively, p < 0.01), and increased the PPR of IML-evoked EPSCs (IML: 0.90 ± 0.04 to 1.36 ± 0.19, p < 0.05; MML: 0.88 ± 0.06 to 0.94 ± 0.10, p = 0.25, data not shown). Pharmacologically isolated NMDAR mediated EPSCs evoked by IML stimulation (recorded at +40 mV) were blocked by WIN to a similar extent (78 ± 3% block, n = 4, p < 0.05; data not shown). Thus, sensitivity to DCG-IV and WIN can be used to distinguish between glutamate release from MPP and MC terminals.

We next tested the pharmacological sensitivity of gluta-
mate release onto POMC-EGFP newborn GCs. Since glutama-
tergic transmission at this stage is primarily mediated by NMDARs (Chancey et al., 2013), we monitored glutamatergic release at +40 mV in response to focal stimulation near newborn GC dendrites. We detected small-amplitude NMDAR EPSCs in 50 of 92 cells (8.7 ± 1.3 pA). NMDAR EPSCs were relatively insensitive to the mGluR agonist DCG-IV (Fig. 1A; 21 ± 5% reduction, p > 0.05 compared with IML EPSCs in mature GCs) but were reduced by the CB1 agonist WIN (Fig. 1B; 61 ± 4% reduction, p > 0.05 compared with IML-evoked EPSCs in mature GCs). In addition, WIN increased the PPR from 0.78 ± 0.09 to 1.21 ± 0.19 (p < 0.05), whereas DCG-IV had no effect on PPR (0.74 ± 0.06 to 0.70 ± 0.05, p = 0.32). These results suggest that the initial glutamatergic input to adult-born GCs is from MCs. Since MF synapses from mature GCs also express Group II mGluRs (Manzoni et al., 1995;
mCherry (ChR2-mCherry) or EYFP (ChR2-EYFP) into the right hilus of POMC-EGFP mice. Many cells near the injection site were infected, including MCs, interneurons, and GCs (Fig. 2A). MCs that expressed ChR2 were identified by characteristic morphology including thorny excrescences and physiological properties including a high frequency of spontaneous EPSPs and nonaccommodating action potentials (APs) with little afterhyperpolarization (Scharfman and Myers, 2012). ChR2-expressing MCs depolarized in response to brief pulses of blue light and fired APs (Fig. 2A, inset). AP firing, but not ChR2-mediated depolarization, was blocked by TTX (data not shown).

MCs project axons along the septo-temporal axis to distal areas of the ipsilateral dentate gyrus (DG; associational pathway) and to the contralateral DG (Ribak et al., 1985; Ratzliff et al., 2004; Scharfman and Myers, 2012; commissural pathway). We found robust ChR2 expression in the hilus and IML of the contralateral (left) DG (Fig. 2B). In the presence of the GABA<sub>A</sub> receptor antagonist picrotoxin (PTX), blue light pulses (1 or 10 ms) evoked EPSCs in mature GCs (Fig. 2C). Light-evoked EPSCs were blocked by WIN to a degree similar to that of electrically evoked IML EPSCs (70 ± 6% reduction; n = 4; p > 0.05). Furthermore, light-activated NMDAR EPSCs recorded at +40 mV were blocked by WIN to a similar extent (67 ± 10% block, n = 4, p > 0.05; data not shown).

We also injected AAV-ChR2 into the entorhinal cortex to drive expression in the MPP, resulting in axonal labeling in the MML of the ipsilateral DG (Fig. 2D,E). Light-evoked EPSCs in mature GCs were sensitive to DCG-IV to an extent similar to electrically evoked MML EPSCs (Fig. 2F, 66% reduction, n = 3, p > 0.05). Thus, ChR2 can be used to selectively activate extrahippocampal and intrahippocampal excitatory inputs (Kumamoto et al., 2012).

**Mossy cells innervate newborn GCs**

To determine whether selective activation of MCs could evoke EPSCs in newborn GCs, we first found contralateral slices with sufficient MC axonal ChR2 expression to evoke light-activated EPSCs in mature GCs (as in Fig. 2B,C). Light-evoked EPSCs were smaller than electrically evoked EPSCs (Fig. 3A; 34 ± 6 pA and 128 ± 27 pA, respectively; p < 0.01, paired t test, n = 15), suggesting that not all IML fibers expressed ChR2. After testing for ChR2 expression by first recording from mature GCs, we subsequently identified neighboring newborn GCs that had electrically evoked NMDAR EPSCs (Fig. 3A, bottom). Under these conditions, we found light-activated EPSCs in 62% of newborn GCs (13 of 21 newborn GCs tested; 2351).

**Optogenetic activation of MCs and MPP**

To selectively activate MCs, we injected AAV carrying synapsin promoter-driven ChR2 tagged with either mCherry (ChR2-mCherry) or EYFP (ChR2-EYFP) into the right hilus of POMC-EGFP mice. Many cells near the injection site were infected, including MCs, interneurons, and GCs (Fig. 2A). MCs that expressed ChR2 were identified by characteristic morphology including thorny excrescences and physiological properties including a high frequency of spontaneous EPSPs and nonaccommodating action potentials (APs) with little afterhyperpolarization (Scharfman and Myers, 2012). ChR2-expressing MCs depolarized in response to brief pulses of blue light and fired APs (Fig. 2A, inset). AP firing, but not ChR2-mediated depolarization, was blocked by TTX (data not shown).

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cells), confirming MCs provide early glutamatergic inputs. Light-activated responses in newborn GCs were blocked by NBQX and AP5 (n = 3; data not shown), as well as WIN (n = 4). Unlike mature GCs, light-activated EPSCs in newborn GCs had the same amplitude as electrically evoked EPSCs (12 ± 2 pA and 15 ± 3 pA; p = 0.3, paired t test, n = 9), indicative of the largely all-or-none nature of the initial glutamatergic inputs (Chancey et al., 2013).

To determine whether MPP also contributes to glutamate release to newborn GCs, we followed the same experimental paradigm with MEC-injected mice. We first found ipsilateral slices with sufficient MPP ChR2 expression to induce light-evoked EPSCs in mature GCs (Fig. 3B; 30 ± 11 pA compared with 145 ± 20 pA for electrically evoked EPSCs; n = 14, p < 0.001). In neighboring newborn GCs that had EPSCs evoked by electrical stimulation, we never detected light-activated EPSCs (Fig. 3B, bottom; 0 of 14 cells). In addition, ChR2 expression in the lateral perforant path did not drive EPSCs in newborn GCs (n = 0 of 6; data not shown), suggesting that connectivity to the perforant path was not yet established.

**Mossy cells drive GABA release onto newborn GCs**

Although the MPP does not innervate POMC-EGFP⁺ newborn GCs, MPP stimulation can provide depolarization through feedforward GABA release (Overstreet Wadiche et al., 2005). We therefore asked whether MCs could also provide GABA release to newborn GCs. In contralateral slices from AAV-ChR2 hilar-injected mice (in the absence of PTX), we first found that light evoked both EPSCs (recorded at E_GABA = −70 mV) and IPSCs (recorded at E_Glutamate = 0 mV) in mature GCs. Consistent with MC-evoked disynaptic inhibition of GCs (Jinde et al., 2012), light-evoked IPSCs were completely blocked by either NBQX or PTX (Fig. 4A; n = 4 and 3, respectively). Neighboring newborn GCs also showed light-evoked disynaptic GABA_A receptor-mediated PSCs that were blocked by either NBQX or PTX (Fig. 4B; n = 3 and 4, respectively). In PTX, subsequent recording at +40 mV revealed monosynaptic NMDA EPSCs (Fig. 4B, bottom). Thus, MCs generate both monosynaptic glutamate and disynaptic GABA release to newborn GCs. As expected, the latency of disynaptic GABA PSCs was longer than that of monosynaptic NMDAR EPSCs (6.7 ± 1.4 ms vs 3.4 ± 0.5 ms, p < 0.05). Yet, the duration of GABA PSCs and NMDA EPSCs was similar (Fig. 4C; decay τ: 78 ± 15 vs 81 ± 11 ms, p = 0.8; n = 7 and 10), suggesting that MC-evoked GABAergic depolarization could potentially contribute to relief of the voltage-dependent Mg²⁺ block at initially silent MC synapses (Chancey et al., 2013).

**Discussion**

Hilar MCs are innervated by the perforant path, CA3 pyramidal cells, and GC MFs and, in turn, provide translaminar excitatory connections in the septo-temporal axis to ipsilateral and contralateral GCs and interneurons (Williams et al., 2007; Scharfman and Myers, 2012; Jinde et al., 2013). Our results show that MCs are poised to translate intrahippocampal and extrahippocampal activity to adult-born neurons during a critical period for survival when the first excitatory synapses are established (Chancey et al., 2013).

Consistent with our findings, Kumamoto et al. (2012) reported EPSCs arising from both MCs and perforant path axons in newly generated GCs at 14 d post-retroviral labeling.
Rabies virus–mediated trans-synaptic tracing also reveals time-dependent innervation from multiple cell types and brain areas. Vivar et al. (2012) show trans-synaptic labeling from 21-d-old GC cells in hilar MCs, local interneurons, astrocytes, and the entorhinal cortex. Surprisingly, they also found evidence for transient innervation from mature GCs and CA3 pyramidal cells. We found no functional evidence for innervation by mature GCs, but we cannot rule out a potential contribution by CA3 cells that can project to the IML (Li et al., 1994). Our results are consistent with trans-synaptic viral labeling of hilar MCs during the second postmitotic week followed by MEC labeling in the third postmitotic week (Deshpande et al., 2013). Since mechanisms of viral tracing are unclear and synaptic signaling can occur via spillover from distantly located release sites (Markwardt et al., 2009, 2011; Capogna and Pearce, 2011; Coddington et al., 2013), a combination of functional and anatomical approaches provides the clearest understanding of synaptic communication.

Our results suggest that MCs play an important role in regulating neurogenesis by providing early glutamatergic synapses to newly generated GCs. At this developmental stage, GABAergic depolarization allows NMDAR activation required for AMPA receptor incorporation at silent synapses (Chancey et al., 2013), suggesting a need for coordinated GABAergic and glutamatergic signaling. MC-evoked disynaptic GABA signaling to newborn GCs thus potentially provides the circuitry to synchronize glutamate and GABA release for activity-dependent synapse unsilencing (Chancey et al., 2013). Interestingly, selective loss of MCs impairs pattern separation (Jinde et al., 2012), a DG-associated task that has been linked to adult-born neurons (Clelland et al., 2009; Sahay et al., 2011; Nakashiba et al., 2012; Niabori et al., 2012). MC innervation of adult-generated neurons could also promote the participation of adult-generated neurons in DG network activity.

References


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