Correction

**NEUROSCIENCE.** For the article “Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain,” by Regina L. Faulkner, Mi-Hyeon Jang, Xiao-Bo Liu, Xin Duan, Kurt A. Sailor, Ju Young Kim, Shaoyu Ge, Edward G. Jones, Guo-li Ming, Hongjun Song, and Hwai-Jong Cheng, which appeared in issue 37, September 16, 2008, of *Proc Natl Acad Sci USA* (105:14157–14162; first published September 9, 2008; 10.1073/pnas.0806658105), the authors note that due to a printer’s error, the equal contributions footnotes appeared incorrectly. The corrected author line and footnotes appear below.

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Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain

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New neurons are continuously generated in restricted regions of the adult mammalian brain. Although these adult-born neurons have been shown to receive synaptic inputs, little is known about their synaptic outputs. Using retrovirus-mediated birth-dating and labeling in combination with serial section electron microscopic reconstruction, we report that mossy fiber en passant boutons of adult-born dentate granule cells form initial synaptic contacts with CA3 pyramidal cells within 2 weeks after their birth and reach morphologic maturity within 8 weeks in the adult hippocampus. Knockdown of Disrupted in Schizophrenia-1 (DISC1) in newborn granule cells leads to defects in axonal targeting and development of synaptic outputs in the adult brain. Together with previous reports of synaptic inputs, these results demonstrate that adult-born neurons are fully integrated into the existing neuronal circuitry. Our results also indicate a role for DISC1 in presynaptic development and may have implications for the etiology of schizophrenia and related mental disorders.

Results

Axonal Targeting of Newborn DGCs in the Adult Hippocampus. An oncoretrovirus-mediated approach was used to express GFP for birth-dating and genetic labeling of newborn DGCs in the adult mouse hippocampus (see Methods) (5, 7, 8, 15). We first characterized the axonal targeting of GFP-expressing (GFP+) DGCs with confocal microscopy. Consistent with early findings (8, 13, 14), GFP+ axons reached the stratum lucidum of CA3 within 1 week postinjection (wpi; Fig. 1), although these axons were restricted to proximal CA3 and were relatively few in number. By 1.5 wpi, GFP+ axons were at the curve of CA3, and en passant expansions began to form (data not shown). GFP+ axons did not extend beyond the CA3 border to other subfields at later stages (Fig. 1B). Thus, mossy fibers of adult-born DGCs seem to extend along the same trajectory as preexisting, mature mossy fibers. The presence of bouton-like expansions, which grew visibly larger over time (Fig. 1A), suggests the formation of synaptic complexes by new neurons.

Development of Mossy Fiber Boutons by Newborn DGCs in the Adult Brain. To further characterize synaptic outputs, we performed quantitative immuno-EM with a focus on the large boutons that form synaptic complexes with the apical dendrites of CA3 pyramidal cells (see Methods). We focused our analysis on these boutons because they are one of the major stops for information transfer through the hippocampal trisynaptic circuit. In the hilus and CA3 regions, mossy fibers also target interneurons (28). However, the DGCs with reduced DISC1 expression in the adult hippocampus exhibit neuronal positioning defects and accelerated development of dendrites and formation of synaptic inputs (15). Although a number of in vitro studies have implicated DISC1 in regulating neurite outgrowth of primary neurons and PC12 cells (26, 27), nothing is known about DISC1 function in axonal development, targeting, and presynaptic differentiation in vivo. In this study, we use retrovirus-mediated expression of an shRNA against mouse disc1 (15) to knock down DISC1 expression in newborn DGCs in the adult hippocampus. We here show that knockdown of DISC1 in newborn DGCs results in mistargeting of mossy fibers and accelerated formation of synaptic outputs, pointing to an important role of DISC1 in regulating the development of axons and synaptic outputs of newborn neurons in the adult brain.
mosaic boutons that contact interneurons are generally small in size and contain one synapse. In contrast, the synaptic complexes analyzed in this study are composed of a large presynaptic mossy fiber bouton and postsynaptic dendritic excrescence from a CA3 pyramidal cell with multiple synaptic contacts forming between the two (16, 17, 29). Given the small percentage of new neurons among all of the existing mature DGCs in the adult hippocampus, unlabeled mossy fiber boutons adjacent to GFP⁺ boutons were analyzed and used as a control for mature bouton structure.

The changes in bouton structure over development were assessed according to a classic staging paradigm (16, 17) (see Methods). Briefly, stage 1 boutons form either no or few synaptic contacts exclusively onto dendritic shafts (nonsynaptic dendritic contacts), stage 2 boutons have synaptic contacts onto immature dendritic spines (finger-like structures without discernible organelles), and stage 3 boutons have synaptic contacts onto mature dendritic spines containing organelles, with stage 3a boutons having fewer synaptic contacts onto each dendritic spine (<2 synaptic contacts per spine) than stage 3b boutons (>2 synaptic contacts per spine).

The unlabeled control boutons exhibited characteristics similar to mature mossy fiber boutons reported previously (16) (Fig. 2D). All of these boutons were assigned to stage 3, with the majority being of the most mature stage of development, stage 3b (Fig. 2F). We next analyzed GFP⁺ boutons across development. All 1.5-wpi and 2-wpi boutons were immature stage 1 boutons with no invading dendritic spines (Fig. 2F). There were no synaptic contacts in 1.5-wpi boutons, and all synaptic contacts at 2 wpi were formed with dendritic shafts (Fig. 2F and data not shown). By 4 wpi, all boutons were beyond this initial stage of development, and approximately one third of boutons fell into each of the remaining stages (2, 3a, and 3b) (Fig. 2F). These boutons contained invading dendritic spines that varied in maturity level, and all had synaptic contacts onto them (Fig. 2B). At the time points thereafter, boutons continued to mature and increasingly contained mature dendritic spines with a number of synaptic contacts (Fig. 2C and D). At both 8 and 16 wpi, the morphologic maturity of GFP⁺ boutons was similar to the mature control boutons that surround them (Fig. 2F).

We further quantified the development of the adult-born boutons with several morphologic features that have been used in previous studies (16, 17) [see Methods; all morphologic data are summarized in supporting information (SI) Table S1]. To focus on the synaptic outputs of adult-born mossy fibers, we excluded nonsynaptic puncta adherentia in this analysis (see Methods). At 1.5 wpi, the GFP⁺ boutons were simple in shape, with no visible mitochondria. They were commonly located apposed to dendritic shafts but did not form any synaptic contacts (n = 4 boutons; Fig. 3 and data not shown). At 2 wpi, the GFP⁺ boutons were still simple in structure, but they were larger and began to contain mitochondria. Fifty percent of these boutons started to form synaptic contacts, which were exclusively with the dendritic shafts of CA3 pyramidal cells (n = 8 boutons; Figs. 2A and 3). Thus, new DGCs establish nonsynaptic synaptic outputs as early as 2 weeks after they are born in the adult brain.

At 4 wpi, the GFP⁺ boutons had reached a mature size and had a mature number of invading dendritic spines when compared with control boutons (n = 8 boutons; Figs. 2B and 3A and B). However, even though 80% of their synaptic contacts were localized to dendritic spines, the number of spinous synaptic contacts was significantly smaller than control boutons, indicating that the 4-wpi boutons had not yet reached morphologic maturity (Fig. 3C and D). At 8 wpi (n = 8 boutons) and 16 wpi (n = 6 boutons), GFP⁺ boutons were similar to mature control boutons across all parameters (Figs. 2C and D). Thus, results from both staging and quantitative analyses indicate that by 8 weeks the axons of new DGCs have developed morphologically mature synaptic outputs onto CA3 pyramidal neurons.
Axonal Targeting of Newborn DGCs with DISC1 Knockdown in the Adult Brain. We have recently shown that DISC1 is required for proper dendritic development and formation of synaptic inputs onto newborn DGCs in the adult brain using retrovirus-mediated expression of an shRNA against mouse disc1 (shRNA-D1) (15). Lentivirus-mediated expression of shRNA-D1, but not control lentivirus, led to a significant reduction in the expression of the endogenous full-length DISC1 in primary hippocampal neurons, as shown by Western blot (Fig. 4B). To investigate whether DISC1 is also required for the development of mossy fiber outputs, we used retrovirus-mediated expression of shRNA-D1 to examine axonal targeting and bouton development. A control shRNA against DsRed (shRNA-C1) was used for comparison (15).

We first examined the growth of adult-born mossy fibers with DISC1 knockdown under the fluorescent microscope. At 1 wpi, the axons with DISC1 knockdown were significantly longer than control axons and had begun to form bouton-like expansions in CA3 (Fig. 4C and data not shown). At 1.5 wpi and all later time points, these axons extended beyond the CA3 border and projected into the CA1 subfield (Fig. 4A and data not shown), which was never observed in control axons. In addition, the axons with DISC1 knockdown seemed to be less tightly restricted to the stratum lucidum of CA3 (Fig. 4A). Thus, DISC1 is required for proper axonal targeting of newborn neurons in the adult brain. It is interesting to note that the aberrant axons with DISC1 knockdown found in CA1 did not form discernible bouton-like expansions until 2 wpi. We characterized these aberrant 2-wpi boutons in more detail using immuno-EM (n = 3 boutons; Fig. 5A’ and found that they made no clear synaptic contacts and were apparently less developed than the 2-wpi boutons with DISC1 knockdown localized within CA3 (Fig. 5A). These observations may imply that, while DISC1 is required for proper axonal targeting of adult-born neurons, its knockdown does not result in the formation of synaptic outputs by aberrant axons with incorrect targets.

Development of Mossy Fiber Boutons by Newborn DGCs with DISC1 Knockdown in the Adult Brain. We used immuno-EM to analyze the development of the adult-born mossy fiber boutons with DISC1 knockdown that were appropriately localized to the stratum lucidum of CA3. The staging paradigm revealed that the development of boutons with DISC1 knockdown was accelerated compared with WT boutons. At both 1 and 1.5 wpi, the boutons with DISC1 knockdown were at stage 1 (Fig. 5E and data not shown). By 2 wpi, 80% of the boutons with knockdown had developed into stage 2, 3a, or 3b (Fig. 5E), which was significantly accelerated when compared with 2-wpi WT boutons (Fig. 2F). At 4 wpi, 50% of the boutons with knockdown were mature stage 3b boutons (Fig. 5E), which was almost identical to the mature 8-wpi WT boutons (Fig. 2F). At 8 and 16 wpi, there was no further increase in the number of stage 3b boutons with DISC1 knockdown, even though a larger percentage of boutons with knockdown came to stage 3a. This suggests that, although DISC1 knockdown accelerates the overall maturation process of adult-born boutons, some of these boutons may not reach the most mature stage of development.

We then performed quantitative analysis on the boutons with DISC1 knockdown. At 1 wpi, boutons were commonly apposed to dendritic shafts and had begun to form synaptic contacts (n = 1 of 5 boutons; data not shown). By 1.5 wpi, the percentage of boutons with knockdown that had synaptic contacts on apposed dendritic shafts had increased to 71% (n = 7 boutons; data not shown). In addition, 29% of boutons had synaptic contacts onto abutting
dendritic spines that had yet to invade the mossy fiber boutons (Fig. 6C). Compared with WT boutons, which did not form synaptic contacts until 2 wpi, these results indicate that boutons with DISC1 knockdown form synaptic contacts 4–7 days earlier than WT boutons. At 2 wpi, boutons with DISC1 knockdown already had several invading spines with synaptic contacts, although they were similar in size to 2-wpi WT boutons, which all lacked invading dendritic spines (n = 7 boutons; Figs. 5A and 6). By 4 wpi, the boutons with knockdown were comparable in all respects to mature control boutons (n = 4 boutons; Figs. 5B and 6). Thus, knockdown of DISC1 leads to accelerated synaptic integration and precocious maturation of mossy fiber boutons by newborn DGCs in the adult brain. In accordance with this, we previously found that the formation of synaptic inputs onto the dendrites of adult-born DGCs with DISC1 knockdown was also accelerated (15).

Finally, we quantified the shRNA-D1/GFP− boutons at 8 and 16 wpi to explore the effects of DISC1 knockdown after these boutons reached maturity (Fig. 5C and D). Although the average size of boutons with DISC1 knockdown at 8 wpi was the same as control boutons (n = 11 boutons; Fig. 6A), they had significantly fewer spinous synaptic contacts than control boutons (Fig. 6C). However, when we quantified boutons with DISC1 knockdown at 16 wpi (n = 10 boutons; Fig. 5D), they were very similar to control boutons in all respects, even though more stage 3a boutons were noted in boutons with knockdown (Fig. 5E). Taken together, these findings demonstrate that DISC1 knockdown accelerates the development of adult-born mossy fiber boutons, and implies that at least some of the boutons are unable to fully develop without DISC1.

Discussion

Adult neurogenesis poses a unique challenge to new neurons in that they have to incorporate into a fully functional and active circuit. Integration of a new neuron into an existing neural circuit requires proper development of synaptic inputs onto its dendrites and synaptic outputs from its axon. Together with recent findings on dendritic synaptic inputs (8, 9, 15), our study demonstrates that new neurons in the adult hippocampus become fully incorporated into the existing neuronal circuit through a coordinated maturation process of their synaptic inputs and outputs. Our results also indicate that DISC1 is a key player in regulating the maturation rate of newborn neurons in the adult brain in vivo.

Our snapshot ultrastructural analysis of the time course of mossy fiber bouton development suggests the following model (Fig. S1): (i) axons of new DGCs reach the CA3 subfield by 1 to 2 weeks; (ii)
development of adult-born DGC mossy fibers is examined, we find here that DISC1 knockdown accelerates the development of neuronal development in the adult brain. In agreement with this observation, we have previously characterized both presynaptic differentiation occurs and initial synaptic contacts are formed on dendritic shafts by 2 weeks; (iii) mossy fiber boutons grow in size, spine invasion occurs, and spinous synaptic contacts are formed between 2 and 4 weeks; (iv) mossy fiber boutons reach a mature size and contain a mature number of invading dendritic spines at 4 weeks while the number of synaptic contacts within each bouton continues to increase; (v) mossy fiber boutons reach morphologic maturity by 8 weeks and remain stable at 16 wpi. Such a time course is very similar to what has been reported for synaptic inputs onto the dendrites of adult-born DGCs (4, 8–10). In the developing rodent brain, mossy fiber boutons reach maturity by the beginning of the third postnatal week, when the oldest granule cells in the hippocampus would be ∼4 weeks of age (16). In contrast, the synaptic inputs and outputs of adult-born DGCs are mature by 8 weeks and not 4 weeks as in the neonatal brain. This prolonged time-course of synaptic integration sets a time constraint to when newborn neurons can contribute to neuronal circuitry in the adult brain.

The detailed molecular mechanisms that regulate the synaptic integration of new neurons in the adult brain remain to be determined (12). DISC1 regulates several aspects of synaptic integration of newborn neurons (15). Our previous in vivo analysis showed that DISC1 knockdown in adult-born DGCs accelerates the development of dendrites, synaptic inputs, and intrinsic neuronal excitability (15) and suggests that DISC1 serves as a regulator of the temporal constraints to when newborn neurons can contribute to neuronal circuitry in the adult brain. In agreement with this view, we find here that DISC1 knockdown accelerates the development of adult-born DGC mossy fibers in vivo (Fig. S1). Axons with DISC1 knockdown exhibit accelerated presynaptic differentiation: initial synaptic contacts are formed by 1 week, synaptic contacts onto dendritic spines are formed by 1.5 weeks, and spine invasion into the bouton occurs by 2 weeks. Thus, synaptic formation occurs 4–7 days earlier in boutons with DISC1 knockdown than in WT boutons, and the boutons with DISC1 knockdown precociously reach morphologic maturity by 4 weeks.

We also unexpectedly find that DISC1 regulates axonal targeting of newborn neurons in the adult brain. Adult-born mossy fibers with DISC1 knockdown tend to grow outside the stratum lucidum of CA3 and inappropriately overshoot into the CA1 subfield (8). Our previous work has shown that DISC1 knockdown has no apparent effect on the targeting of adult-born DGC dendrites (15). Together, this new finding may suggest a differential role for DISC1 in regulating development of axons and dendrites of newborn neurons in the adult brain. However, DISC1 has been shown to play a role in the dendritic orientation of embryonic cortical neurons in vivo (23), and in vitro experiments have shown that DISC1 knockdown in embryonic hippocampal neurons results in a decrease in axonal outgrowth (26, 27). It is therefore likely that the function of DISC1 is diverse and is highly dependent on the context.

After becoming morphologically mature, the adult-born mossy fiber boutons with DISC1 knockdown tend to have fewer total spinous synaptic contacts at 8 wpi. However, this difference is not apparent in boutons with DISC1 knockdown at 16 wpi. Although additional experiments are needed, these observations suggest that DISC1 may regulate the maturation or stability of these boutons. It is interesting to note that a recent EM analysis of mossy fiber boutons from postmortem schizophrenic brains revealed that, although mossy fiber boutons from schizophrenics are the same size as in controls, the boutons in schizophrenic brains reduce in number in both spine and synaptic contact number (50, 51), suggesting that destabilization of the mossy fiber boutons may be important for the pathology of schizophrenia in general.

In summary, the results described here identify new roles for DISC1 in the axonal development of adult-born neurons and suggest that DISC1 may regulate the maturation or stability of these boutons. It is therefore likely that the function of DISC1 is diverse and is highly dependent on the context.

**Methods**

**Construction, Production, and Stereotaxic Injection of Engineered Retroviruses.** Engineered self-inactivating murine onc-retroviruses were used to express EGFP or coexpress GFP and shRNA specifically in proliferating cells and their progeny (5, 13, 14). As described previously (15), shRNA expression results in the complete inhibition of GluR2 mRNA and protein in primary hippocampal cultures at 2 days in vitro, which was then analyzed for endogenous DISC1 expression at 9 days in vitro by Western blot using anti-DISC1 antibody (goat, Santa Cruz Biotechnology, N-16). High titers of engineered retroviruses (1 × 10^7 U/ml) were produced as previously described (15). Adult (7- to 8-week-old) female C57BL/6 mice (Charles River) housed under standard conditions were anesthetized, and retroviruses were stereotaxically injected at 4 sites (0.5 μl per site at 0.25 mm with the 2.5 mm coordinates (millimeters), as previously described (15): anteroposterior, −2; lateral, ±1.6; ventral, 2.5; and anteroposterior, −3; lateral, ±2.6; ventral, 3. All animal procedures were in accordance with institutional guidelines.

**Immunostaining, Confocal Imaging, and Electron Microscopy.** For confocal analysis, horizontal or coronal brain sections (40 μm) were prepared from injected mice and processed for immunostaining for GFP (rabbit, 1:1,000–2,000, Invitrogen, Carlsbad, CA) and DAB reaction. Sections were slightly permeabilized, allowing the specific labeling of newborn granule cells (32). GFP and shRNA were coexpressed from the same viral vector under the control of the EF1α and U6 promoter, respectively (15). The following short-hairpin sequences were used: GGCTACATGAGAAGCAG (shRNA-D1) and ATGTTCACTGAGGCTC (shRNA-C1). The specificity and efficiency of these shRNAs have been previously characterized both in vitro and in vivo (15). To further confirm the efficacy, untransduced shRNA-D1/GFP or GFP alone was used to infect primary hippocampal cultures at 2 days in vitro, which was then analyzed for endogenous DISC1 expression at 9 days in vitro by Western blot using anti-DISC1 antibody (goat, Santa Cruz Biotechnology, N-16). High titers of engineered retroviruses (1 × 10^7 U/ml) were produced as previously described (15). Adult (7- to 8-week-old) female C57BL/6 mice (Charles River) housed under standard conditions were anesthetized, and retroviruses were stereotaxically injected at 4 sites (0.5 μl per site at 0.25 mm with the 2.5 mm coordinates (millimeters), as previously described (15): anteroposterior, −2; lateral, ±1.6; ventral, 2.5; and anteroposterior, −3; lateral, ±2.6; ventral, 3. All animal procedures were in accordance with institutional guidelines.
enhance the signal. Sections were imaged with confocal microscopy at 1-μm intervals, and projection images were used for analysis of axonal growth as previously described (8). Measurements were carried out in a blind fashion to the genetic manipulation. Data are presented in a distribution plot, and statistical significance was assessed using the Kolmogorov–Smirnov test as previously described (15).

For EM, all boutons analyzed were from serial ultrathin section reconstructions. In selecting boutons of newborn neurons for analysis, we preferentially selected boutons that were visible at the light level, because large boutons tend to exhibit more mature characteristics (16) and our aim was to describe the structure of the most mature boutons at each developmental time point. This selection was done consistently across experimental groups. Given the small percentage of new neurons among all DGCs in the adult hippocampus, the unlabeled boutons adjacent to GFP+ boutons were likely from mature DGCs. Thus, we analyzed neighboring unlabeled mature boutons as controls. These unlabeled boutons were all of similar size and complexity and we did not preferentially select the largest ones.

Reconstructions consisted of anywhere from 3 to 41 serial ultrathin sections and from −13 serial sections on average. The perimeter of each bouton was measured from the serial section in which the bouton was largest using Image J (National Institutes of Health). Because the serial sections from analyzed boutons did not overlap, reconstructions and the number of serial sections in reconstructions varied, synaptic contact and spine densities are reported normalized for reconstruction size (unit volume). We calculated the volume for each reconstructed bouton by multiplying bouton perimeter by bouton thickness (the number of serial sections in a reconstruction multiplied by the thickness of each serial section [70 nm]). Synaptic contact and spine densities were normalized for this calculated bouton volume. Synaptic contacts were defined by a postsynaptic density of 80 nm or less and synaptic contacts localized only to the dendritic shaft and had no spines or one spine that lacked organelles. Boutons classified as stage 2 contained immature invading dendritic spines that lacked organelles, and these dendritic spines had synaptic contacts onto them. Boutons classified as stage 3 contained invading dendritic spines with organelles and synaptic contacts. The second modification to bouton staging in our study was as follows. We found that stage 3 boutons could be further divided into two subcategories according to the number of synaptic contacts per contact. When boutons reached stage 3, the parameters of the bouton remained relatively constant except for an apparent increase in the number of synaptic contacts per mature bouton. Moreover, there was an average of just over two synaptic contacts per spine, which is consistent with a previous report (16). We divided stage 3 boutons into stages 3a and 3b using this value of two synaptic contacts per spine to reflect this developmental change. Stage 3a boutons had fewer than two synaptic contacts per spine, and stage 3b boutons had two or more synaptic contacts per spine.

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