High-Frequency Stimulation Induces Gradual Immediate Early Gene Expression in Maturing Adult-Generated Hippocampal Granule Cells

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Increasing evidence shows that adult neurogenesis of hippocampal granule cells is advantageous for learning and memory. We examined at which stage of structural maturation and age new granule cells can be activated by strong synaptic stimulation. High-frequency stimulation of the perforant pathway in urethane-anesthetized rats elicited expression of the immediate early genes c-fos, Arc, zif268 and pCREB133 in almost 100% of mature, calbindin-positive granule cells. In contrast, it failed to induce immediate early gene expression in immature doublecortin-positive granule cells. Furthermore, doublecortin-positive neurons did not react with c-fos or Arc expression to mild theta-burst stimulation or novel environment exposure. Endogenous expression of pCREB133 was increasingly present in young cells with more elaborated dendrites, revealing a close correlation to structural maturation. Labeling with bromodeoxyuridine revealed cell age dependence of stimulation-induced c-fos, Arc and zif268 expression, with only a few cells reacting at 21 days, but with up to 75% of cells activated at 35–77 days of cell age. Our results indicate an increasing synaptic integration of maturing granule cells, starting at 21 days of cell age, but suggest a lack of ability to respond to activation with synaptic potentiation on the transcriptional level as long as immature cells express doublecortin.

Keywords: adult neurogenesis, Arc, BrdU, c-fos, LTP, pCREB, rat, zif268

Introduction

New hippocampal granule cells (GCs) are continuously generated from neural stem and progenitor cells throughout life in the mammalian dentate gyrus (Altman and Das 1965; Gage 2010). Neurogenesis within the adult hippocampus contributes to learning and memory, although the precise function is only beginning to be understood (Drapeau and Abrous 2008; Zhao et al. 2008). Increasing evidence indicates that newly generated GCs play a crucial role in spatial and episodic memory, particularly in pattern separation (Clelland et al. 2009; Alme et al. 2010; Nakashiba et al. 2012). Furthermore, suppression of granule cell neurogenesis in mice leads to deficits in various learning tasks that are related to hippocampus-specific forms of spatial memory (Clelland et al. 2009; Jessberger et al. 2009).

The survival of maturing GCs depends on their functional integration into the existing network and can be increased by behavioral stimuli, such as exposure to an enriched environment (Van Praag et al. 1999; Tashiro et al. 2007), hippocampus-dependent learning paradigms (Gould et al. 1999; Dupret et al. 2007; Kee et al. 2007), and acute stimulation of the entorhinal cortex (Stone et al. 2011). About 50% of postmitotic GCs in the adult dentate gyrus die within the first 4 weeks after their birth (Dayer et al. 2003; Snyder et al. 2009). Surviving GCs become synaptically integrated into the neuronal network by spreading axons with synapses to hilar neurons and CA3 pyramidal cells, as well as extending dendrites with spines into the molecular layer, and receiving excitatory input from perforant path afferents (Van Praag et al. 2002; Espósito et al. 2005; Kee et al. 2007; Zhao et al. 2007; Toni et al. 2008). However, it has remained an open issue at which stage of structural maturation newly generated GCs become functionally integrated, and at which time-point new neurons start to contribute to hippocampal learning and memory (Snyder et al. 2009, 2012). Whereas facilitated induction of associative long-term potentiation has been demonstrated for young maturing GCs (Schmidt-Hieber et al. 2004; Ge et al. 2007), new GCs are increasingly likely to be incorporated into circuits supporting spatial memory at a more mature state of 6–12 weeks (Kee et al. 2007).

There is ample evidence that induction of effective synaptic plasticity associated with learning and memory requires de novo transcription in the cell nucleus (Sanes and Lichtman 1999). This process is initiated by immediate early genes (IEGs), which serve as reliable markers for synaptic activity and plasticity (Jones et al. 2001; Fleischmann et al. 2003; Reymann and Frey 2007; Bramham et al. 2010; Chen et al. 2010). Thus, it should be expected that newborn GCs respond to afferent stimulation with IEG expression during their critical period of dendritic arborization, spinogenesis, and enhanced synaptic excitability and plasticity (Schmidt-Hieber et al. 2004; Espósito et al. 2005; Ge et al. 2007; Zhao et al. 2008; Häussler et al. 2012). However, previous studies using behavioral tests or electrical stimulation with LTP induction protocols have failed to induce IEGs in immature GCs (Kuipers et al. 2009; Sandoval et al. 2011). Here, we performed extensive high-frequency stimulation (HFS) of the perforant path in adult rats under in vivo conditions (Steward et al. 1998) in order to induce maximal expression of IEGs in hippocampal GCs as a precondition to analyze the potential transcriptional response in maturing GCs.

Importantly, we found that young GCs are only synaptically activated on the transcriptional level after the phase of doublecortin (DCX) expression. Thereafter they become gradually activated with increasing age, but do not reach the activation state of the mature population.

Materials and Methods

Animals

Adult male Sprague Dawley rats (8–13 weeks, 220–450 g; Charles River, Sulzfeld, Germany) were housed under standard laboratory conditions in large rat cages (30 cm x 40 cm) with selected objects to assemble an enriched environment. All animal experiments were performed in conformance with German guidelines for the use of laboratory animals.
BrdU Injections

To analyze newborn cells of a specific age (7, 14, 21, 28, 35, 49, 63, and 77 days), 5-bromo-2′-deoxyuridine (BrdU, AppliChem) was injected intraperitoneally (i.p.) at 200 mg/kg body weight at a single time point.

Surgical Procedures, Perforant Path Stimulation

All surgical procedures were performed under deep urethane anesthesia (1.25 g/kg body weight s.c. initially and additional injections as needed, 250 mg urethane/ml 0.9% saline), in agreement with the German law on the use of laboratory animals. Surgery and stimulation procedures were performed as previously described (Schwarzacher et al. 2006; Jedlicka et al. 2009). In short, animals were placed in a Kopf stereotaxic device (Kopf instruments, Tujunga, CA, USA.). Rectal temperature was maintained at 37.0 ± 0.5 °C. Two small holes (1.5–2.0 mm diameter) were drilled in the skull and a bipolar stainless steel-stimulating electrode (NE-200, Rhodes Medical, Woodland Hills, CA, USA.) was placed in the angular bundle of the perforant path (coordinates from lambda: L: 4.5 mm; AP: +0.5 mm; V: –3.5 mm measured from the surface of the brain). Glass microelectrodes (1.5 mm outer diameter) were pulled on a Zeitz (München, Germany) electrode puller, filled with 0.9% saline, and placed in the dorsal blade of the granule cell layer (coordinates from bregma: L: 2.0 mm, AP: –3.5 mm, V: –3.5 mm). The vertical tip position was optimized under perforant path control stimulation using the characteristic shape of the evoked potentials.

HFS was used to maximally evoke population spikes and induce robust long-term potentiation (LTP) as has been described in detail (Steward et al. 1998). HFS was applied for 30 min or for 2 h. One HFS train consisted of 8 pulses (500 µA, 0.1-ms pulse duration) of 400 Hz once per 10 s. A baseline fEPSP slope was calculated from the average of responses over the 10 min prior to the theta-burst stimulation (TBS). Baseline stimulus intensity was set to evoke a population spike of ∼1 mV before LTP induction. The potentiation of the fEPSP slope was expressed as percentage change relative to baseline.

TBS for LTP induction consisted of 6 series of 6 trains of stimuli at 400 Hz, 200 ms between trains, 20 s between series (Jones et al. 2001). Pulse width and stimulus intensity was doubled during the TBS in comparison to baseline recordings.

In sham-operated rats, stimulation and recording electrodes were placed with the aid of small voltage stimulation pulses and left in place for 2 h without stimulation. All animals were perfused 2 h after the start of stimulation or sham procedure.

Open-Field Exposure

For novelty-induced activation, rats were conditioned to fearless handling several days in advance. Rats were exposed to a novel environment in a large box (70 cm × 40 cm) for 1 h and were then transferred back into the cage and perfused after 2 h.

Immunohistochemistry

Rats were deeply anesthetized with an overdose of pentobarbital (300 mg/kg body weight) and transcardially perfused with a 0.1 M PBS. Serial frontal sections of the hippocampus (50 µm) were cut with a vibratome, washed in PBS, and stored 4°C for 3 days in 4% paraformaldehyde in 0.1 M PBS. The following antibodies were used: Anti-Arc (rabbit, polyclonal, 1:1000, Synaptic Systems, Ctl. 156-0020) or mouse, monoclonal, 1:200, Dako, Ctl. M0744) for 24 h at room temperature. Fluorescence-immunohistological detection of BrdU was performed with secondary fluorescence-labeled antibodies (1:1000; Alexa 488 and 568; Vector Labs., Burlingame, CA, USA.) for 24 h at room temperature.

Following immunohistological staining of Arc, CB, c-fos, DCX or zif268, some sections were further processed for BrdU detection. Free-floating sections were incubated in 2 N HCl at 37 °C for 30 min and 0.1 M boracic acid (pH 8.5) at room temperature for 10 min, followed by incubation with anti-BrdU (rat, monoclonal, 1:200; Abcam, Ctl. 6326–250 or mouse, monoclonal, 1:200, Dako, Ctl. M0744) for 24 h at room temperature. Fluorescence-immunohistological detection of BrdU was performed with secondary fluorescence-labeled antibodies (1:1000; Alexa 488 and 568; Vector Labs., Burlingame, CA, USA.) for 24 h at room temperature. For staining of cell nuclei TO-PRO-3 (Invitrogen, Ctl. T3605) was used at a concentration of 1:30 000 at room temperature for 10 min.

Results

Mature Dentate Granule Cells Express c-fos and Arc Following High-Frequency Stimulation of the Perforant Path

In order to maximally stimulate GCS, we applied a strong HFS paradigm developed by Steward et al. (1998). Two-hour HFS induced a robust LTP of the slope and population spike (data
Following 2-h HFS, expression of a marker of synaptic activity, c-fos, and a marker of synaptic plasticity, activity-related cytoprotein (Arc), was significantly enhanced in the great majority of calbindin (CB)+-GCs in the ipsilateral dentate gyrus (DG) (97.57 ± 1.72%, n = 27, for c-fos; 95.57 ± 4.42%, n = 29, for Arc; Fig. 1A–D, G, and H) compared with control animals, in which c-fos and Arc expression was restricted to a few GCs, all of them being CB+ (0.38 ± 0.11%)

Figure 1. Expression of c-fos and Arc in hippocampal granule cells (GCs) following 2-h high-frequency stimulation (HFS) of the perforant path. HFS induced the expression of markers for synaptic activity, c-fos (A), and synaptic plasticity, Arc (B), in the ipsilateral (ipsi) granule cell layer (GCL) of the dentate gyrus (DG). Colabeling for calbindin (CB), a marker of mature GCs, revealed that most CB+ cells were c-fos (C and G) and Arc (D and H) positive following 2-h HFS. Newly born GCs labeled with doublecortin (DCX) did not exhibit c-fos (E and F) nor Arc (F and H), neither in the control, nor ipsi- or contralateral (contra) sides following 2-h HFS. (G) c-fos/CB 2-h HFS ipsi n = 27, contra n = 3, control n = 10; c-fos/DCX, 2-h HFS ipsi n = 3, contra n = 3, control n = 3. (H) Arc/CB 2-h HFS ipsi n = 29, contra n = 6, control n = 8; Arc/DCX 2-h HFS ipsi n = 6, contra n = 6, control n = 3. Scale bars: (A and B) 100 µm; (C–F) 10 µm. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.
for c-fos, 1.03 ± 0.1% for Arc Fig. 1C,D and G,H). A similar ipsilateral activation of GCs was observed following 30 min of HFS (data not shown). In the hippocampus, both, c-fos and Arc stainings were restricted to the ipsilateral DG, indicating a selective synaptic activation of ipsilateral GCs. In the contralateral DG, no increase of c-fos or Arc expression over control levels could be detected after HFS. Triple stainings with CB, the neuronal marker NeuN and the cell nuclei marker TO-PRO-3 revealed a co-expression of NeuN in 100 ± 0.0% of CB+ cells (n = 3), and a co-expression of CB in 95.61 ± 0.74% of NeuN+ cells (n = 3), indicating an almost complete CB staining of mature GCs (Supplementary Fig. S1A,B). TO-PRO3-staining served for delineation of the granule cell layer (GCL). CB−/NeuN+ cells of the GCL likely represented immature GCs (e.g., DCX+ cells, see later) and interneurons that are sparsely located within the GCL (Woodson et al. 1989).

**Immature Dentate Granule Cells do not Express c-fos and Arc Following High-Frequency Stimulation of the Perforant Path**

Next, we analyzed if immature GCs could be activated by HFS. A variety of chemical markers can be used to label the different maturation stages of adult newborn GCs (Zhao et al. 2008; von Bohlen Und Halbach 2011). Pre- and young postmitotic maturing GCs of neuronal fate can be labeled in the adult DG with doublecortin (DCX) and polysialic acid neural cell adhesion molecule (PSA-NCAM) (Zhao et al. 2008). Both markers produced robust somato-dendritic staining. 100% of PSA-NCAM cells were co-stained with DCX, whereas some additional cells were only DCX+ (7.49 ± 2.03% of all DCX+ cells, n = 3, Supplementary Fig. S1C,D). Therefore, DCX was preferred for labeling of immature GCs in this study. Whereas the majority of DCX+GCs were CB−, a small subpopulation of DCX+GCs showed co-staining with CB (7.43 ± 2.42%, n = 3, data not shown, for a cell age-related analysis, see later). Surprisingly, and in contrast to DCX−/CB+ cells, DCX+ cells did in no case exhibit immunoreactivity for c-fos and/or Arc following 2-h HFS (Fig. 1E–H). Thus, young maturing DCX+ cells appeared not to be responsive for synaptic activation via HFS.

**Young Maturing Adult Newborn Granule Cells Cannot Be Activated by Mild Stimulation or Novel Environment Exposure**

As the HFS protocol represents a massive stimulation that could possibly block physiological responses in immature neurons, we also performed a mild perforant path TBS for LTP induction with a total of 216 pulses (Jones et al. 2001) that elicited c-fos and Arc immunoreactivity in ipsilateral mature CB+GCs, but failed to activate any DCX+ cells (data not shown). In addition, we conducted an open-field exposure to novel environment in awake animals which elicited c-fos and Arc expression in a subfraction of mature CB+GCs (Fig. 2B,D), but again, neither c-fos nor Arc labeling was detected in any DCX+cell. In summary, unlike mature CB+GCs, young maturing DCX+GCs did not react with

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**Figure 2.** C-fos and Arc expression is not induced in young maturing GCs following novel environment exposure. Exposure to novel environment increased the number of c-fos⁺ [A and C] and Arc⁺ [B and D] mature calbindin (CB)⁺-GCs in awake rats, but failed to elicit any c-fos or Arc immunoreactivity in young maturing doublecortin (DCX)⁺-GCs of the subgranular zone (SGZ) and granule cell layer (GCL). [C] c-fos/CB novelty n = 4, control n = 7; c-fos/DCX novelty n = 4, control n = 3. [D] Arc/CB novelty n = 4, control n = 5; Arc/DCX novelty n = 4, control n = 6. Two-sided Wilcoxon rank-sum test: *P < 0.05. Scale bar: 10 µm. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.
Increasing Synaptic Activation of Maturing Dentate Granule Cells with Cell age

Next, we wanted to analyze at which postmitotic age newborn cells become responsive to HFS. We performed intraperitoneal injections of the mitosis marker bromodeoxyuridine (BrdU) and studied the age-dependent expression of the 2 maturation markers DCX and CB. DCX was present in most BrdU+ cells 7 days post-BrdU injection (dpi) and declined to low levels at 21 dpi (Fig. 3). CB was already present in some BrdU+ cells at 7 and 14 dpi, and almost all of these cells were CB+/DCX+. CB/DCX+ cells represented the major population of BrdU+ cells from 21 dpi on. Following 2-h HFS, expression of c-fos and Arc in BrdU+ cells was observed earliest at 21 dpi (15.39 ± 6.25% of BrdU+ cells for c-fos (n = 5) and 7.18 ± 3.59% of BrdU+ cells for Arc (n = 3). The percentage of c-fos+ and Arc+/BrdU+ cells after 2-h HFS increased with cell age and reached ~75% at 35 dpi (83.96 ± 5.29% of BrdU+ cells for c-fos (n = 5) and 74.18 ± 4.93% of BrdU+ cells for Arc (n = 4) with no further increase until 77 dpi (77.12 ± 10.07% of BrdU+ cells for c-fos (n = 3) and 80.04 ± 6.28% of BrdU+ cells for Arc (n = 3). Statistical analysis revealed a significant difference between the amount of activated BrdU+/CB+ cells (i.e., the total population of mature and maturing CB+-GCs except the BrdU+ cells) and the subpopulations of BrdU+-tagged GCs (for c-fos: BrdU21–28 dpi = 33.06 ± 8.61%, n = 8; BrdU35–77 dpi = 78.07 ± 2.79%, n = 16; BrdU− = 95.02 ± 0.4%, n = 24; for Arc: BrdU21–28 dpi = 25.65 ± 6.32%, n = 8; BrdU35–77 dpi = 73.87 ± 3%, n = 15; BrdU− = 96.6 ± 0.28%, n = 23; 2-tailed Wilcoxon rank-sum test, P < 0.05, for c-fos and Arc). On the basis of these findings, we concluded that even after 77 days of cell development, functional maturation and integration was not completed in all adult newborn dentate GCs.

pCREB133 Expression Does not Increase in Young Dentate Granule Cells Following High-Frequency Perforant Path Stimulation

Next, we wanted to examine whether young maturing DCX+ cells exhibited a detectable general reactivity to HFS. We took advantage of the fact that the transcription factor cAMP response element-binding protein (CREB) is a rapidly responding intracellular effector of neurotransmitter signaling and is endogenously activated in nonstimulated young maturing granule neurons (Nakagawa et al. 2002; Fujioka et al. 2004). Two-hour HFS elicited pCREB133 expression bilaterally in principal cells of the DG, CA1, and CA3 hippocampal regions (Fig. 4A). Whereas virtually all CB+-GCs in the ipsilateral (99.85 ± 0.07%, n = 3) and contralateral (99.73 ± 0.05%, n = 3) GCL of the DG were pCREB133+, only a very few cells showed weak pCREB133 immunoreactivity in the control group (8.05 ± 3.6%, n = 3; Fig. 4C,D). In contrast, about 40% (39.66 ± 1.76%, n = 3) of young maturing DCX+-GCs partly expressed pCREB133 endogenously in control animals, but the

Figure 3. Increasing synaptic activation of maturing dentate GCs with age. Following 2-h HFS, c-fos and Arc immunoreactivity was detected in calbindin (CB)+/BrdU+GCs 21–77 days postinjection (dpi) of BrdU (A, 35 dpi). The expression of DCX and CB in maturing BrdU+ cells correlated strongly with cell age. During the first 14 days, the majority of cells were DCX+, with an ongoing transition towards CB+ cells and a decline of DCX+ cells after 14 days (B). Two-hour HFS-induced expression of c-fos and Arc was present in BrdU+CB−GCs from 21 dpi on with increasing age and resulted in a plateau from 35 to 77 dpi (C and D). Even at 77 dpi, only about 8% of analyzed BrdU+ cells exhibited c-fos (C) or Arc (D). Younger (21–28 dpi) and older (35–77 dpi) BrdU+GCs expressed significantly less c-fos+ and Arc+ compared with the total population of BrdU+GCs following 2-h HFS (insets in C and D). (B) BrdU/CB/DCX, n7 dpi = 3, n21 dpi = 3, n28 dpi = 3, n35 dpi = 5; (C) c-fos/BrdU/CB, n21 dpi = 3, n28 dpi = 3, n35 dpi = 5, n49 dpi = 5, n63 dpi = 3, n77 dpi = 3, n21–28 dpi = 8, n35–77 dpi = 16, nBrdU+ = 24; D, Arc/BrdU/CB, n21 dpi = 3, n28 dpi = 3, n49 dpi = 5, n63 dpi = 3, n77 dpi = 3, n21–28 dpi = 8, n35–77 dpi = 15, nBrdU+ = 23. Two-sided Wilcoxon rank-sum test: *P < 0.05. Scale bar: 10 μm. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.
expression could not be further enhanced following 2-h HFS (35.78 ± 4.36%, n = 3; Fig. 4E,F).

We subsequently investigated if HFS could have an effect on pCREB133-expression of DCX⁺-positive cells depending on their structural maturation. This concerned in particular the subpopulation of maturing DCX⁺ GCs, which already showed elaborated dendrites toward the outer molecular layer, the zone of perforant path afferent input, and thus might be responsive to HFS. DCX, as a microtubule-associated protein, is expressed throughout the somata and dendrites of GCs during the phase of structural maturation (Francis et al. 1999; Gleeson et al. 1999). We introduced a classification of DCX⁺-GCs into 6 different stages, according to orientation and outgrowth of dendritic processes in the subgranular zone (stage 1, no processes; stage 2, processes in the subgranular zone), the granule cell layer (stage 3, inner half of the granule cell layer; and stage 4, outer half of the granule cell layer), inner molecular layer (stage 5) and outer molecular layer (stage 6; Fig. 5A, see also Materials and Methods section). The 6 DCX⁺ maturation stages correlated with cell age. At the age of 7 days, most DCX⁺ cells represented stage 1–4 neurons (≥85%), and only a few cells exhibited processes that reached the IML (stage 5) and OML (stage 6). At the age of 14 days, a few stage 1–4 DCX⁺ were left, whereas the majority of DCX⁺ cells (≥85%) represented stage 5–6 (Fig. 5C and D). Furthermore, DCX⁺ cells exhibited the marker Neuronal Nuclei (NeuN), an indicator for postmitotic neurons (Mullen et al. 1992; Kempermann et al. 2003) with increasing numbers in later DCX⁺-cell stages (Fig. 5E,F). Therefore, DCX⁺-cell staging appeared to be a useful classification system for structural maturation of young GCs.

Endogenous expression of pCREB133 closely correlated to structural maturation, with only a few pCREB133⁺ cells in stage 1 DCX⁺ cells, and >90% pCREB133⁺ cells in stage 5 and 6 DCX⁺ cells (Fig. 5G). Following 2-h HFS, there was no indication of an increased number of activated pCREB133⁺ cells at any structural stage (Fig. 5H). Therefore, endogenous pCREB133 expression appears to be closely correlated to structural maturation of young GCs, but cannot be further enhanced through synaptic activation.

Figure 4. Increased pCREB133 expression in dentate GCs following HFS. Two-hour HFS elicited bilateral pCREB133 expression in principal cells of the dentate gyrus (DG), and of the CA1 and CA3 hippocampal regions (A and B). Whereas most CB⁺-GCs in the ipsi- and contralateral granule cell layer (GCL) of the DG were pCREB133⁺ following 2-h HFS, only a few cells showed weak pCREB133 immunoreactivity under control conditions (C and D). pCREB133 was endogenously expressed in about 36% of immature DCX⁺ cells of control animals, but was not further enhanced by 2-h HFS (E and F). (D) pCREB133/CB, n = 3; (F) pCREB133/DCX, n = 3. Scale bar: (A and B) 100 µm; (C and E) 10 µm. ML, molecular layer; SGZ, subgranular zone.
**zif268 Expression Increases in Maturing Dentate Granule Cells Following High-Frequency Perforant Path Stimulation**

Similar to pCREB133, zinc finger binding protein clone 268 (zif268) is a transcription factor that can be enhanced by increased neuronal activity (Bozon et al. 2002) and is required for long-term synaptic plasticity (Jones et al. 2001). Two-hour HFS strongly increased zif268 expression in the ipsilateral GCL (Fig. 6A). 97.12 ± 2.3% (n = 3) of CB+ GCs in the ipsilateral DG exhibited strong zif268 expression following HFS, whereas GCs only exhibited weak zif268 immunoreactivity in the contralateral GCL and in the control condition (Fig. 6B–D). In addition, endogenous zif268 expression was found in some DCX+ cells, but was restricted to the more mature stages 5 (≤2% of DCX+) and 6 (>20% of DCX+). Application of 2-h HFS led to no significant increase of zif268 expression in

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**Figure 5.** pCREB133 expression is not enhanced in maturing DCX+ cells following HFS. Young maturing adult newborn DCX+-GCs were classified into 6 stages according to orientation and outgrowth of dendritic processes in the subgranular zone (SGZ; stage 1, no processes; stage 2, processes in the SGZ), the granule cell layer (GCL; stage 3, inner half of the GCL; stage 4, outer half of the GCL), the inner molecular layer (IML, stage 5), and outer molecular layer (OML, stage 6; A and B). Classification of BrdU-tagged DCX+ cells showed an age-dependent progression of dendritic outgrowth within the first 14 days (C and D). The neuronal marker Neuronal Nuclei (NeuN) was increasingly expressed with structural maturation and was expressed in all stage 6 DCX+ cells (E and F). pCREB133 expression in DCX+ cells tightly correlated to structural maturation in un-stimulated controls, but no indication of an enhancement of pCREB+ following 2-h HFS was found at any DCX+ stage (G and H). (B, D, F, and H) n = 3 for all groups. Scale bars 10µm. ML, molecular layer.
stage 5 and 6 DCX+ cells (2-sided Wilcoxon rank-sum test: stage 5 \( P = 0.74 \), stage 6 \( P = 0.08 \), \( n_{2\text{h HFS}} = 6 \), \( n_{\text{control}} = 8 \); Fig. 6E,F).

To study the time course of zif268 expression in maturing GCs beyond the phase of DCX expression, we measured the zif268 immunofluorescence intensities of BrdU-tagged cells at various dpi, and compared them to non-BrdU-tagged, but CB+ cells within the proximity of the ipsilateral GCL. A stimulation-induced increase of zif268 immunoreactivity in CB+ cells was first observed at 21 dpi (32.06 ± 3.8%, \( n = 3 \)), and then strongly increased to 67.97 ± 5.2% of the total CB+ cell population at 35 dpi (\( n = 3 \)). Thenceforth, no further increase in zif268 immunofluorescence intensity was observed. Younger (21–28 dpi) and older (35–77 dpi) BrdU+/CB+GCs showed significantly weaker intensities compared with the BrdU−/CB+GCs population (BrdU\(_{21-28 \text{ dpi}}\) = 44.48 ± 5.1%, \( n = 8 \); BrdU\(_{35-77 \text{ dpi}}\) = 69.01 ± 2.25%, \( n = 16 \); BrdU− = 100%, \( n = 24 \); 2-sided Wilcoxon signed-rank test, \( P < 0.05 \); Fig. 6G,H). Therefore, young maturing CB+ cells appear to be responsive to synaptic activation starting at 3 weeks of cell age, but show considerable less IEG expression following HFS than the total population of mature and maturing CB+ cells.

Figure 6. Increased zif268 expression in dentate GCs following HFS. Two-hour HFS strongly increased ipsilateral zif268 expression in principal cells of the dentate gyrus (DG), and of the CA1 and CA3 hippocampal regions in comparison to a weak endogenous zif268 expression on the contralateral side, and in sham control sections (A and B). After 2-h HFS, most CB+GCs in the ipsilateral granule cell layer of the DG exhibited strong zif268 expression (+++), whereas contralateral and control GCs showed weak (+) or medium (+++) zif268 immunoreactivity (C and D). Endogenous zif268 expression was only present in stage 5 and stage 6 of young maturing DCX+ cells (arrowheads in E), and was not significantly enhanced following 2-h HFS (E, F; 2 sample, 2-sided Wilcoxon test: stage 5 \( P = 0.74 \), stage 6 \( P = 0.08 \), \( n_{2\text{h HFS}} = 6 \), \( n_{\text{control}} = 8 \)). Following 2-h HFS, a comparison between CB+/BrdU+ (arrowheads in G; 21 and 77 dpi BrdU), and CB+/BrdU− cells revealed an age-correlated increase of zif268 immunofluorescence intensity in maturing CB+ cells reaching a plateau at 35–77 dpi (BrdU) with 70% zif268 immunofluorescence intensity of the total population of BrdU−/CB+ cells (G and H). Younger (21–28 dpi) and older (35–77 dpi) BrdU−GCs showed significantly less zif268 immunofluorescence intensity than BrdU−/GCs (inset in H). Zif268/BrdU/CB, \( n_{21 \text{ dpi}} = 3 \), \( n_{28 \text{ dpi}} = 5 \), \( n_{21-28 \text{ dpi}} = 8 \), \( n_{35-77 \text{ dpi}} = 16 \), \( n_{2\text{h HFS}} = 6 \), \( n_{\text{control}} = 8 \). Two-sided Wilcoxon signed-rank test: *\( P < 0.05 \). Scale bar: (A and B) 100µm; (C, E, and G) 10µm. GCL, granule cell layer; IML, inner molecular layer; OML, outer molecular layer; SGZ, subgranular zone.
Discussion

In this study, we examined correlations between the structural maturation, chemical marker expression, and age of newly born GCs with their capability to express various IEGs following strong synaptic stimulation, as an indication of successful functional integration in the adult network under in vivo conditions. The applied HFS protocol led to c-fos and Arc expression in nearly all mature GCs, but failed to induce c-fos and Arc expression in young maturing DCX⁺-GCs. Individual young GCs varied considerably in their time course of structural maturation. Once maturing GCs started to express CB and lost DCX expression, they gradually became responsive to HFS from 21 days on and showed IEG expression in about 75% of the maturing GCs at 35–77 days of cell age (Fig. 7). We conclude that structural and functional integration varies between individual young GCs and needs 3 weeks for the earliest maturation GCs, but requires at least 5 weeks until the majority of new GCs are able to respond to synaptic activation with signs of synaptic plasticity, and thus may contribute to processes of learning and memory formation.

Delayed Onset of Synaptic Activation and IEG Expression in Young GCs

Two-hour HFS induced LTP with robust c-fos and Arc expression in practically all mature CB⁺-GCs in the ipsilateral DG. Only a few CB⁺-GCs could not be activated, but those likely represent young CB⁺-GCs. Importantly, neither c-fos nor Arc expression was observed in young maturing DCX⁺-GCs after HFS. In addition, shorter HFS (30 min) or a TBS-LTP protocol, and novel environment exposure in awake animals failed to elicit c-fos and Arc in DCX⁺-GCs, whereas CB⁺-GCs were activated in all tests. Furthermore, some CB⁺-GCs in control animals expressed c-fos or Arc, attributed to home-cage activity (Marrone et al. 2008), but no spontaneously activated DCX⁺-GCs were found. Thus, the lack of activated immature DCX⁺-GCs is probably not due to an unphysiological or inadequate stimulation. In line with these findings, Kuipers et al. (2009) reported resistance to Arc activation in 1- to 28-day-old GCs with a TBS-LTP protocol. Similarly, spatial exploration induced Arc expression in GCs only from 30 days on (Sandoval et al. 2011).

Following HFS, we observed a gradual increase of c-fos, Arc, and zif268 expression in BrdU⁺/CB⁺-GCs starting at 21 days of cell age. Interestingly, Snyder et al. (2009) found earliest c-fos expression in newborn GCs at 2 weeks and in all BrdU⁺-GCs at 4 weeks following seizure induction by intraperitoneal kainate injection, demonstrating that immature neurons have the capability to express IEGs under pathophysiological conditions. The time course of stimulation-induced zif268 expression preceded c-fos and Arc expression. An early onset of zif268 expression in 2- to 4-week-old GCs was

![Figure 7](https://academic.oup.com/cercor/article-abstract/24/7/1845/295591)
reported following a TBS-LTP protocol (Bruel-Jungerman et al. 2006; Kuipers et al. 2009) or physiological stimulation by water maze training as well as by kainate-induced seizures (Snyder et al. 2009, 2012). In our study, basal zif268 expression was endogenously present in CB⁺-GCs and also in some DCX⁺-GCs with elaborated dendrites. In some contrast to Snyder et al. (2009), who found a peak of stimulation-induced zif268 expression in 3-week-old GCs, we observed a continuous increase of inducible zif268 expression until the fifth week of cell age. From this, we conclude that young GCs become gradually integrated in the existing adult network (Fig. 7).

Our experiments were performed in young adult rats (8–13 weeks). The time course of maturation and IEG expression could be different and might decrease in older animals. However, Morgenstern et al. (2008) found no difference in spine density of newborn granule cells between young (2 months) and senescent mice. Furthermore, Marrone et al. (2012) could not detect a different rate of Arc expression in newborn granule cells of senescent rats in response to spatial exploration. These findings suggest that while the total number of newborn granule cells decreases with increasing age of the animal (Dayer et al. 2003), the general time course of integration into the network remains remarkably constant.

**Gradual Structural and Functional Integration of Maturing GCs**

Expression of IEGs such as c-fos, Arc, and zif268 are considered as a prerequisite for a successful synaptic activation and biosynthesis dependent late phase of LTP (Guzowski et al. 2001; Jones et al. 2001; Davis 2003; Fleischmann et al. 2003). Numerous studies have shown that Arc plays an essential role in this process that requires upregulation and trafficking of Arc mRNA to GC dendrites and local Arc translation at sites of synaptic activity, as well as sustained Arc synthesis for LTP consolidation and silencing of inactive synapses (Steward et al. 1998; Steward and Worley 2001; Messaoudi et al. 2007; Bramham et al. 2008; Jedlicka et al. 2008; Okuno et al. 2012). Therefore, maturing GCs that are involved in synaptic plasticity and learning should be responsive to LTP induction protocols and react with nuclear IEG expression. However, the lack of stimulation-induced c-fos, Arc, and zif268 expression in young maturing GCs does not exclude forms of synaptic plasticity that do not require IEG expression (Bramham 2010). We observed HFS-induced c-fos and Arc expression in some maturing CB⁺ cells already at 3 weeks. On the other hand, c-fos and Arc expression as well as zif268 immunofluorescence intensity increased gradually and reached only 75% of the values for CB⁺-GCs even after 11 weeks. Consistent with this delayed responsibility, Zhao et al., (2006) provided evidence for a sustained time course of 4–18 weeks for spino-genesis, especially of mature mushroom spines in new GCs. Furthermore, functional glutamatergic perforant path-GC synapses were detected from 3 weeks on (Espósito et al. 2005; Zhao et al. 2008). In contrast, several studies in adult rats have suggested that new GCs contribute to hippocampal function as early as 2–3 weeks of age (Shors et al. 2001, 2002; Madsen et al. 2003; Snyder et al. 2005). In these studies, adult neurogenesis was suppressed by systemic toxin application or local hippocampal irradiation prior to behavioral testing. Taken together, this would suggest that specific functions attributed to young maturing granule cells would not demand strong synaptic perforant path–GC contacts and would not rely on IEG expression. Interestingly, following hippocampal irradiation, Lacefield et al. (2010) found decreased perforant path responses, whereas spontaneous γ-frequency bursts increased in the dentate gyrus, indicative of a more modulatory role of young granule cells within the hippocampal network. Furthermore, in a recent study using rabies virus-mediated retrograde tracing, Vivar et al. (2012) provided evidence for a pronounced input to new granule cells from the perirhinal and the lateral entorhinal cortex from day 21 on. This points to distinct characteristics in network function of young granule cells that might be independent of transcriptional activation of IEGs required for classical forms of synaptic plasticity (Bramham 2010).

In adult mice, electrophysiological studies (Van Prag et al. 2002; Espósito et al. 2005; Ge et al. 2006, 2007), as well as morphological (Zhao et al. 2006; Jessberger et al. 2007) and behavioral (Jessberger and Kempermann 2003; Kee et al. 2007) reports, suggest that new neurons are minimally functional, and would be unlikely to contribute to behavior until at least 6–8 weeks of age. However, a direct comparison of adult neurogenesis in mice and rat (Snyder et al. 2009) revealed that newborn GCs in rats show faster maturation and signs of earlier and stronger activation than mice (Snyder et al. 2009).

In summary, the gradual reactivity of newborn GCs to HFS observed in our study indicates that any network function attributed to very young GCs would likely be performed by a small number of early mature GCs. As the total number of new GCs represents only a subfraction of the mature GC population, the actual number of young GCs responsive to functional activity appears to be limited.

**Correlation of Chemical and Structural GC Maturation with Age**

Young GCs endogenously expressed pCREB133, as described previously (Nakagawa et al. 2002). Interestingly, pCREB135 expression correlated with structural staging, being expressed in about 10% of stage 2, but in 100% of stage 6 DCX⁺-GCs. This is in line with the role of CREB-signaling as an important factor for survival and dendritic development of GCs (Fujikawa et al. 2004). However, we could not detect any enhancement of pCREB133 expression in DCX⁺-GCs following HFS, although GABA-mediated activation of pCREB133 has been shown in maturing GCs (Nakagawa et al. 2002; Ge et al. 2006; Merz et al. 2011). In contrast to DCX⁺-GCs, endogenous pCREB133 expression was hardly detectable in mature CB⁺-GCs, but was strongly activated following HFS. Thus, the role of pCREB133 signaling appears to change from a growth-related function in maturing GCs to an excitation-related function in mature GCs.

In general, chemical and structural development of new GCs corresponded to cell age. Our data from Sprague Dawley rats closely correlate to similar labelings in Long-Evans rats (Snyder et al. 2009). We found a change from DCX to CB expression in new GCs within 3–4 weeks. Double-labeling revealed a subpopulation of DCX⁺/CB⁺ cells that probably represented early CB⁺ cells. Remarkably, these cells showed no enhancement of IEGs following HFS. Although structural development of DCX⁺ cells increased with age, some neurons...
showed dendrites in the inner and outer molecular layer (stage 5 and 6) already at 1 week, whereas other neurons remained in early structural stages (stage 3–4) at 4 weeks. Thus, the individual structural development of young maturing GCs is subject to considerable variation. In addition, it has to be considered that BrdU labeling does not necessarily, and in all cases, reflect the exact cell age. Using a comparable BrdU protocol in rats (a single injection of 300-mg/kg body weight compared with 200-mg/kg body weight in our study), Dayer et al. (2003) detected BrdU-labeled granule cells that divided within a range of 4 days postinjection. In our study, the postmitotic neuronal marker NeuN (Mullen et al. 1992; Kempermann et al. 2003) was expressed in 100% of the CB+ cells (Supplementary Fig. S1A,B), and NeuN expression increased with structural maturation in DCX+ cells (Fig. 5F), although some stage 5 and 6 DCX+ cells did not show NeuN expression. This leaves the possibility open that some BrdU-labeled cells could represent a population of granule cells divided from later stage DCX cells.

In comparison to rodents, the time course of granule cell maturation appears to be substantially prolonged in higher mammals such as primates and also in humans (Knoth et al. 2010; Kohler et al. 2011). Although markers of the different phases of neurogenesis have been found in a sequence well comparable to rats and mice, new granule cells take more than 6 times longer to mature in macaque monkeys (Kohler et al. 2011). In these animals, the onset of DCX and NeuN expression in BrdU+ cells was delayed and the majority of BrdU+/NeuN+ cells still expressed DCX after 23 weeks of cell age (Kohler et al. 2011). In a human postmortem analyses, Knoth et al. (2010) detected DCX expression in granule cells of individuals between birth and 100 years of age. DCX was co-expressed with a variety of markers of proliferating and immature cells, in addition to NeuN expression, indicating largely extended granule cell maturation in humans. Given the similar patterns of neurogenesis-associated features in rodents, the gradual maturation and delayed susceptibility to stimulation of new granule cells found in our rat study suggest that functional integration of newly generated granule cells in primates and humans would be severely protracted. Together with the fact, that the rate of adult hippocampal neurogenesis is small and strongly decreases with age in monkeys and humans (Eriksson et al. 1998; Knoth et al. 2010; Kohler et al. 2011), this would point to a very limited functional role of human adult neurogenesis at least under physiological conditions.

The Role of Adult Newborn GCs

The functional significance of adult newborn GCs remains a highly discussed topic (Ming and Song 2011; Bergami and Berninger 2012). Increasing evidence suggests that newly formed GCs contribute to hippocampus-specific forms of learning and memory, which might be different to the function of mature (or permanent) GCs (Clelland et al. 2009; Aimone et al. 2011). Recent studies suggested a model of DG function, in which young GCs may support pattern separation, whereas old GCs contribute to rapid pattern completion (Nakashiba et al. 2012) or even may retire to a silent population (Alme et al. 2010). We found no indication for a functional silencing of old GCs, as more than 95% of the mature GC appeared to be highly responsive to synaptic activation. On the other hand, a small number of young CB+-GCs exhibited signs of structural and functional maturation already as early as 3 weeks of cell age, but at least 5 weeks seem to be necessary, until the majority of newly generated GCs are responsive to successful synaptic activation and synaptic plasticity including late LTP, that requires de novo transcription. Therefore, young granule cells appear to be gradually integrated into the existing neuronal network, in order to contribute to different forms of hippocampal learning.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References


