Neuronal Activity–Induced Gadd45b Promotes Epigenetic DNA Demethylation and Adult Neurogenesis

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The mammalian brain exhibits diverse types of neural plasticity, including activity-dependent neurogenesis in the adult hippocampus. How transient activation of mature neurons leads to long-lasting modulation of adult neurogenesis is unknown. Here we identify Gadd45b as a neural activity-induced immediate early gene in mature hippocampal neurons. Mice with Gadd45b deletion exhibit specific deficits in neural activity-induced proliferation of neural progenitors and dendritic growth of newborn neurons in the adult hippocampus. Mechanistically, Gadd45b is required for activity-induced DNA demethylation of specific promoters and expression of corresponding genes critical for adult neurogenesis, including BDNF and FGF. Thus, Gadd45b links neuronal circuit activity to epigenetic DNA modification and expression of secreted factors in mature neurons for extrinsic modulation of neurogenesis in the adult brain.

Adult neurogenesis represents a prominent form of structural plasticity through continuous generation of new neurons in the mature mammalian brain (1, 2). Similar to other neural activity-induced plasticity with fine structural changes within individual neurons, adult neurogenesis is modulated by a plethora of external stimuli (1, 2). For example, synchronized activation of mature dentate neurons by electroconvulsive treatment (ECT) in adult mice causes sustained up-regulation of hippocampal neurogenesis (3) without any detectable cell damage (fig. S1). How transient activation of mature neuronal circuits modulates adult neurogenesis over days and weeks is largely unknown.

Epigenetic mechanisms potentially provide a basis for such long-lasting modulation (4). We examined the expression profiles of known epigenetic regulators in response to ECT, including those involved in chromatin modification (5). One gene we found to be strongly induced by ECT was Gadd45b (Fig. 1A) (6), a member of the Gadd45 family previously implicated in DNA repair, adaptive immune response (7–10), and DNA 5-methylcytosine excision in cultured cells (11). We first characterized Gadd45b induction by neuronal activity in the adult hippocampus (5). Analysis of micro-dissected dentate gyrus tissue showed robust, transient induction of Gadd45b expression by a single ECT (Fig. 1A; fig. S2; table S1). In situ analysis revealed induction largely in NeuN+ mature dentate granule cells (Fig. 1B; fig. S3). Spatial exploration of novel environment, a behavioral paradigm that activates immediate early genes (IEGs) (12), also led to significant induction of Gadd45b, but not Gadd45a or Gadd45g (Fig. 1, C-D). The majority of Gadd45b-positive cells also expressed Arc (Fig. 1D; 88 ± 3%; n = 4), a classic activity-induced IEG. Thus, physiological stimulation is sufficient to induce Gadd45b expression in dentate granule cells. Experiments with pharmacological manipulations of primary hippocampal neurons further suggested that Gadd45b induction by activity requires the N-methyl D-aspartate receptor (NMDAR), Ca2+, and CaMK signaling (fig. S4; Supporting Text). In vivo injection of the NMDAR antagonist CPP abolished ECT-induced Gadd45b and Arc expression in the adult dentate gyrus (Fig. 1E). Taken together, these results suggest that Gadd45b shares the same induction pathway as classic activity-induced IEGs (13).

We next assessed whether Gadd45b induction is required for neural activity-dependent adult neurogenesis. Adult Gadd45b knockout (KO) (10) mice appeared anatomically normal (fig. S5) and exhibited identical NMDAR-dependent induction of known IEGs at 1 hr after ECT (Fig. 1E). To
examine neural progenitor proliferation, adult mice at 3 days after ECT or sham treatment were injected with bromodeoxyuridine (BrdU) and sacrificed 2 hrs later (5). Stereological counting showed similar densities of BrdU+ cells in the dentate gyrus between WT and KO mice without ECT (Fig. 2). After ECT, however, there was a 140% increase in the density of BrdU+ cells in WT mice with only 40% increase in KO littermates (Fig. 2). Little caspase-3 activation was detected within the dentate gyrus under all these conditions (figs. S1 and S6), ruling out a potential contribution from cell death. To confirm this finding with a manipulation of better spatiotemporal control, we developed effective lentiviruses to knockdown the expression of endogenous Gadd45b with short-hairpin RNA (shRNA; fig. S7). Expression of shRNA-Gadd45b through stereotactic viral injection largely abolished ECT-induced proliferation of adult neural progenitors, whereas the basal level was similar to that of shRNA-control (fig. S7). We also examined exercise-induced adult neurogenesis, a physiological stimulation that induced a modest Gadd45b elevation (fig. S8A). A 7-day running program led to a dramatic increase of neural progenitor proliferation in adult WT mice, but was significantly less effective in their KO littermates (fig. S8B). Taken together, these results demonstrate a specific and essential role of Gadd45b in activity-induced, but not basal level of neural progenitor proliferation in the adult dentate gyrus.

We next examined the role of Gadd45b induction in the dendritic development of newborn neurons. Retroviruses expressing GFP were stereotaxically injected into the dentate gyrus of adult WT and KO mice to label proliferating neural progenitors and their progeny (5, 14). A single ECT was given at 3 days after injection, when the majority of GFP-labeled cells already became post-mitotic neurons (14). Quantitative analysis showed that ECT led to marked increase in the total dendritic length and complexity of GFP+ newborn neurons at 14 days after retroviral labeling (Fig. 3). This ECT-induced dendritic growth was significantly attenuated in KO mice, while the basal level of dendritic growth was similar (Fig. 3). Thus, Gadd45b is also essential for activity-induced dendritic development of newborn neurons in the adult brain.

How does transient Gadd45b induction regulate activity-dependent adult neurogenesis over the long-term? Gadd45a has been implicated in promoting global DNA demethylation in cultured cells, yet the finding remains controversial (11, 15). To examine whether Gadd45b induction may confer long-lasting epigenetic modulation in the expression of neurogenic niche signals, we analyzed DNA methylation status using micro-dissected adult dentate tissue enriched in NeuN+ mature neurons (5). No significant global DNA demethylation was detected after ECT in vivo (figs. S9 and S10B; Supporting Text). We next used methylated DNA immunoprecipitation (MeDIP) analysis in a preliminary screen for region-specific DNA demethylation, with a focus on growth factor families that have been implicated in regulating adult neurogenesis (2). Significant demethylation was found at specific regulatory regions of brain-derived neurotrophic factor (BDNF) and fibroblast growth factor-1 (FGF-1; fig. S10B). Bisulfite sequencing analysis further confirmed ECT-induced demethylation within the regulatory region IX of BDNF (16) and the brain-specific promoter B of FGF-1 (17) (Fig. 4, A-B; fig. S11; table S2). Every CpG site within these regions exhibited marked reduction in the frequency of methylation (Fig. 4A). Time-course analysis further revealed the temporal dynamics of DNA methylation status at these CpG sites (figs. S12 and S13). In contrast, no significant change was induced by ECT in the pluripotent cell specific Oct4 promoter or the kidney and liver-specific FGF-1G promoter (18) (Fig. 4B; fig. S11B). Comparison of adult Gadd45b WT and KO mice without ECT showed no significant difference in the basal levels of DNA methylation within BDNF IX and FGF-1B regulatory regions (Fig. 4B; fig. S14). In contrast, ECT-induced DNA demethylation of these regions was almost completely abolished in KO mice (Fig. 4, A-B; figs. S10C and S11A). In addition, overexpression of Gadd45b appeared to promote DNA demethylation in vivo (Fig. 4C) and to activate methylation-silenced reporters in cultured post-mitotic neurons (fig. S15). Chromatin immunoprecipitation (ChIP) analysis further showed specific binding of Gadd45b to the FGF-1B and BDNF IX regulatory regions (fig. S16). ECT-induced gene expression from these regions and total expression of BDNF and FGF-1, were largely absent in Gadd45b KO mice at 4 hrs (Fig. 4D; fig. S17), consistent with a critical role of DNA methylation status in regulating gene expression. Thus, Gadd45b is essential for activity-dependent demethylation and late-onset expression of specific secreted factors in the adult dentate gyrus.

In summary, Gadd45b links neuronal circuit activity to region-specific DNA demethylation and expression of paracrine neurogenic niche factors from mature neurons in controlling key aspects of activity-dependent adult neurogenesis (fig. S18). As endogenous targets of Gadd45b-dependent demethylation pathway, BDNF is known to promote dendritic growth of neurons in vivo and FGF-1 exhibited robust mitogenic activity as FGF-2 on neural progenitor proliferation in vitro (fig. S19). The presence of Gadd45b in chromatin associated with BDNF IX and FGF-1B regulatory regions in neurons (fig. S16) points to its direct role in gene regulation and potentially in a demethylation complex (fig. S18) (19). The known role of Gadd45 family in 5-methylcytosine excision (7, 8, 11) is consistent with the emerging notion that region-specific demethylation can be
mediated through DNA repair-like mechanisms as supported by genetic and biochemical studies in both Arabidopsis and mammalian cells (20, 21) (Supporting Text).

How transient neuronal activation achieves long-lasting effects in neural plasticity and memory has been a long-standing question; enzymatic modification of cytosine in DNA was proposed as a means to provide such necessary stability with reversibility (22). Although DNA demethylation can occur passively during cell division, emerging evidence suggests the existence of active demethylation in post-mitotic cells (23–25). DNA demethylation in neurons represents an extra layer of activity-dependent regulation, in addition to transcription factors and histone-modifying enzymes (13). Gadd45b expression is altered in some autistic patients (26) and is induced by light in the suprachiasmatic nucleus (27), by induction of long-term potentiation in vivo (28). Gadd45b is also associated with critical period plasticity in the visual cortex (29). Thus, Gadd45b may represent a common target of physiological stimuli in different neurons in vivo and mechanisms involving epigenetic DNA modification may be fundamental for activity-dependent neural plasticity.

References and Notes
5. Material and Methods and supporting data are available on Supporting Online Material.
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Supporting Online Material
Materials and Methods
SOM Text
Figs. S1 to S19
Tables S1 and S2
References
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Fig. 1. Activity-induced neuronal Gadd45b expression. (A) Q-PCR analysis of ECT-induced expression of Gadd45a, Gadd45b and Gadd45g in the adult dentate gyrus after a single ECT. (B) Sample images of Gadd45b in situ hybridization of the adult hippocampus after ECT. Scale bar: 0.5 mm. (C, D) Gadd45 induction in the dentate gyrus after 1 hr spatial exploration of novel environment. Shown in (C) is a summary from Q-PCR analysis. Shown in (D) are sample confocal images of Gadd45b in situ hybridization, DAPI and Arc immunostaining. Note that the majority of Gadd45b-positive cells (open and closed arrowheads) were Arc-positive (closed arrowheads). Scale bar: 50 μm. (E) NMDAR-dependent induction of Gadd45b, Arc and Homer1 in the adult dentate gyrus at 1 hr after ECT. The NMDAR antagonist 3-(2-carboxypiperizin-4-yl)-propyl-1-phosphonic acid (CPP) was injected at 1 hr before ECT (10 mg/kg body weight, i.p.). Values represent mean ± SEM (n = 4; *: P < 0.01, ANOVA).

Fig. 2. Essential role of Gadd45b in activity-induced proliferation of adult neural progenitors. (A) Sample projected confocal images of BrdU immunostaining (red) and DAPI (Blue). Scale bar: 50 μm. (B) Summary of stereological
quantification of BrdU+ cells in the dentate gyrus. Values represent mean ± SEM (n = 4-5 animals as indicated; *: P < 0.01, ANOVA).

**Fig. 3.** Essential role of Gadd45b in activity-induced dendritic development of newborn neurons in the adult brain. (A) Sample projected Z-series confocal images of GFP+ dentate granule cells at 14 days after viral labelling. Scale bar: 50 μm. (B) Quantification of the total dendritic length of GFP+ dentate granule cells. Values represent mean ± SEM (n = 23-45 neurons for each condition; *: P < 0.01, ANOVA). (C) Analysis of dendritic complexity of the same group of cells as in (B; *: P < 0.01, Student t-test).

**Fig. 4.** Essential role of Gadd45b in activity-induced specific DNA demethylation and gene expression in the adult dentate gyrus. (A-B) Bisulfite sequencing analysis of adult dentate gyrus tissue before or at 4 hrs after ECT. Shown in (A) is a schematic diagram of the genomic region subjected to analysis and a summary of methylation frequency at individual CpG sites. Shown in (B) is a summary of mean DNA methylation levels of individual alleles. Values represent mean ± SEM (n = 10-15; **: P < 0.01; *: P < 0.05; #: P > 0.1, ANOVA; See exact P values in Table-S2). (C) Methylation-specific PCR analysis from the dentate gyrus of WT mice after one or two ECTs (“24+4”: 4 hrs after two ECTs at 24 hrs apart), or 7 days after lentivirus-mediated expression of Gadd45b-GFP or GFP alone without ECT. Primers are specific for methylated (M) and un-methylated alleles (UM) of the FGF-1B promoter, or for bisulfite sequencing without CpGs (Input). (D) Summary of the mRNA and protein expression in the dentate gyrus of adult Gadd45b WT and KO mice at 4 hrs after ECT and sham controls. Values represent mean ± SEM (n = 4; *: P < 0.01, ANOVA).
A) BDNF IX and FGF-1B gene structures with methylation analysis.

B) Frequency of methylation at individual CpG sites for Gadd45b WT and KO in ECT and normal conditions.

B) Bar graph showing mean methylation levels across different conditions:
- WT
- WT (ECT)
- KO
- KO (ECT)

C) Table showing ECT (hours) for 0, 4, 24, and 24+4.

D) Graphs showing normalized mRNA and protein levels:
- BDNF IX
- Total BDNF
- FGF-1B
- Total FGF-1
- total BDNF
- total FGF-1
- Arc