Chapter 6
The Role of the Gadd45 Family in the Nervous System: A Focus on Neurodevelopment, Neuronal Injury, and Cognitive Neuroepigenetics

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Abstract The growth arrest and DNA damage-inducible (Gadd)45 proteins have been associated with numerous cellular mechanisms including cell-cycle control, DNA damage sensation and repair, genotoxic stress, neoplasia, and molecular epigenetics. The genes were originally identified in in vitro screens of irradiation- and interleukin-induced transcription and have since been implicated in a host of normal and aberrant central nervous system processes. These include early and postnatal development, injury, cancer, memory, aging, and neurodegenerative and psychiatric disease states. The proteins act through a variety of molecular signaling cascades including the MAPK cascade, cell-cycle control mechanisms, histone regulation, and epigenetic DNA demethylation. In this review, we provide a comprehensive discussion of the literature implicating each of the three members of the Gadd45 family in these processes.

6.1 Introduction

The growth arrest and DNA damage-inducible (Gadd)45 family includes the related Gadd45α, Gadd45β/Myd118, and Gadd45γ/CR6 proteins, referred to here as Gadd45a, Gadd45b, and Gadd45g, respectively. The corresponding genes were identified in different cell lines following irradiation stress and interleukin treatment (Fornace et al. 1988; Abdollahi et al. 1991; Beadling et al. 1993). These evolutionarily conserved proteins are small (~18 kDa), acidic homologs with both nuclear and cytoplasmic expression (Tamura et al. 2012). Expression of the *gadd45* genes was confirmed in a variety of tissues including the skeletal muscle, heart, kidney, lungs, brain, and testis (Zhang et al. 1999). Consistent with their discovery, the *gadd45*

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genes were shown to be sensitive to a wide variety of stressors in diverse cell lines (Tamura et al. 2012). In the central nervous system (CNS), the genes have been investigated as critical contributors to neuronal and glial stress responses, apoptosis, and mitosis. This is consistent with their long-established role in cell-cycle checkpoint regulation (Kearsey et al. 1995; Hildesheim et al. 2002). More recently, Gadd45a and Gadd45b were implicated in epigenetic control of gene expression, and this discovery prompted a growing literature documenting their role as players in adult cognitive function and CNS diseases (Barreto et al. 2007; Ma et al. 2009; Day and Sweatt 2011). In this review, we summarize the body of evidence showing the Gadd45 proteins regulate nervous system development, injury responses, and cognitive neuroepigenetics.

6.2 Nervous System Development

6.2.1 Expression Patterns of the Gadd45 Genes in Neural Development

We begin with a discussion of the role of the Gadd45 family in the development of the CNS. One study in particular provides a comprehensive assessment of the expression patterns of the Gadd45 genes throughout murine embryonic development (Kaufmann et al. 2011). The authors began by assessing expression in mouse embryos. Gadd45a mRNA was found to be expressed at low levels in the gastrula stage, and this expression increased during neurulation, plateauing by embryonic day 9. In contrast, while strong *gadd45b* transcription was found during gastrulation, expression decreased rapidly during neurulation. Gadd45g mRNA expression begins at low levels and increases continuously following gastrulation. Relative quantification of the three transcripts at embryonic day 10.5 revealed very minute *gadd45b* expression and robust expression of *gadd45a* and *gadd45g*. These results suggest Gadd45b may play the least critical function among the three genes in embryogenesis. In *Xenopus* embryogenesis, similar relative expression patterns were found. *Gadd45a* expression peaks between embryonic days 10.5 and 12.5, *gadd45b* expression is minimal at embryonic day 10.5 but increases thereafter, and *gadd45g* expression peaks between embryonic days 12.5 and 18 (Kaufmann and Niehrs 2011).

Region-specific analysis of transcription in mice showed prominent *gadd45a* expression early in the mesoderm and primitive streak, the region through which cell migration gives rise to the three germ layers. *Gadd45b*, in contrast, is strongly localized to the chorion but not epiblast cells at embryonic day 7.5. However, *gadd45b* is mildly expressed later in the posterior remnants of the primitive streak. *Gadd45g* expression is also excluded from the epiblast cells at this stage. It is instead found in the ectoplacental cone and the extraembryonic ectoderm.

Upon neurulation, Gadd45a mRNA is highly enriched in the neural folds, suggesting a significant role in initial formation of the neural tube. Supporting this hypothesis is the finding that a fraction of homozygous Gadd45a-null mutants

exhibit exencephaly, a condition in which the brain grows outside of the skull due to improper neural tube closure (Hollander et al. 1999). However, this finding may not be due to Gadd45a-regulated apoptosis, as the requirement of apoptosis for neural tube closure has been challenged (Massa et al. 2009). At this stage, Gadd45b is not found in the lips of the neural folds but is expressed in the progenitors of the midbrain and hindbrain. Between embryonic days 8.5 and 8.75, *gadd45g* transcription continues in the extraembryonic allantois, and embryonic expression is found in the presomitic mesoderm. Notably, expression begins in neural fold precursors and then becomes prominent in dorsal midbrain. Milder expression is found in the facio-acoustic and dorsal root ganglia at this stage as well.

By embryonic day 9, *gadd45a* expression builds in placodes of the trigeminal and dorsal root ganglia (Kaufmann et al. 2011). By day 9.5, expression is found in the facio-acoustic, glossopharyngeal, and vagal ganglia as well as the olfactory epithelium. Transcripts in the neural tube, however, begin to fade but persist in the caudal neuropore and lamina terminalis, the final regions to undergo closure. After embryonic day 9.5, a ubiquitous basal expression pattern of *gadd45a* persists, and this includes the cranial ganglia VII–X. At day 10.5, expression appears in the epithelium of the telencephalic ventricles. Expression was not detected in the midbrain and hindbrain regions. In contrast, by embryonic day 9, expression of *gadd45b* builds in the dorsal midbrain and somites. In light of this pattern of embryonic Gadd45b transcriptional patterning, it is not surprising that prominent expression in the striatum was found in the adult brain (Sultan et al. 2012). Somitic expression of Gadd45b persists in the trunk somites by embryonic day 10.5, and additional expression occurs in the dorsal aorta, first branchial arches, and forelimb bud. Relative to expression in these regions, Gadd45b mRNA was only weakly detected in the forebrain, suggesting it plays a less significant role in neural development. Finally, *gadd45g* transcription after embryonic day 9 was found to be the most pronounced of the three loci (Kaufmann et al. 2011). Highly pronounced staining was found in the dorsal midbrain, the cranial and dorsal root ganglia, and neural tube. At day 9.5, *gadd45g* was expressed robustly at the forebrain-midbrain junction, the trigeminal and facio-acoustic ganglia, the otic cup, and the latero-ventral hindbrain. However, expression in the neural tube was not found to be homogenous; instead, *gadd45g* is predominantly transcribed in the dorsal and ventral peripheral cells of the neural tube. By embryonic day 10.5, expression remains pronounced throughout the neural tube, extending from the most caudal tip to the midbrain rostrally. At this point, diffuse expression in the forebrain and dense expression in the dorsal root ganglia begin to appear as well. Murine expression of the *gadd45* genes is summarized in Fig. 6.1.

In situ hybridization analysis of expression patterns of the *gadd45* genes in *Xenopus* also revealed differential transcriptional profiles (Kaufmann and Niehrs 2011). *Gadd45a* transcripts are present homogenously in gastrulae in both ectoderm and mesoderm layers. Upon neurulation, significant expression continues in the ectoderm and neuroectoderm, but little expression is seen in the neural tube as in murine development. However, *gadd45b* expression was shown to be relatively concentrated in the neural tube as well as the initial ectoderm during gastrulation even

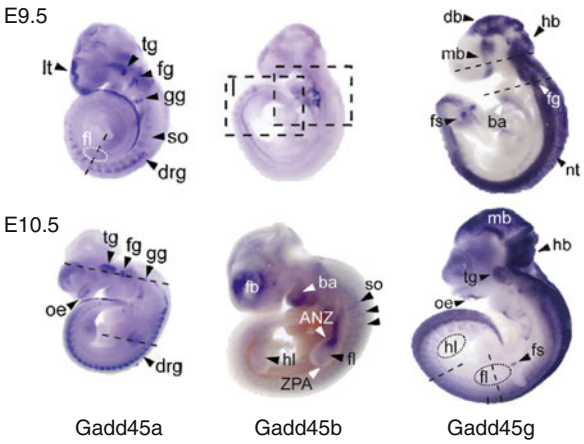

Fig. 6.1 Expression of the *gadd45* genes in mouse embryonic development. Whole mount in situ hybridization on E9.5 (*top row*) and E10.5 (*bottom row*) murine embryos was used to visualize expression of *gadd45a* (*left*), *gadd45b* (*middle*), and *gadd45g* (*right*). Lateral views are shown. Key: *ANZ* anterior necrotic zone, *ba* first branchial arch, *db* dorsal midbrain, *drg* dorsal root ganglia, *fb* forebrain, *fg* facio-acoustic ganglia, *fl* forelimb bud, *fs* forming somite, *gg* glossopharyngeal ganglia, *hb* hindbrain, *hl* hindlimb bud, *lt* lamina terminalis, *mb* midbrain, *nt* neural tube, *oe* olfactory epithelium, *so* somitic mesoderm, *tg* trigeminal ganglia, *ZPA* zone of polarizing activity. Reproduced with permission from Kaufmann et al. (2011)

though overall expression was very low compared to *gadd45a* and *gadd45g*. Similar to its murine homolog, the *Xenopus gadd45g* gene showed the most prominent neural expression, beginning in primary neuron precursors and later in the brain and eye. Although the expression of *Xenopus gadd45a* and *gadd45b* differ somewhat from their respective mouse homologs, it should be noted that the frog *gadd45b* gene shares under 60 % homology with mouse *gadd45b*. The frog *gadd45a* and *gadd45g* genes, however, share over 70 % homology with murine orthologs. Finally, neural expression of *gadd45g* was similarly confirmed in the medaka, *Oryzias latipes* (Candal et al. 2004). Expression was found in the neurula, and this spreads throughout the entire brain, lens, olfactory bulbs, and optic tectum.

Together, these results implicate each of the Gadd45 family genes in embryogenesis and neurulation but to differing extents. By far, *gadd45g* expression is the most robust in nervous tissue progenitors in both mice and frogs, suggesting it plays the strongest role in the ontogeny of the central and peripheral nervous system. In mice, *gadd45b* expression in nervous tissue is relatively weak, especially outside of the dorsal midbrain, and is instead more selectively localized to somites, mesodermal tissue that later gives rise to skeletal muscle, dermis, and vertebrae (Kawahara et al. 2005; Kaufmann et al. 2011). *Gadd45a* expression likely plays the strongest role in neural tube closure in mice. Beyond embryogenesis, *gadd45a* transcription and protein expression persist in the adult murine cerebral cortex (Sarkisian and Siebzehnrubl 2012). Interestingly, cortical expression in the human fetus was similarly observed,

suggesting a conserved developmental transcriptional profile for Gadd45a. Future studies are needed to assess the transcriptional profile of *gadd45b* and *gadd45g* beyond embryogenesis.

6.2.2 Regulation of Nervous System Development

There is also evidence from recent studies that both Gadd45a and Gadd45g play functional roles in neural development. In addition to the proclivity for exencephaly in Gadd45a-null mice, both knockdown and overexpression of *gadd45a* in *Xenopus* embryos, for instance, produced a range of developmental defects including gastrulation defects, reduced pigmentation, and head defects (Kaufmann and Niehrs 2011). Surprisingly, *gadd45b* manipulations failed to produce a developmental phenotype, suggesting that its low but detectable expression in the embryo is less consequential. In contrast, increases or decreases in *gadd45g* gene product led to pleiotropic phenotypes similar to those of *gadd45a* manipulation (de la Calle-Mustienes et al. 2002; Kaufmann and Niehrs 2011). In medaka, overexpression of *gadd45g* attenuated embryonic development and cell number, and knockdown produced developmental and morphological abnormalities after the neurula stage (Candal et al. 2004).

The functionality of Gadd45a in neurodevelopment extends beyond gross anatomical features and pertains to cell differentiation as well. An in vitro assay of cortical neuron development found that both overexpression and knockdown of *gadd45a* transcription suppress the formation of distal neurite processes and often promote aberrantly shaped and sized cell bodies (Sarkisian and Siebzehnrubl 2012). The authors similarly found that reduced *gadd45a* expression in the cortex in vivo impairs dendritic arborization and neuronal migration to superficial cortical layers. Overexpression failed to affect migration but caused irregular and hypertrophied cell body development. Additionally, enhanced expression reduced survival and impaired development in a rat glioma cell line in vitro, suggesting Gadd45a regulates apoptosis. Likewise, overexpression of *gadd45g* in an embryonic carcinoma cell line produced a neuronal phenotype, suggesting Gadd45g regulates not only anatomical development but also differentiation of neurons (Huang et al. 2010). Together, these studies demonstrate a significant role of the Gadd45 genes, particularly Gadd45a and Gadd45g in the development of the nervous system at both a gross and cellular scale.

6.2.3 Molecular Mechanisms in Neurodevelopment

The molecular pathways mediated by the Gadd45 family in neurodevelopment are under investigation, but some studies have uncovered a link with the mechanisms of cell-cycle regulation and apoptosis (Fig. 6.7a, b). The low-penetrance exencephaly finding in Gadd45a-null homozygotes, for instance, has been linked to the

XPC-associated pathway (Patterson et al. 2006). XPC functions as an oxidative DNA damage repair factor, acting through both nucleotide- and base-excision repair (BER) mechanisms at a genome-wide scale. It also modulates tumor formation and redox homeostasis (Hollander et al. 1999; Melis et al. 2011). Indeed, *gadd45a*/*XPC-null* double mutant mice displayed no further increase in exencephaly rate compared to single mutants, suggesting an overlapping mechanism. In contrast, homozygous deletion of *Trp53*, the gene encoding the tumor-suppressing p53 protein, caused a substantial increase in neural tube closure rate compared to *gadd45a* homozygous single mutants (Patterson et al. 2006). This finding suggests Gadd45a and p53 operate through separate pathways in development, a surprising result in light of the indirect induction of the Gadd45a promoter by p53 (Zhan et al. 1998). Additionally, both genes contribute to genome stability, apoptosis, G1 cell-cycle checkpoint control through p21, and global genome repair. Concurrently, *gadd45a*/*Cdkn1a*-null mutants, those lacking both Gadd45a and the cyclin-dependent kinase inhibitor p21, also exhibited pronounced exencephaly rates versus single mutants (Patterson et al. 2006). At a subcellular level, Gadd45a-null mice exhibit genomic instability including aneuploidy, chromosomal aberrations, centrosome instability, and abnormal growth and mitosis. The lack of observed alterations in apoptosis rate in most cell lines and lack of a phenotype in induction of JNK and p38 kinase cascades upon cytotoxic stress in Gadd45a-null cells indicate that the mechanism for the observed phenotype may involve perturbed G2/M progression rather than p53-dependent cell death (Hollander et al. 1999). Accordingly, Gadd45a-null cells exhibit attenuated G2 checkpoint activation upon DNA damage (Wang et al. 1999). Future studies are needed to determine whether this mechanism underlies neural tube defects.

A second proposed mechanism for Gadd45a function in brain development involves the MAPK kinase kinase (mitogen-activated protein kinase kinase kinase), MEKK4, which is known to promote neuronal migration and maturation (Sarkisian et al. 2006; Yamauchi et al. 2007). Gadd45a directly binds and activates MEKK4 (Takekawa and Saito 1998). Furthermore, knockdown of MEKK4 reduced neurite arborization in cortical neurons in vitro, recapitulating the effect of Gadd45a reduction and suggesting a common functional pathway in neuron maturation (Sarkisian and Siebzehnrubl 2012). The Gadd45a-MEKK4 pathway furthermore has been shown to stimulate phosphorylation of c-Jun N-terminal kinase (JNK), a subfamily of MAPK proteins, and this pathway modulates neurite outgrowth in a neuroblastoma cell culture (Yamauchi et al. 2007). This study additionally identified this linear pathway as a target of the mood-stabilizer valproic acid (VPA). VPA is used to treat manic-depressive states by regulating a number of mechanisms targeting neurotransmitter uptake and catabolism, postsynaptic receptors, and histone deacetylation. VPA has been shown to stimulate adult neurogenesis, neurite extension, and neuroprotective mechanisms (Coyle and Duman 2003). VPA directly induces *gadd45a* expression in fibroblast and neuroblastoma cultures and adult cortical neurons (Yamauchi et al. 2007; Sarkisian and Siebzehnrubl 2012). Furthermore, in neuroblastoma cells, VPA was shown to induce *gadd45a* expression in association with neurite extension. Gadd45a is necessary for the VPA-induced neurite

extension effect, and overexpression of *gadd45a* is sufficient to recapitulate this phenotype. Both VPA and Gadd45a require MEKK4 expression to stimulate cell maturation, suggesting a conserved role of the Gadd45a–MEKK4 interaction in neuronal maturation. Additionally, this interaction promotes the canonical MAPK cascade upon VPA exposure: Both VPA and *gadd45a* overexpression were found to enhance phosphorylation of JNK and its focal adhesion protein effector, paxillin, and this is dependent on MEKK4. Finally, neurite extension induced by both VPA and Gadd45a requires the ability to phosphorylate both JNK and paxillin. Interestingly, the activity of ERK, a separate MAPK, is necessary for maturation by VPA but not Gadd45a, suggesting VPA requires a broader range of MAPK function to exert a morphological effect. In a separate paradigm in which neurite extension is promoted by depriving cells of serum, JNK and paxillin phosphorylation is necessary but only partially sensitive to Gadd45a knockdown (Yamauchi et al. 2006). Taken together, these findings reveal that Gadd45a mediates neuronal maturation to differing extents in a context-dependent manner. The in vivo developmental implications of this result, however, are not fully clear. Furthermore, the VPA- and serum deprivation-induced pathways may have a convergent point, but it is unclear where this lies.

Gadd45a has also been identified as a downstream target of Sonic hedgehog (Shh), a morphogen with diverse neurodevelopmental roles (Galvin et al. 2008). Shh is known to signal through the Gli family of transcription factors; in neural stem cells, Shh was found to induce Gli1 selectively, and overexpression of Gli1 strongly induced *gadd45a* transcription. *Gadd45a* overexpression in neural stem cells induces the G2/M phase arrest and the prevalence of the proapoptotic marker, activated caspase-3. Gli1 may bind the *gadd45a* promoter directly or may act indirectly, such as via the p53 pathway. It is also hypothesized that Gadd45a functions in neural stem cell mitosis may coordinate the activity of the Cdc2 kinase and cyclin B1 as it does in other cell lines (Jin et al. 2002; Maeda et al. 2002). In light of the potential role of the Shh–Gli1 pathway in adult neurogenesis within the hippocampus and subventricular zones of the brain, it is additionally possible that Gadd45a may influence this process. However, adult neurogenesis has not yet been assessed in *gadd45a* mutants. Taken together, these results show Gadd45a likely functions through at least two signaling pathways which may regulate different aspects of nervous system development at both an anatomical and cellular scale.

The mechanistic basis for Gadd45b and Gadd45g in nervous tissue development has been less thoroughly studied. However, both proteins are also involved in MEKK4 activation in human cells, suggesting possible roles in development that may also be redundant with those of Gadd45a (Takekawa and Saito 1998). In a murine embryonic carcinoma cell line, each of the *gadd45* genes was robustly upregulated in response to retinoic acid (RA), a universal morphogen critical to mammalian embryonic patterning (Sheng et al. 2010). RA binds a heterodimeric nuclear complex and coordinates a host of transcriptional events in development. In neural development, RA was shown to antagonize signaling by bone morphogenetic protein (BMP), a crucial developmental player whose activation stimulates a cascade of phosphorylation of Smad cytoplasmic effectors. Activated Smad

complexes translocate to the nucleus to orchestrate a pattern of gene expression affecting the developmental trajectory of the tissue. In the mammalian dorsal–ventral axis, BMP signaling is restricted to the dorsal region whereas RA signaling resides in the intermediate region. During neurulation, BMP naturally promotes proliferation of progenitor cells and inhibits neurogenesis. In the chick neural tube, it was confirmed that RA suppresses BMP signaling and promotes proliferation and differentiation of neural progenitor cells (Sheng et al. 2010). In the dorsal neural tube, the interplay between these factors also coordinates specification of neural progenitors in the primitive spinal cord. The induction of *gadd45b* and *gadd45g* in vitro appears to be functional in the RA-induced downregulation of phospho-Smad1. Indeed, reduction in Gadd45b and Gadd45g expression restores pSmad1 in the presence of RA and disrupts the RA-induced association between pSmad1 and Smurf2, an E3 ubiquitin ligase. Since MAPK signaling was similarly shown to mediate the reduction in pSmad1, it is a reasonable to hypothesize that RA-mediated induction of the *gadd45* genes triggers a MAPK cascade which leads to the reduction in pSmad1 levels and that the resulting alterations in transcriptional programming promotes a neuronal cell phenotype. The mechanisms by which Gadd45b and Gadd45g regulate neuronal differentiation are also not fully clear. Indeed, it appears Gadd45g influences molecular pathways of cell-cycle arrest and hence promotes neuronal development indirectly rather than directly (Candal et al. 2005).

The breadth of upstream regulators of transcription of the Gadd45 family in neurogenesis is complex and not fully understood. For instance, in a similar culture system, the proneurogenic protein Ascl1, a member of the basic helix-loop-helix family of transcription factors, was shown to induce *gadd45g* by binding two E-box elements in its promoter (Huang et al. 2010). Neurogenin-2 and Mash1, regulators of dorsal and ventral telencephalon development, respectively, similarly coordinate *gadd45g* transcription (Gohlke et al. 2008). Since these transcriptional factors regulate glutamatergic and GABAergic neuron development, respectively, additional studies are needed to address the possible function of Gadd45g in either or both of these classes. Additionally, the in vivo function of Ascl1 during neuronal differentiation in the embryo has not yet been confirmed. One additional pathway regulating Gadd45g expression in conjunction with *Xenopus* neuronal development involves the *Iroquois* family of genes encoding repressors of neural differentiation (de la Calle-Mustienes et al. 2002). Indeed, *Xiro1* overexpression suppresses *gadd45g* transcription, but this may be an indirect consequence of its induction of neuronal repressors. In early *Xenopus* neural development, *gadd45g* expression prefigures many territories where cells will stop dividing, and it is hypothesized that it links cell-cycle arrest, neuronal differentiation, and notch signaling. In the neural plate, cells expressing the notch receptor XDl1 escape lateral inhibition from nearby cells; concurrent expression of *gadd45g* in these cells permits them to exit the cell cycle and differentiate into neurons through a process likely dependent on cyclin and cyclin-dependent kinases. Indeed, knockdown of Gadd45a and Gadd45g produced growth arrest and upregulation of cell-cycle inhibitors p21, p15, and cyclin G1, as well as p53 (Kaufmann and Niehrs 2011). In contrast, neighboring cells undergoing notch signaling exhibit decreased expression of *gadd45g* and proneural genes, and

these fail to undergo differentiation (Gohlke et al. 2008). Finally, it should be noted that the phenotypes observed from single gene manipulation studies may belie the full effect of the Gadd45 family because of compensatory autoregulation (Kaufmann and Niehrs 2011).

6.3 Neuronal Lesions

The majority of studies of the Gadd45 genes in nervous system focuses on their dynamic regulation and function in pathological states. Here, we review the role of the Gadd45 family in conditions of ischemia, physical and chemical injury, neoplasm, and seizure-associated excitotoxicity.

6.3.1 Ischemia

Early studies of Gadd45a in the brain argued for a key role in regulating neuronal response to damage by conditions of low perfusion. In cases of global or localized ischemia, oxygen and glucose deprivation result in ATP reduction and energy depletion, and this often triggers excitotoxicity and cell death due to excessive glutamate release and stimulation of intracellular calcium signaling (Taoufik and Probert 2008). This condition is most prevalent in human stroke, and ongoing studies aim to uncover the underlying aberrant signaling mechanisms and novel therapeutic avenues.

In a neonatal rodent model of focal ischemia involving unilateral, transient occlusion of the common carotid artery, a well-delineated cortical infarct showing characteristic molecular and morphological features of apoptosis is produced after reperfusion (Charriaut-Marlangue et al. 1999). Gadd45a protein levels were elevated in cortical layers II and III, but the most salient feature of this upregulation is its association with cells that did not experience DNA fragmentation as evidenced by the TUNEL stain. Furthermore, these cells were largely localized in the penumbra, the border of the infarct. In contrast, adjacent TUNEL-positive cells largely failed to show upregulation of Gadd45a (Fig. 6.2). The authors posed that Gadd45a plays a protective function in cells experiencing ischemic stress. However, this expression may decrease once double-stranded DNA breaks become prevalent and defy DNA repair mechanisms; these cells may then become committed to apoptosis. This model predicts that the growing infarct is always delineated by a Gadd45a-positive border which demarcates the extent of cerebral damage and that the border expands as damaged cells lose Gadd45a expression. Similarly, another study of transient focal ischemia confirmed that Gadd45a mRNA increases broadly throughout the ischemic cortex 4 h after the onset of damage but is restricted to the penumbra after 24 h (Jin et al. 1996). At both time points, Gadd45a protein is more selectively expressed in sublethally injured cells of the penumbra. This also suggests a pretranslational mechanism controls Gadd45a levels in association with

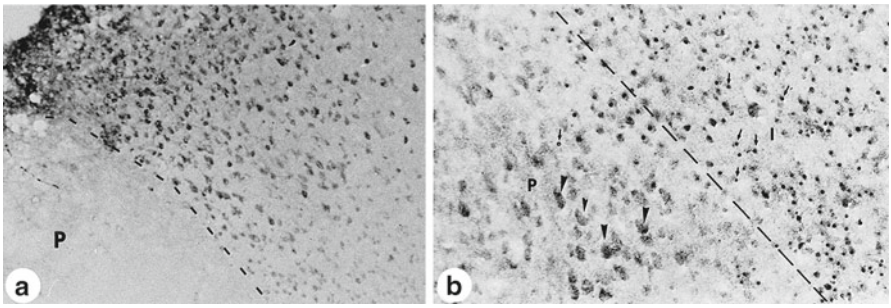

Fig. 6.2 Expression of Gadd45a protein in the cortex after ischemia induced by unilateral MCA occlusion in the neonatal rat. (**a**) After 24 h, Gadd45a was detected in layers II and III and largely absent from the penumbra (P). (**b**) Double staining for Gadd45a immunoreactivity and TUNEL assay performed at 48 h. *Small arrows* indicate TUNEL-positive nuclei, which are mostly localized to the infarct (I) rather than the penumbra. *Arrowheads* delineate Gadd45a-positive cells in the penumbra, suggesting a protective function of Gadd45a. Reproduced with permission from Charriaut-Marlangue et al. (1999)

sublethal degrees of ischemic damage. In similar models, increases in Gadd45a transcripts were found in pyramidal neurons, the principal excitatory units of the cortex, at the edge of the infarct; additionally, this elevation was found in reversibly damaged cells (Hou et al. 1997; Li et al. 1997b). Expression of the Gadd45 family in relation to the survival rate of interneurons after ischemic damage, however, has not been investigated. The pattern of Gadd45a expression broadly during induction of neuronal damage and more focally during the recovery phase was replicated in a similar focal ischemia model (Schmidt-Kastner et al. 1998). *Gadd45a* expression was most strongly associated with modestly damaged brain regions, suggesting a protective function. Even the dorsomedial cortex and dorsal hippocampus, regions spared from damage, exhibited enhanced Gadd45a levels. These responses could represent a prophylactic mechanism by cells sensing distant ischemic damage possibly because of spreading waves of perifocal depolarization and resultant gene induction in unaffected brain regions. Accordingly, in the hippocampus, forebrain ischemia produces strong Gadd45a induction in apoptotic granule cells of the dentate gyrus but only weak induction in largely necrotic CA1 pyramidal cells (Li et al. 1997a).

The hippocampus is still subject to ischemic damage in other rodent models and shows similar patterns of Gadd45a induction, particularly 24 h after ischemia (Wang et al. 2011). Additionally, in a model of transient global ischemia in the rodent brain, Gadd45a transcripts were rapidly induced in the dentate gyrus and, shortly afterwards, in the CA1 and CA3 subfields (Chen et al. 1998). Expression remained elevated 24–48 h after ischemia only in CA1. Interestingly, while protein expression was induced in all three regions, by 72 h, expression was reduced below baseline in CA1. Similarly, neurons with double-stranded breaks failed to show Gadd45a expression in the striatum and thalamus. This finding correlates with strong evidence of DNA fragmentation at this time point, suggesting again that Gadd45a expression in global ischemia plays a protective role after sensing DNA damage but is no longer induced when cells, predominantly neurons, are largely damaged.

Little is known of the molecular mechanisms by which the Gadd45 genes potentially influence post-ischemia recovery. One study, however, points to NF-κB in a model of neonatal hypoxia–ischemia (Nijboer et al. 2009). This transcription factor regulates numerous target genes during inflammation and influences cell death and survival. In ischemia, NF-κB inhibition protects the brain from injury and blocks ischemia-induced upregulation of *gadd45b*. Since Gadd45b is known to inhibit JNK signaling and subsequent activation of the transcription factor AP-1, the authors speculate that Gadd45b mediates signaling between NF-κB and the JNK cascade in association with cell survival (De Smaele et al. 2001). Indeed, inhibition of JNK abrogated the neuroprotective effect of NF-κB inhibition. However, a causative role of Gadd45b or Gadd45a, which similarly mediates MAPK signaling, in cerebral ischemia has not yet been established.

Given this speculative role in recovery from ischemia, Gadd45b inhibition could serve as a novel therapeutic target in stroke. However, one study which showed cerebral ischemia induces *gadd45b* expression in the cortex contradicts this notion (Liu et al. 2012). Electrical stimulation of the fastigial nucleus of the cerebellum was found to induce further cortical Gadd45b expression, particularly in cell nuclei. This treatment improved recovery from the injury as assessed by a motor task 1 month after the injury. This suggests a positive association between Gadd45b expression and stroke recovery. Further studies are needed to delineate the precise role of the Gadd45 genes in ischemic recovery and to investigate the therapeutic potential of targeting their expression and function.

6.3.2 Neuronal Injury

Numerous studies have investigated expression patterns and functionality of the Gadd45 family after neuronal lesions. Here, we summarize results pertaining to both physical and chemical lesion models.

6.3.2.1 Physical Neuronal Lesions

A number of studies have assessed Gadd45 transcription in association with peripheral nerve injury in rodent models. Transection of the sciatic nerve (axotomy) has been shown to induce *gadd45a* transcription robustly in dorsal root ganglion (DRG) cells, the primary afferent stream for peripheral sensory information, days after the injury (Costigan et al. 2002; Xiao et al. 2002; Befort et al. 2003; Lin et al. 2011). *Gadd45g* is induced to a lesser extent, whereas *gadd45b* is only modestly upregulated in the DRG, suggesting all three Gadd45 genes may mediate the injury response but that Gadd45a plays the strongest role (Befort et al. 2003). Supporting this hypothesis is the co-localization of Gadd45a transcripts with c-jun and ATF3, markers of injury, in primary sensory neurons of the DRG as well as motor neurons in the ventral spinal cord after sciatic nerve transection (Fig. 6.3). This is a surprising finding because these motor neurons and DRG cells have cell bodies located in

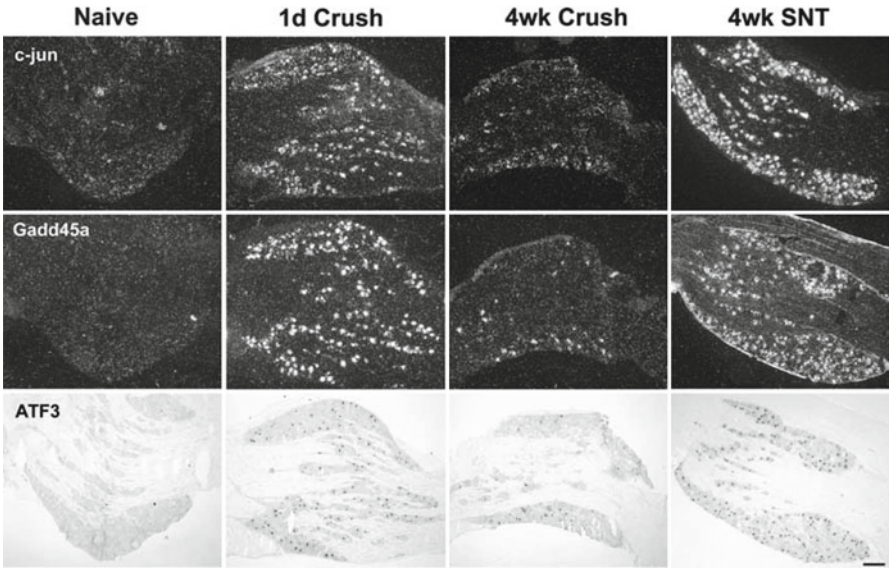

Fig. 6.3 Expression of *gadd45a* mRNA in the dorsal root ganglion (DRG) after sciatic nerve injury. Shown are sections of L4 DRGs from naïve, 1 day or 4 weeks after crush injury sections. Additionally, 4-week post-sciatic nerve transection (SNT) sections are displayed. Transcripts of c-jun (*top row*), Gadd45a (*middle row*), and ATF3 (*bottom row*) are presented. One day after crush injury, all markers are upregulated, and Gadd45a expression remains elevated when regrowth is prevented (4 weeks transection with ligation). Gadd45a expression is downregulated in the DRG when peripheral nerve regrowth after crush injury has completed (4-week crush). *Scale bar*, 200 **μ**m. Reproduced with permission from Befort et al. (2003)

different regions, but both cell types have axons running in the periphery and the ability to survive and regenerate following injury. Similarly, Gadd45a is induced across all cell types broadly in the DRG after other peripheral nerve lesions such as the more proximal spinal segmental nerve lesion and sciatic nerve crush. Likewise, Gadd45a is upregulated after more distal lesions including chronic constriction of the sciatic nerve and spared nerve injury in which the tibial and common peroneal nerves are ligated and sectioned.

In contrast, a dorsal rhizotomy lesion involving transection of dorsal roots proximal to the DRG cell bodies only modestly induced Gadd45a, suggesting Gadd45a plays different roles in transcriptional networks prompted by central and peripheral injuries (Befort et al. 2003). Indeed, the dorsal rhizotomy spares axonal contact between the periphery and DRG neurons and results in very little cell death and biochemical changes in comparison to those spurred by peripheral damage. It is interesting to note that injury-associated *gadd45a* induction in the DRG reverts to its pre-injury, virtually undetectable baseline level when injured axons are allowed to regrow and reinnervate peripheral targets. This reduction is not seen when repair is physically impaired, and this suggests a retrograde signal from the target cells attenuates *gadd45a* expression when the injury is repaired. Similarly, direct injury

to the spinal cord enhanced the expression of cell-cycle regulators including *gadd45a*, particularly in neurons (Di Giovanni et al. 2003). We also note the unique role of Gadd45a expression after injury in that embryonic and postnatal DRG cells do not express the gene at detectable levels (Befort et al. 2003). Hence, the injury-induced response is truly a de novo phenomenon and not merely a recapitulation of expression patterns in early ontogeny as is the case with other regeneration- and survival-associated genes such as GAP-43 and Hsp27.

These models are clinically relevant in relation to molecular mechanisms of neuropathic pain, defined as pain initiated by primary lesions or dysfunction of the nervous system. Indeed, *gadd45a* is induced along with other cell-cycle and apoptosis regulators in the DRG following spinal nerve transection or ligation (Wang et al. 2002; Xiao et al. 2002). However, one study challenges the notion that direct injury to afferent sensory nerves is required for pain-associated transcriptional programming; the authors examined the possibility that incisional pain induced by skin lesions that spare nerve injury could induce regeneration-associated transcription in the DRG (Hill et al. 2010). Indeed, DRG neurons innervating the affected skin region exhibited moderately enhanced *gadd45a* expression. This effect likely follows from sensitization of the neurons exposed to skin wound-related processes and inflammation and suggests tissue injury even in the absence of direct nerve injury can promote a state of enhanced growth capacity in sensory neurons. Gadd45a may regulate this process. However, induced acute local inflammation failed to upregulate *gadd45a* in the DRG cells innervating the corresponding peripheral site (Befort et al. 2003). One explanation for this discrepancy is that inflammation alone is insufficient to induce *gadd45a*; this reinforces the notion that *gadd45a* induction reflects a highly regulated response to the presence and maintenance of peripheral nerve injury and not merely the presence of cellular stress.

Recently, it has become clear that Gadd45a expression in the DRG not only correlates with peripheral nerve injury but also mediates survival potential of the afferents. After spinal nerve ligation, adult DRG cells remain largely intact and correlate with strong Gadd45a mRNA and protein induction patterns, whereas neonatal DRG cells, which show undetectable *gadd45a* expression, are highly susceptible to cell death (Lin et al. 2011). Knockdown of Gadd45a in the DRG impairs survival and promotes apoptosis after injury. Concomitantly, overexpression of *gadd45a* in vitro protects DRG cells from nerve growth factor (NGF) withdrawal-induced apoptosis, and this effect may be mediated by maintenance of anti-apoptotic Bcl-x_L levels.

These studies of peripheral nerve lesion-induced *gadd45a* regulation suggest that Gadd45a-regulated survival mechanisms may be a promising target of therapy in neuropathic pain and denervation conditions. The utility of such therapy may not be limited to neurons, though, as skeletal muscle similarly experiences a prolific enhancement in *gadd45a* transcription after denervation (lower motor neuron loss) or spinal cord injury (upper motor neuron loss) (Zeman et al. 2009). Accordingly, the finding of Gadd45a elevation extends to other nerve injury models as well. After optic nerve transection, both primary (initial lesion site) and secondary (regions beyond the initial lesion site) neurodegeneration exhibits enhanced *gadd45a* transcripts (Levkovitch-Verbin et al. 2011). Interestingly, elevated protein expression

persists longer in the secondary region, a similar finding to the previously discussed reports of Gadd45a elevation in the penumbra of tissue damaged by ischemia. Elevation and nuclear translocation of Gadd45a and known binding partner PCNA were also found in apoptotic cells, localized to the margins of the cortical contusion and hippocampus, in a rodent traumatic brain injury model (Kaya et al. 1999). These studies further support the protective role of Gadd45a after neuronal insult. It is possible, however, that injury-related *gadd45b* expression plays an opposing role. For instance, mice carrying a mutation that confers protection against Wallerian degeneration, which involves fragmentation of axon segments separated from their somas, myelin sheath segmentation, and removal of debris by Schwann and immune cells, show reduced expression of *gadd45b* and other plasticity-associated genes compared to wild-type mice after sciatic nerve lesion (Barrette et al. 2010). Still, a causative role of Gadd45b in injury-related processes has not been established, and altered expression is not necessarily functional. Collectively, these results strongly implicate Gadd45a in the protection of neurons after physical nerve injury both proximal and distal to the affected cells.

6.3.2.2 Neuronal Injury by Nonphysical Insults

Nerve damage results not only from physical insults such as trauma and skin incisions discussed above but also from exposure to other environmental neurotoxins. A number of reports document damage-associated regulation of the Gadd45 family. Early studies, for instance, showed induction of *gadd45a* transcripts following gamma irradiation exposure in various tissue including the brain (Yoshida et al. 1994, 1996). This confirmed previous reports of similar *gadd45a* upregulation following ionizing radiation in vitro, including the original report identifying the *gadd45a* gene in irradiated Chinese hamster ovarian cells (Fornace et al. 1988; Papathanasiou et al. 1991). Irradiation-associated DNA damage is known to modulate p53, and it is postulated that this mechanism is responsible for Gadd45a signaling in response to cell damage (Yoshida et al. 1996).

Chemical injury associated with neuropathology produces similar induction patterns. Neurodegeneration characterized by ataxia, paralysis, and axonopathy follows from exposure to organophosphorus-ester chemicals, and a study of this condition in hens found enhanced *gadd45a* and *Bcl-2* expression in the cerebrum, cerebellum, brain stem, and spinal cord (Damodaran et al. 2011). Enhanced *gadd45g* expression was also associated with exposure to the environmental neurotoxin, carbonyl sulfide, in the posterior colliculi, an especially susceptible brain region that regulates auditory processing (Morrison et al. 2009). While few studies have investigated damage-induced Gadd45g induction in the brain, it is likely to play a similar role in cellular stress response as previously shown in chemical-, radiation- and inflammation-associated induction in other cell lines (Zhang et al. 1999; Jung et al. 2000). Chemical damage to the auditory system is also associated with altered *gadd45a* expression. For example, salicylic acid, the main ingredient in aspirin, impairs hearing at high concentrations; salicylic acid damaged spiral ganglion

neurons and peripheral fibers in rodent cochlear organotypic cultures and induced *gadd45a* in association with apoptosis (Wei et al. 2010). Its temporal expression recapitulates the protective pattern found in ischemia and physical nerve lesion studies in the auditory system in a model of noise-induced apoptosis in the cochlear epithelium and lateral wall (Hu et al. 2009). Namely, *gadd45a* expression is enhanced shortly after noise exposure but is reduced even below baseline after 1 week during a broad proapoptotic response. Thus, *gadd45a* expression peaks when cells are trying to stay alive but falls once they "give up" and enter apoptosis.

An alternative mechanism of chemical injury associated with altered Gadd45 signaling involves toxicity resulting from excessive neuronal activity. This can be induced by pharmacological agents that boost excitatory, glutamatergic signaling or impair inhibitory tone. For instance, quinolinic acid, an NMDA receptor agonist and driver of neuronal activity, injected into the striatum produces DNA fragmentation, p53 activation, and enhanced Gadd45a transcript and protein expression in the striatum and cortex (Hughes et al. 1996). Gadd45a exhibited both rapid and prolonged upregulation, and it is hypothesized to be regulated initially by immediate early gene (IEG) transcription factors such as Fos and Jun-B and later by nuclear p53. In a different model, enhancing glutamatergic signaling by chemical inhibition of glutamate reuptake in the striatum prompted *gadd45a* expression (Lievens et al. 2000). Enhanced transcripts were found in the periphery of the lesion after short-term lesion, reflecting both neuronal and glial expression, but after long-term quinolinic acid treatment, Gadd45a was only upregulated within the lesion core, composed mostly of reactive astrocytes. These results suggest Gadd45a is associated with neuroprotection and preventing the core lesion site from expanding and that its protective effects may not be limited to neurons.

6.3.3 Neoplasia

An extensive literature characterizes the aberrant function and regulation of the Gadd45 family in various cancers including pancreatic, hepatocellular, lung, cervical, and gastrointestinal carcinomas and different lymphomas (Tamura et al. 2012). The *gadd45* genes contain genetic or epigenetic alterations in these conditions. Accordingly, the Gadd45 family has emerged as a potential target in antitumor therapy including drugs that promote expression by upstream signaling control and epigenetic regulation.

Studies examining the Gadd45 proteins in nervous system neoplasias have predominantly focused on pituitary adenoma, pilocytic astrocytoma, and medulloblastoma. Pituitary adenomas are monoclonal tumors and the most prevalent intracranial neoplasms and present clinically in relation to the specific subclass of cells undergoing growth; these include cells that secrete prolactin, growth hormone, ACTH, and gonadotropes. Postmortem analysis of human gonadotrope tumors revealed substantially reduced Gadd45b and Gadd45g mRNA and protein but normal Gadd45a expression (Zhang et al. 2002; Michaelis et al. 2011). Additionally, most

nonfunctioning, growth hormone-secreting, and prolactin-secreting pituitary tumors failed to show detectable Gadd45g transcripts whereas expression was found unanimously in normal pituitary tissue (Zhang et al. 2002). These reductions likely play a functional role in tumorigenesis because overexpression of *gadd45g* in various pituitary tumor lines and *gadd45b* in gonadotrope tumors attenuates colony formation in vitro. Gadd45b was also shown to promote apoptosis in gonadotrope cells. These proteins likely act through common G1/S and G2/M growth arrest mechanisms. However, their upstream regulation is unclear, as p53 gene mutations are not common and NF-κB is not upregulated in pituitary tumors (Michaelis et al. 2011). Additionally, these mechanisms exhibit cell origin-specificity, as ACTH-releasing tumors demonstrated a slight upregulation, not reduction, of *gadd45b*.

Although Gadd45a has not been shown to modulate pituitary neoplasms, its expression is enhanced in pilocytic astrocytoma tissue (Jacob et al. 2011). These low-grade, sporadic tumors predominate childhood CNS neoplasms and tend to affect the cerebellum and optic nerve pathways. Altered MAPK activation in pilocytic astrocytomas is thought to drive senescence, a hallmark of these tumor cells. Gadd45a and other senescence markers are speculated to drive this activity; of note, Gadd45a has been shown to promote MAPK-induced senescence in skin cancer (Hildesheim et al. 2002).

Altered Gadd45a signaling has also been implicated in cerebellum-originating medulloblastoma, the most common malignant CNS tumor in children (Chou et al. 2001; Asuthkar et al. 2011). In tumor cells, NGF causes apoptosis by binding the receptor, TrkA, and this interaction is necessary for coincident NGF-induced *gadd45a* expression (Chou et al. 2001). Mutations in TrkA that block apoptosis also abolish *gadd45a* induction, providing correlational evidence that Gadd45a regulates NGF-induced apoptosis. This study intriguingly supports evidence for an entirely novel pathway for Gadd45a regulation by NGF; NGF-induced ERK, p38, and JNK activation (all MAPK proteins) is similar in medulloblastoma and pheochromocytoma lines in which NGF produces opposite effects on apoptosis. Additionally, p38 was shown to be unnecessary for NGF-induced apoptosis in medulloblastoma cells. This suggests some CNS tumor lines harbor different mechanisms of *gadd45a* regulation that do not necessary rely on p53 and MAPK signaling. One possibility is a pathway mediated by the medulloblastoma-associated tumor suppressor gene, PATCHED1, and Sonic hedgehog signaling (Kappler et al. 2004).

Medulloblastoma cells also appear to utilize novel Gadd45a-dependent mechanisms in association with radiation exposure (Asuthkar et al. 2011). Radiotherapy attenuates tumor growth but also activates sporadic recurrences in part by activating matrix metallopeptidase (MMP)-9, which helps dissolve extracellular matrix to aid in cell migration (Goc et al. 2013). IR-induced growth arrest in cultured medulloblastoma cells was associated with enhanced expression of Gadd45a and its binding partner, Cdc2, and Gadd45a was shown to promote arrest and apoptosis (Asuthkar et al. 2011). The Gadd45a–Cdc2 interaction is likely important for growth arrest in these cells as was in colon carcinoma cells (Jin et al. 2000). *Gadd45a* overexpression blocked invasion potential of irradiated medulloblastoma cells and reduced MMP9 expression in vitro and in vivo (Asuthkar et al. 2011). Gadd45a also

promotes p53 activation and modulates the membrane expression of β-catenin and its binding partners E- and N-cadherin. This effect on transmembrane proteins likely affects invasion potential by regulating cell–cell contact. Together these findings implicate Gadd45a as a critical regulator of tumorigenesis that acts through diverse signaling pathways affecting cell growth, apoptosis, and cell–cell interaction.

6.3.4 Seizures

We previously discussed the involvement of the Gadd45 family in excitotoxicity induced by chemical lesions. Related to these findings are a number of studies documenting altered expression of these genes in association with seizures. Here we review these results, highlighting the neuroprotective role of these genes in excitotoxic conditions.

Seizures are defined as brief behavioral changes in response to abnormal, synchronized, and repetitive burst activity of neuron populations, and epilepsy is characterized as a syndrome of recurrent, spontaneous seizures (Shin and McNamara 1994). Partial seizures emanate from a localized brain region whereas generalized seizures exhibit diverse, bilateral activity. Excessive glutamatergic signaling characterizes seizure activity and associated necrotica, apoptotic, and autophagic cell death (Wang and Qin 2010). Aberrant downstream mechanisms include intracellular calcium homeostasis, free radical production, kinase and protease activity, transcription factor activity, and IEG activation.

Human temporal lobe epilepsy, in which excitotoxic cell death appears in the limbic system and related structures, is commonly modeled in the rodent by kainate treatment (Zhu et al. 1997). Kainic acid (KA) activates a subclass of glutamate receptors and likely produces excitotoxicity by inducing sodium influx, depolarization and subsequent activation of NMDA receptors, and calcium influx. KA toxicity is associated with apoptosis and autophagy more than necrosis which often follows NMDA receptor-mediated cell death (Wang and Qin 2010). Subcutaneous KA injection was shown to induce Gadd45a expression in the parietal and piriform cortex, hippocampus, striatum, and thalamus, and focal intra-amygdala KA similarly induced expression in the limbic system, thalamus, and cortex (Zhu et al. 1997; Henshall et al. 1999). Moreover, intracerebral KA induced *gadd45a* expression in the hippocampus (Choi et al. 2011). Transcript expression was largely localized to neurons, and enhanced protein expression was also confirmed (Zhu et al. 1997). Moreover, both studies showed evidence of DNA fragmentation and apoptosis, especially in the CA3 subfield. *Gadd45a* transcription after subcutaneous KA is initially widespread but later remains elevated only in vulnerable regions. However, these regions exhibit decreased Gadd45a protein levels that coincide with the extent of neurodegeneration, suggesting that dying cells experience a translational block of Gadd45a as with other cell survival genes (Zhu et al. 1997). Amygdala-evoked seizures produced cell death and DNA fragmentation only in CA3 even though *gadd45a* transcription was widespread (Henshall et al. 1999). Still, transcript expression

was highest in CA3; these findings reinforce the conclusion that cells upregulate *gadd45a* in response to insults in an effort to remain viable but that translation may eventually be impaired as cells commit to apoptosis. KA-induced *gadd45a* induction may occur in part through p53 signaling, but p53 is only activated in vulnerable regions after systemic KA (Zhu et al. 1997). Alternatively, KA-induced DNA modification in the form of 8-hydoxyl-2-deoxyguanosine may spur *gadd45a* expression as previously shown (Henshall et al. 1999).

Nevertheless, as with other forms of neurotoxicity, glutamate-driven *gadd45a* expression plays an ambiguous role in cell survival. In murine hippocampal cultures, for instance, glutamate treatment induces Gadd45a mRNA and protein expression in association with cell death, but knockdown of Gadd45a or p53, which is also phosphorylated by the treatment, rescues cell viability (Fig. 6.4) (Choi et al. 2011). However, this cell line lacks ionotropic glutamate receptors but is still subject to oxidative neurotoxicity. Accordingly, inhibition of reactive oxidative species signaling prevented glutamate-induced *gadd45a* induction. This study also provides evidence for a cell death model in which glutamate signaling induces oxidative species which then activates the MKK4-JNK-p53 pathway, triggering *gadd45a* transcription and apoptosis. It appears, therefore, that Gadd45a promotes cell death under certain conditions such as oxidative stress but may prevent excitotoxic cell death in vivo, a scenario that likely drives its expression through multiple pathways including non-p53 signaling. At a molecular level, however, it is still unclear what characterizes the protective or death-inducing role of Gadd45a.

In contrast, Gadd45b and Gadd45g appear to play a less equivocal role in neuronal survival after excitotoxic insults. In hippocampal neurons, cAMP response element-binding protein (CREB), a key mediator of synaptic activity-dependent gene expression, promotes expression of both of these genes and coincidently confers protection against excitotoxic cell death (Tan et al. 2012). Synaptic neuronal activity exerts a neuroprotective effect against future insults in part through regulation of a class of activity-regulated inhibitor of death (AID) genes including *gadd45b* and *gadd45g* (Zhang et al. 2009). Glutamate-driven calcium signaling activates nuclear calmodulin-dependent protein kinase IV (CAMKIV), a critical mediator of CREB-dependent transcription of AID genes after periods of action potential bursting. Indeed, manipulations of *gadd45b* and *gadd45g* confirmed that these genes confer protection against chemically and growth factor withdrawal-induced cell death in vitro and mediate activity-dependent resistance to cell death. Moreover, these genes ameliorate KA-induced hippocampal cell death in vivo. However, expression of the Gadd45 proteins may not always change, as KA-induced seizure preconditioning, a protective paradigm against future insults, failed to induce Gadd45b protein in the hippocampus (Miller-Delaney et al. 2012).

In adult rodents, seizures are associated with enhanced neurogenesis in the dentate gyrus, a unique niche of postnatal mitosis in the CNS (Naegele 2009). However, a consequence of hyperactivity-driven neurogenesis is that newborn neurons fail to integrate normally into granule cell layer targets and instead migrate to ectopic locations in the hilus; improper hippocampal rewiring often spurs hyperexcitability and

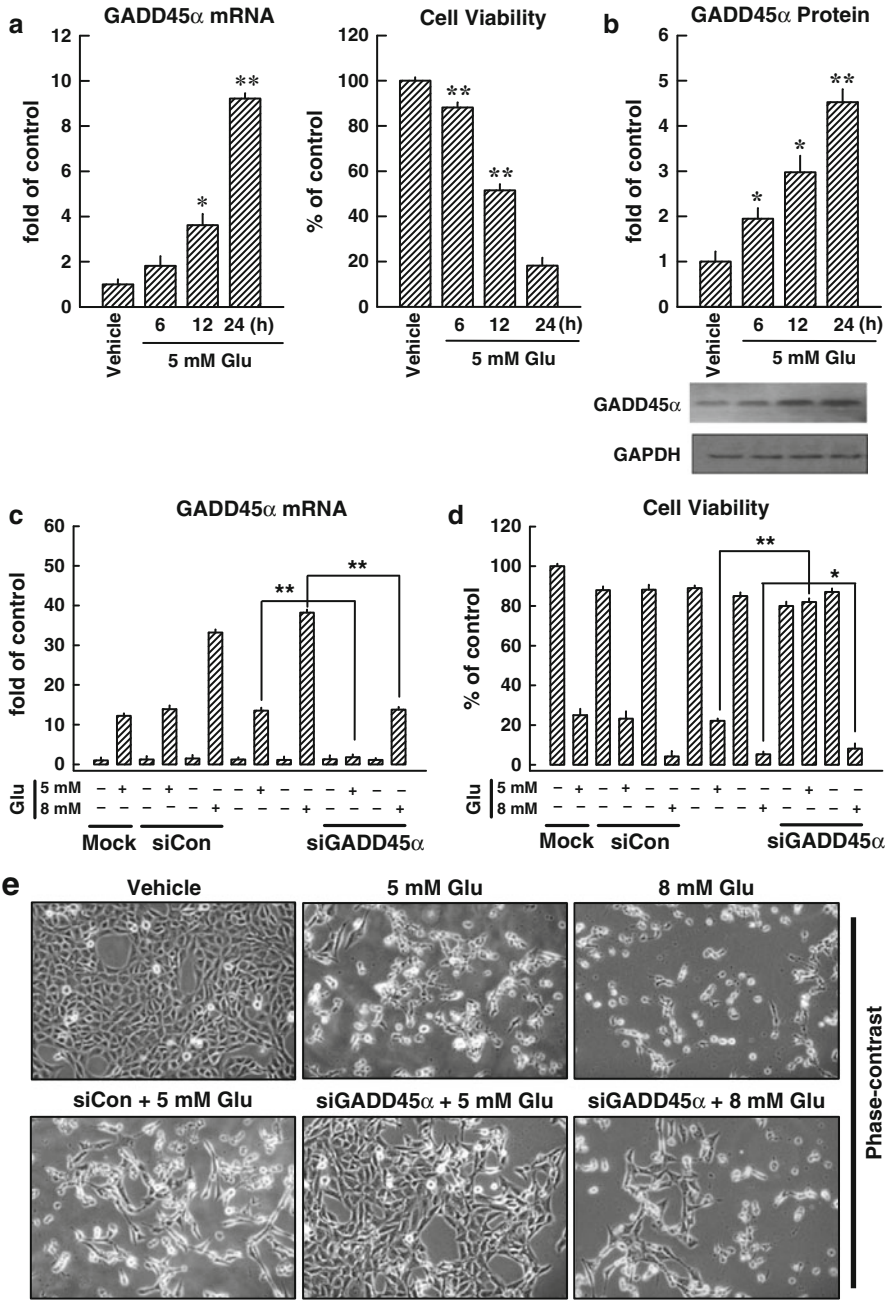

Fig. 6.4 Gadd45a mediates glutamate-induced oxidative cytotoxicity in HT22 hippocampal neuronal cell line. (**a**) *Gadd45a* transcripts and (**b**) Gadd45a protein levels were quantified after 5 mM glutamate treatment. Enhanced expression coincided with attenuated cell viability, assessed through MTT assay. (**c**–**e**) Cells were treated with 5 or 8 mM glutamate for 24 h after 24 h of pre-treatment with Gadd45a siRNA. RT-PCR, MTT assay, and phase contrast microscopy were used to confirm knockdown of *gadd45a* and rescue of cytotoxicity induced by 5 mM glutamate. Reproduced with permission from Choi et al. (2011)

* - $p < 0.05$, ** - $p < 0.01$

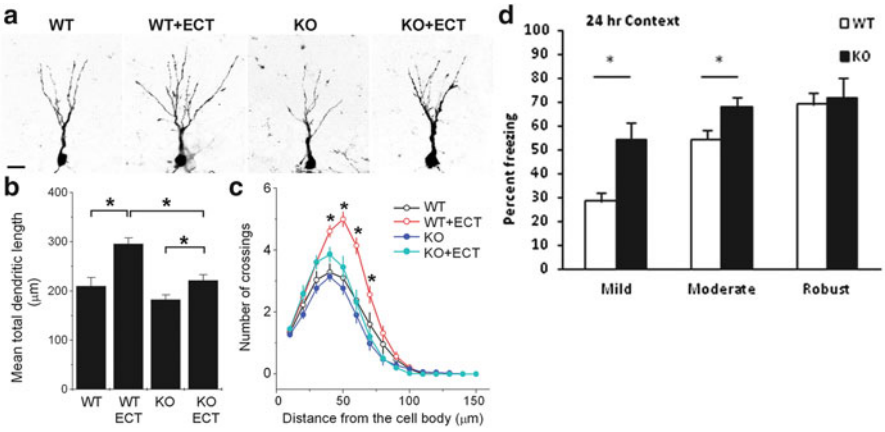

Fig. 6.5 Essential role of Gadd45b in activity-associated phenotypes in the adult CNS. (**a**–**c**) Wild-type and *gadd45b*-null mutant mice were injected with retroviruses expressing GFP to label adult-generated neural progenitors and their progeny. A single episode of electroconvulsive treatment (ECT) or sham treatment was given after 3 days, and dendritic morphology was assessed 14 days after virus injection. Mean dendritic length and dendritic complexity were impaired in knockout mutants after ECT but not in naïve mice. (**d**) Wild-type and *gadd45b*-null mutants were subjected to one of three background contextual fear conditioning paradigms of increasing robustness. Mutants exhibited heightened contextual fear memory 24 h after training, and this phenotype was most pronounced after mild conditioning. Reproduced with permission from Ma et al. (2009) (**a**–**c**) and Sultan et al. (2012) (**d**)
In sections b and c, * means $p < 0.01$; In d, * means $p < 0.05$

epileptogenesis. Seizures were found to upregulate *gadd45g* and, more so, *gadd45b* in the dentate gyrus granule cell layer (Ma et al. 2009). Furthermore, Gadd45b knockdown or knockout impaired activity-driven proliferation of neural progenitors and dendritic development of newborn neurons (Fig. 6.5a–c). Together, these findings show Gadd45b and Gadd45g are especially critical in periods of hyperexcitability in guarding against cell death and, in the case of Gadd45b, postnatal mitosis and development.

6.4 Cognitive Neuroepigenetics

In the preceding discussion, we focused on mechanisms of the Gadd45 family in a host of conditions of cell proliferation and injury in the nervous system. Here we focus on the relatively recently appreciated contribution of the Gadd45 family to the emerging field of cognitive neuroepigenetics (Day and Sweatt 2010). The most salient difference between these and the previously discussed mechanisms is their function in normal, senescent adult neurons rather than those undergoing programmed cell death or proliferation. Neuroepigenetic mechanisms have instead

been studied most exquisitely in relation to plasticity-associated mechanisms in neuronal function.

The term "epigenetics" was coined by Conrad Waddington who speculated that a landscape of pretranscriptional mechanisms mediates gene–environment interactions which control the phenotype of the cell. Epigenetic mechanisms were traditionally defined as heritable alterations in a chromosome that affect gene expression without affecting the underlying DNA sequence (Berger et al. 2009). A number of distinct molecular mechanisms fit this definition, but posttranslational modifications of histones and DNA methylation are considered the two canonical epigenetic phenomena.

In the nucleus, DNA is packaged in an elegant, hierarchical fashion. The first layer of compaction involves demarcation of nucleosomes, each of which is comprised of 147 bp of DNA wrapped around an octomer of histone proteins and a linker region with variable length in accordance with cell type and organism (Sadeh and Allis 2011). Nucleosomes are organized in nonrandom, regularly spaced arrays throughout the genome, and complex molecular regulators of nucleosome positioning and density are potent mediators of cis-acting transcriptional dynamics. Core histone units are composed of central globular domains and projecting N-terminal tails which bear several residues subject to modification by acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (Sultan and Day 2011). A number of residue-specific "writer" and "eraser" enzymes corresponding to these marks have been characterized; likewise, "reader" proteins help translate the mark into an enhancement or suppression of transcription (Maze et al. 2013).

In addition to core histones and regulatory DNA-binding proteins, DNA itself also constitutes chromatin and can undergo covalent modification (Day and Sweatt 2011). Epigenetic DNA methylation usually refers to the addition of a methyl group to the 5′ position on a cytosine ring. Usually the target cytosine is followed by a guanine, termed a "CpG site." CpG sites occur at a lower frequency than statistically expected and tend to cluster in regions called "CpG islands," spans of DNA containing a high frequency of CpGs that are largely unmethylated. This is unsurprising, as CpG islands tend to exist at promoter regions of active genes, and DNA methylation usually (although not exclusively) represses transcription. DNA methylation is catalyzed by DNA methyltransferase enzymes, DNMT1, DNMT3a, and DNMT3b in adults (Grayson and Guidotti 2013). DNMT1 maintains DNA methylation in hemimethylated DNA strands after cell division so that complementary CpGs both carry the methyl mark. DNMT3a and DNMT3b catalyze de novo methylation by single carbon transfer from *S*-adenosylmethionine (SAM) to unmethylated cytosines. Methylcytosine readers include numerous methyl-binding domain (MBD) proteins that facilitate a transition of local chromatin to a transcription-permissive state or repress it (Chahrour et al. 2008; Grayson and Guidotti 2013). Methylated cytosines can be passively demethylated after cell division if DNMT1 activity is suppressed, but reversing methylation in senescent cells, termed "active DNA demethylation," remains a vexing question and subject of future research (Wu and Sun 2009).

6.4.1 Memory

Memory formation proceeds in a sequence of steps of increasing stability after the learning event (Miyashita et al. 2008). During consolidation, a hippocampus-dependent process for certain types of learning paradigms, information progresses beyond the initially labile phase into a long-term, stable trace. Both consolidation and maintenance, a cortical event, rely on a diverse array of orchestrated de novo gene transcription (Sweatt 2009). This well-replicated finding motivated a number of studies that implicate dynamic histone modifications in memory formation (Day and Sweatt 2011). Contextual fear conditioning, for instance, induces global changes in both permissive and repressive marks including H3K9 dimethylation, H3K4 trimethylation, H3S10 phosphorylation, and numerous H3 and H4 acetylation events (Chwang et al. 2006; Gupta et al. 2010; Peleg et al. 2010). Of particular interest clinical interest is the memory-boosting effect of inhibiting histone deacetylase complex (HDAC) 2 in hippocampus-dependent memory (Levenson et al. 2004; Guan et al. 2009).

Persisting molecular signatures are theoretically required for memory trace stabilization; the need to identify such mechanisms that defy erasure during normal neuronal metabolism prompted investigations into the role of DNA methylation, long thought to be a stable epigenetic mark, in memory formation (Day and Sweatt 2010). Active methylation in the hippocampus was indeed shown to regulate fear memory consolidation and synaptic potentiation, a cellular correlate of behavioral memory (Levenson et al. 2006; Miller and Sweatt 2007; Feng et al. 2010). Although gene-specific methylation and demethylation events were found after training, these marks reverted to baseline levels after 1 day, suggesting hippocampal methylation dynamics do not modulate memory persistence (Miller and Sweatt 2007). Additionally, active demethylation of genes including *reelin* and *bdnf*, active memory regulators, spurred the hunt for a demethylation signaling cascade (Miller and Sweatt 2007; Lubin et al. 2008).

Gadd45a was then implicated in active DNA demethylation of exogenous genes including the Oct4 promoter in an in vitro system, but this finding remains equivocal (Barreto et al. 2007; Jin et al. 2008). In the nervous system, robust induction of Gadd45b expression was similarly implicated in activity-induced DNA demethylation of the fibroblast growth factor (FGF)-1B and BDNF exon IXa promoters in the dentate gyrus (Fig. 6.6a) (Ma et al. 2009). Moreover, induction of the corresponding genes was impaired in Gadd45b-null mice, suggesting Gadd45b-mediated demethylation functionally regulates gene transcription. Importantly, seizure induction did not produce significant cell death or excitotoxicity in wild-type or mutant mice. Therefore, the effects of Gadd45b deletion are likely to reflect only plasticity-related induction of trophic factors by mature granule cells rather than seizure-associated reductions in cell viability.

The characterization of Gadd45b as a hippocampal IEG that facilitates epigenetic regulation of BDNF encouraged studies of Gadd45b in hippocampus-dependent memory. Indeed, *gadd45b* and *gadd45g* but not *gadd45a* transcripts were found to

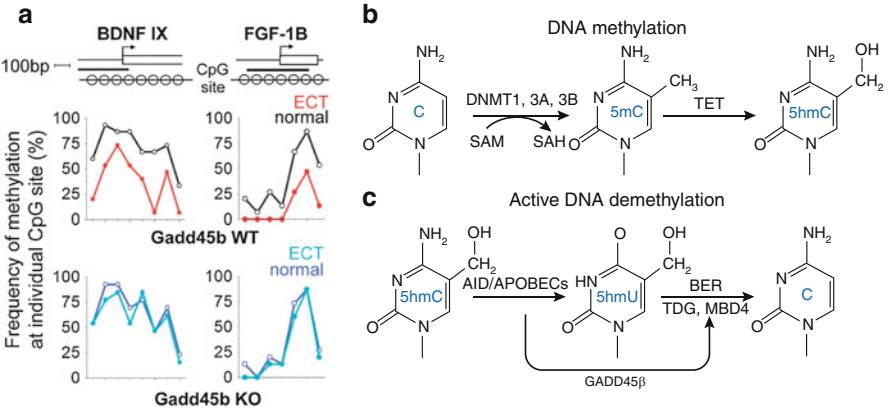

Fig. 6.6 Gadd45b regulates active DNA demethylation in an activity-associated manner. (**a**) Wild-type and *gadd45b*-null mutant mice were subjected to ECT or sham treatment. After 4 h, demethylation was found at the transcriptional start sites of *BDNF exon IX* and *FGF-1B* genes by sodium bisulfite sequencing of dentate gyrus tissue. Normal baseline DNA methylation was found between genotypes, but knockouts were impaired demethylation after ECT. (**b**, **c**) Schematics of the putative cytosine demethylation mechanism. DNMT enzymes catalyze single carbon transfer to produce 5-methylcytosine (5mC). The ten-eleven-translocase (TET) family of enzymes catalyze oxidation to 5-hydroxymethylcytosine (5hmC). TET1, TET3, and DNMT1 contain –CXXC– domains which bind clustered, unmethylated CpG dinucleotides. Oxidative deamination of 5hmC to 5-hydroxymethyluridine (5hmU) may occur through the AID (activity-induced cytidine deaminase)/APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) family of deaminases. Subsequently, the uracil-DNA glycosylase (UDG) family including thymine-DNA glycosylase (TDG, MBD4) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) are thought to process 5hmU through a base-excision repair (BER) mechanism. Additional intermediates such as 5-formylcytosine and 5-carboxylcytosine may be generated as well. Gadd45b may facilitate this process although the mechanism is unknown. Reproduced with permission from Ma et al. (2009) (**a**) and Grayson and Guidotti (2013) (**b**, **c**)

be upregulated following fear memory learning in the hippocampus and amygdala (Leach et al. 2012; Sultan et al. 2012). Both transcripts were also induced by potassium depolarization and tetrodotoxin withdrawal, a means of inducing quasi-synchronous activity, in neuron cultures (Saha et al. 2011; Sultan et al. 2012). Furthermore, Gadd45b-null mutants were found to exhibit enhanced hippocampus-dependent fear and spatial memory and long-term synaptic potentiation (Sultan et al. 2012). In particular, these enhancements were most prominent in conditions of mild behavioral and synaptic activity, suggesting Gadd45b regulates the threshold for memory formation but not capacity (Fig. 6.5d). Furthermore, mice performed normally in most baseline behavioral tasks and a test of amygdala-dependent fear memory, suggesting Gadd45b primarily functions in hippocampus-dependent tasks. One study, however, found contextual fear memory deficits in mutants (Leach et al. 2012). These conflicting results may reflect differences in task parameters or epigenome–genome interactions during backcrossing. Still, these studies agree that Gadd45b selectively regulates hippocampus-dependent memory.

The breadth of loci targeted by Gadd45b for epigenetic regulated is largely unknown and a subject for future investigation (Fig. 6.7c). A large number of putative of targets exist, as synchronous neuronal activation was shown to modulate methylation in over 1 % of CpGs assessed throughout the genome (Guo et al. 2011a). In this model, Gadd45b was found necessary for active demethylation for candidate sites including CREB-binding protein (CBP), a critical memory-associated transcription factor and epigenetic regulator itself, and Grip1, an AMPA receptor-binding protein necessary for synaptic depression. Further investigation is also needed to address the potential contributions of Gadd45a and Gadd45g to memory-related signaling.

An understanding of molecular epigenetic dynamics requires an appreciation for extensive cross-talk between canonical mechanisms, especially DNA methylation, histone acetylation, and histone methylation (Kondo 2009; Brinkman et al. 2012). These horizontal interactions along with complex, sequence-specific, and activity-tuned functions of epigenetic writers and erasers engender unique permutations of epigenetic signatures at gene regulatory regions. The possibility that these permutations selectively drive changes in local transcription defines the "epigenetic code," a speculative concept that is the subject of current investigation (Day and Sweatt 2011). In memory consolidation, Gadd45b may play a significant role in neuronal epigenetic coding. For instance, mice treated with an HDAC inhibitor showed enhanced fear memory and a trend towards reduced *gadd45b* expression (Vecsey et al. 2007).

A recent finding of great interest is the discovery that 5-hydroxymethylcytosine (5hmC) is present at considerable frequency in the genome of principal cerebellar nuclei (Kriaucionis and Heintz 2009). Catalyzed by the ten-eleven translocase (TET) protein family, 5hmC is formed by hydroxylation of 5mC and is present at particularly high levels in the brain (Khare et al. 2012). Its function is not fully understood, but there is evidence that 5hmC represents an oxidated intermediate in active demethylation of 5mC in neurons (Guo et al. 2011b). The conversion of 5mC to 5hmC may be followed by BER immediately or after conversion of 5hmC to 5-hydroxymethyluracil (5hmU) by the activation-induced deaminase (AID) and apolipoprotein B mRNA-editing enzyme complex (APOBEC) family of deaminases (Bhutani et al. 2010; Guo et al. 2011b). Gadd45a and Gadd45b are involved in BER of mutated cytosines and may act similarly in neurons (Liebermann and Hoffman 2008; Rai et al. 2008). As evidence of this hypothesis, Tet1 and APOBEC regulate seizure-induced demethylation and upregulation of BDNF IX and Fgf-1B, known Gadd45b-binding targets (Ma et al. 2009; Guo et al. 2011b). Gadd45b may therefore coordinate the conversion of 5hmC to unmethylated cytosine by recruiting deaminase and BER factors (Fig. 6.6b, c). Alternatively, at some loci, a direct mutation-BER route may bypass 5hmC. Finally, an interesting finding is that DNA methylation itself appears to control expression of the *gadd45* genes in cancer (Tamura et al. 2012). Should a similar mechanism mediate expression in the brain, this would imply the *gadd45* genes function in a complex, epigenetic positive or negative feedback loop in association with cognitive processing. Future studies are needed to investigate these possibilities in periods of high neuronal activity.

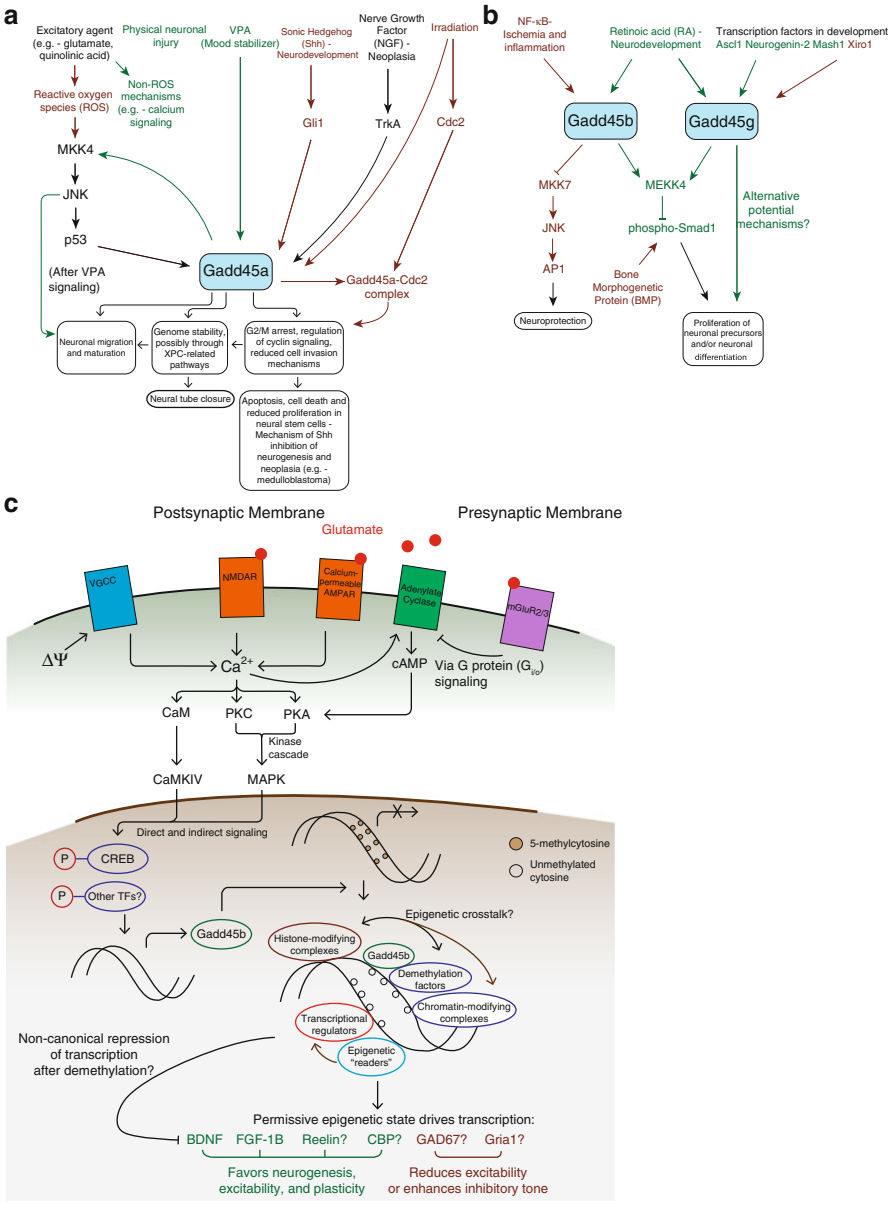

Fig. 6.7 Neuronal molecular signaling by the Gadd45 proteins. (**a**) Numerous upstream cascades impinge on *gadd45a* expression in neuronal development, injury, and tumor formation. Still, the function of Gadd45a in the balance between cell survival and death is largely dependent on the cell type and context. *Green arrows* indicate pathways in which Gadd45a generally plays a protective function. *Red arrows* indicate a predominant role of Gadd45a in promoting cell death or apoptosis. *Black arrows* preceding Gadd45a represent ambiguous, context-dependent signaling pathways. (**b**) Schematic similarly outlining potential regulation and function of Gadd45b and Gadd45g in neurodevelopment and injury. (**c**) Potential mechanisms for Gadd45b regulation and function in neuroepigenetic dynamics during activity-associated states such as seizures and memory formation. Gadd45b has been shown or hypothesized to regulate each of the genes listed at the bottom, illustrating a potentially diverse breadth of function in tuning experience-dependent neuronal output. See text for details and references

6.4.2 Autism

Spatial memory assessment of Gadd45b-null mice in the Morris water maze task revealed a surprising finding; in a control, pretraining phase, mice are taught to associate a visible flag with the escape platform. Although mutant and wild-type mice learned the task rapidly, mutants exhibited significant aversion to swim towards the flag in initial trials (Sultan et al. 2012). Since mutants did not show impairments in swim speed or other performance factors, we surmised that mutants bear a mild neophobia phenotype. As neophobia is associated with autism spectrum disorders, Gadd45b may plausibly regulate autistic features (Patterson 2011). Autism disorders are characterized by pathological neurodevelopment, and patients present with deficits in social interaction and cognition, aberrant communication and language skills, and stereotyped behaviors (Nguyen et al. 2010). A microarray study revealed elevated *gadd45b* transcripts in the superior temporal gyrus of autistic patients (Garbett et al. 2008). Studies have also uncovered dysregulation of DNA methylation in lymphoblastoid cells and histone methylation prefrontal cortex neurons in autistic patients (Nguyen et al. 2010; Shulha et al. 2012). The functionality of Gadd45b in autistic behavior and epigenetically targeted loci has yet to be uncovered. Potential autism-associated genes include SHANK3, which encodes a synaptic scaffolding protein, and neuroligin3, which encodes a neuronal transmembrane signaling protein; furthermore, both are sensitive to DNA methylation (Guo et al. 2011a; Uchino and Waga 2013). Additionally, serum BDNF is reduced in patients (Hashimoto et al. 2006; Abdallah et al. 2013).

6.4.3 Alzheimer's Disease and Aging

Aging is associated with oxidative stress, altered calcium homeostasis, chromosomal abnormalities, deficits in DNA repair, and nuclear and mitochondrial damage (Irier and Jin 2012). These features accelerate neurodegeneration and probably mediate age-related cognitive decline, most notably in Alzheimer's disease (AD). AD patients suffer from debilitating deficits in memory, decision-making, and language. Age is the strongest risk factor for developing AD, and it is likely that AD and aging are sensitive to disturbances in overlapping molecular pathways.

The deposition of extracellular plaques of amyloid β-peptide (Aβ) in the brain is a hallmark pathological feature of AD (Lambert et al. 1998). In a human preneuron cell line, Aβ exposure induced DNA damage and robust *gadd45a* expression, suggesting *gadd45a* is sensitive to genotoxic stress in AD and mediates a repair response as in the previously discussed injury models (Santiard-Baron et al. 1999, 2001). AD patients' brains also exhibit enhanced Gadd45a and Bcl-2 expression in DNA-damaged cells (Torp et al. 1998). Gadd45a also appears to confer protection against DNA damage-induced apoptosis. However, in a study of skin fibroblasts, which show impaired oxidative metabolism in AD, oxidative stress applied to patient cells produced less cell death than in control cells (Uberti et al. 2002).

Additionally, while normal cells showed stress-induced Gadd45a and p53 protein upregulation, AD cells showed a blunted response. These findings suggest Gadd45a regulates DNA damage-associated stress responses in AD, but its net effect on cell viability may vary by cell type and treatment paradigm.

Future studies of the Gadd45 proteins in AD may focus on epigenetic mechanisms. Indeed, global DNA methylation in the cortex is reduced in AD patients, and 5mC levels inversely correlate with neurofibrillary tangles, a canonical intracellular hallmark of AD pathology (Mastroeni et al. 2010). Similarly, 5hmC was shown to be enriched in pathways associated with age-related neurodegeneration (Song et al. 2011). Site-specific alterations in methylation of disease-related genes have also been documented in apolipoprotein E (APOEε4) in patients and presenilin 1 (PSEN1) in a mouse model (Wang et al. 2008; Fuso et al. 2012). Similarly, altered histone modifications are associated with AD, and enhancement in histone acetylation alleviates memory deficits (Francis et al. 2009; Kilgore et al. 2010; Stilling and Fischer 2011; Sultan and Day 2011; Gräff et al. 2012). In addition, HDAC inhibitors are speculated to boost cognitive function in a number of neurodegenerative disease states (Gräff and Tsai 2013). In light of the procognitive and epigenetic roles of Gadd45b in memory and dysregulation of one-carbon transfer pathways in AD, the Gadd45 family and associated active demethylation regulators may emerge as a second class of targets in neuroepigenetic pharmacotherapy (Fuso and Scarpa 2011; Sultan et al. 2012). The protective function of the Gadd45 genes in excitotoxicity discussed above may also mediate broader protection against neurodegeneration; accordingly, aberrant neuronal excitation is present in numerous disease states including AD (Mehta et al. 2013).

The role of the Gadd45 proteins in stress responses spurred investigations into their role in aging. In fruit flies, expression of the Gadd45a ortholog (*D-GADD45*) is reduced in the nervous system with age, and overexpression of the gene in the nervous system prolongs life span (Plyusnina et al. 2011, 2012; Moskalev et al. 2012). The longevity phenotype is likely conferred by more efficient DNA repair as evidenced by reduced spontaneous DNA aberrations in overexpressing mutants. Importantly, overexpression of *gadd45a* does not affect fecundity or motor behavior, suggesting the gene selectively attenuates the metabolic effects of aging. Moreover, *gadd45a* is upregulated in response to thermal, oxidative, and food deprivation stressors and regulates the effects of stress on longevity (Moskalev et al. 2012). Flies with *gadd45a* mutations also exhibited impaired hormesis, in which preexposure to low-dose radiation attenuates the life span reduction caused by subsequent high-dose radiation. Gadd45a likely acts through damage control mechanisms similar to those described previously, including the MAPK cascade, apoptosis pathways, and oxidative damage signaling. However, it is reasonable to speculate that the Gadd45 proteins also affect aging-related epigenetic changes. Indeed, a plethora of studies have investigated DNA methylation changes in human aging (Johnson et al. 2012). Methylation profiles of whole blood may even serve as a biomarker reflecting advancing age and age-related disease states (Hannum et al. 2013). Additional studies are needed to delineate the potential contribution of the *gadd45* genes to age-associated epigenetic drift.

6.4.4 Psychosis

Major psychosis refers predominantly to schizophrenia (Sz), a neurodevelopmental disorder characterized by multiple symptom types, and bipolar disorder (BP), a condition of bouts of mania and depression (Peedicayil 2011; Grayson and Guidotti 2013). Sz affects up to 1 % of the global population and usually produces clinically notable impairments in late adolescence and early adulthood (Lewis and Sweet 2009). Sz patients present with three categories of symptoms. Positive symptoms include delusions, rigidly held false beliefs, hallucinations and other perceptual difficulties, aberrant thought, and psychomotor activity in the form of disorganized behavior, posturing, and catatonia, a condition of severely altered motor function (Lewis and Sweet 2009). Negative symptoms include social withdrawal, impaired volition, disturbed affect, poverty in speech, and anhedonia (impaired ability to experience pleasure). Cognitive symptoms reflect dysfunction in selective attention, working and episodic memory, executive function, language, and social and emotional processing. These are considered the most significant clinical features of Sz, occurring for longer periods of time than positive symptoms in patients. The degree of cognitive impairment is also the most accurate indicator of prognosis. SZ comorbidities include depression, emotional disability, cardiovascular disease, substance abuse, and heightened risk of suicide. Affected individuals' families are likely to experience emotional distress, and the disease is associated with substantial economic effects due to lost productivity and medical expenses.

Studies of schizophrenia pathophysiology show that alterations in cortical circuit function largely underlie the clinical features of the disease. SZ is accordingly associated with diminished cortical neuropil and pyramidal neuron spine density and soma volume (Akbarian et al. 1995; Kolluri et al. 2005). Changes in the function of interneurons, the principal regulators of inhibition in the brain, have also been associated with Sz (Lewis 2012). Interneurons broadly modulate circuit tone and synchronized oscillations, which are thought to contribute to normal cognitive function and are altered in psychosis, by releasing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Gonzalez-Burgos et al. 2010). A collection of studies has confirmed that transcription of glutamic acid decarboxylase (GAD67), the primary enzyme responsible for GABA production, is reduced in cortices from patients (Spencer et al. 2003; Ford et al. 2007; Gonzalez-Burgos et al. 2010; Lewis 2012). The consequent impairment in inhibitory tone is likely a factor in symptoms such as memory impairment and hallucinations.

A growing collection of studies have investigated alterations in epigenetic programming in association with major psychosis, and it is in this context that aberrant Gadd45 signaling has been hypothesized to modulate the disease phenotype (Peedicayil 2011). These have been predominantly assessed in cortex biopsies from patients. Utilizing a candidate gene approach, these have robustly uncovered alterations in DNA methylation in promoters of *reelin* and *gad1*, the gene encoding GAD67, in relation to psychosis. For example, one study found enhanced *DNMT1* and reduced *reelin* transcription in Sz cortex (Veldic et al. 2004). Notably, DNMT1

protein co-localizes with Reelin, but transcription of each respective gene was almost exclusively localized to interneurons. Enhanced DNA methylation in the *reelin* gene promoter was also found in Sz brains (Abdolmaleky et al. 2005; Grayson et al. 2005). Although it is unclear whether this epigenetic mark directly influences transcription in the Sz cortex, it is interesting to note that higher methylation was found in close association with a putative cycle AMP response element and stimulating protein-1 site in the *reelin* promoter. In light of the localization of Reelin to GABAergic interneurons and its role in the integrity of the extracellular matrix, the neuropil and synaptic plasticity, studies have also sought to examine GAD67 epigenetic regulation in order to dissect a broader function link between epigenetic dysfunction and GABAergic tone. For example, the fastest spiking interneurons in the cortex, those in layers I, II, and IV, also contain the highest DNMT1 transcripts, suggesting a positive association between DNA methylation and GABAergic tone (Veldic et al. 2004). Additionally, GAD67 transcription negatively correlates with DNMT1 transcription in psychotic patient cortices (Veldic et al. 2005). To the contrary, one finding suggests demethylation of *gad1* may be noncanonically associated with downregulation of GAD67 or that other gene regulatory elements outside the promoter may be more critical in modulating expression (Huang and Akbarian 2007).

Still, the finding of aberrant methylation in Sz suggests Gadd45 function may affect neural tone alterations in patients. Two studies in particular have examined this hypothesis. The first assessed DNA methylation dynamics in response to altered activity of metabotropic glutamate receptors (mGlu), as activation of the group II subclass (composed of types mGlu2 and mGlu3) attenuates presynaptic glutamatergic activity, and agonists have been associated with antipsychotic effects (Matrisciano et al. 2011). The study first found enhanced *gadd45b* and *gadd45g* mRNA and Gadd45b protein expression in the frontal cortex of naïve mice in response to a single or repeated doses of systemic mGlu2/3 activation. Both VPA, a weak HDAC inhibitor, and the atypical antipsychotic clozapine but not the typical antipsychotic haloperidol similarly induce *gadd45b* transcription, suggesting Gadd45b may play a role in certain form of antipsychotic therapy. Because Gadd45b was shown to bind *bdnf exon IX* and *fgf-1B* promoters in association with activity-induced demethylation (Ma et al. 2009), the authors examined possible binding to *reelin*, *bdnf exon IX*, and *gad1* promoters after mGlu2/3 activation. Enhanced binding was confirmed along with active demethylation of each of these promoters following methionine-induced hypermethylation. VPA and clozapine similarly reduced *reelin* baseline methylation as well as the methionine-induced hypermethylated promoter.

The second study analyzed cortical tissue from psychotic subject from two brain banks (Gavin et al. 2012). The study found selectively reduced Gadd45b recruitment in the proximal promoter but not in a downstream region of *bdnf exon IXabcd* in psychotic patients. The authors then elegantly demonstrated strong hypermethylation of the same promoter region in affected subjects and a similar increase in 5-hmC signal. Concomitantly, reduced total BDNF expression was confirmed. If Gadd45b plays a causative role in demethylation in association with the psychosis phenotype, its reduced binding to target loci such as *bdnf exon IX* may have caused

the demethylation cascade to become "stuck" in the hydroxymethylated state. According to this model of *bdnf* demethylation, Gadd45b would promote the conversion of 5-hmC to unmethylated cytosine, but this has not been confirmed (Guo et al. 2011b). Although Gavin et al. (2012) dissected the epigenetics of BDNF expression, it is not clear whether attenuated BDNF signaling is a key mediator of the disease phenotype or whether it is only one of many epigenetically dysregulated genes. It is also not clear which cortical cells specifically harbor the alterations in Gadd45b-mediated signaling; indeed both excitatory and inhibitory cortical cells but not glia express Gadd45b in the normal prefrontal cortex (Gavin et al. 2012). Finally, the authors uncovered a counterintuitive finding that *gadd45b* transcripts and protein are both elevated in psychotic subjects' cortices. Of course, this conflicts with the finding of suppressed DNA binding and elevated methylation of *bdnf exon IX*, and this suggests that global expression differences in Gadd45b and possibly other potent modulators of the epigenome do not necessarily drive commensurate changes in site-specific binding patterns. It remains to be seen which specific characteristics of promoter sequences and associated chromatin influence the recruitment of the demethylation machinery. Alternatively, enhanced expression could represent a compensatory effect to other factors associated with a more restrictive chromatin state in Sz such as enhanced DNMT expression, DNA methylation, and repressive histone modifications (Veldic et al. 2004, 2005; Grayson et al. 2005; Gavin and Sharma 2010).

Together, these results provide evidence that Gadd45b influences altered neuronal signaling in Sz and mediates certain forms of antipsychotic therapy. Future studies, such as with Gadd45b-mutant mice, are needed, however, to confirm a functional effect of the protein. It should be noted, however, that Gadd45b-null mutants did not exhibit alterations in prepulse inhibition, an index of sensorimotor gating that is affected in Sz models (Sultan et al. 2012). Additionally, it remains to be seen that Gadd45b specifically drives demethylation and upregulation of *reelin*, *gad1*, and *bdnf* in psychosis. Of particular interest is the possible role of Gadd45b in epigenetically altered *gad1* expression and inhibitory tone. If the Gadd45 proteins do indeed influence cortical inhibition in this manner, demethylation mechanisms could become a novel molecular target in Sz treatment.

Few studies have sought to profile the span of methylation changes in psychosis, but Mill et al. (2008) utilized an epigenome-wide effort to address this problem. Genomic DNA from frontal cortex biopsies of schizophrenic and bipolar patients differed at a number of loci from control brains when global methylation was assessed. These epigenetic modifications corresponded with changes in steady-state transcripts encoding regulators of glutamatergic and GABAergic signaling and neurodevelopment and other transcripts highlighted in genetic linkage studies. For example, loci proximal to the NMDA receptor subunit gene *NR3B* and the AMPA receptor subunit gene *Gria2* were hypomethylated in patients, suggesting aberrantly high demethylation activity, possibly due to altered Gadd45 signaling could drive excessive excitatory activity. Likewise, genes encoding vesicular glutamate transporters VGLUT1 and VGLUT2 also showed altered methylation patterns associated with downregulation of the former and demethylation and upregulation of the

latter. In hypothesizing a functional link between DNA methylation changes and psychosis etiology, the authors emphasize the complex, interactive effects of epigenomic marks in the global transcriptional network. It is important, therefore, not to place excessive weight on single transcriptional or epigenetic changes in psychosis or other disease states, and future studies will need to take this into account. In addition, a recent study found elevated *TET1* and suppressed *APOBEC* expression in psychosis patient cortices and associated increases in 5hmC throughout the genome and at *bdnf* and *gad1* promoters (Dong et al. 2012). The associated decreases in BDNF and GAD67 expression may result from similar reductions in the ability of neurons to convert 5hmC to unmethylated cytosine and hence accumulation of 5hmC or from a direct repressive function of TET1 independent of its enzymatic activity (Grayson and Guidotti 2013). Therefore, future studies of Gadd45 proteins in Sz will need to address potential alterations in 5hmC levels as well. Finally, the functional interactions between canonical epigenetic cascades suggest Gadd45 proteins may also mediate the contributions of histone modifications to psychosis pathology and treatment (Sharma et al. 2008; Guidotti et al. 2009; Kurita et al. 2012; Labrie et al. 2012).

6.5 Conclusions

We have summarized the key findings of Gadd45 protein function in the CNS. The Gadd45 family is expressed in distinct patterns during the development of the nervous system and likely mediates cell-cycle control during mitosis. In a variety of nervous system stressors including physical and chemical injury to nerves and neoplasia, the Gadd45 family has been implicated in sensing DNA damage and controlling the balance between apoptosis and cell survival. More recently, an exciting literature has identified the Gadd45 family as regulators of active DNA demethylation, a still elusive molecular epigenetic mechanism that appears to control adult cognitive function and neuropsychiatric dysfunction. Future work is needed to delineate the breadth of mechanisms that stimulate Gadd45 expression and those by which the proteins mediate DNA repair, cell-cycle control, and epigenetic regulation of transcription. Studies will also need to identify small molecule regulators of Gadd45 function and to investigate their potential uses in conditions including CNS cancers, nerve damage, and cognition.

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