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Folate regulation of axonal regeneration in the rodent central nervous system through DNA methylation

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The folate pathway plays a crucial role in the regeneration and repair of the adult CNS after injury. Here, we have shown in rodents that such repair occurs at least in part through DNA methylation. In animals with combined spinal cord and sciatic nerve injury, folate-mediated CNS axon regeneration was found to depend on injury-related induction of the high-affinity folate receptor 1 (Folr1). The activity of folate was dependent on its activation by the enzyme dihydrofolate reductase (Dhfr) and a functional methylation cycle. The effect of folate on the regeneration of afferent spinal neurons was biphasic and dose dependent and correlated closely over its dose range with global and gene-specific DNA methylation and with expression of both the folate receptor Folr1 and the de novo DNA methyltransferases. These data implicate an epigenetic mechanism in CNS repair. Folic acid and possibly other nontoxic dietary methyl donors may therefore be useful in clinical interventions to promote brain and spinal cord healing. If indeed the benefit of folate is mediated by epigenetic mechanisms that promote endogenous axonal regeneration, this provides possible avenues for new pharmacologic approaches to treating CNS injuries.

Introduction

The mature CNS is among tissues most refractory to healing after injury. In contrast to neuronal genesis in the fetal CNS, regeneration of axons in the adult brain and spinal cord is impaired by inhibitory molecules expressed in CNS myelin and glial cells in response to injury (1–3). In turn, damaged neurons in the adult CNS often fail to express genes required for effective axon regrowth and repair (1, 4). Recently, it has been observed that both the developing and injured adult mammalian CNS benefit from folic acid (FA) supplementation. First, periconceptional FA supplementation markedly reduces the incidence of neural tube and other malformations (5, 6). Furthermore, we have shown that parenteral FA produces up to a 10-fold, dose-dependent improvement in axon regrowth and functional recovery after injury to the adult CNS, an effect well in excess of other interventions (7). Corresponding efforts to improve neurological function through folate administration in neurodevelopmental diseases, including autism and depression, and other neuropsychiatric disorders are underway (8). However, the mechanisms underlying the efficacy of folate intervention remain unclear and may be related to diverse pathways in which single-carbon transfers participate.

Recognition that gene expression may be modified without altering genomic sequence via heritable covalent changes has contributed to an improved understanding of CNS pathophysiology in a number of inborn disorders (9–12). DNA methylation, an epigenetic mechanism ascribed mainly to the folate pathway and related metabolites, is a major gene-expression-modulating mechanism. Accordingly, we’ve reasoned that if genetic regulation of CNS development, regeneration, and repair is mediated by DNA methylation, it may also be responsive to FA and other factors that influence single-carbon transfers. We aimed, therefore, to determine whether interventions that modify single-carbon metabolism result in changes to genomic DNA methylation, which in turn correspond to the extent of axonal recovery and growth after experimental injury in the CNS. Herein, we report the consequences of specific interventions on enzymatic steps that comprise the folate pathway from cellular entry to methylation on axonal regeneration in the adult CNS after sharp injury. For this purpose, we use the spinal cord regeneration model (SCRM), which combines a bilateral dorsal column transection with a conditioning left sciatic nerve injury (7, 13). As depicted in a folate pathway schematic diagram (Figure 1), we show that agonists and antagonists of sequential reactions in folate metabolism exhibit dose-dependent inhibition and promotion of CNS repair, respectively, and that axonal regeneration relies upon the integrity of DNA methylation pathways. Additionally, we report for what we believe is the first time that axonal injury induces expression of the folate surface receptor folate receptor 1 (Folr1), thereby potentially enabling enhanced intracellular accumulation of folate; that suppression of the bioactivation of FA through dihydrofolate reductase (Dhfr) inhibits the regeneration of injured CNS axons; that global demethylation of spinal cord DNA accompanies injury, an effect prevented by folate supplementation; and that there is a direct, biphasic correlation between the proportion of regenerat-

Conflict of interest: Teodoro Bottiglieri reports having been the chairman of the Advisory Board for Methylation Sciences Inc., holding stock options in Methylation Sciences Inc., and having received grant/research funding from Pamlab LLC, distributor of B vitamins as a medical food.

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ing afferent axons in the spinal cord, Folr1 expression, global DNA methylation, and the specific methylation of the Gadd45a gene associated with spinal injury and neurite outgrowth. Finally, we show that the dose-dependent association between regeneration after injury and the extent of spinal cord DNA methylation correlates with the expression of the de novo DNA methyltransferase enzymes Dnmt3a and Dnmt3b, but not with the expression of Dnmt1 that is responsible for maintenance of DNA methylation.

Results
The SCRM model of spinal cord axonal regeneration combines a sharp bilateral cervical dorsal column transection with a unilateral “conditioning” sciatic nerve injury (spinal cord and sciatic nerve injury [SCSN]) (7, 13). Injuring the peripheral axon of the dorsal root ganglia (DRG) enhances growth of both the peripheral (sciatic) and central (dorsal column) components of the DRG (13). In addition, we have previously shown that the combination of peripheral nerve injury and i.p. FA supplementation is additive or synergistic, increasing the number of regenerating axons on the conditioned side nearly 10-fold (7). Accordingly, the SCRM results reported in this study refer to the conditioned side of the spinal cord. For consistency, the combined bilateral dorsal columns and unilateral sciatic nerve injury model was used for all regeneration, molecular, and biochemical protocols.


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Combined spinal cord and peripheral nerve injury induces expression of Folr1 but not the reduced folate carrier. Folic acid does not diffuse into the cell. Therefore, specific active transport is essential. Two folate transport systems are available to the neuron: a transmembrane bidirectional anion channel, the reduced folate carrier (Rfc), with 2 isoforms, Rfc1 and Rfc2; and a receptor-mediated endocytotic pathway, the folate receptor (Folr) (previous nomenclature FR or folate-binding protein-FOLBP), with 4 known isoforms, α, β, γ, and δ (14–16). Rfc1 is an anion exchanger ubiquitously expressed in diverse cells and tissues with low affinity for FA (14, 16). In contrast, the α isoform of Folr (Folr1) is a high-affinity, low-capacity system, operating at low concentrations of exogenous FA. To determine whether a sharp transection injury to the dorsal columns of the spinal cord and the sciatic nerve alters Folr1 and Rfc1 expression, mRNA transcript levels of the 2 genes were measured in the lumbar DRG cell bodies corresponding to the injured spinal and peripheral axons using in situ hybridization and in the injured spinal cord using RT-PCR. We observed a 2-fold increase in the percentage of neurons expressing Folr1 mRNA following injury to the adult spinal cord and sciatic nerve in rats (Figure 2, A, B, G, and H). There was no change in Rfc1 mRNA expression either by RT-PCR or in situ hybridization (Figure 2, D and E). Next, we sought to correlate these results with immunohistochemical assays of Folr1 protein expression in the DRG neurons corresponding to the injured axons. A 6-fold increase in the percentage of neurons immunostaining for Folr1 was observed in the injured animals compared with uninjured controls (Figure 2, C, I, and J), with minimal change seen in Rfc1 protein levels in either group (Figure 2F). Confocal microscopy showed colocalization of the Folr1 expression with a neuronal marker indicating that the cells expressing the Folr are neurons rather than glia (Figure 2K).

Mice with reduced expression of Folr1 do not regenerate spinal or optic axons in vivo or DRG axons in vitro after injury. To determine whether increased Folr1 expression after axonal injury is essential for the

Figure 1
Summary of experimental interventions within the folate and methylation pathways. Folic acid enters the cell through Folr1, which is upregulated with injury. It is then converted to the active tetrahydrofolate (THF) form by Dhfr. This allows eventual production of nucleotides and certain amino acids as well as transfer of the methyl group into the methionine methylation cycle. The latter occurs through the B12-dependent MS step. Subsequently, SAM is the substrate used by the methyltransferase enzymes for the methylation reactions. Inhibition of Folr1, Dhfr, MS, and Dnmt suppresses CNS regeneration. In turn, activation of Dnmt enhances CNS regeneration.
The proregenerative effect of FA, a sharp injury was administered to the cervical dorsal columns and left sciatic nerve of heterozygous Folr1 mice (17). After 48 hours, the left lumbar DRGs were removed to initiate in vitro neuronal cultures, as previously described (4). Whereas WT control mice demonstrated significant axonal elongation in the dissociated cells at 24 hours, Folr1+/– animals exhibited little or no growth of long axons (>299 μm) in culture (Figure 3).

Absence of glia from the culture medium indicates that axonal regeneration is independent of the glial microenvironment. Sharp transection injuries were subsequently administered to the optic nerve and cervical dorsal columns of Folr1+/– and WT mice, and a sciatic nerve graft was attached to the optic or spinal cord injury sites respectively, as previously reported (7, 13, 18). After an interval of time adequate for growth of axons a distance of at least 1 cm into the graft (i.e., 2 weeks in the spinal model and 2 months in the optic model), the end of the graft was backfilled with a fluorescent tracer, and the corresponding cell bodies in the retina and DRG were counted. Supplementation of WT animals with i.p. FA at a dose previously shown to enhance regeneration (80 μg/kg) (7) produced at least a 2-fold increase in the percentage of regenerating spinal neurons into the graft and almost a 10-fold increase in the number of regenerating retinal ganglion cell (RGC) neurons compared with the WT animals without folate treatment. Conversely, no axonal regeneration was observed in the Folr1+/– heterozygous animals receiving supplemental FA (Figure 4). Taken together, these results suggest that injury-induced augmentation of Folr1 receptor expression plays an essential role in the effects of FA on both spinal cord and optic axonal regeneration.

Figure 2
Combined spinal cord and peripheral nerve injury induces expression of Folr1 but not Rfc1. 4 days after sharp transection of both dorsal columns and the left sciatic nerve in rats, the left L5 and L6 DRGs were removed, sectioned, and mounted to undergo in situ hybridization (A, D) and immunohistochemistry (C, F) of the Folr1 and Rfc1 receptors. The spinal cord was removed for RT-PCR (B, E) and Western immunoblot (Figure 7, A and E). Note the significant upregulation of Folr1 with injury, with no change in the Rfc1 levels both in the spinal cord (SC) and DRG: in situ Folr1 n = 8 (SC), 8 (DRG); in situ Rfc1 n = 5 (SC), 5 (DRG); Folr1 RT-PCR n = 3 (SC), 3 (DRG); Rfc1 RT-PCR n = 3 (SC), 3 (DRG); immuno Folr1 n = 4 (SC), 4 (DRG); immuno Rfc1 n = 4 (SC), 4 (DRG), 2-tailed Student’s t test; mean ± SEM; *P < 0.05. (G, H) Folr1 in situ hybridization of DRG sections without (G) and with (H) preceding combined spinal cord and left sciatic nerve injury. (I, J) Folr1 immunostaining of DRG sections without (I) and with (J) preceding combined spinal cord and left sciatic nerve injury. (K) Confocal microscopy showing colocalization of the red Folr1 stain with the green neuronal (neurofilament) stain. Original magnification, ×40.
**Dhfr bioactivation of FA is essential for folate-mediated CNS regeneration.**
Upon entry into the cell, FA is converted into active tetrahydrofolate form via 2 sequential reductions catalyzed by Dhfr (Figure 1). To determine whether the beneficial effects of FA on axonal regeneration depend upon its initial activation steps, the optic nerve and spinal cord experiments described above were conducted with and without FA in animals pretreated with the Dhfr antagonist methotrexate (MTX). A 10:1 inhibition of Dhfr activity was confirmed via Dhfr assay with only a mild change in protein levels observed on Western blot, indicating that MTX suppresses the enzymatic activity rather than the protein expression of Dhfr (Figure 5, A, C, and D). Compared with control animals, in which regrowth of injured axons into the grafts took place as described above, little or no spinal axonal regeneration in response to FA was observed in the MTX-treated animals (Figure 5B). In the absence of MTX, injury increased the protein and activity levels of Dhfr minimally, if at all (Figure 5, C and D). These results suggest that unencumbered intracellular conversion of FA to tetrahydrofolate in tandem with injury-mediated expression of Folr1 receptors are critical steps in folate-mediated axonal regeneration.

**Combined spinal cord and peripheral nerve injury inhibits methyl cycling and S-adenosylmethionine bioavailability.** Through its 5-methyltetrahydrofolate intermediary, the folate pathway donates methyl groups to the methylcyylation cycle, enabling synthesis of S-adenosylmethionine (SAM), the primary methyl donor in neurons and all other cells of the CNS, upon its conversion to S-adenosylhomocysteine (SAH) (Figure 1) (19). SAH is hydrolyzed to replenish homocysteine stores. However, if homocysteine accumulates in excess, SAH formation is favored. In turn, SAH inhibits methyltransferase reactions, thereby exerting negative feedback on methylation reactions (20). Accordingly, measurement of the SAM/SAH ratio is an index of both cofactor of methionine synthase (MS), N_{2}O irreversibly inhibits MS activity (21). To determine whether methylfolate-dependent synthesis of methionine from homocysteine by MS is a critical step in folate-mediated axonal regeneration, N_{2}O was used to inactive MS in vivo in animals with and without spinal cord injury and with and without FA supplementation. In animals breathing N_{2}O, axonal regeneration declined to baseline in the SCRM model, demonstrating that the single-carbon cycle is an essential component of the proregenerative effect of FA (Figure 6B).

**Combined spinal cord and peripheral nerve injury suppresses and FA restores Dnmt3a and Dnmt3b, but not Dnmt1, protein levels in a biphasic fashion.** DNA methyltransferases (Dnmts) participate in direct methylation of CpG dinucleotides in the mammalian genome, using methyl group provided by SAM. Dnmt1 maintains the pattern of DNA methylation (i.e., methylation of hemimethylated sites), while Dnmt3a and Dnmt3b participate in de novo methylation (i.e., methylation of previously unmethylated sites). To determine the relative contributions of the Dnmts to CNS injury and repair, Dnmt1, Dnmt3a, and Dnmt3b protein levels were measured prior to and following combined spinal cord and peripheral nerve injury using Western blot analysis. No detectable effect on Dnmt1 protein levels after injury was observed (Figure 7, A and D). However, injury suppressed Dnmt3a and Dnmt3b protein levels in the spinal cord, and i.p. FA treatment restored both Dnmt3a and Dnmt3b levels to preinjury baselines. The magnitude of de novo Dnmt protein expression in response to increasing doses of parenteral folate was biphasic (Figure 7, A–C). Interestingly, this correlates closely with our earlier observations (7), confirmed here in a different group of animals (Figure 7F), in which we show that the proregenerative effect of FA is dose dependent, with peak effects observed at 80 μg/kg. No toxicity is observed at higher doses, but beneficial effects gradually diminish to baseline in a dose-dependent fashion. Moreover, the protein levels of the methyltransferases were measured in response to antagonists of Dhfr and MS. Dnmt1 levels again show minimal change with any intervention, while Dnmt3a and Dnmt3b protein levels decrease with MTX and are eliminated with nitrous oxide (Figure 7, A–C).

**Figure 3**
Mice with reduced expression of the *Folr1* gene do not regenerate injured DRG axons in vitro. Forty-eight hours after an in vivo sharp injury to the spinal cord (dorsal columns) and left sciatic nerve in mice, the left lumbar DRGs that contain the cell bodies of the injured axons were removed, dissociated, and placed in a culture medium that sustains growth. In the WT animals (A, C), a significant number of axons reached a length of 300 μm; conversely, neurons from the *Folr1*−/− mice were less able to extend long axons. Instead, these neurons were more likely to show sprouting of short axons (B, D), indicating a decreased ability of the neurons to regenerate, as previously described (4, 24). Whereas 41% of neurons were able to extend axons greater than 299 μm in the WT animals, only 12% of neurons showed such growth in the *Folr1*−/− mice. Original magnification, ×20.
Although Folr1 expression increases after neural injury, the response of Folr1 expression to increasing doses of FA is biphasic, peaking at 80 μg/kg as well (Figure 7, A and E).

Combined spinal cord and peripheral nerve injury suppresses spinal cord DNA methylation, and FA restores methylation levels in a biphasic fashion. To determine whether injury- and folate-dependent alterations in Dnmt levels correlate with global DNA methylation in the spinal cord, methyl group incorporation was measured by radioactive assay (23) in spinal cords 3 days after sharp bilateral cervical dorsal column combined with left sciatic nerve injury to replicate the injury used in the SCRM regeneration model. Injury decreases global genomic methylation by 40%, an effect that is prevented by FA supplementation (Figure 8). Moreover, a dose-dependent biphasic increase and subsequent decrease in spinal cord global methylation was also observed (Figure 9), closely tracking the biphasic curves of FA dose-dependent afferent spinal axon regeneration (Figure 7F and previous results in ref. 7) as well as de novo methyltransferase expression. It is important to note that lower counts in Figures 8 and 9 indicate higher methylation values. Although global methylation is measured 3 days after the injury whereas regeneration is tested at 2 weeks, there is no difference in methylation levels between these 2 time points (data not shown). “Regenerative competence” is thought to be initiated 24–48 hours after injury and persist until target reinnervation (24).

Thus, FA inhibits a hypomethylation response created by injury in a dose-dependent fashion, and DNA methylation together with Dnmt3a and Dnmt3b protein levels correlate with the capacity of the spinal neurons to regenerate their injured axons to a ceiling at the dose that produces a peak methylation response (80 μg/kg). Thereafter, increments in FA reverse axonal regeneration to baseline for reasons that remain unclear.
Figure 5

Dhfr bioactivation is essential for folate-mediated CNS regeneration. Using the SCRM model of afferent spinal regeneration in rats, i.p. MTX was used to inhibit Dhfr, thus preventing the conversion of FA into the bioactive form tetrahydrofolate, as demonstrated in the Dhfr activity assay (A) (n = 5 [uninjured]; n = 5 [injured]; n = 5 [injured and exposed to MTX]; Student’s t test; mean ± SEM; *P = 0.006), with only a small change in protein levels on Western immunoblot (C, D). N, normal (uninjured); Inj, combined spinal cord and sciatic nerve injury. Note that there is minimal or no increase in Dhfr protein and activity levels after spinal cord/sciatic nerve injury. The dose of MTX (400 μg/kg) that determined maximal inhibition with minimal toxicity was selected from a literature review (46, 51, 52). MTX suppressed the regeneration of spinal axons into a nerve graft to below baseline levels (B) (n = 10 [untreated controls]; n = 7 [FA]; n = 7 [MTX]; n = 6 [MTX and FA]). 1-way ANOVA with Bonferroni’s correction; mean ± SEM; *P < 0.05.

Discussion

Parenteral FA administration prior to and following injury improves the regeneration in vivo of injured sensory spinal and optic axons into transplanted peripheral nerve grafts (8). Herein, we report the effects of interventions in folate pathways from absorption and activation to utilization that are targeted to resolve the cellular mechanisms responsible for the proregenerative effects of folate. We demonstrate for what we believe is the first time that a combined spinal cord and peripheral nerve injury induces spinal cord expression of the high-affinity FollR receptor (but not the low-affinity Rfc1 receptor) and triggers global DNA hypomethylation of the postinjury spinal cord genome. Accordingly, we propose that increased FollR expression enables increased FA to enter the cell and that FA in turn regulates receptor activation in a dose-dependent fashion. In addition, we demonstrate that reduction of FA to its active form by Dhfr is essential to its proregenerative effects. In turn, the dose dependency of global DNA methylation in the CNS via de novo methylation enzymes Dnmt3a and Dnmt3b, but not maintenance enzyme Dnmt1, parallels the dose dependency of folate-mediated recovery.

FA agonist promotes, and FA antagonist inhibits, growth of injured sensory spinal axons into a peripheral nerve graft. To determine whether the Dnmt3a and Dnmt3b methyltransferases are critical to postinjury folate-mediated recovery, the regeneration of afferent spinal axons into a graft was assessed as above in ratspretreated with the Dnmt agonist 3-aminobenzamide (3-ABA) (15 mg/kg i.p.) (25) and antagonist 5-azacytidine (5-AzaC) (0.1 mg/kg i.p.) (26) with and without FA. 5-AzaC partially blocked folate-mediated regeneration (Figure 10A). 3-ABA, which enhances the activity of the methyltransferases through inhibition of poly (ADP-ribose) polymerases (25), promoted regeneration when administered alone, but it did not add to folate-mediated recovery in the single dose studied (Figure 10B).

Combined spinal cord and peripheral nerve injury increases methylation of the Gadd45a promoter, and FA increases methylation in a biphasic fashion in 12 of 18 CpG sites. To determine whether the effect of FA on global methylation is associated with methylation of specific genes associated with axonal regeneration, we examined the methylation status of 18 CpG sites on the Gadd45a promoter. The percentage of methylation of 12 of these sites increased with spinal cord injury and increased further in a biphasic fashion with FA supplementation. As with global methylation, the dose-dependent configuration of these changes was similar in peak folate doses and overall morphology to those observed with folate-mediated spinal cord regeneration (Figure 11).

Inhibition of enzymatic steps along the folate pathway impairs axonal regeneration. For reasons yet to be elucidated, the beneficial effects of folate supplementation after CNS injury also exhibit a biphasic dose response curve with optimal regeneration occurring at a dose of 80 μg/kg. A strikingly parallel biphasic response is also observed in both the methylation status of the CNS genome and the protein levels of the Folr as well as in the de novo methyltransferases in response to folate after injury. Specifically, 80 μg/kg of parenteral FA optimizes Folr1 expression, Dnmt3a and Dnmt3b protein levels, the methylation status of the adult CNS, and its ability to regenerate after injury. Biphasic methylation changes are global across the genome and in the promoter of at least 1 gene, Gadd45a. Gadd45 is a cell-cycle gene linked to both neuronal activity (27, 28) and DNA methylation (29). Gadd45 controls neurite outgrowth (28) and is induced by spinal cord injury to control neuronal damage and cell death (27).

Doses of FA higher than 80 μg/kg induce relative hypomethylation associated with reduced de novo methyltransferase levels and decreased axonal regeneration. The effect, which reaches levels comparable to those seen in untreated groups only at very high doses, suggests a loss of benefit from higher folate doses rather than toxicity. Others have reported biphasic effects related to the folate and methylation pathways in tissues of distinct germline derivation, e.g., monocytes and vascular smooth muscle, suggesting that the effect is not confined to neuronal cells (30–32). While the prore-
generative effect of FA is greater than that of other interventions reported to date, the declining effect with doses higher than 80 μg/kg indicates a ceiling on the regenerative potential provided by FA supplementation. Such a limitation may be related to transcriptional regulation of the de novo methyltransferases and/or the Folr. Furthermore, a recent report shows that FA inhibits the uptake of 5-methyltetrahydrofolate by the Folr in a dose-dependent fashion and suggests that FR-α (Folr1) is the major uptake mechanism at low concentrations, whereas the Rfc is the major uptake mechanism at higher concentrations (33). Experiments are underway to identify specific factors that affect the transcriptional regulation of the methyltransferases and Folr1 receptor and to determine how these are in turn affected by folate supplementation.

The ability to impart epigenetic methylcytosine signals to DNA depends on substrate availability (e.g., FA), Folr function, the enzymes of methionine metabolism, and the ability to transfer methyl groups to DNA strands via specific methyltransferases. We propose that FA-induced axonal regeneration operates through enhanced DNA methylation, which in turn may result in biphasic consequences in the expression of proteins underlying axonal growth and regeneration. Evidence in favor of this hypothesis includes the following: (a) inhibition of sequential steps within the folate and methylation pathways strongly inhibits the proregenerative effect of FA after spinal and optic injury; (b) enhancing methyltransferase function increases the regeneration of injured spinal neurons; and (c) the effective dose of FA has a narrow biphasic range with an optimal dose response at 80 μg/kg in maximizing spinal regeneration, restoring normal levels of global methylation in the injured spinal cord, and amplifying the expression of the de novo methyltransferases. This is consistent with earlier reports showing a relationship between DNA methylation and the CNS. Aberrant methylation is associated with reduced neurogenesis, neural stem cell differentiation, and increased genomic instability (34); DNA methylation is altered in brain ischemia (35) and in at least 3 clinical disorders associated with mental impairment, including Rett syndrome, ICF syndrome (immunodeficiency, centromeric heterochromatin, facial anomalies), and fragile X syndromes (36); and de novo Dnmt activity is necessary for memory consolidation in the hippocampus (37). However, the present hypothesis does not exclude alternative mechanisms. For example, hypomethylation may be a bystander to toxic consequences of homocysteine accumulation. Increasing doses of homocysteine in vascular smooth muscles has been shown to increase the expression of Dnmt3a and Dnmt3b as
Figure 7
Combined spinal cord and peripheral nerve injury suppresses the protein levels of the de novo methyltransferases Dnmt3a and Dnmt3b, but not the maintenance methyltransferase Dnmt1, and FA treatment restores the levels in a biphasic fashion that parallels the biphasic response to FA treatment seen with spinal regeneration and Folr1 expression. (A–D) Combined spinal cord and peripheral nerve injury suppresses Dnmt3a and Dnmt3b protein levels in mouse spinal cords, with no effect on Dnmt1. Furthermore, FA treatment restores these levels in both Dnmt3a and Dnmt3b. When increasing doses of FA are given i.p., a biphasic response is observed in the de novo methyltransferases Dnmt3a and Dnmt3b, with maximal protein levels occurring at 80 μg/kg. (F) Folic acid supplementation was previously shown to affect rat CNS regeneration following injury in a dose-dependent manner that corresponds tightly to the de novo Dnmt response. These results, obtained from our laboratory and previously reported by Iskandar et al. (7), are confirmed here in a different group of animals. One-way ANOVA with Bonferroni’s correction; n (FA dose) = 9 (0 μg/kg); 9 (40 μg/kg); 5 (80 μg/kg); 9 (400 μg/kg); 9 (800 μg/kg). Mean ± SEM; *P < 0.05 for all comparisons except 0 versus 400, 0 versus 800, and 40 versus 400. Conversely, treatment with MTX significantly decreases Dnmt3a and Dnmt3b protein levels, while treatment with N₂O nearly eliminates Dnmt3a and Dnmt3b protein expression. Neither injury nor folate, MTX, nor N₂O affect the levels of the maintenance methyltransferase Dnmt1 (A, E). Furthermore, although Folr1 expression increases after injury, its response to increasing doses of FA is similarly biphasic. (A) Western blots. All lanes were run on the same gel. Noncontiguous lanes are separated by a thin white line in the figure. (B–D, E) Plots of the band densities relative to the corresponding actin bands. One-way ANOVA with Bonferroni’s correction; n = 3 in each group; mean ± SEM; *P < 0.05.
As demonstrated previously (7, 13), optimal conditions for spinal axonal regeneration in this model (SCRM below) require a conditioning sciatic nerve injury. As demonstrated previously (7, 13), optimal conditions for spinal axonal regeneration in this model (SCRM below) require a conditioning sciatic nerve injury. A complete transection of the left sciatic nerve was performed through a separate incision to replicate the injury in the regeneration (SCRM) model. Four days postoperatively, the rats were anesthetized and perfused intracardially with 4% PFA. The L5 DRGs were removed bilaterally and fixed using 4% paraformaldehyde. The L5 DRGs were removed bilaterally, and fixed using 4% paraformaldehyde. The cervical cord was exposed through a C3 laminectomy and dural opening. Using a pair of sharp jeweler forceps, both posterior columns were removed bilaterally and fixed using 4% PFA for 10 hours followed by C3 laminectomy and dural opening. Using a pair of sharp jeweler forceps, both posterior columns were removed bilaterally and fixed using 4% PFA for 10 hours followed by 80°C cryostat. Sections were cut at a thickness of 12 μm, placed onto glass slides, and stored at –80°C for later in situ hybridization and immunohistochemistry (below). For RT-PCR, the unperfused spinal cord was removed 4 days after the injury and processed as detailed below.

SCRM. Adult male Sprague-Dawley rats or male Folr1+/– or Folr1+/+ mice were subjected to surgery under ketamine/xylazine anesthesia, as previously described (Figure 4) (4, 38, 39). The cervical cord was exposed through a C3 laminectomy and dural opening. Using a pair of sharp jeweler forceps, a well-defined 0.5-mm–deep injury was made in both posterior columns. A 2-cm sciatic nerve segment harvested from the left hind limb was then implanted at the injury site using 10-0 nylon sutures in the pia. The other end of the graft was allowed to lie freely under the skin. Two weeks later, the incision was reopened under a similar anesthetic and a gelfoam soaked in 5 μl of the retrograde tracer Fluoro-Gold was placed at the free end of the nerve graft. The fluorescent tracer is picked up solely by axons that have grown into the graft and undergoes retrograde transport (13). 24 hours later, the animal was deeply anesthetized and perfused through the heart with 4% paraformaldehyde. The L5 DRGs were removed bilaterally, post-fixed, incubated in 30% sucrose overnight, and quick frozen in OCT. Because, in our previous reports, very little axonal regeneration had been noted on the nonconditioned side (the side without prior sciatic nerve injury) (7), counting and analysis (below) were reported here only on the conditioned side. Sections were cut at 10 μm with a cryostat, floated on prewetted glass slides, and stored at –80°C. For analysis, sections were examined under a fluorescence microscope, and the number of fluorescently labeled cells was counted by 2 independent observers blinded to treatment. The same sections were then counterstained with cresyl violet to reveal the cell somas of all neurons in the DRG. Fluorescently labeled cells and total cells were counted in treated and control animals by an observer unaware of the treatment conditions. The percentage of labeled DRG cells in each animal was calculated. In order to determine labeled neuronal numbers accurately, the assumption-based method of Abercrombie was used, in which only cells with visible nucleoli are counted (40, 41). The expected total number of lumbar DRG neurons in mice is between 3,000 and 4,000 (42). A 1-way ANOVA with Bonferroni’s correction; mean ± SEM; *P < 0.05.
was placed into the graft at a distance of 1 cm from the globe. Twenty-four hours later, the animal was deeply anesthetized and perfused intracardially with 4% paraformaldehyde. The globe was removed and postfixed with 4% PFA fixative, and DEPC-PBS for 20 minutes each. The slides were then placed in an RNase-free glass container with acetic anhydride in triethanolamine (TEA) buffer for 15 minutes with stirring for acetylation. Following a 5-minute PBS wash, the slides were dehydrated with successive methanol washes from 30%-100%. The slides were then exposed to a prehybridization solution consisting of ×20 SSC, 50% formamide, 10% SDS, 5% lauryl sarcosine, blocking reagent, and DEPC water. After 1 hour, the solution was replaced with the hybridization solution including the probe at the concentration determined by the dot blot direct detection assay, 1:25, and allowed to hybridize overnight at 42°C in a humid container.

The following day, the slides were immersed in successive washes of 1× SSC-0.3% CHAPS and 1.5× SSC-0.3% CHAPS at 60°C for 10 minutes each and then cooled to 37°C. Next, the solution was replaced with 2× SSC-0.3% CHAPS and 2× SSC at 37°C for 20 minutes each. The slides were allowed to cool to room temperature, and 2× SSC-0.3% CHAPS and 0.2× SSC-0.3% CHAPS were administered at 60°C for 30 minutes each. PTW-0.3% (where PTW consists of 0.1% [v/v] Tween 20 in PBS) CHAPS was then applied to cool to room temperature, and 2× SSC-0.3% CHAPS was then applied at 60°C for 2 minutes. At room temperature, PTW was applied for an additional 10 minutes followed by PBT (0.1 M PBS, pH 7.4, 0.1% BSA, 0.2% Triton X-100) for 15 minutes. The slides were then incubated with 10% goat serum in PBT with 2 mM levamisole for 1 hour at room temperature. The solution was then replaced with anti-digoxigenin-AP (1:1,000) in 2.5% goat serum, PBT, and levamisole and incubated overnight at 4°C.

On the final day, the slides were washed 3 times with PBT for 30 minutes each at room temperature. The slides were then washed with an alkaline phosphatase buffer for 5 minutes followed by the alkaline phosphatase buffer with levamisole for another 5 minutes. To stain with the hybridized probe, the slides were then incubated in alkaline phosphatase buffer with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP 90 µl/ml), producing a color substrate to quantify hybridization efficiency. The slides were developed until color was evident and then immersed in deionized water. The slides were dried, mounted, and cover-slipped for preservation. Cells hybridized with the probe were counted using a fluorescence microscope by a reader blinded to the treatment status of the specimens. Specificity of the probe was controlled for by comparison of antisense-labeled slides with sense-labeled slides of corresponding DRGs. The number of stained neurons was counted by an observer unaware of the treatment conditions. Random sections in each of the control and experimental groups were then counterstained with cresyl violet to reveal the cell somas of all neurons in the DRG. The total cells were then counted in treated and control animals by an observer unaware of the treatment conditions.

The percentage of labeled DRG cells in each animal was calculated.

**Folr molecular experiments in rats**

*In situ hybridization.* All steps preparing for and including in situ hybridization were completed in RNase-free environments using sterile equipment, solutions, and techniques. Preparation of the probe was accomplished following the oligonucleotide 3’ end labeling protocol per manufacturer’s instructions (Roche Applied Science). The oligonucleotide was combined with double-distilled water, x5 reaction buffer, CoCl₂ solution, DIG-dUTP solution, and terminal transferase, mixed, centrifuged briefly, and incubated at 37°C for 15 minutes. The reaction mixture was then placed on ice, and the reaction was stopped using 0.2 M EDTA. The labeling efficiency was determined by dot blot direct detection method according to the manufacturer’s instructions (Roche Applied Science).
The following day, the sections were rinsed with 0.1 M PBS 3 times for 10 minutes each time. This rinse was applied prior to and following each incubation step. The first incubation consisted of goat anti-mouse biotinylated secondary antibody (BA-2001; Vector Laboratories) (1:200) in antibody-diluting buffer for 1 hour at room temperature. An incubation with avidin-peroxidase followed (1:100 with antibody diluting buffer prepared 30 minutes prior, prepared using Vector Vectastain working solution protocol; Vector Laboratories) for 45 minutes at room temperature. After rinsing, the color reaction was developed with Vector DAB kit for 5–10 minutes. The slides were rinsed a final time with the 0.1 M PBS 3 times for 5 minutes each time and allowed to air dry at room temperature overnight. On day 3, the slides were dehydrated in alcohol and xylene, dried, and coverslipped using Permount (Fisher Scientific) kit for 5–10 minutes. The slides were rinsed a final time with the 0.1 M PBS 3 times for 10 minutes each time. On day 2, the sections were rinsed with 0.1 M PBS (pH 7.4) for 10 minutes, 3 times, then cover-slipped with Fluor Mount G for confocal microscopy.

**Immunohistochemistry for confocal microscopy.** On day 1, the DRG slides were rinsed with 0.1 M PBS (pH 7.4) 3 times for 5 minutes each time and incubated in blocking buffer (5% horse serum and 0.3% Triton X-100 in PBS) for 60 minutes at room temperature. Sections were then incubated in goat polyclonal α-FR (M-19) 1 in 50 and mouse monoclonal neurofilament 1 in 500 in antibody-diluting buffer (2% horse serum, 0.2% Triton X-100 in PBS) overnight at 4°C. On day 2, the sections were rinsed with 0.1 M PBS for 10 minutes, 3 times. Sections were then incubated with donkey anti-goat Alexa Fluor 596 (1:500) (red fluorescence for α-FR [M-19]) and donkey anti-mouse Alexa Fluor 488 (1:1000) (green fluorescence for neurofilament) in antibody-diluting buffer for 1.5 hours at room temperature. The sections were then rinsed with 0.1 M PBS (pH 7.4) for 10 minutes, 3 times, then cover-slipped with Fluor Mount G for confocal microscopy.

### Figures

**Figure 10**

Dnmt agonist promotes, and Dnmt antagonist inhibits, the regeneration of injured sensory spinal axons into a peripheral nerve graft. Spinal axonal regeneration was measured in the SCRM model on the “conditioned” side after i.p. treatment with a Dnmt agonist 3-ABA (15 mg/kg) or antagonist 5-AzaC (0.1 mg/kg) with and without FA at 80 μg/kg. Treatment with the antagonist suppresses the proregenerative effect of FA (A). Conversely, treatment with the agonist at this dose significantly improves spinal regeneration into a nerve graft in vivo. The effect of 3-ABA at 15 mg/kg is equivalent to the 60 μg/kg dose of FA (7) (B). n = 7 (FA); n = 9 (FA and 5-AzaC); n = 11 (vehicle control); n = 9 (3-ABA); n = 7 (FA); n = 9 (FA and 3-ABA). One-way ANOVA with Bonferroni’s correction; mean ± SEM; * P < 0.05.
Wisconsin). The doses of MTX (400 μg/kg, i.p.) and nitrous oxide (80% N₂O–20% O₂; 4-hour exposure 3 days prior to the injury, then 2-hour exposures every other day for 2 weeks) were selected from a literature review (45–48) and preliminary confirmatory experiments in our laboratory.

Dhfr assay. The Dhfr assay is based on the ability of Dhfr to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to tetrahydrofolic acid. Rats were treated with daily i.p. doses of MTX (400 μg/kg) for 3 days prior to spinal cord injury and for an additional 3 days after the injury. The injured spinal cords were then removed, homogenized, and subjected to the Dihydrofolate Reductase Assay Kit (CS0340; Sigma-Aldrich) according to the manufacturer's instructions.

MS assay. Frozen spinal cord samples were pulverized in liquid nitrogen using a porcelain mortar and pestle, and the powdered tissue was resuspended in a buffer containing 100 mM potassium phosphate, pH 7.2, 0.24 M sucrose, aprotinin (2 μg/ml), PMSF (25 μg/ml), tosyl-L-lysine chloromethyl ketone (TLCK) (3 μg/ml), and trypsin inhibitor (3 μg/ml). Homogenates were centrifuged, and MS activity was measured in cell-free extracts as described previously (22).

DNA methylation experiments

Treatments with FA, 3-ABA, or antagonist 5-AzaC. Sprague-Dawley rats were given i.p. injections of the following agents alone or in combination:

Figure 11

Combined spinal cord and peripheral nerve injury increases methylation of the Gadd45a promoter, and FA increases methylation further in a biphasic fashion in 12 of 18 CpG sites. The spinal cords of animals subjected to combined spinal cord and left sciatic nerve injury (SCSN) and increasing i.p. concentrations of FA were removed for analysis. The percentage of methylation at each of 18 CpG sites of the Gadd45a promoter was measured in triplicate using pyrosequencing. Note the biphasic response to FA in 12 of the sites studied. Most of the missing data on the graphs are caused by failed analysis by both software and manual analysis. For the samples with no data on any of the CpG sites, this is caused by failed PCR. Overall, the quadratic term in the dose-response models was statistically significant ($P = 0.0001; n = 3$), confirming that there is a U-shaped dose-response to the folate dose. There is no evidence of differential dose responses among sites ($P = 0.8$), although power is low for identifying specific CpG sites with differing dose responses.
Dnmt agonist, 3-ABA (15 mg/kg; Sigma-Aldrich); Dnmt antagonist, 5-AzaC (0.1 mg/kg; Sigma-Aldrich); and FA at these concentrations: 0 μg/kg (untreated), 20 μg/kg, 40 μg/kg, 80 μg/kg, 160 μg/kg, 400 μg/kg, and 800 μg/kg. The daily i.p. injections, given under aseptic conditions, began 3 days prior to and continued until 4 days following the spinal cord injury. Throughout all protocols reported in this paper, 20 μl of FA 5 mg/ml (APP Pharmaceuticals) was diluted in DDH₂O as needed to obtain the final concentration.

*Combined spinal cord and sciatic nerve injury surgery.* The rats were subjected to a combined cervical spinal cord and sciatic nerve injury as detailed above (SCSN). Other rats were used as unjured unreated controls. Four days following the injury, the animals were anesthetized, and the entire spinal cord was harvested for use in the experiments below.

**DNA extraction and purification.** Spinal cord tissue was lysed with proteinase K by vortexing. The tissue was then incubated at 55°C for 1–3 hours. Buffer AL (QIAGEN) and 100% ethanol were then aliquoted in succession with alternating vortexing and incubation periods until the solutions were completely homogenous. The mixtures were then transferred to DNeasy Mini Spin columns for washes with Buffer AW1 and Buffer AW2 (QIAGEN).

With each wash, the mixture was centrifuged at 8,000 g, 20,000 g respectively, and flow-through was discarded. A final 2 aliquots of Buffer AE was then used to elute the purified total DNA from the final dry membrane.

**Methylation incorporation assay.** For methylation incorporation (23), 1 μg of genomic DNA was combined with 1H-SAM, SsI buffer, double-distilled water, and SssI enzyme. Control reactions lacking the enzyme were also prepared. After heating for 1 hour at 37°C, the reaction mixture was aspirated through a Whatman DE-81 2.3-cm filter disk and successively washed with 50 mM sodium phosphate buffer, 70% ethanol, and 100% ethanol. The filter disk was then air dried and placed in a vial with scintillant fluid. The number of incorporated radioactively labeled methyl groups in the DNA sample was scored by a programmed scintillation counter. For statistical analysis, see below.

**Biochemical determination of SAM and SAH spinal cord levels and SAM/SAH ratio.** Spinal cord tissue was dissected and immediately homogenized with 5 volumes of perchloric acid (0.4 M). Homogenates were centrifuged and the clear PCA extract stored at -20°C until analysis. The SAM and SAH measurements were determined by HPLC coupled to UV detection as previously reported (49).

**Methyltransferase Western blot analysis in mouse spinal cord.** Protein (30 μg) was separated by SDS-PAGE electrophoresis and transferred to the polyvinylidene difluoride membrane. The membranes were blocked with 5% milk in TBS containing 0.1% Tween-20 followed by overnight incubation at 4°C with primary monoclonal antibodies diluted 1:500 (Dnmt1) and 1:250 (Dnmt3a and Dnmt3b) in washing buffer (0.05 M Tris-base, 0.5 M NaCl, 0.05% Tween-20 in pH 7.6). After several washes, secondary antibody goat anti-mouse IgG-HRP was added (1:4,000 in washing buffer) and incubated at room temperature for 1 hour. The membrane was washed and specific bands were observed using super signal detection reagents (Thermo Fisher Scientific). The total protein content was normalized using mouse anti-actin antibodies (1:5,000). Primary mouse monoclonal antibodies were as follows (Abcam): Dnmt1 (ab13573); Dnmt3a (ab13888); and Dnmt3b (ab13604). Secondary antibody goat anti-mouse antibody was as follows: IgG-HRP SC2005 (Santa Cruz Biotechnology Inc.). The densities of the bands were then measured using NIH Imagej software relative to the densities of the corresponding actin bands. Data were plotted and statistical analyses performed using a 1-way ANOVA with Bonferroni’s correction for pairwise comparisons, and statistical significance was defined as P < 0.05 for all comparisons.

**Spinal cord regeneration in response to Dnmt agonist or antagonist.** In the rat SCRM above, the Dnmt agonist 3-ABA (15 mg/kg) or the antagonist 5-AzaC (0.1 mg/kg) was given i.p. with or without 80 μg/kg of FA. All treatments were made with i.p. injections starting 3 days prior to surgery and given daily until 2 weeks after surgery.

**Gadd45a methylation analysis.** Pyrosequencing for allele quantification (PSQ H96A; QIAGEN Pyrosequencing) is a real-time sequencing-based DNA analysis that quantifies multiple and consecutive CpG sites individually (50). Rat Gadd45a methylation assay covers eighteen CG dinucleotides in the 5’ upstream region ranging from −211 to −12 in reference to the translational start site (start codon ATG’ Ensembl: ENSRNOG00000005615). 500 ng of DNA samples was bisulfite modified using the EZ Methylation Kit (Zymo Research Inc.) PCR was performed using 10× PCR buffer, 3.0 mM MgCl₂, 200 μM of each dNTP, 0.2 μM each of forward and reverse primers, 1.25 U HotStar DNA polymerase (QIAGEN), and approximately 10 ng of bisulfite-converted DNA per 50 μl reaction. PCR cycling conditions were as follows: 95°C, 15 minutes; 45 cycles of 94°C, 30 seconds; 56°C, 30 seconds; 72°C, 30 seconds; and 72°C, 5 minutes. Products were then held at 4°C.

PCR was performed with 1 of the PCR primers biotinylated to convert the PCR product to single-stranded DNA templates. PCR products (each 10 μl) were sequenced using the Pyrosequencing PSQ96 HS System (QIAGEN Pyrosequencing) following the manufacturer’s instructions. Three separated sequencing reactions are needed to sequence the PCR amplicon. The methylation status of each locus was analyzed individually as an artificial T/C SNP using QcPcG software (QIAGEN Pyrosequencing).

Samples were run in triplicate for each of the experimental conditions. All pyrosequencing services were performed by EpigenDx Inc.

**Statistics.** All data are mean ± SEM. Statistical significance was determined by Student’s t test or 1-way ANOVA with Bonferroni’s correction for all pairwise comparisons, and a P value less than 0.05 was considered significant. For the DNA methylation incorporation assay, a dose-response regression model was fit to the responses for injured animals that were treated with a folate dose of at least 20 μg/kg, including linear and quadratic terms for log dose. The quadratic term is intended to capture a U-shape dose-response curve. All P values are reported without correction for multiple comparisons. Note that decreased counts indicate increased methylation and vice versa. For pyrosequencing, a mixed-effects model with a random effect for animal was used to assess the overall relationship between folate dose and CpG site methylation. Linear and quadratic terms for log dose were again included in the model as fixed effects. For each site, percentage of methylation was first normalized by the overall site mean in order to account for site-to-site variations in response. Variation between sites in dose response was assessed using mixed-model–based ANOVA.

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