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IMMEDIATE COMMUNICATION

Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal

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Methylation events play a critical role in the ability of growth factors to promote normal development. Neurodevelopmental toxins, such as ethanol and heavy metals, interrupt growth factor signaling, raising the possibility that they might exert adverse effects on methylation. We found that insulin-like growth factor-1 (IGF-1)- and dopamine-stimulated methionine synthase (MS) activity and folate-dependent methylation of phospholipids in SH-SY5Y human neuroblastoma cells, via a PI3-kinase- and MAP-kinase-dependent mechanism. The stimulation of this pathway increased DNA methylation, while its inhibition increased methylation-sensitive gene expression. Ethanol potently interfered with IGF-1 activation of MS and blocked its effect on DNA methylation, whereas it did not inhibit the effects of dopamine. Metal ions potently affected IGF-1 and dopamine-stimulated MS activity, as well as folate-dependent phospholipid methylation: Cu^{2+} promoted enzyme activity and methylation, while Cu^{+}, Pb^{2+}, Hg^{2+} and Al^{3+} were inhibitory. The ethylmercury-containing preservative thimerosal inhibited both IGF-1- and dopamine-stimulated methylation with an IC_{50} of 1 nM and eliminated MS activity. Our findings outline a novel growth factor signaling pathway that regulates MS activity and thereby modulates methylation reactions, including DNA methylation. The potent inhibition of this pathway by ethanol, lead, mercury, aluminum and thimerosal suggests that it may be an important target of neurodevelopmental toxins.
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Introduction

Developmental disorders include a spectrum of neurological conditions characterized by deficits in attention, cognition and learning, frequently accompanied by abnormal behaviors. Severe deficits may be recognized at birth, but a failure to achieve standard milestones during initial years of life remains the primary basis of diagnosis in most cases. While the underlying cause(s) remains obscure for many developmental disorders, metabolic abnormalities involving purine synthesis (eg Lesch–Nyhan Syndrome and adenylsuccinate lyase deficiency)[1,2] or impaired methylation-dependent gene silencing and/or imprinting (Rett and Fragile-X syndromes)[3,4] suggest biochemical mechanisms that may be involved. The development disorders can also be caused by exposure to toxins (eg ethanol, in fetal alcohol syndrome; heavy metals, in lead poisoning),[5,6] although the precise mechanisms underlying their toxicity are not known. The recent increase in the incidence of autism has led to the speculation that environmental exposures including vaccine additives (ie aluminum and the ethylmercury-containing preservative thimerosal) might contribute to the triggering of this developmental disorder.[7]

Normal development is closely related to cellular differentiation, and growth factor-initiated signaling promotes differentiation of pluripotent cells.[8] Furthermore, altered patterns of DNA methylation and associated gene silencing underlie phenotypic differences between undifferentiated and differentiated cells.[9] Together, these observations suggest that growth factors promote cellular differentiation by producing effects on DNA methylation. This suggestion is reinforced by the observation that

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blocking methylation interferes with growth factor response.[10,11]

Methylation reactions, including DNA methylation, are generally controlled by the ratio of the methyl donor *S*-adenosylmethionine (SAM) to its demethylated product *S*-adenosylhomocysteine (SAH), since SAH retains considerable affinity for methyltransferase enzymes.[12,13] Methionine synthase (MS) exerts an important influence on the [SAM] to [SAH] ratio by efficiently converting homocysteine to methionine, using 5-methyltetrahydrofolate as the methyl donor. This prohibits the reversion of homocysteine to SAH, which is otherwise thermodynamically favored.[14] In some tissues, but not the brain, homocysteine can also be converted to methionine by a betaine-dependent methyltransferase. Thus methylation reactions in the brain are highly dependent on MS activity.

In addition to the methylation of homocysteine, MS is also essential for folate-dependent methylation of membrane phospholipids carried out by the D4 dopamine receptor.[15–17] Dopamine activation of the D4 receptor initiates a four-step cycle of phospholipid methylation (PLM) in which the side chain of a methionine residue in the receptor is adenosylated, enabling transfer of its methyl group to the head group of an adjacent phospholipid. Following the removal of the adenosyl group by SAH hydrolase, MS provides a new, folate-derived methyl group to the side chain, thereby supporting dopamine-stimulated PLM. In light of studies linking attention-deficit hyperactivity disorder (ADHD) to genetic variants of the D4 receptor found only in primates,[18] we have proposed that dopamine-stimulated PLM might play an important role in attention and in attention-initiated learning.[19]

In the current study, we investigated the ability of dopamine and insulin-like growth factor-1 (IGF-1) to regulate MS activity and folate-dependent PLM in SH-SY5Y human neuroblastoma cells, and found that they stimulated activity via a PI3-kinase- and MAP-kinase-dependent signaling pathway. Furthermore, we examined the ability of several neurodevelopmental toxins to interfere with this novel mode of regulation. Their potent inhibitory effects raise the possibility that impaired MS activity may contribute to developmental disorders and to disorders of attention.

Materials and methods

Phospholipid methylation

SH-SY5Y cells were grown in six-well plates in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin/fungizone. After a wash with Hank's balanced salt solution, cells were incubated for 30 min in 600 μl of Hank's solution containing 1 μCi/ml [^{14}C]formate (or [^{3}H-*methyl*]methionine), in the presence of IGF-1 or dopamine. Drugs or metal salts were added 30 min prior to the period of radiolabeling. The reaction was terminated by an initial wash with ice-cold unlabeled Hank's solution followed by 500 μl ice-cold 10% TCA. After scraping, cells were sonicated and an aliquot was removed for protein assay. Following centrifugation, the pellet was dispersed in 1.5 ml of 2 N HCl/MeOH/$CHCl_3$ (1 : 3 : 6), vortexed and allowed to separate. The lower $CHCl_3$ layer was washed twice with 400 μl of 0.1 N KCl in 50% MeOH and an aliquot counted for radioactivity after evaporation.

MS activity

SH-SY5Y cells were scraped, pelleted and frozen at −80°C prior to assay for MS activity. Approximately 10^8 cells were resuspended in 1 ml of 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. Cells were disrupted by sonication on ice and the homogenate centrifuged at 4°C. Assays were performed under anaerobic conditions, as described previously.[20] The reaction mixture contained 100 mM potassium phosphate, pH 7.2, 500 μM homocysteine, 152 μM SAM, 2 mM titanium citrate, 250 μM (6*R,S*)-5-$^{14}CH_3$-H_4folate and enzyme in a final volume of 1 ml. The reaction was initiated by the addition of CH_3-H_4folate, incubated for 60 min at 37°C and terminated by heating at 98°C for 2 min. Radiolabeled methionine was separated on a Dowex 1-X8 column, which was eluted with 2 ml of water. Control assays, in which sample enzyme was omitted, served as blanks.

[^{14}C] Formate Autoradiography

SH-SY5Y cells in six-well plates were incubated with Hank's solution containing [^{14}C] formate (5 μCi/ml) for 30 min and the reaction terminated by the addition of 1 ml of ice-cold lysis buffer. After scrapping, the lysate was centrifuged at 30 000 × *g* for 30 min and the pellet resuspended in 1 ml of PBS after which an aliquot was dissolved in sample buffer and separated by SDS-PAGE. A blot containing [^{14}C]-labeled membrane proteins was subsequently analyzed by phosphorimaging.

Global DNA methylation

As described previously[21] DNA was extracted from cultured cell pellets using a phenol : chloroform : isoamyl alcohol protocol. DNA (1 μg) was enzymatically hydrolyzed by sequential digestion with nuclease P1, venom phosphodiesterase I and alkaline phosphatase, and 20 μl of the digest was injected onto a reversed-phase analytical HPLC column (Suplex pKb 100). Isocratic elution was carried out with a mobile phase of ammonium acetate (7 mM; pH = 6.7) and methanol (5% v/v) in water. For mass spectrometry, stable isotopes 15N3 2′-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2′-deoxycytidine were used as internal standards. Ions of m/z 126 and 130 were used to detect 5-methyl-2′-deoxycytidine and its isotopomer, and ions of m/z 112 and 115 were used to detect 2′-deoxycytidine and its stable isotope, respectively. DNA methylation status was computed as the amount of 5-methylcytidine/μg DNA.

Methylation-sensitive PCR

MDA-MB-231, MDA-MB-435 and MCF-7 cells (1.5×10^6) were plated onto 10 cm dishes and allowed to attach overnight prior to treatment with wortmannin or LY 294002 for either 16 h or 30 h. Cells were then scraped, divided into two aliquots and pelleted. One pellet was extracted with TRI REAGENT, to isolate RNA (for RT-PCR), and the other was lysed in 500 μl TNES (10 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 0.5% SDS), for DNA extraction (for methylation-sensitive PCR). RNA samples were reverse transcribed by an initial reaction with dNTPs and oligo-DT at 65°C followed by the addition of DTT, RNase inhibitor and Superscript II at 42°C for 50 min and subsequently 72°C for 15 min to yield cDNA. RT-PCR was performed on the cyclin D2 gene using primers 5′-CATGGAGCTGCTGTGCCACG (sense) and 5′-CCGACCTACCTCCAGCATCC (antisense) and, as a control, the 36B4 primers 5′-GATTGGCTACC-CAACTGTTGCA (sense) and 5′-CAGGGGCAGCAGC-CACAAAGGC (antisense) as described previously.[22] For methylation-sensitive PCR, samples in TNES were incubated with proteinase K (20 μl of 20 mg/ml) overnight at 55°C prior to DNA extraction and resuspension in TE buffer. DNA samples were then treated with sodium bisulfite and subsequently extracted using a Wizard DNA cleanup kit, followed by ethanol precipitation and resuspension in ddH_2O. Methylation-sensitive PCR studies were then performed on cyclin D2 using primers specific for methylated and unmethylated DNA. Products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

Results

IGF-1 stimulates MS

MS utilizes 5-methyltetrahydrofolate as a required cofactor, so it is possible to assess its activity in intact cells by labeling the single-carbon folate pool with [^{14}C]formate and measuring the subsequent appearance of label in methylated products (Figure 1). Using this strategy, we evaluated the effect of IGF-1 on folate-dependent PLM in SH-SY5Y human neuroblastoma cells. Exposure to IGF-1 produced a dose-dependent increase in folate-dependent PLM with an $EC_{50} = 0.4$ nM, but insulin and IGF-2 did not share this activity (Figure 2a). However, when PLM was measured using [^{3}H-*methyl*]methionine, which bypasses MS (Figure 1), IGF-1 had no effect, although cycloleucine, an inhibitor of methionine adenosylation, reduced methylation (Figure 2b). This specificity of IGF-1 for the stimulation of folate-dependent PLM suggests an action at the level of MS. The tyrosine kinase inhibitor genistein blocked IGF-1 stimulation of folate-dependent PLM (Figure 2c), consistent with an essential role for receptor autophosphorylation.

IGF-1 activates both PI3-kinase and MAP-kinase signaling in SH-SY5Y cells.[23] The selective PI3-kinase inhibitors wortmannin and LY294002 caused dose-dependent decreases in the basal level of MS-dependent PLM and blocked the IGF-1-induced increase (Figure 2d). PD98059, a specific inhibitor of MEK (MAP-kinase kinase), also inhibited the IGF-1-induced increase of PLM (Figure 2e). In contrast, inhibition of PI3-kinase or MAP-kinase pathways did not affect folate-independent PLM, measured with [^{3}H-*methyl*]methionine (Figure 2f). Thus, both PI3-kinase and MAP-kinase activities are required for the IGF-1 stimulation of folate-dependent PLM.

To assess the influence of IGF-1 on MS, SH-SY5Y cells were treated identically to PLM studies and assayed for enzyme activity, measured as the conversion of homocysteine to methionine. As shown in Table 1, IGF-1 increased MS activity to 212% of the basal level. Remarkably, wortmannin and PD98059 each not only blocked the IGF-1-induced increase but also eliminated basal enzyme activity. These effects are in close accord with PLM results and indicate a critical role for PI3-kinase and MAP-kinase signaling pathways in regulating MS.

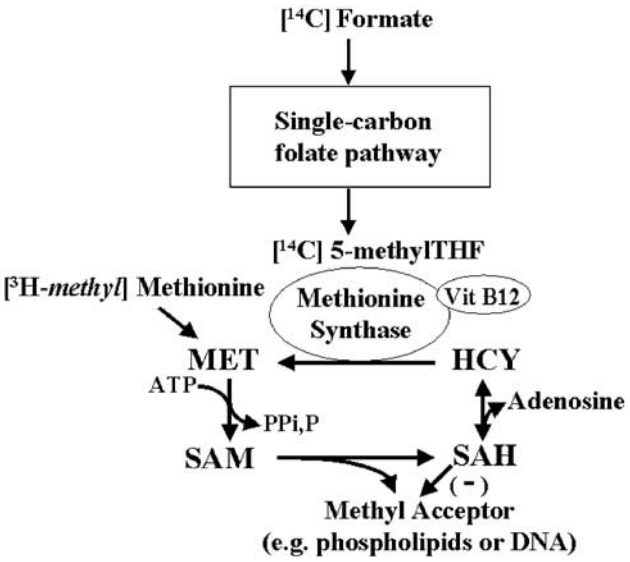

Figure 1 Folate-dependent and folate-independent PLM. SAM provides methyl groups to numerous acceptors, including phospholipids and DNA. Its methyl group originates from either the folate pathway in the form of 5-methyltetrahydrofolate (5-methylTHF), via MS-dependent methylation of homocysteine (HCY) or from methionine (MET). SAH, an inhibitor of methyl transfer reactions, is reversibly hydrolyzed to homocysteine and adenosine. By decreasing homocysteine levels, MS can promote methylation.

Dopamine stimulates MS

Dopamine caused a dose-dependent increase of folate-dependent PLM in SH-SY5Y cells with an EC_{50} of 0.8 μM (Figure 3a). Similar to IGF-1, dopamine-stimulated PLM was dependent on both PI3 kinase and MAP kinase, as indicated by the inhibitory effects of wortmannin, LY294002 and PD98059 (Figures 3b and c).

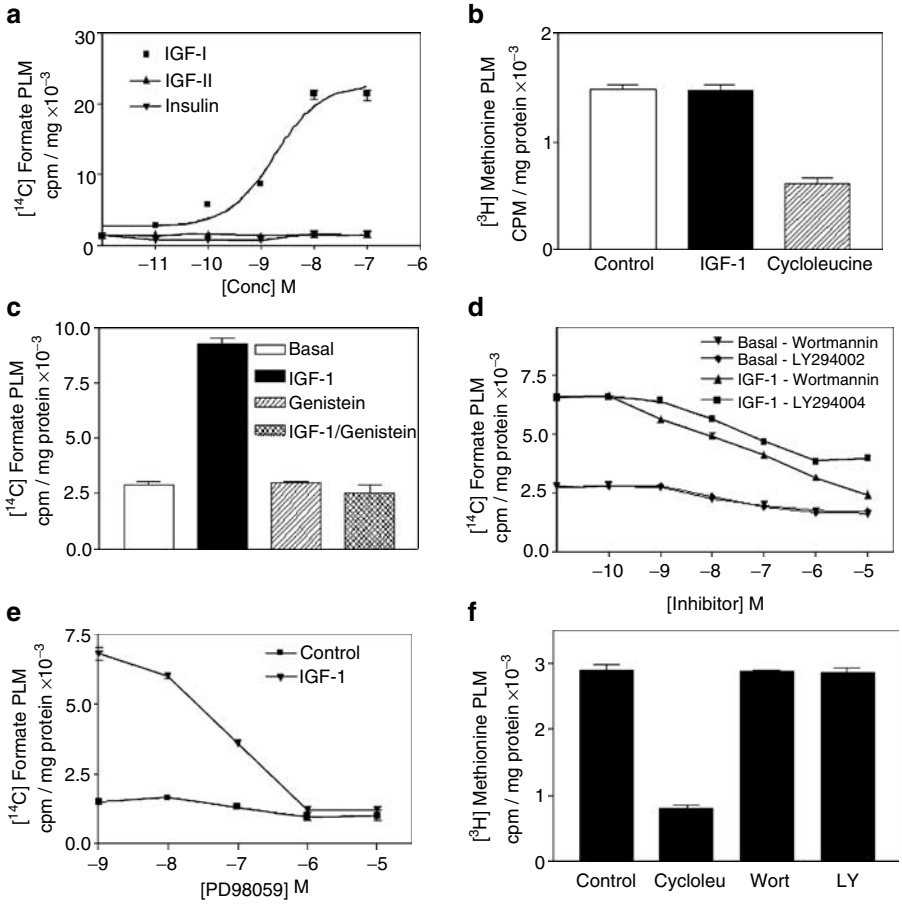

Figure 2 IGF-1 stimulates folate-dependent PLM. **(a)** Folate-dependent PLM, measured with [^{14}C]formate, ±IGF-1, IGF-2 or insulin. **(b)** Folate-independent PLM, measured with [^{3}H-*methyl*]methionine, ±IGF-1 (10 nM) or the methionine adenosyltransferase inhibitor cycloleucine (2 mM). **(c)** Folate-dependent PLM±IGF-1 (10 nM) or genistein (10 μM). **(d)** Folate-dependent PLM±PI3-kinase inhibitors wortmannin and LY294002. (e) Folate-dependent PLM± the MEK inhibitor PD98059. **(f)** Folate-independent PLM±wortmannin (1 μM) or LY294002 (1 μM).

To confirm direct D4 receptor involvement in folate-dependent PLM, cells were labeled with [^{14}C]formate for 30 min and cell membrane proteins were separated via SDS-PAGE and then transferred to a blot for autoradiography. As shown in Figure 3d, only a single 41 kDa protein, corresponding to the D4 receptor, was radiolabeled under these conditions. Dopamine increased receptor labeling, while labeling was reduced by clozapine, a D4 receptor antagonist, and by cycloleucine, an inhibitor of methionine adenosyltransferase. IGF-1 increased D4 receptor-associated radiolabel, consistent with its activation of MS, while wortmannin and PD98059 blocked both dopamine- and IGF-1-stimulated labeling (Figure 3e). Dopamine increased MS activity 2.5-fold and the increase was blocked by wortmannin (Table 1). When added in combination, dopamine and IGF-1 increased MS activity 4.5-fold, indicative of separate but additive mechanisms of PI3-kinase activation. Thus IGF-1 and dopamine both regulate MS activity, and IGF-1 promotes folate-dependent methylation of the D4 dopamine receptor.

Effects of ethanol

Ethanol inhibits PI3-kinase-dependent IGF-1 signaling in SH-SY5Y cells,[24] and we evaluated its effect on basal, IGF-1- and dopamine-stimulated, folate-dependent PLM. Ethanol potently reduced folate-dependent PLM activity, and at the highest concentration tested (0.5% v/v), basal- and IGF-1-stimulated PLM were reduced by 67 and 65%, respectively (Figure 4a). The IC_{50} for the inhibition of folate-dependent PLM (0.04% or 8.8 mM) reflects one of the most highly ethanol-sensitive responses reported to date. Conversely, ethanol had no effect on folate-independent PLM, measured with [^{3}H-*methyl*]methionine, at concentrations up to 0.5% (Figure 4b). When combined with wortmannin or LY294002, ethanol produced no additional decrease in PLM (Figure 4c). In MS assays, a 60 min exposure to 0.1% ethanol reduced the activity to an undetectable level and there was no response to IGF-1 (Table 1). Thus, ethanol potently interferes with the ability of the IGF-1/PI3-kinase system to augment MS activity directed toward homocysteine.

Table 1 MS activity in SH-SY5Y human neuroblastoma cells

Treatment	*MS activity[a] (pmol/min/mg)*
Basal	29.1 ± 2.7 (100%)
IGF-1 (10 nM; 30 min)	61.9 ± 2.6 (212%)
Wortmannin (0.1 μM; 60 min)	ND
IGF-1 + wortmannin	ND
Dopamine (10 μM; 30 min)	74.1 ± 2.6 (254%)
Dopamine + wortmannin	ND
Dopamine + IGF-1	132.1 ± 7.7 (454%)
PD98059 (1 μM; 60 min)	ND
IGF-1 + PD98059	ND
Dopamine + PD98059	ND
Ethanol (0.1%; 60 min)	ND
IGF-1 + ethanol	ND
Dopamine + ethanol	ND
$CuCl_2$ (10 μM; 60 min)	37.0 ± 3.4 (127%)
IGF-1 + $CuCl_2$	56.6 ± 4.7 (194%)
Dopamine + $CuCl_2$	71.2 ± 5.8 (244%)
CuCl (10 μM; 60 min)	1.9 ± 1.6 (7%)
IGF-1 + CuCl	33.1 ± 3.5 (114%)
Dopamine + CuCl	32.3 ± 3.3 (111%)
$HgCl_2$ (10 μM; 60 min)	ND
IGF-1 + $HgCl_2$	ND
Dopamine + $HgCl_2$	ND
$PbNO_3$ (10 μM; 60 min)	2.7 ± 0.1 (9%)
IGF-1 + $PbNO_3$	26.4 ± 0.1 (90%)
Dopamine + $PbNO_3$	35.4 ± 2.5 (122%)
Thimerosal (10 nM; 60 min)	ND
IGF-1 + thimerosal	ND
Dopamine + thimerosal	ND

[a]Results are the mean ± sem of replicate measurements from two to four separate experiments. ND = no detectable enzyme activity.

In contrast to its inhibition of basal- and IGF-1-stimulated, folate-dependent PLM, ethanol did not reduce dopamine-stimulated PLM, but instead caused a modest increase (Figure 4d). Since dopamine-stimulated PLM involves the methylation of the D4 receptor, not homocysteine, this implies that ethanol impairs the methylation of homocysteine, but not the methylation of the D4 receptor. Indeed, after exposure to 0.1% ethanol, dopamine no longer stimulated homocysteine methylation (Table 1). Ethanol there-

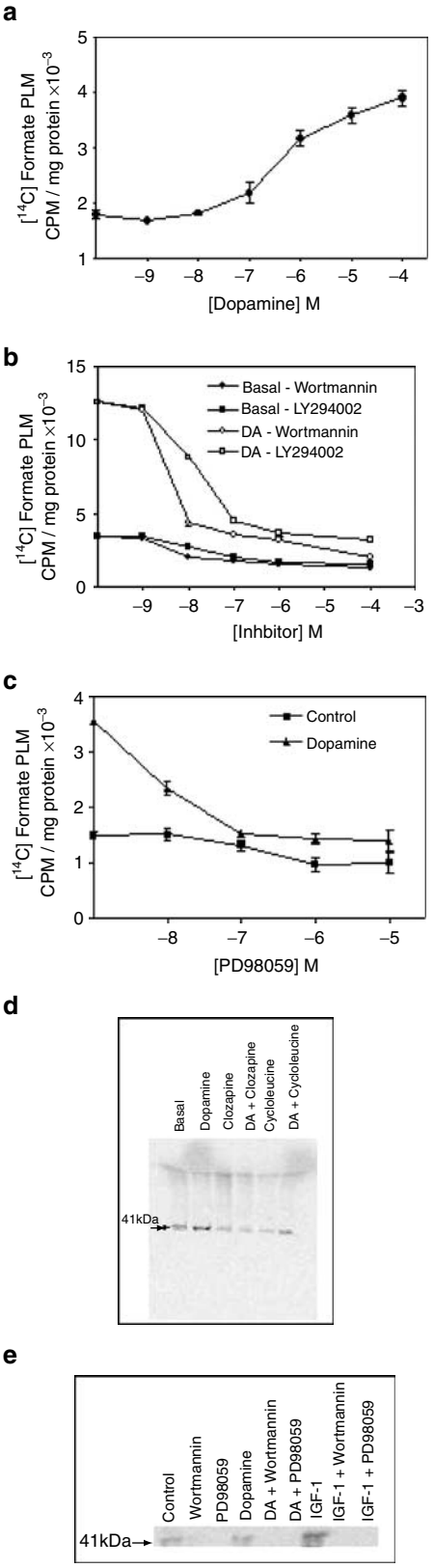

Figure 3 D4 dopamine receptor-mediated, folate-dependent PLM. (a) Dopamine-stimulated PLM measured with [^{14}C]formate. (b) Folate-dependent PLM ± dopamine (10 μM) ± wortmannin and LY294002. (c) Folate-dependent PLM ± dopamine (10 μM) ± PD98059. (d, e) Autoradiograms of SH-SY5Y membrane proteins after a 30 min incubation with [^{14}C]formate. **(d)** Dopamine (DA) (10 μM), clozapine (1 μM), cycloleucine (2 mM). **(e)** Wortmannin (1 μM), PD98059 (1 μM), dopamine (10 μM) and IGF-1 (10 nM).

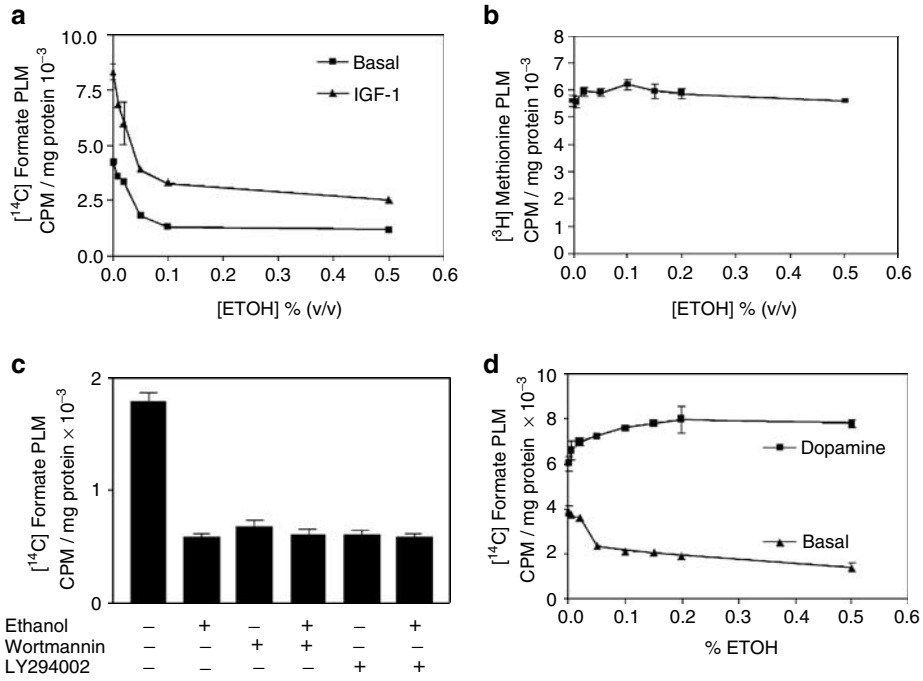

Figure 4 Effects of ethanol on PLM. (a) Folate-dependent PLM ± IGF-1 (10 nM) ± ethanol (ETOH). (b) Folate-independent PLM ± ethanol. (c). Folate-dependent PLM ± ethanol (0.1%), wortmannin (1 μM), LY294002 (1 μM). (d). Folate-dependent PLM ± dopamine (10 μM) ± ethanol.

fore diverts folate-derived methyl groups toward D4R-mediated PLM, away from homocysteine methylation.

Effects of metal ions and thimerosal

Heavy metal exposure during early development is associated with impaired neurological and cognitive function,[25–28] and Cu^{2+} has been previously shown to increase PI3-kinase activity.[29] To evaluate a possible effect of metal ions on MS-dependent PLM, SH-SY5Y cells were incubated with Cu^{2+}, Cu^{+}, Hg^{2+} and Pb^{2+} at a concentration of 10 μM for 30 min prior to and during [^{14}C]formate labeling in the presence or absence of IGF-1. As shown in Figure 5a, Cu^{2+} increased basal PLM while other metal ions had no effect. All metals, with the exception of Cu^{2+}, inhibited the stimulatory action of IGF-1. Pretreatment with penicillamine, which binds Cu^{2+}, completely eliminated the IGF-1 response. None of the metal ions affected PLM measured with [^{3}H-*methyl*]-methionine (Figure 5b), indicating their specificity for MS-related methylation events. Cu^{+} blocked radiolabeling of the D4 receptor, while Cu^{2+} was without effect (Figure 5g).

In dose–response studies, Hg^{2+} and Pb^{2+} potently inhibited IGF-1-stimulated, folate-dependent PLM with IC_{50} values of 15 and 100 nM, respectively (Figure 5c). Aluminum inhibited IGF-1-stimulated PLM in a biphasic manner, with IC_{50} values of 0.1 and 200 nM (Figure 5d). Against dopamine-stimulated PLM, however, Al^{3+} exhibited monophasic inhibition with an IC_{50} of 150 nM.

It has been suggested that increases in the incidence of ADHD and autism might be linked to the ethylmercury-containing preservative thimerosal,[7,30,31] a component of vaccines formulated in multidose containers. Thimerosal potently inhibited basal, IGF-1- and dopamine-stimulated, folate-dependent PLM, with a threshold of approximately 10 pM and an IC_{50} of 1 nM (Figure 5e), and also blocked folate-dependent radiolabeling of the D4R (Figure 5h). Similar to metal ions, thimerosal had no effect on folate-independent PLM (Figure 5b). When Cu^{2+} (1 μM) was added, the extent of thimerosal inhibition was reduced (Figure 5e), suggesting that heavy metals compete with Cu^{2+} in the PI3-kinase pathway leading to MS activation.

Thimerosal is composed of ethylmercury bound to thiosalicylate, a metal chelator that is similar in structure to penicillamine. Thiosalicylate inhibited IGF-1-stimulated, folate-dependent PLM, albeit with 500- to 100-fold lower potency than thimerosal, but did not affect basal PLM (Figure 5f). This effect of thiosalicylate was greatly reduced in the presence of 1 μM Cu^{2+}, suggesting that the chelation of Cu^{2+} may underlie its inhibitory effect.

Cu^{2+} modestly increased MS activity and did not interfere with stimulation by IGF-1 and dopamine (Table 1). Cu^{+} and Pb^{2+} reduced the basal activity by more than 90%, but allowed nearly normal increases by IGF-1 and dopamine. Thimerosal and Hg^{2+} each reduced MS activity to an undetectable level and completely blocked stimulatory effects of IGF-1 and dopamine (Table 1). Based on these results, the

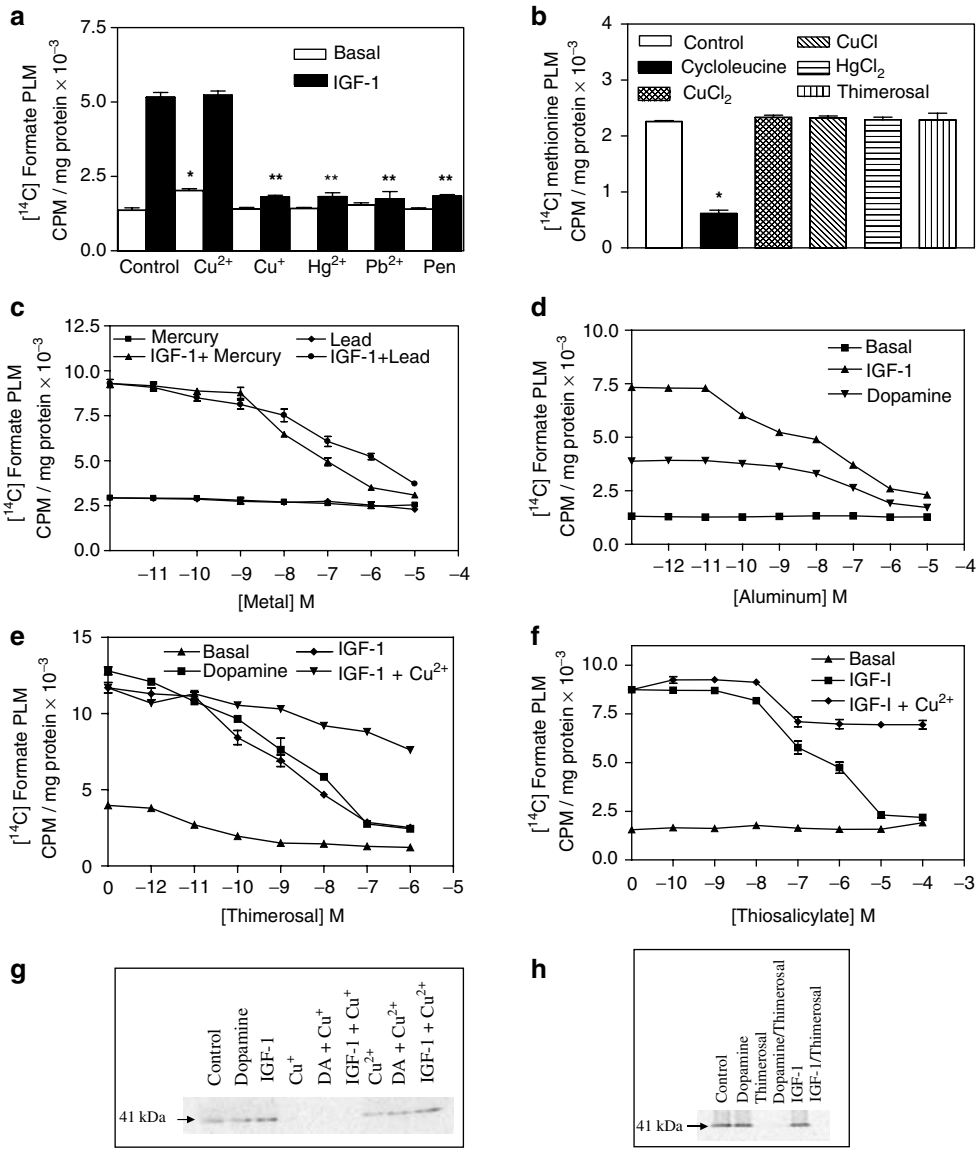

Figure 5 Effects of heavy metal ions and thimerosal on PLM. (a) Folate-dependent PLM ± IGF-1 (10 nM) in the presence of $CuCl_2$, CuCl, $HgCl_2$ or $PbCl_2$ (10 μM), or penicillamine (1 mM). *Significant increase from control ($P<0.05$); **Significant decrease from IGF-1 only ($P<0.01$). **(**b**)** Folate-independent PLM ± cycloleucine (2 mM), $CuCl_2$, CuCl, $HgCl_2$, thimerosal (10 μM). *Significant decrease from control ($P<0.01$). (c–f) Folate-dependent PLM in the presence of IGF-1 (10 nM) or dopamine (10 μM) ± mercury, lead, aluminum, Cu^{2+} (1 μM), thimerosal or thiosalicylate. (g) Radiolabeling of the D4 dopamine receptor in the presence of dopamine (10 μM), IGF-1 (10 nM), CuCl (1 μM), $CuCl_2$ (1 μM). **(**h**)** Dopamine (10 μM), thimerosal (10 nM) and IGF-1 (10 nM).

inhibitory effects of metal ions and thimerosal on folate-dependent PLM can be attributed to the inhibition of MS activity.

DNA methylation

Since increased MS activity can lower the levels of SAH, an inhibitor of methylation reactions, we examined the influence of IGF-1 and dopamine on global DNA methylation status. After a 6 h exposure, IGF-1 increased global DNA methylation by 107%, while dopamine caused an increase of 41% (Table 2). Wortmannin caused a modest increase in DNA methylation, and blocked IGF-1- and dopamine-induced increases. Ethanol had no effect on its own but, similar to wortmannin, blocked the ability of IGF-1 to increase DNA methylation. In contrast, ethanol did not block the stimulatory effect of dopamine. Thus the ability of both IGF-1 and dopamine to increase MS activity is associated with substantial increases in DNA methylation, suggesting that PI3-kinase signaling may alter gene expression via this mechanism. Moreover, changes in DNA methylation parallel the effects of these agents on folate-dependent PLM.

To evaluate the ability of PI3 kinase to affect DNA methylation and gene expression, we used methyla-

Table 2 Global DNA methylation in SH-SY5Y cells

Drug treatment	*Global DNA methylation[a] (ng MeCyt/μg DNA)*
Control	1.38 (100%)
IGF-1 (0.1 nM)	2.87 (207%)
Wortmannin (0.1 μM)	1.69 (123%)
IGF-1 + wortmannin	1.23 (89%)
Dopamine (10 μM)	1.94 (140%)
Dopamine + wortmannin	1.39 (101%)
Ethanol (0.1% v/v)	1.40 (102%)
IGF-1 + ethanol	0.91 (66%)
Dopamine + ethanol	2.78 (201%)

[a]Each data point is the mean of replicate determinations from duplicate samples.

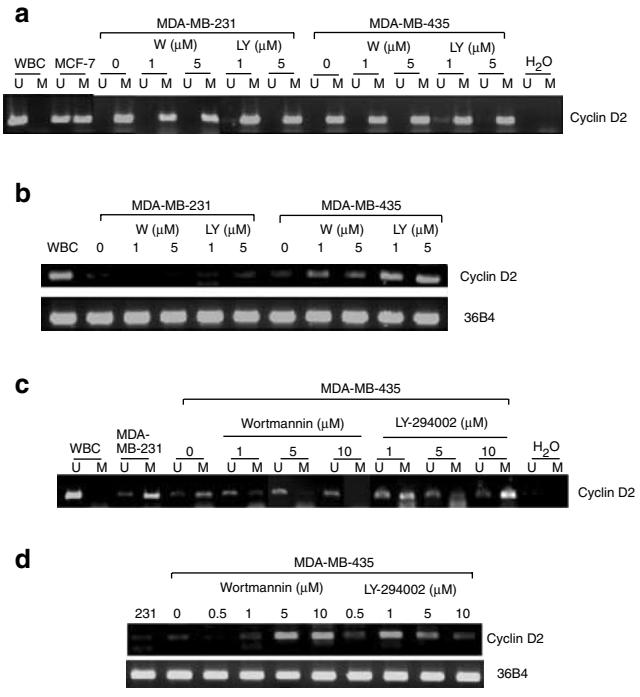

Figure 6 Methylation-specific PCR (MSP) and RT-PCR analysis of cyclin D2 following wortmannin and LY 294002 treatment. Cells (MDA-MB-231 and MDA-MB-435) were treated with 0–5 μM wortmannin (W) or LY 294002 (LY) for 16 h. The MSP analysis of cyclin D2 was performed using primers specific for unmethylated (U) or methylated (M) DNA amplification (a). RT-PCR was performed to detect cyclin D2 expression and, as a control, the housekeeping gene 36B4 (b). MDA-MB-435 cells were treated with 0–10 μM of Wortmannin and LY-294002 for the longer time period of 30 h. MSP analysis (c) and RT-PCR (d) was then performed as above on the cyclin D2 gene. WBC: white blood cell control that is unmethylated and expresses cyclin D2. Untreated MCF-7 cells are hemimethylated for cyclin D2 (a).

tion-sensitive PCR to determine the methylation status of the cyclin D2 gene, which contains a methylation-sensitive promoter.[32,33] As illustrated in Figure 6a, a 16 h treatment with LY294002, but not wortmannin, increased the proportion of demethylated cyclin D2 promoter in the breast cancer-derived MDA-MB-231 and MDA-MB-435 cell lines. RT-PCR showed that both wortmannin and LY294002 increased gene expression in MDA-MB-435 cells (Figure 6b). After a 30 h incubation, wortmannin and LY294002 both caused demethylation of the cyclin D2 promoter in MDA-MB-435 cells, along with an increase in transcription (Figures 6c and d). This confirms the ability of PI3 kinase to regulate DNA methylation and gene expression in a non-neural cell line.

Discussion

MS links the single-carbon folate pathway to the methionine cycle, and is a potentially important site for metabolic control. Nonetheless, there have been no prior reports of its regulation by extracellular signaling pathways. Our studies demonstrate the ability of IGF-1 and dopamine to increase MS activity via a mechanism that requires the activity of both PI3-kinase and MAP-kinase pathways. MS activity is a major determinant of both homocysteine and SAH levels, and the efficiency of methylation reactions is governed by the [SAM] to [SAH] ratio.[12,13] These relationships imply that growth factors, by increasing PI3- and MAP-kinase activity, can facilitate transmethylation reactions, via activation of MS. Conversely, agents interfering with this mechanism will impair methylation.

Our studies also provide evidence that ethanol, heavy metals and the vaccine preservative thimerosal potently interfere with MS activation and impair folate-dependent methylation. Since each of these agents has been linked to developmental disorders, our findings suggest that impaired methylation, particularly impaired DNA methylation in response to growth factors, may be an important molecular mechanism leading to developmental disorders.

DNA methylation is a crucial regulator of gene expression that has been linked to several developmental disorders. The majority of Rett syndrome cases are caused by *MeCp2* mutations that interfere with MeCP2 to binding to methylated CpG sites in the genome.[3] As a consequence, the protein complex necessary for histone modification and gene silencing fails to form, leading to dysregulated gene expression. Fragile-X syndrome is associated with localized hypermethylation of unstable CGG repeats at fragile sites on the X-chromosome (Xq27.3).[4] Impaired MS activity could therefore contribute to developmental disorders via altered patterns of DNA methylation.

Growth factors (eg nerve growth factor, brain-derived neurotrophic factor and IGF-1) promote development of the neuronal phenotype and support the function and survival of differentiated nerves.[34–36]

The capacity to activate simultaneously both PI3-kinase and MAP-kinase pathways is a feature of many growth factors.[37–39] Blocking the methionine cycle (eg with inhibitors of SAH hydrolase) interferes with neurotrophic responses,[10,11] indicating an essential role for methylation in growth factor action. Since differences in cellular phenotype reflect varied patterns of methylation-dependent gene silencing, it is reasonable to hypothesize that growth factors might directly or indirectly modulate genomic methylation status during development.

IGF-1 exerts trophic and antiapoptotic effects on a wide variety of cell types, and its involvement in brain development is well documented.[40,41] In addition to its neurotrophic action, IGF-1 promotes differentiation and survival of myelin-producing oligodendrocytes,[42] an action in which divalent copper plays an integral role. Thus the chelation of copper causes demyelination and an upregulation of IGF-1.[43] Vitamin B12 deficiency[44] and chronic nitrous oxide exposure,[45] both of which impair MS, also cause demyelination. We found that Cu^{2+} promotes MS activity (Table 1) and protects against the inhibitory effects of other metals (Figure 5e), while Cu^{2+} chelation has an opposite effect (Figures 5a and f). Thus oligodendrocytes provide a specific example of how IGF-1, metal ions and methylation can combine to affect cellular differentiation and brain development.

During postnatal development, myelination is critical for the specification of fixed connections between brain regions (ie hard-wiring), and there have been a number of reports of abnormal white matter (ie myelination) in autism.[46–48] Neurodevelopmental insults affecting myelination could lead to abnormal neural connections, resulting in the enhancement of certain relationships, but deficiencies in others, as is frequently observed in autism. Reduced IGF-1 levels have been reported in autism,[49] which may also contribute to impaired myelination.

Fetal ethanol exposure, consequent to maternal alcohol use, leads to the complex disorder known as fetal alcohol syndrome.[5] In humans and animal models, IGF-1 levels are reduced after fetal ethanol exposure, and the decrease is sustained through postnatal development.[50,51] Ethanol increases homocysteine levels in animals and man,[52,53] in association with impaired MS activity.[54] Ethanol potently inhibits basal- and IGF-1-stimulated MS activity (Table 1), reduces folate-dependent methylation (Figure 3a), and blocks the ability of IGF-1 to increase DNA methylation (Table 2). The IC_{50} for ethanol inhibition of methylation (8 mM) corresponds to blood levels produced by only one or two drinks, indicating a potential for adverse effects on methylation events from only moderate drinking. In a related finding, IGF-1 has been shown to promote recovery from carbon tetrachloride-induced cirrhosis, by increasing DNA methylation and normalizing gene expression.[55]

As illustrated in Figure 7, MS has two substrates, homocysteine and the dopamine D4 receptor in its

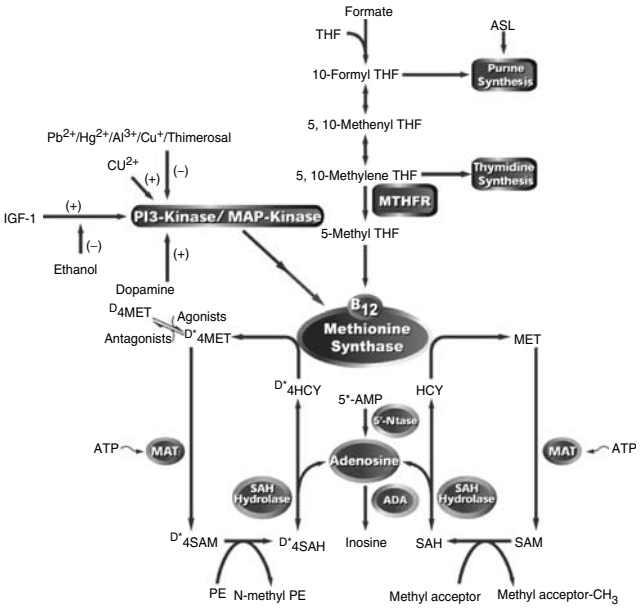

Figure 7 IGF-1 and dopamine regulate MS. Formate provides carbon atoms to the folate pathway that are used for purine and thymidine synthesis or are irreversibly reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to 5-methylTHF. MS utilizes 5-methylTHF for the methylation of homocysteine (HCY; right) and the D4 dopamine receptor during dopamine-stimulated PLM (D4R; left). D4R-mediated PLM requires the active receptor conformation (indicated by *) and is promoted or inhibited by receptor agonists or antagonists, respectively. IGF-1 and dopamine augment MS activity via a PI3-kinase- and MAP kinase-dependent mechanism, increasing methionine synthesis and lowering SAH levels. Cu^{2+} promotes MS activity, while Pb^{2+}, Hg^{2+}, Al^{3+}, Cu^{+} and thimerosal reduce activity. ATP-dependent adenosylation of methionine by methionine adenosyltransferase (MAT) forms SAM, the universal methyl donor for many reactions, including DNA methylation. SAH hydrolase reversibly catalyzes adenosine removal from SAH. Abnormalities involving purine synthesis (eg ASL mutations) or adenosine metabolism (increased 5′-nucleotidase (5′-Ntase) or decreased adenosine deaminase (ADA) activity) can adversely affect the capacity for methylation and thereby synergize with reduced MS activity.

homocysteine state. Dopamine-stimulated PLM, measured with [^{14}C]formate, reflects D4 receptor-directed MS activity and ethanol increases dopamine-stimulated PLM (Figure 4d), in contrast to its inhibition of homocysteine-directed MS activity (Table 1). These results indicate that ethanol promotes the ability of MS to utilize the D4 receptor as a substrate, while simultaneously decreasing homocysteine methylation.

Dopamine increases MS activity and folate-dependent PLM in SH-SY5Y cells via a mechanism requiring both PI3-kinase and MAP-kinase activation (Table 1, Figures 3b and c), and this increase will promote the efficiency of the D4 receptor-mediated PLM cycle. Although the functional role of dopamine-

stimulated PLM is not fully understood, the incidence of ADHD is linked to genetic variations within the D4 receptor gene,[18] and it has been proposed that dopamine-stimulated PLM plays a central role in attention.[19] Impairment of MS could therefore adversely affect the capacity for attention and could contribute to the risk of ADHD.

Lead exposure, particularly early in life, causes growth retardation along with impairments in attention and cognitive development,[6] and government guidelines establish blood concentrations exceeding 500 nM as indicative of lead poisoning.[56] An increase in blood lead levels from 1 to 10 μg/dl (120–1200 nM) is associated with an IQ decrease of 7.4 points.[28] Since lead inhibits IGF-1-stimulated methylation with an IC_{50} value of 100 nM (Figure 5c), impaired MS could contribute to developmental delay and impaired cognition associated with lead poisoning.

Mercury exposure has been suggested as a possible cause of autism[7] and methylmercury is a well-recognized neurotoxin.[57] A blood mercury level of 29 nM has been recommended by the Environmental Protection Agency as a reference value for defining toxic exposure.[58] We found that inorganic mercury inhibits IGF-1-stimulated methylation with an IC_{50} of 15 nM (Figure 5c).

Aluminum salts are used as vaccine adjuvants, based on their ability to improve dendritic cell response to presented antigens. The aluminum content of vaccines varies from 0.125 to 0.85 mg/dose, which would produce concentrations of approximately 0.7 to 4.5 μM, if uniformly distributed in the body water of a 7 kg infant. These concentrations produce greater than 50% inhibition of both IGF-1- and dopamine-stimulated methylation, raising the possibility that aluminum concentrations produced by vaccination might adversely affect methylation events. In light of the importance of MS in regulating DNA methylation[59,60] and the central role of DNA methylation in development,[9] we propose that metal exposures, including lead, mercury and aluminum, may contribute to developmental syndromes via their inhibitory effects on signaling pathways that regulate MS activity.

Thimerosal is an ethylmercury derivative of thiosalicylate, widely used as a preservative to block the growth of contaminating organisms in biological products. It was included in most vaccines in the US until 1999, when the FDA initiated a precautionary program calling for 'thimerosal-free' vaccines. Most, but not all, vaccines are now 'thimerosal-free', meaning that they contain less than 0.5 μg thimerosal/dose.[61] Thimerosal inhibits IGF-1 and dopamine-stimulated methylation with an IC_{50} near 1 nM, (Figures 5e and f), indicating its potential for disrupting normal growth factor control over methylation. Thiosalicylate itself also inhibited methylation, presumably by chelating Cu^{2+}, but was more than 100-fold less potent than thimerosal (Figure 5f), indicating that the ethylmercury in thimerosal is responsible for its inhibitory effect. The presence of added Cu^{2+}, however, significantly offsets thimerosal-induced inhibition, reflecting competition between promotional and inhibitory effects of metals on the PI3-kinase MS pathway. Thus, the toxicity of thimerosal in the body may depend upon the concentrations of metal ions that provide either additive toxicity or protective effects on PI3-kinase signaling. Thimerosal has been reported to activate apoptosis in lymphocytes[62] and in cultured human cortical neurons,[63] consistent with the inhibition of the PI3-kinase signaling pathway.

A single thimerosal-containing vaccination p roduces acute ethylmercury blood levels of 10–30 nM,[64] and blood samples in 2-month-old infants, obtained 3–20 days after vaccination, contain 3.8–20.6 nM ethylmercury.[65] Our studies therefore indicate the potential for thimerosal to cause adverse effects on MS activity at concentrations well below the levels produced by individual thimerosal-containing vaccines.

If impaired MS activity does indeed contribute to neurodevelopmental toxicity, limitations in other pathways that support homocysteine methylation could predispose individuals to higher risk. Since SAH hydrolase is reversible, the concentration of adenosine determines the probability that homocysteine will be reconverted to SAH (Figure 7). Adenosine deaminase activity is reduced in autism,[66] which would lead to higher adenosine levels and enhanced SAH formation. A polymorphism in the adenosine deaminase gene, that gives rise to a lower activity enzyme, is over-represented in autism.[67,68] Adenosine is formed by the action of 5′-nucleotidase on AMP, and Page *et al*[69] found eight- to 10-fold higher 5′-nucleotidase activity in association with an 'autism-like' developmental disorder. Each of these autism-associated metabolic abnormalities could synergize with reduced MS activity to impair methylation.

Mutations in the adenylosuccinate lyase (ASL) gene are a rare but penetrant cause of autism.[2] Lower enzyme activity blocks *de novo* purine synthesis in conjunction with a massive buildup of preblock intermediates that are ultimately excreted in the urine. As illustrated in Figure 7, increased flux of folate-derived single-carbon groups to purine synthesis restricts the availability of 5-methylTHF for MS. Moreover, increased flux of single-carbon groups toward *de novo* purine synthesis is common in autism,[70] as well as in Lesch–Nyhan Syndrome,[71] and this may increase sensitivity to neurodevelopmental toxins acting on MS.

A recent rise in autism incidence[72] has triggered concerns that an environmental factor might be promoting developmental disorders. Attention has been directed towards vaccines as a possible cause of the rise, since there has been a significant increase in the number of required vaccinations since the early 1980s.[7,28,29] Depending on infant weight and vaccination schedule, the vaccine-associated dosage of ethylmercury during the initial 24 months of life approached or exceeded federal guidelines for

methylmercury exposure.[61] A recent analysis of data from the Vaccine Adverse Event Reporting System, maintained by the Centers for Disease Control, found a significant correlation between the use of the thimerosal-containing formulation (*vs* the thimerosal-free formulation) of the Diphtheria, Tetanus and acellular Pertussis (DTaP) vaccine and autism.[31] The discovery of the PI3-kinase/MAP-kinase/MS pathway, and its potent inhibition by developmental neurotoxins, including vaccine components thimerosal and aluminum, provides a potential molecular explanation for how increased use of vaccines could promote an increase in the incidence of autism. The increased incidence of ADHD, which preceded the more recent rise in autism, could represent an alternative manifestation of vaccine-associated neurodevelopmental toxicity, since the D4 dopamine receptor is linked to ADHD[18] and its PLM function depends on MS.[15]

There are important limitations to our findings. We utilized a transformed cell line, and molecular events in tumor-derived cells might not mirror those in normal cells. SH-SY5Ycells are undifferentiated neuronal precursor cells, so it remains unclear whether growth factors and/or dopamine modulate MS activity and DNA methylation in fully differentiated cells. On the other hand, undifferentiated cells may provide a particularly appropriate model system for the study of developmental disorders. It is obvious that biochemical studies under cultured cells conditions do not replicate the complex *in vivo* environment, in terms of ambient metal ion concentrations, redox conditions and other factors that could influence methylation events. Further investigation of the *in vivo* and *in vitro* effects of heavy metals on growth factor-induced cellular differentiation is needed. While our studies focused exclusively on MS- and methylation-related events, we can speculate that other PI3-kinase signaling pathways may also be affected by metal ions.

In summary, IGF-1 and dopamine activate methionine kinase in SH-SY5Y human neuroblastoma cells via a PI3-kinase and MAP-kinase-dependent mechanism, and the activation is associated with increased DNA methylation. Several neurodevelopmental toxins inhibit this newly recognized pathway with remarkable potency, suggesting that their pathological effects might result from interruption of growth factor-initiated increases in DNA methylation and normal epigenetic regulation of gene expression. Further studies are needed to establish the functional significance of regulated MS activity and to evaluate the possibility that vaccine components (ie thimerosal and aluminum) may have contributed to the risk of autism, ADHD and other developmental disorders.

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