Stress-induced gene expression and behavior are controlled by DNA methylation and methyl donor availability in the dentate gyrus

Emily A. Saunderson[a,1], Helen Spiers[b], Karen R. Mifsud[a], Maria Gutierrez-Mecinas[a,2], Alexandra F. Trollope[a,3], Abeera Shaikh[a], Jonathan Mill[b,c], and Johannes M. H. M. Reul[a,4]

[a]Neuro-Epigenetics Research Group, University of Bristol, Bristol BS1 3NY, United Kingdom; [b]Institute of Psychiatry, King's College London, London SE5 8AF, United Kingdom; and [c]University of Exeter Medical School, University of Exeter, Exeter EX2 5DW, United Kingdom

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Stressful events evoke long-term changes in behavioral responses; however, the underlying mechanisms in the brain are not well understood. Previous work has shown that epigenetic changes and immediate-early gene (IEG) induction in stress-activated dentate gyrus (DG) granule neurons play a crucial role in these behavioral responses. Here, we show that an acute stressful challenge [i.e., forced swimming (FS)] results in DNA demethylation at specific CpG (5′-cytosine–phosphate–guanine-3′) sites close to the *c-Fos* (FBJ murine osteosarcoma viral oncogene homolog) transcriptional start site and within the gene promoter region of *Egr-1* (early growth response protein 1) specifically in the DG. Administration of the (endogenous) methyl donor *S*-adenosyl methionine (SAM) did not affect CpG methylation and IEG gene expression at baseline. However, administration of SAM before the FS challenge resulted in an enhanced CpG methylation at the IEG loci and suppression of IEG induction specifically in the DG and an impaired behavioral immobility response 24 h later. The stressor also specifically increased the expression of the de novo DNA methyltransferase *Dnmt3a* [DNA (cytosine-5-)-methyltransferase 3 alpha] in this hippocampus region. Moreover, stress resulted in an increased association of Dnmt3a enzyme with the affected CpG loci within the IEG genes. No effects of SAM were observed on stress-evoked histone modifications, including H3S10p-K14ac (histone H3, phosphorylated serine 10 and acetylated lysine-14), H3K4me3 (histone H3, trimethylated lysine-4), H3K9me3 (histone H3, trimethylated lysine-9), and H3K27me3 (histone H3, trimethylated lysine-27). We conclude that the DNA methylation status of IEGs plays a crucial role in FS-induced IEG induction in DG granule neurons and associated behavioral responses. In addition, the concentration of available methyl donor, possibly in conjunction with Dnmt3a, is critical for the responsiveness of dentate neurons to environmental stimuli in terms of gene expression and behavior.

stress | behavior | DNA methylation | immediate-early gene | hippocampus

Adaptation to stressful challenges is crucial for maintaining health and well-being. These events induce physiological and behavioral responses that enable the individual to cope with the challenge. In the brain, molecular mechanisms are initiated that facilitate learning of adaptive behavioral responses and the consolidation of memories of the event. Inappropriate responses to stress have been linked with psychiatric disorders, such as major depression and anxiety (1–3).

Glucocorticoid hormones, secreted in response to a stressful challenge, in conjunction with activated intracellular signaling pathways in neurons of the hippocampus, play a key role in consolidating behavioral responses to stress (4, 5). The hippocampal extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) pathway, activated through *N*-methyl D-aspartate receptors (NMDA-Rs) and other membrane receptors, is involved in behavioral responses seen in Morris water maze learning, contextual fear conditioning, and the forced swim (FS) test. In these behavioral paradigms, phosphorylated ERK1/2 in hippocampal neurons activate the chromatin-modifying enzymes mitogen- and stress-activated kinase 1 (MSK1) and ETS domain protein 1 (Elk-1), resulting in changes in gene transcription (5–7). Glucocorticoid hormones, via the glucocorticoid receptor (GR), facilitate the activation (phosphorylation) of MSK1 and Elk-1 by ERK1/2. MSK1 and Elk-1 activation leads to phosphorylation of serine-10 and acetylation of lysine-14, respectively, in histone H3 in multiple gene promoters, such as *c-Fos* (FBJ murine osteosarcoma viral oncogene homolog) and early growth response protein 1 (*Egr-1*), resulting in transcriptional activation of these genes (5, 7). Blocking NMDA-Rs or GRs, inhibition of ERK MAPK signaling, or gene deletion of MSK1 all prevent histone H3 phosphorylation and acetylation and the induction of c-Fos and Egr-1 in the hippocampus and impair behavioral responses in the Morris water maze test, contextual fear conditioning, and the FS test (5, 8–12). Regarding the FS test, the dentate gyrus (DG) was identified as the hippocampal region conferring these molecular and behavioral responses (4, 5, 11).

In addition to histone H3 phosphorylation and acetylation, other epigenetic mechanisms, including histone methylation and DNA methylation, are thought to be involved in behavioral responses to stress. Acute and chronic restraint stress evokes distinct effects in histone H3 methylation in various subregions of the hippocampus (13). Contextual fear conditioning results in histone H3 methylation [e.g., dimethylation of lysine-9 (K9) or trimethylation of K4 in histone H3] and DNA methylation changes in the hippocampus (13–16). Although *c-Fos* and *Egr-1* gene induction has been shown to be of

Significance

Appropriate behavioral responses to psychologically stressful events are important for maintaining mental health and well-being. The consolidation of these behavioral responses critically depends on the induction of the immediate-early gene products FBJ murine osteosarcoma viral oncogene homolog (c-Fos) and early growth response protein 1 (Egr-1) in dentate gyrus neurons. In this report, we found that an intricate balance between DNA methylation, DNA demethylation, and availability of the methyl donor *S*-adenosyl methionine governs the induction of these genes as well as the behavioral responses after stress. These findings provide insights into the epigenetic control of gene expression underlying stress-induced behavioral adaptation.

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[1]Present address: Barts Cancer Institute, Queen Mary University of London, London EC1M 6BQ, United Kingdom.

[2]Present address: Institute of Neuroscience and Psychology, University of Glasgow, Glasgow G12 8QQ, United Kingdom.

[3]Present address: Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Townsville, QLD 4811, Australia.

[4]To whom correspondence should be addressed. Email: Hans.Reul@bristol.ac.uk.

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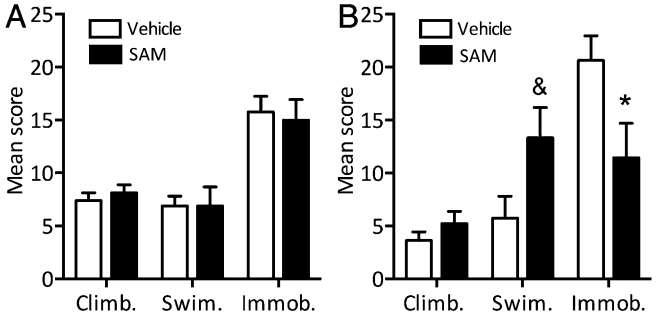

Fig. 1. The effect of SAM on FS-induced behavior. Rats were given one injection of vehicle or SAM (100 mg/kg, s.c.) 30 min before FS (15 min, 25 °C) and 24 h later were forced to swim again under the same conditions. The graphs show the climbing, swimming, and immobility behavior scored in 10-s bins during the first 5 min of the initial test (*A*) and retest (*B*). Data are shown as the mean behavioral score (mean ± SEM, $n = 8$–9). $^{*}P < 0.05$ compared with the respective vehicle-treated group; $^{\&}P = 0.072$ compared with the respective vehicle-treated group. For more information on statistical analyses in Figs. 1–7, see *SI Statistics Information to Main Manuscript Figs. 1–7*.

critical importance for the consolidation of behavioral responses in the Morris water maze, fear conditioning, and FS (5, 9, 17), the role of histone and DNA methylation changes at these immediate-early genes (IEGs) is still unclear. Interestingly, administration of the endogenous methyl donor *S*-adenosyl methionine (SAM) disrupts the consolidation of behavioral responses in the FS test (18), suggesting a requirement of methylation-dependent epigenetic mechanisms. Therefore, we postulated that, in addition to histone H3 phosphorylation and acetylation, histone H3 methylation and/or DNA methylation changes may represent a prerequisite for FS-induced c-Fos and Egr-1 induction in DG neurons and subsequent behavioral responses.

This study shows that FS results in reduced DNA methylation at specific 5′-cytosine–phosphate–guanine-3′ (CpG)s within *c-Fos* and *Egr-1* gene promoters and untranslated regions in DG neurons. Furthermore, our data show that administration of SAM significantly increases DNA methylation at these *c-Fos* and *Egr-1* gene loci and inhibits c-Fos and Egr-1 induction in DG neurons, impairing consolidation of behavioral responses after FS. Our results indicate that behavioral responses to stress are governed by an intricate balance between methyl donor availability, DNA methylation, and DNA demethylation processes.

Results

SAM Impairs Consolidation of FS-Induced Behavioral Responses. To investigate the role of methylation-dependent epigenetic mechanisms in stress-induced behavioral responses, we treated rats with the endogenous methyl donor SAM and measured changes in FS-induced behavior (Fig. 1). Because endogenous SAM is produced by the liver, we chose to administer the methyl donor systemically. In the initial FS session, no (acute) effect of SAM on behavior was found (Fig. 1*A*). In the retest, however, animals treated with the methyl donor showed significantly less immobility behavior than the vehicle-injected controls (Fig. 1*B*), indicating that SAM treatment disrupted consolidation of this behavioral response after the initial FS session.

SAM Attenuates FS-Induced c-Fos and Egr-1 Induction in DG Neurons. Previously, we have shown that induction of the IEG products c-Fos and Egr-1 in dentate granule neurons is required for the behavioral immobility response observed after FS (5, 11). Therefore, because SAM impaired this behavioral response, we determined whether the methyl donor disrupted c-Fos and Egr-1 induction. Rats received a single injection of SAM 30 min before FS and were killed 60 min after the start of the challenge, a time point when the numbers of c-Fos-positive (c-Fos^{+}) and Egr-1^{+} DG granule neurons have reached peak levels after stress (5). SAM significantly attenuated the FS-induced increase in c-Fos and completely abolished the rise in Egr-1 among dentate granule neurons (Fig. 2 *A* and *B* and Fig. S1). These effects occurred specifically among neurons within the dorsal blade of the DG (Fig. S2 *A* and *B*). The ventral blade neurons were not affected by the stressor (as shown previously) (5, 19, 20) or SAM treatment. The effect of SAM was unique to the DG because stress-induced c-Fos and Egr-1 expression in the CA1 and CA3 regions of the hippocampus was unaffected by the injected methyl donor (Fig. S3).

FS-Induced Histone H3, Phosphorylated Serine 10 and Acetylated Lysine-14 Formation Is Not Affected by SAM. FS-evoked induction of IEG products c-Fos and Egr-1 in DG granule neurons critically requires H3S10p-K14ac (histone H3, phosphorylated serine 10 and acetylated lysine-14) formation (5, 10, 11, 21). Therefore, we investigated whether the inhibitory action of SAM on c-Fos and Egr-1 induction was due to an effect on H3S10p-K14ac formation. For instance, SAM has been shown to increase protein phosphatase activity via methylation (22). Treating rats with SAM before FS however did not affect the formation of H3S10p-K14ac in DG granule neurons (Fig. 3). Furthermore, no effect was seen when immunopositive neurons in the dorsal and ventral blades were analyzed separately (Fig. S4*A*). These observations indicate that the methyl donor may attenuate the stress-evoked c-Fos and Egr-1 induction through a mechanism downstream from H3S10p-K14ac and/or via an alternative, most likely methylation-associated, epigenetic mechanism. Therefore, the role of histone and DNA methylation processes in FS-induced c-Fos and Egr-1 expression was investigated.

FS Evokes CpG-Specific Demethylation at the *c-Fos* and *Egr-1* Gene Promoters Specifically in the DG. Next, we asked whether the effect of FS on c-Fos and Egr-1 involved changes in DNA methylation at specific CpG dinucleotides in the gene promoter and in an area coding for the mRNA's 5′ untranslated region (UTR) downstream from the transcriptional start site (TSS) (Figs. S5*A* and S6*A*). In the DG, within area 2 of the *c-Fos* UTR, CpGs 3 and 4 showed significant hypomethylation after FS, with a trend in the same direction at CpG 5 (Fig. 4). No significant FS-induced changes occurred within area 1 (Fig. 4). Moreover, in the CA regions, no significant changes in CpG methylation after stress were observed in areas 1 and 2 within the *c-Fos* gene promoter and UTR (Fig. S5*B*), indicating the neuroanatomical specificity of stress-induced CpG methylation changes.

FS resulted in significantly reduced DNA methylation at CpGs 5, 11, 13, and 15 in area A of the *Egr-1* gene promoter in the DG, with near-significant differences at CpGs 7, 8, 14, 16, and 17 (Fig. 5). In area B, we found a trend of a FS effect on the methylation of CpGs

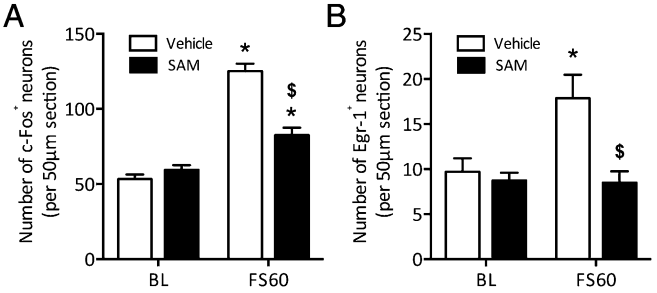

Fig. 2. The effect of SAM on FS-evoked c-Fos and Egr-1 induction in the DG. Rats were given one injection of vehicle or SAM (100 mg/kg, s.c.) 30 min before FS (15 min, 25 °C) and killed 60 min after the start of the challenge (FS60). The baseline (BL) groups were killed 90 min after injection. The graphs show the number of c-Fos^{+} and Egr-1^{+} neurons in the whole DG within a 50-µm section (*A* and *B*, respectively). Data are shown as the average number of c-Fos^{+} or Egr-1^{+} neurons from three 50-µm-thick coronal brain slices per animal (mean ± SEM, $n = 5$–6). $^{*}P < 0.05$ compared with the respective BL group; $^{\$}P < 0.05$ compared with the respective vehicle/FS60 group.

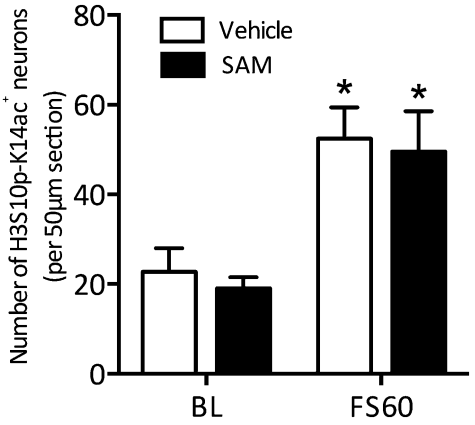

Fig. 3. The effect of SAM on H3S10p-K14ac formation in the DG after FS. Rats were given one injection of vehicle or SAM (100 mg/kg, s.c.) 30 min before FS (15 min, 25 °C) and killed at FS60. The BL groups were killed 90 min after the injection. The graphs show the number of H3S10p-K14ac$^+$ neurons in the DG. Data are shown as an average number of H3S10p-K14ac$^+$ neurons from three 50-µm-thick coronal brain slices per animal (mean ± SEM, n = 4–6). *$P < 0.05$ compared with the respective BL group.

6 and 8 (Fig. 5). In the CA regions, however, CpG methylation in areas A and B was not affected by the stressor (Fig. S6*B*).

SAM Treatment Before FS Stress Increases DNA Methylation at Specific CpG Sites in the *c-Fos* UTR and the *Egr-1* Gene Promoter in the DG. Given the reduction of DNA methylation observed within the UTR/promoter region of the IEGs in the DG after FS, we examined whether SAM treatment inhibited c-Fos and Egr-1 induction by preventing stress-evoked CpG demethylation. Our analyses focused on area 2 in the *c-Fos* UTR (Fig. S5*A*) and area A in the *Egr-1* gene promoter (Fig. S6*A*) because these regions showed the largest stress-induced CpG demethylation.

SAM treatment followed by FS significantly increased methylation of CpGs 1 and 2 in the *c-Fos* UTR (Fig. 6) and CpGs 4–8 and 13 in the *Egr-1* gene promoter (Fig. 7). CpG methylation did not increase in the SAM-injected animals killed under baseline conditions, indicating that increased availability of the methyl donor, in the absence of a stressful challenge, is insufficient to increase CpG methylation in these DG neurons. Furthermore, except for an increase in CpG 1 methylation in the baseline group and a decrease in CpG 1 and 2 methylation in the stressed group in c-Fos area 2, SAM administration did not change CpG methylation at either IEG gene promoter/UTR in the CA regions (Fig. S7).

Although we observed a significant main effect of stress on DNA methylation levels, post hoc analyses did not identify a significant effect of FS on DNA methylation levels at individual CpGs in the vehicle-treated stressed rats compared with vehicle-treated baseline controls (Figs. 6 and 7). Given the apparent stress sensitivity of CpG methylation levels in the UTR/promoters of IEGs, it is likely that the psychological stress associated with the injection has masked the effect of FS in the vehicle-treated groups.

Because SAM is the universal methyl donor, we checked whether FS and SAM would affect histone methylation processes within the IEG gene loci under study. We studied histone H3 methylation changes known to be involved in either gene activity [histone H3 trimethylated at lysine-4 (H3K4me3)] or gene suppression (H3K9me3, H3K27me3). Fig. S8 shows that SAM and FS did not alter these methylated histone marks within the *c-Fos* UTR and the *Egr-1* gene promoter.

FS Increases DNA (Cytosine-5-)-Methyltransferase 3 alpha mRNA Expression in the DG. Because FS in conjunction with SAM treatment resulted in increased DNA methylation at specific CpG sites in the *c-Fos* and *Egr-1* genes, we investigated the effect of FS on mRNA expression of several members of the *Dnmt* family, as possible mediators of the observed increase in DNA methylation, as well as mRNA expression of *Tet1* (Tet methylcytosine dioxygenase 1), a key enzyme in DNA demethylation. In the DG, *Dnmt3a* [DNA (cytosine-5-)-methyltransferase 3 alpha] expression was significantly increased immediately after the 15-min FS session (Fig. 8*A*) whereas no forced swim effect on *Dnmt3a* expression was found in the CA regions of the hippocampus (Fig. 8*B*). The expression of *Dnmt3b*, *Dnmt1*, and *Tet1* mRNA remained unchanged after FS in both the DG and CA regions (Fig. S9).

Increased Association of Dnmt3a with the *c-Fos* UTR and *Egr-1* Gene Promoter After FS. To investigate whether the increased *Dnmt3a* mRNA results in increased association of this Dnmt with the *c-Fos* UTR and *Egr-1* gene promoter regions, we conducted ChIP assays for Dnmt3a. We also conducted Dnmt3b and Tet1 ChIP assays to check whether FS might induce enrollment of (de-)methylating proteins to the chromatin independent of increased expression. We found that FS resulted in a significantly increased association of Dnmt3a, but not Dnmt3b or Tet1, with these regions in the IEG genes (Fig. 9). Association of Dnmt3b was significantly reduced at the *Egr-1* promoter after stress, indicating that there is a locus-specific decrease in Dnmt3b binding in the absence of gene expression changes (Fig. 9*B*). Thus, increased Dnmt3a expression and gene association after FS, together with the elevated levels of SAM, may underlie the increased CpG methylation at the *c-Fos* UTR and *Egr-1* gene promoter in the DG, resulting in suppressed c-Fos and Egr-1 gene expression and impaired behavioral responses to the stressor.

Discussion

A sole traumatic event has long-term implications for future behavioral responses to similar incidents. Here, we show that the DNA methylation status at the *c-Fos* and *Egr-1* gene promoters, specifically in sparsely activated DG neurons, plays a crucial role in the consolidation of immobility behavior after FS. The stressful event evoked the demethylation of distinct CpGs within the promoter and UTR of these IEGS. Conversely, elevation of methyl donor availability led, in the stressed animals, to markedly elevated CpG methylation, inhibition of IEG expression, and impaired immobility behavior. The observed changes in DNA methylation may be due to the increased *Dnmt3a* expression and the increased association of this Dnmt with the IEG loci in these DG neurons.

FS evoked CpG-specific demethylation events in the DG but not in the hippocampal CA region. Region-specific active DNA demethylation has been shown to play a role in activity-induced gene expression in DG granule neurons, likely mediated by Tet1 and/or Gadd45b (23–25). Furthermore, DNA demethylation in the

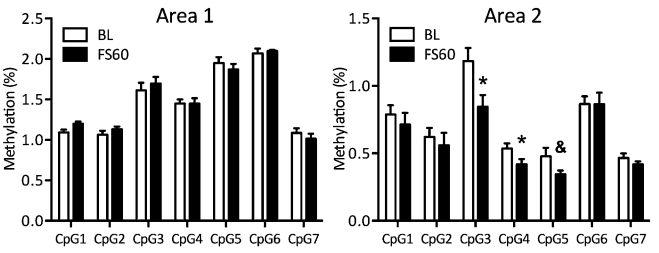

Fig. 4. FS-induced CpG-specific DNA methylation changes in the *c-Fos* promoter region. Rats were killed immediately (BL group) or subjected to FS (15 min, 25 °C) and killed at FS60. The location of CpGs within areas 1 and 2 with respect to the rat *c-Fos* gene are shown in Fig. S5. The graphs show DNA methylation changes at CpGs in area 1 and area 2 in the DG. Data are shown as percentage methylation (mean ± SEM, n = 3–6). *$P < 0.05$; &, $P < 0.1$, compared with the respective BL group.

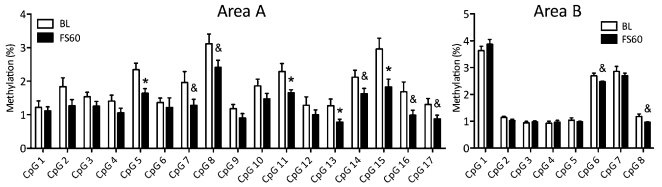

Fig. 5. FS-induced CpG-specific DNA methylation changes in the *Egr-1* promoter region. Rats were killed immediately (BL group) or subjected to FS (15 min, 25 °C) and killed at FS60. The location of CpGs within areas A and B with respect to the rat *Egr-1* gene are shown in Fig. S6. The graphs show DNA methylation changes at CpGs in area A and area B in the DG. Data are shown as percentage methylation (mean ± SEM, $n = 5–6$). $^{*}P < 0.05$; $^{\&}P < 0.1$, compared with the respective BL group.

DG also occurred after voluntary running (24). Thus, DG neurons have been found to be rather susceptible to DNA methylation changes in response to environmental stimuli. The gene expression changes in the DG after such stimuli are known to occur in sparsely distributed neurons (5, 10, 11), suggesting that the observed DNA demethylation events are also occurring in these neurons.

The methyl donor SAM had a strong effect on FS-induced gene expression and behavioral responses. The disruption of the behavioral immobility response by SAM corresponds with earlier observations made in both rats and mice (18). Until now, the underlying molecular mechanism of action of SAM on this behavioral response was unknown. SAM had no effect on baseline c-Fos and Egr-1 expression but strongly inhibited the FS-evoked IEG responses specifically in DG granule neurons. No effect of the methyl donor was observed on IEGs in the CA1 and CA3 regions of the hippocampus. Furthermore, there was no effect of SAM and FS on histone methylation at these genes. Previously, we have shown that FS-induced c-Fos and Egr-1 in DG neurons are critically involved in the consolidation of the behavioral immobility response (5). The neuroanatomically selective effect of SAM further underscores the importance of IEG expression in DG neurons for this stress-induced behavioral response. Furthermore, the induction of these IEGs in DG neurons requires the formation of the dual histone mark H3S10p-K14ac within the promoter regions of these genes. The formation of this epigenetic mark is the result of concomitant GR and NMDA-ERK-MSK1-Elk-1 signaling in these DG neurons (5). Clearly, SAM had no effect on the formation of this dual histone mark, indicating that the methyl donor did not produce its effects on gene expression and behavior through interference with these signaling pathways. Therefore, the methyl donor seems to act via a methylation/demethylation mechanism downstream of the dual histone modifications.

In our studies, SAM affected DNA methylation only under stress conditions. Administration of SAM before FS resulted in significant increases in DNA methylation within areas of the *c-Fos* 5′ UTR and *Egr-1* promoter in the DG that had previously shown demethylation after the stressor only. In vehicle-injected rats, FS failed to result in significant demethylation possibly due to (restraint) stress associated with the injection, underlining that the DNA methylation status of these CpGs is highly stress-sensitive. Because SAM is an endogenous methyl donor synthesized by *S*-adenosyl methionine synthetase mainly in the liver, the observation that this methyl donor markedly affects stress-induced gene expression and behavioral responses has greater physiological implications. We show that the impact of stressful events like FS on gene expression and behavior may depend on the cellular concentration of SAM. Presently, little is known of the regulation of *S*-adenosyl methionine synthetase activity and the control of SAM uptake in the brain. In yeast, a mechanism for sensing SAM levels was revealed that would determine metabolic processes underlying growth (22). Possibly, the mammalian brain also has a mechanism for monitoring SAM levels that determines the neuronal response to environmental stimuli.

The exact mechanism through which FS in the presence of elevated SAM levels inhibits IEG expression still needs to be clarified. Within the *c-Fos* gene, SAM- and stress-evoked CpG methylation changes occurred mainly within area 2, which is located downstream from the TSS in a region that codes for the 5′ UTR of the mRNA molecule. RNA-polymerase II and associated factors assemble upstream of the TSS and produce short RNA fragments; however, additional mechanisms are required before full-length transcripts can be produced. This poised state of gene transcription allows a rapid induction of c-Fos in response to stimuli (26). The CpGs within area 2 of the *c-Fos* gene reside within the window of elongation termination, which is between +30 and +60 bp after the TSS (27). Therefore, because DNA methylation can prevent transcriptional elongation (28), the SAM and FS-induced CpG methylation increases may result in premature termination of the *c-Fos* transcript in the DG neurons. Area A within the *Egr-1* promoter is ∼500 bp upstream from the TSS. Increased DNA methylation in this region could influence transcription factor binding and disrupt chromatin remodeling and/or assembly of transcriptional machinery. For instance, in silico analysis of transcription factor binding sites presented specificity protein 1 (Sp1) and Krüppel-like factor 9 (Klf9) sites within the DNA sequence of area A. The *Klf9* gene contains glucocorticoid response elements (GREs), and expression is induced in response to elevated corticosterone levels (29), which are known to occur after FS (30). Thus, the SAM and FS-induced increases in CpG methylation in this region of the *Egr-1* gene may have disrupted Klf9-mediated (and possibly Sp1-mediated) transcriptional activation, but confirmation of this postulate requires further investigation.

FS resulted in an increased expression of the de novo DNA methyltransferase *Dnmt3a* (but not *Dnmt3b*, *Dnmt1*, and *Tet1*) specifically in the DG. This enhanced expression after stress, in the presence of elevated SAM levels, may be responsible for the increased CpG methylation in the *c-Fos* UTR and *Egr-1* gene promoter, resulting in inhibition of gene expression and impaired behavioral responses. The increased recruitment of Dnmt3a at these IEG loci after FS supports this notion; however, in the context of normal SAM levels, this observation seems to be contradictory because FS results in DNA demethylation at the IEG loci and increased expression of c-Fos and Egr-1. Observations made in vitro may explain this apparent paradox. Dnmt3a has been shown to function as a DNA demethylase under conditions of elevated Ca^{2+} levels whereas the methyltransferase activity was reinstated after raising SAM levels (31–33). Induction of IEGs in DG neurons in vivo requires the opening of NMDA receptors, allowing a sustained rise in intracellular Ca^{2+} levels (11). In view

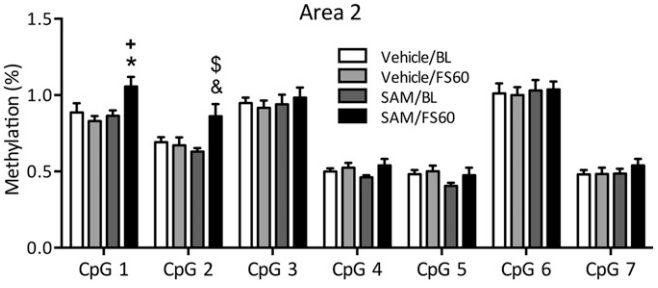

Fig. 6. The effect of SAM treatment on FS-induced DNA methylation changes at CpGs within the *c-Fos* UTR in the DG. Rats were given one injection of vehicle or SAM (100 mg/kg, s.c.) 30 min before FS (15 min, 25 °C) and killed at FS60. The BL groups were killed 90 min after the injection. The graph shows methylation of CpGs in area 2 of the *c-Fos* UTR in the DG. Data are shown as percentage methylation (mean ± SEM, $n = 4–6$). $^{*}P < 0.05$ compared with the respective vehicle/FS60 group; $^{\$}P < 0.05$ compared with the respective SAM/BL group; $^{+}P = 0.076$ compared with the respective SAM/BL group; $^{\&}P = 0.076$ compared with the respective vehicle/FS60 group.

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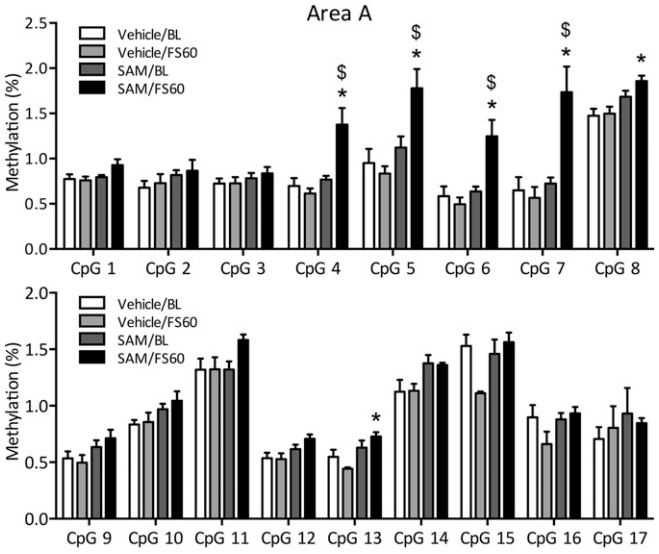

Fig. 7. The effect of SAM treatment on FS-induced DNA methylation changes at CpGs within the *Egr-1* gene promoter in the DG. Rats were given one injection of vehicle or SAM (100 mg/kg, s.c.) 30 min before FS (15 min, 25 °C) and killed at FS60. The BL groups were killed 90 min after the injection. The location of CpGs within area A with respect to the *Egr-1* gene is shown in Fig. S6. The graph shows methylation of CpGs in area A of the *Egr-1* gene promoter in the DG. Data are shown as percentage methylation (mean ± SEM, $n = 4$–6). $^{*}P < 0.05$ compared with the respective vehicle/FS60 group; $^{\$}P < 0.05$ compared with the respective SAM/BL group.

of the findings of Chen et al. (33), it may be expected that the risen Ca^{2+} levels favor the DNA demethylase activity of the recruited Dnmt3a enzyme, resulting in the demethylation at the IEG loci that we observed. When SAM levels were elevated in our study, apparently the enzyme activity of the recruited Dnmt3a shifted to a methyltransferase activity, resulting in DNA methylation of the IEG loci. Together, these data suggest that the DNA methylation status is governed not only by the recruitment (and expression levels) of DNA methylating/demethylating enzymes but also by the concentration of SAM and other physiological factors (e.g., Ca^{2+}).

The mechanisms controlling the increased association of Dnmt3a with the *c-Fos* and *Egr-1* gene loci after FS are unknown but may involve changes in the chromatin structure (e.g., through local H3S10p-K14ac formation), as well as posttranslational modifications of Dnmt3a that regulate its affinity for binding partners (34, 35). Previous work has shown that Dnmts play an important role in hippocampus-dependent learning (15, 36) Furthermore, *Dnmt3a* expression increases in the hippocampus after contextual fear conditioning (14) and is up-regulated in the DG after electroconvulsive shock (24), highlighting the importance of this de novo methyltransferase in activity-induced neuronal function. The possibility that Dnmt3a may function as a DNA demethylase in FS-activated neurons suggests additional layers of complexity to stressor-induced epigenomic regulation that should be explored.

Our work shows that, after FS, not only is *Dnmt3a* expression increased in the DG but so is its association with IEG loci; nevertheless, the levels of SAM determine the impact on IEG induction and the consolidation of the behavioral immobility response. Presently, it is unknown to what extent SAM levels determine responses in other hippocampus-dependent behavioral models like Morris water maze learning and contextual fear condition. Our results indicate that the neuronal concentration of SAM is a key factor in the molecular and behavioral responses evoked by environmental challenges. This notion is supported by work in vitro that demonstrates that inhibition of Dnmts disrupts hippocampal neuron function, but this disruption is rescuable by elevating SAM levels (37), indicating that a tightly controlled balance between Dnmt activity and SAM is important for normal hippocampal neuron function. Accordingly, it seems that a tight control of SAM synthesis (and Dnmt function) is of pivotal physiological importance.

In summary, the induction of IEGs in DG granule neurons is highly complex. Neuronal activation due to stressful stimuli is regulated by NMDA, by GABA-A and glucocorticoid receptors, by ERK MAPK signaling, by H3S10p-K14ac formation (5, 11, 21), and, as shown in the present study, by distinct CpG methylation events. It seems that IEG induction is checked by multilevel control mechanisms whereby the CpG methylation status plays a go/no-go role. The control of IEG induction in DG neurons is reflected in the (long-term) consolidation of the behavioral response after FS. The stressful challenge also resulted in increased expression of the de novo methyltransferase *Dnmt3a*, which may act as a DNA demethylase in the context of normal SAM levels and elevated Ca^{2+} in activated DG neurons. In contrast, if SAM levels were elevated, stress led to an increased methylation of CpGs within the gene/gene promoter of the IEGs, resulting in suppressed gene expression and impaired behavioral responses. Thus, our study shows that CpG methylation status is an important controller of IEG expression in DG

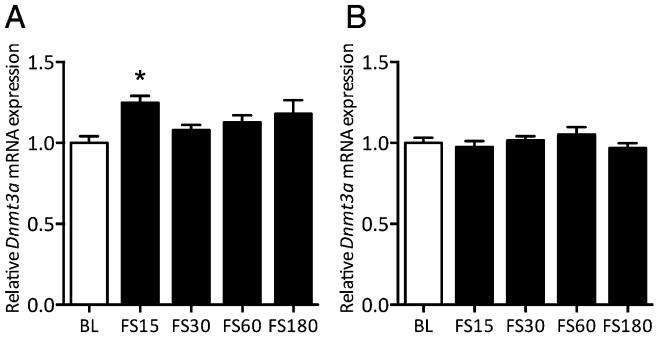

Fig. 8. Effect of FS on *Dnmt3a* mRNA expression in the DG and CA regions of the hippocampus. Rats were killed immediately (BL group) or subjected to FS (15 min, 25 °C) and killed immediately (FS15), 30 min (FS30), 60 min (FS60), or 180 min (FS180) after the start of the challenge. The graphs show *Dnmt3a* mRNA expression in the DG (*A*) and the CA regions (*B*) of the hippocampus. Data are shown as relative mRNA copy number standardized to the expression of the housekeeping genes *Hprt1* and *Ywhaz* (mean ± SEM, $n = 8$–9). Statistical analysis (one-way ANOVA): (*A*) $F_{(5,38)} = 3.0$, $P < 0.05$; (*B*) $F_{(5,39)} = 0.97$, $P = 0.43$. Dunnett's post hoc test: $^{*}P < 0.05$ compared with the BL group.

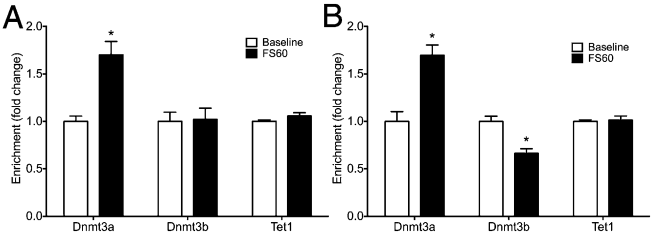

Fig. 9. Association of Dnmt3a, Dnmt1, and Tet1 with c-Fos and Egr-1 gene loci after FS. Rats were killed under baseline conditions or at 60 min after the start of a 15-min FS session (FS60). ChIP for Dnmt3a, Dnmt3b, and Tet1 was conducted on hippocampus tissue, followed by quantitative PCR (qPCR) for the c-Fos (*A*) and Egr-1 (*B*) loci studied for DNA methylation changes after SAM and FS. Data are expressed as the enrichment of the respective enzymes at the loci at FS60 relative to the enrichment in the baseline situation (mean ± SEM, $n = 4$). $^{*}P < 0.01$, Student's t test.

neurons. Moreover, we revealed that the levels of available SAM, possibly in conjunction with Dnmt3a expression and action, are a determining factor in the responsiveness of DG neurons to environmental stimuli, with significant consequences for the organism in terms of gene expression and behavior.

Materials and Methods

Animals and Drug Treatment. Male Wistar rats (150–175 g) were purchased from Harlan and group housed. All procedures were approved by the University of Bristol Ethical Committee and by the Home Office of the United Kingdom (UK Animal Scientific Procedures Act 1986). Rats were forced to swim for 15 min in 25 °C water or left undisturbed (5, 10, 11). Some animals received pretreatment with a drug or the vehicle 30 min before FS. Rats were killed at the indicated times (see figure legends) after FS or were kept until 24 h later to undergo another FS test (retest) for 5 min. Behavior was scored every 10 s during the first 5 min of the test and retest. The drug used was SAM (100 mg/kg body weight) to raise levels of the endogenous methyl donor. For more information, see *SI Materials and Methods*.

Tissue Preparation. For immunohistochemistry, rats were perfused with saline and 4% (wt/vol) paraformaldehyde and inhibitors. Brains were cut into 50-μm coronal sections and kept at 4 °C. For other studies, after decapitation, the entire hippocampus was dissected or the DG and CA regions were microdissected from the dorsal hippocampus in 1-mm coronal brain slices. Tissues were snap frozen in liquid N_2 and stored at −80 °C. For more information, see *SI Materials and Methods*.

Immunohistochemistry. Immunohistochemistry was conducted using published methods (5). For more information, see *SI Materials and Methods*.

Bisulfite Pyrosequencing. Genomic DNA from DG and CA regions was subjected to bisulfite conversion and pyrosequenced as described in *SI Materials and Methods*.

ChIP, RNA Analysis, and Quantitative PCR. ChIP and RNA extraction were performed using published methods (7, 38, 39). For a complete description, see *SI Materials and Methods*.

Statistical Analysis. Data were analyzed by ANOVA, Student's *t* test, and appropriate post hoc tests. For more information, see *SI Materials and Methods*.

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