ORIGINAL ARTICLE

The genome-wide landscape of DNA methylation and hydroxymethylation in response to sleep deprivation impacts on synaptic plasticity genes

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Sleep is critical for normal brain function and mental health. However, the molecular mechanisms mediating the impact of sleep loss on both cognition and the sleep electroencephalogram remain mostly unknown. Acute sleep loss impacts brain gene expression broadly. These data contributed to current hypotheses regarding the role for sleep in metabolism, synaptic plasticity and neuroprotection. These changes in gene expression likely underlie increased sleep intensity following sleep deprivation (SD). Here we tested the hypothesis that epigenetic mechanisms coordinate the gene expression response driven by SD. We found that SD altered the cortical genome-wide distribution of two major epigenetic marks: DNA methylation and hydroxymethylation. DNA methylation differences were enriched in gene pathways involved in neurogenesis and synaptic plasticity, whereas large changes (>4000 sites) in hydroxymethylation were observed in genes linked to cytoskeleton, signaling and neurotransmission, which closely matches SD-dependent changes in the transcriptome. Moreover, this epigenetic remodeling applied to elements previously linked to sleep need (for example, *Arc* and *Egr1*) and synaptic partners of Neuroligin-1 (NLGN1; for example, *Dlg4*, *Nrxn1* and *Nlgn3*), which we recently identified as a regulator of sleep intensity following SD. We show here that *Nlgn1* mutant mice display an enhanced slow-wave slope during non-rapid eye movement sleep following SD but this mutation does not affect SD-dependent changes in gene expression, suggesting that the NLGN pathway acts downstream to mechanisms triggering gene expression changes in SD. These data reveal that acute SD reprograms the epigenetic landscape, providing a unique molecular route by which sleep can impact brain function and health.

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INTRODUCTION

Sleep associates with brain health, and this relationship is exemplified by the predominance of alterations in sleep macro- and micro-architecture observed in most psychiatric and neurological conditions such as neurodegenerative diseases, autism, schizophrenia or mood disorders.^{1–4} In parallel, sleep perturbations such as sleep fragmentation or sleep loss modulate cognitive performance and mood,^{5,6} and may either alleviate or exacerbate certain psychiatric conditions.⁷ The identification of mechanisms underlying the relationship between sleep and brain function is crucial to develop targeted interventions in several mental health disorders.

Recovery from sleep loss involves a rebound in electroencephalographic (EEG) delta power (1–4 Hz) measured during non-rapid eye movement (NREM) sleep.^{8–10} More recently, the slope of individual NREM sleep slow waves (SW) was specifically shown to track sleep need, being steeper after prolonged wakefulness^{11–13} indicative of a more synchronized switch between the silent and the burst-firing states of neuronal activity, and likely of increased

synaptic efficacy.¹¹ These markers can be used in animal models of impaired central nervous system functioning to dissect the molecular circuitry underlying sleep need.

Important changes in the brain transcriptome are observed with acute sleep deprivation (SD).^{14–17} These data contributed to current hypotheses regarding the role for sleep in metabolism and energy regulation, synaptic plasticity and neuroprotection. We showed that the glucocorticoid surge during SD importantly contributes to these changes.¹⁷ However, glucocorticoids do not seem to underlie the EEG response to SD given that adrenalectomy does not acutely change delta power rebound.¹⁷ Therefore, the pathways underlying the changes in brain-expressed transcripts that are specifically linked to EEG recovery features remain to be identified. Targeting pathways regulating gene expression in response to neuronal activity and involved in plasticity, cognition and mental health should help defining the molecular elements underlying the effect of prolonged wakefulness on sleep SW.

We recently revealed that *Nlgn1* knockout mice (*Nlgn1*^{−/−}), which exhibit social novelty and fear-conditioning deficits, show

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reduced wakefulness duration and altered EEG during wakefulness and sleep.¹⁸ NLGN1 belongs to a family of adhesion proteins involved in shaping synaptic function that was linked to autism.¹⁹ Notably, patients with autism spectrum disorder present impairments in sleep initiation and maintenance as well as modifications in EEG activity during both wakefulness and sleep.^{3,20} Thus, NLGN1, and likely its interacting partners, may link neuronal activity to the duration and quality of wakefulness and sleep.¹⁸ Because *Nlgn1*^{-/-} mice show an amplified delta power rebound after SD,¹⁸ these represent a model to delineate the pathways involved in recovery sleep that are associated to differences in cognitive function.

Here we tested the hypothesis that changes in NLGN1 driven by sleep pressure may have a role in the brain transcriptional response observed after acute SD. We first confirmed the involvement of NLGN1 in shaping the sleep EEG, as *Nlgn1*^{-/-} mice specifically showed an enhanced synchrony of neurons under high sleep need as indexed by steeper NREM sleep SW slope. However, SD in *Nlgn1*^{-/-} mice induced changes in gene expression similar to those observed in *Nlgn1*^{+/-} mice, including in the expression of DNA methylation-related enzymes. These results suggest that SD-dependent synaptic modifications that involve NLGN1 are downstream of molecular pathways driving the transcriptional response to sleep loss. We thus explored whether epigenetic mechanisms, known to respond to neuronal activity and to regulate the expression of plasticity-related genes,^{21–23} could trigger the SD-dependent changes in synaptic components. We indeed identified differentially methylated and hydroxymethylated regions after SD that were enriched in functional pathways involved in neurotransmission, cellular assembly or metabolism. These results reveal that epigenetic regulation is a unique pathway modulating both the transcriptional and synaptic responses to acute sleep loss.

MATERIALS AND METHODS

For detailed methodological descriptions, see Supplementary information.

Animals and EEG

Male mice from strains C57BL/6J and B6;129-*Nlgn1*^{tm1Bros/J} (*Nlgn1*^{+/-}; *Nlgn1*^{+/-} *Nlgn1*^{-/-}, exons 1 and 2 replaced by a neo cassette²⁴) were used. Mice used for EEG and microarray are the same as those used previously,¹⁸ and were implanted for EEG as previously described.¹⁸ EEG was recorded during a 24-h baseline, during a 6-h SD²⁵ starting at light onset, and during 18 h of recovery. Vigilance states were visually assigned to 4 s epochs.²⁶

Spectral analysis was performed to calculate EEG power during NREM sleep between 0.75 and 20 Hz per 0.25 Hz for the 24-h baseline and the first recovery hour. SW detection was performed during NREM sleep using a home-made detector^{12,13} with criteria adapted from previous work.²⁷ SW properties were averaged per 12 h light and dark periods, and for 12 intervals during baseline light, 8 intervals during the 6 h following SD and 6 intervals during dark periods.

Microarray

A week after EEG recording, half of *Nlgn1*^{+/-} and *Nlgn1*^{-/-} mice were submitted to a second 6-h SD immediately followed by killing together with non-sleep-deprived mice (control). Forebrain (hindbrain excised) RNA was extracted,^{17,18} processed and hybridized on Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) by Genome Quebec (Montreal, Canada). Data were analyzed using GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA). *P*-values were adjusted using Benjamini and Hochberg false discovery rate (FDR).²⁸ Quantitative PCR (qPCR) validations were performed as described elsewhere.¹⁸

DNA enrichment and labeling

An anterior part of the cerebral cortex of C57BL/6J mice submitted to a 6-h SD was sampled, and DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). The remaining cerebral cortex was used for RNA extraction to perform qPCR validations (see above). Six pools of DNA (that is, three SD

and three control) each including the DNA of three SD or three control mice (total nine mice per condition) were fragmented using a bioruptor (Diagenode, Denville, NJ, USA) and used for both methylation (5mC) and hydroxymethylation (5hmC) enrichments. 5mC enrichment was performed as previously described.²⁹ 5hmC enrichment was performed using the Hydroxymethyl collector kit (ActiveMotif, Carlsbad, CA, USA). The DNA input and bound fractions were purified, amplified and labeled using Whole Genome Amplification (Sigma-Aldrich) and CGH Enzymatic Labeling (Agilent Technologies) kits.

5 mC and 5 hmC arrays

Custom 400K promoter tiling arrays were used (Agilent Technologies). For 5mC, probe sequences were selected to tile all genomic regions from -1200 to 2400 bp downstream each transcription start site as defined by Ensembl (release 66 for mouse). For 5hmC, all exons including the 250 bp before and after each gene were tiled with probes at 100 bp spacing (Ensembl release 65 for mouse).

Hybridization, washing, scanning and feature extraction were performed following the Methylated DNA Immunoprecipitation protocol (Agilent Technologies). Extracted intensities were analyzed, and lists of differentially 5mC and 5hmC regions were determined as described elsewhere.²⁹ *P*-values were corrected using FDR (threshold < 0.2 for 5mC and < 0.1 for 5hmC). qPCR validations were performed as described,²⁹ with *Tbp*, *GusB*, *Rps9* or *Gapdh* used as reference genes. All data are expressed as group mean ± s.e.m.

Biological function analysis

Ingenuity Pathway and DAVID bioinformatics resources^{30,31} were used for biological functions analyses. Significance of enrichments was computed using Fisher's Exact Test.

RESULTS

Altered SW in *Nlgn1*^{-/-} mice

As expected, sleep intensity was increased after SD as indexed by NREM sleep low-frequency EEG activity (Figure 1a). This increase was significantly higher in *Nlgn1*^{-/-} than in *Nlgn1*^{+/-} mice between 0.75 and 1.25 Hz. Low-frequency activity reflects individual SW characteristics, which were shown to better define recovery sleep^{11–13} with slope, in particular, specifically thought to unmask synaptic efficacy.^{11,32}

Nlgn1^{-/-} mice showed a higher number of SW (that is, a higher SW density) during light periods, and higher SW amplitude in the light and dark periods than both *Nlgn1*^{+/-} and *Nlgn1*^{-/-} mice (Figure 1b). Because these differences were similar in baseline and recovery, they did not depend on the pressure for sleep. However, only *Nlgn1*^{-/-} and *Nlgn1*^{+/-} mice showed an increase in SW slope after SD during the light period. All genotypes exhibited a decrease in recovery slope compared with baseline in the dark period, with *Nlgn1*^{-/-} showing a steeper slope than other genotypes.

When the time course of SW properties was analyzed in detail (Figure 1c), *Nlgn1*^{-/-} mice showed higher amplitude and slope compared with *Nlgn1*^{+/-} during the light period, especially under high sleep need during recovery. The increase in SW amplitude and slope between the first interval of baseline and the first interval of recovery was significantly higher in *Nlgn1*^{-/-} than in *Nlgn1*^{+/-} mice (*P* < 0.03). In addition, the decrease in amplitude and slope between the first and last intervals of the recovery light period was higher in *Nlgn1*^{-/-} than in both *Nlgn1*^{+/-} and *Nlgn1*^{-/-} mice (Figure 1d). SW durations were increased during recovery in all three genotypes.

Typical brain transcriptome changes after SD in *Nlgn1*^{-/-} mice

To determine whether molecular pathways triggered by SD differed in *Nlgn1*^{-/-} mice, we used microarrays to map changes in the forebrain transcriptome after SD. Combining both genotypes, the expression of 1298 genes was significantly affected

Figure 1. (a) Non-rapid eye movement (NREM) sleep spectral power during the first hour of recovery (REC) after sleep deprivation (SD) expressed relative to the 24-h baseline (BL) ($n=9$ *Nlgn1*^{+/+}, 13 *Nlgn1*^{+/-}, 12 *Nlgn1*^{-/-}). Differences between *Nlgn1*^{-/-} and *Nlgn1*^{+/+} are highlighted by red ($P \leq 0.05$) and pink ($P < 0.1$) symbols (also in c). (b) Slow wave (SW) density and properties averaged during light or dark periods of BL and REC. SW density was higher in *Nlgn1*^{-/-} than in *Nlgn1*^{+/+} and *Nlgn1*^{+/-} for the 12-h light (genotype: $F_{2,24} \geq 3.6$, $P < 0.04$), and was higher in REC than BL for the light period and lower in REC than BL for the dark period (condition: $F_{1,34} \geq 50.7$, $P < 0.0001$). Amplitude was higher in *Nlgn1*^{-/-} than *Nlgn1*^{+/+} and *Nlgn1*^{+/-} for both periods (genotype: $F_{2,34} \geq 3.8$, $P < 0.03$); and higher in REC than BL for the 12-h light, whereas lower in REC than BL for the 12-h dark (condition: $F_{1,34} \geq 5.3$, $P < 0.03$). Duration of positive phase and of negative phase was higher in REC than BL for both periods (condition: $F_{2,34} \geq 46.2$, $P < 0.0001$). For the 12-h light, slope was higher in REC than in BL only in *Nlgn1*^{-/-} and *Nlgn1*^{+/-} (interaction: $F_{2,34} \geq 3.2$, $P \leq 0.05$). For the 12-h dark, slope was lower in REC compared with BL (condition: $F_{1,34} = 66.5$, $P < 0.0001$) and higher in *Nlgn1*^{-/-} than *Nlgn1*^{+/+} or *Nlgn1*^{+/-} (genotype: $F_{2,34} = 5.4$, $P < 0.01$). (c) Time course of SW properties averaged across equal intervals during BL and REC. During light periods, amplitude was higher in *Nlgn1*^{-/-} than *Nlgn1*^{+/+} in the first six intervals of REC sleep as was slope for specific intervals (Interactions: $F_{2,31} \geq 2.3$, $P < 0.03$). During dark periods, *Nlgn1*^{-/-} showed higher slope than *Nlgn1*^{+/+} and *Nlgn1*^{+/-} (genotype: $F_{2,31} = 4.6$, $P < 0.02$). All SW properties varied significantly with time, and SD is indicated by the black rectangle. (d) Decay of SW density, amplitude and slope between the first and the last intervals of REC light. The amplitude and slope decay was higher in *Nlgn1*^{-/-} than *Nlgn1*^{+/-} and *Nlgn1*^{+/+} ($F_{2,31} > 6.6$, $P < 0.01$). *Nlgn1*: Neuroligin-1.

by SD (FDR < 0.05), covering biological functions associated with neurological functions, stress response, circadian rhythms, gene expression and psychological disorders (Supplementary Table S1), with predicted upstream regulators related to metabolism (for example, UDP-D-glucose), synaptic transmission (for example, NMDAR: N-methyl-D-aspartate receptors), circadian rhythms (for example, CLOCK: Circadian Locomotor Output Clocks Kaput) or activity-dependent signaling pathways (for example, CREB1 and CREM) (Supplementary Table S2). No significant genome-wide Genotype $\times$ SD interaction was detected after FDR correction, and only 184 transcripts showed a $P < 0.05$ before correction (Figures 2a and b).

Therefore, a strong overlap was observed in genes responding to SD in *Nlgn1*^{+/+} and *Nlgn1*^{-/-} mice that did not reflect the amplified rebound of SW slope in *Nlgn1*^{-/-} mice. We validated this conclusion by qPCR performed on selected genes associated

to transcription and stress response, as all interactions did not reach statistical significance (Figure 2c), except for *Fgf1* (fibroblast growth factor 1). A significant correlation between qPCR and microarray values was found (Figure 2d). Interestingly, effects of SD were confirmed for genes associated to transcription (for example, *Arid4b* and *Cdkn1a*) and to DNA methylation (5mC) (for example, *Mat2b*, *Dmmt3a1* and *Dnmt3a2*), which suggest a role for these elements in shaping the brain transcriptome in response to SD.

SD changes the epigenome

Thus, the absence of NLGN1 does not importantly contribute to SD-dependent changes in brain transcriptome. However, elevated neuronal activity associated with SD shapes the transcriptome and affects *Nlgn1* expression, which impacts synaptic function. Indeed, in the cerebral cortex specifically, the expression of some *Nlgn1*

Figure 2. (a) Heat maps of the 184 transcripts displaying an interaction Genotype $\times$ sleep deprivation (SD) at $P < 0.05$. Columns refer to individual microarray data ($n = 6$ per group). Transcripts were ordered by hierarchical clustering (complete linkage) with *Nlgn1*^{+/+} fold change taken as a reference. (b) Scatter plot of the fold change in expression induced by SD in *Nlgn1*^{+/+} versus *Nlgn1*^{-/-} mice. Only the 1298 probes significantly affected by SD with a false discovery rate (FDR) < 0.05 (two-way analysis of variance) are shown. The 184 transcripts that showed an interaction with at $P < 0.05$ before FDR correction are highlighted in red. (c) Microarray data and quantitative PCR (qPCR) validations of selected targets showing measurements in *Nlgn1*^{+/+} versus *Nlgn1*^{-/-} mice under control (Ctrl) and SD conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control (and between genotypes for *Arid4b*). (d) Correlation between fold change relative to *Nlgn1*^{+/+} in control condition observed in microarray and qPCR validation data sets. Nlgn1: Neuroligin-1.

transcript variants is decreased by SD (Supplementary Figure S1A), and our previous data support the involvement of clock genes in this cortical decrease.¹⁸ Epigenetic mechanisms could be responsible for this, because they regulate alternative splicing,³³ and are mechanistically linked to functions affected by SD (for example, metabolism and stress response), including clock genes.^{34–36} Moreover, the expression of *Nlgn* and their partners *Neurexins* (*Nrxn*) was recently shown to be regulated by epigenetic regulators.³⁷

DNA methylation (5mC) is an established mechanism of gene silencing when occurring at upstream transcriptional regulatory regions, while methylation in body of genes could modulate additional regulatory regions or transcriptional processes such as splicing.^{33,38–40} DNA hydroxymethylation (5hmC), a further modification of 5mC, is highly abundant in the brain,^{41–43} and could

serve as a stable mark that diversifies the 5mC signal.^{34,44–46} The impact of SD on genome-wide 5mC and 5hmC profiles was thus examined in the mouse cerebral cortex, for which we first replicated the observation of SD-dependent changes in the expression of genes associated to 5mC (*Dnmt3a1*, *Dnmt3a2* and *Mat2b*; Supplementary Figure S1B) as we showed in the forebrain (Figure 2c). DNA immunoprecipitation and chemical labeling^{42,46} were used, for 5mC and 5hmC, respectively, because of their exquisite specificity for the two different modifications.

SD changes DNA methylation. We observed 227 differentially methylated probes (150 less and 79 more methylated after SD) associated with 136 promoters (FDR < 0.2) (Supplementary Table S3). Enrichments were identified in genes related to neurogenesis ($P = 2.91E-5$), synaptic plasticity ($P = 5.7E-3$) and glutathione

Figure 4. (a) Heat map of 4000 more significant probes showing an effect of sleep deprivation (SD) on 5hmC in the mouse cerebral cortex. Columns refer to three pools of DNA of three control (Ctrl) and three SD mice (total nine per group). Transcripts were ordered by hierarchical clustering (complete linkage). (b) Differentially 5hmC sites were enriched in exons ($P < 10E-300$, Fisher's Exact Test); TTS/3'-untranslated region ($P < 10E-300$) but negatively enriched in promoters ($P < 10E-300$), introns ($P < 6.8E-11$) and intergenic regions ($P < 1.6E-43$). (c) Correlation between DNA hydroxymethylation differences measured by 5hmC-IP-arrays and 5hmC-IP-quantitative PCRs for 15 different genomic regions. (d) Changes in 5hmC after a 6-h SD for selected targets. (e) Selected changes in 5hmC and mRNA expression after a 6-h SD. $^{\#}P < 0.1$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with control (Ctrl).

expression supports the hypothesis that NLGN1 acts downstream of the mechanisms triggering transcriptional changes associated with prolonged wakefulness. Our results thus suggest that epigenetic modifications constitute a primary response to elevated sleep pressure showing the potential to integrate different brain processes, and to modulate synaptic function notably by affecting the expression of synaptic elements.

We previously showed that *Nlgn1*^{-/-} mice suffer from impaired wakefulness quality and duration, which associated with enhanced delta power rebound after SD.¹⁸ Here we observed that *Nlgn1*^{-/-} mice exhibit more SW of higher amplitude and slope, but of equivalent duration. SW may have a role in information processing and synaptic plasticity.^{9,11,27,32} A higher

slope associates with more synchronous recruitment of cortical neurons,¹¹ and high amplitude and slope were linked to stronger synaptic strength.^{11,32} However, despite an apparent intensified sleep after SD that may represent stronger synaptic connections, *Nlgn1*^{-/-} mice show deficits in wake duration and quality¹⁸ that suggest either an impaired recovery during sleep or an enhanced damaging impact of wakefulness. The former may be supported by the role of NLGN1 in regulating NMDAR activity,⁵³ which may recover during sleep; whereas the second fits with the role of NLGN1 in tuning down glutamate release under high activity.⁵⁴ We found that SW amplitude and slope decreased in the course of sleep in *Nlgn1*^{-/-} mice, and even more than in *Nlgn1*^{+/+}, suggesting a functional recovery system. Hence, *Nlgn1*^{-/-} mice

may express a steeper increase in sleep need during wakefulness likely because of more damaging wakefulness.

The EEG differences between *Nlgn1*^{-/-} and *Nlgn1*^{+/+} mice were observed without major changes in the brain transcriptome after SD. Of note, *Fgf1* was the only transcript showing an increase strictly in *Nlgn1*^{-/-} mice, suggesting that this gene is downstream to *Nlgn1*. Administration of FGF1 was shown to increase NREM sleep in mammals,^{55,56} and recent observations of a SD-dependent increase in *Fgf1* in oligodendrocytes may indicate that these cells specifically benefit from such increase during SD.⁵⁷ This indicates that specific brain cells may be affected in *Nlgn1*^{-/-} mice and that it can impact EEG synchrony in response to SD.

SD increased, in both *Nlgn1*^{-/-} and *Nlgn1*^{+/+} mice, the expression of the DNA methyltransferases *Dnmt3a1* and *Dnmt3a2*. DNMT3a2, in particular, senses calcium signaling and NMDAR activity.⁵⁸ We also observed a decrease in methionine adenosyltransferase II beta (*Mat2b*) expression, which is part of a methionine transferase complex that catalyzes the synthesis of the methyl donor in DNA 5mC reactions.⁵⁹ These observations, in addition to the fact that DNA methylation is sensitive to neuronal activity,²² regulate the expression of plasticity-related genes,^{21,23,60,61} and is implicated in cognitive processes and psychiatric diseases,^{21,40,60–62} strongly support the hypothesis that changes in epigenetic mechanisms are responsible for the SD-dependent response of the brain transcriptome.

We indeed discovered that SD changes DNA 5mC and 5hmC patterns in the cerebral cortex. Genes differentially methylated relate to neuritogenesis, synaptic plasticity and cellular stress response. Of interest, some of these genes are potential targets of the estrogen receptor, a known modulator of 5mC levels^{63,64} and a proposed regulator of sleep duration.⁶⁵ Furthermore, we observed a widespread impact of SD on the DNA 5hmC landscape, covering genes involved in organization of cytoskeleton, gene expression, neurotransmission, cell signaling and synaptic assembly, which closely reflects functions previously linked to the SD-driven transcriptome.^{14–17} We also observed enrichment in genes associated with organismal death that may reflect the cellular stress induced by SD or, alternatively, a stress hardening process as an adaptation to SD.⁶⁶ Importantly, many transcripts related to synaptic function and adhesion (for example, *Nrxn1-2-3*, *Nlgn3*, *Ephb2-3-4-6* and *Epha7-8*) were enriched in genes differentially hydroxymethylated after SD, providing a pathway by which the 5hmC pattern can feedback on synaptic activity. These include many partners of NLGN1 such as NRXN, NLGN3 and DLG4. Moreover, enriched 5hmC differences in exons and 3'-untranslated region may have a role, as for 5mC,^{33,52} in gene expression, pre-mRNA processing and splicing that is of particular relevance to the *Nrxn*-*Nlgn* family,⁶⁷ especially in the context of elevated sleep need.¹⁸

Functions covered by epigenetic modifications after SD and their association with changes in gene expression strongly suggest that they actively participate in the regulation of sleep need by adjusting the brain transcriptome to the modified internal milieu. In future studies, the direct contribution of both 5mC and 5hmC to the EEG and gene expression changes induced by SD will need to be addressed. Assessing the participation of these changes in the transient and acute response to sleep loss and in more stable events representing chronic adaptation⁶⁸ will reveal basic aspects of brain function and their links to mental health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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