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The intellectual disability risk gene *Kdm5b* regulates long term memory consolidation in the hippocampus

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Abstract

The histone lysine demethylase KDM5B is implicated in recessive intellectual disability disorders and heterozygous, protein truncating variants in *KDM5B* are associated with reduced cognitive function in the population. The KDM5 family of lysine demethylases has developmental and homeostatic functions in the brain, some of which appear to be independent of lysine demethylase activity. To determine the functions of KDM5B in hippocampus-dependent learning and memory, we first studied male and female mice homozygous for a *Kdm5b*$^{\Delta ARID}$ allele that lacks demethylase activity. *Kdm5b*$^{\Delta ARID/\Delta ARID}$ mice exhibited hyperactivity and long-term memory deficits in hippocampus-dependent learning tasks. The expression of immediate early, activity-dependent genes was downregulated in these mice and hyperactivated upon learning stimulus compared to wildtype mice. A number of other learning-associated genes was also significantly dysregulated in the *Kdm5b*$^{\Delta ARID/\Delta ARID}$ hippocampus. Next, we knocked down *Kdm5b* specifically in the adult, wildtype mouse hippocampus with shRNA. *Kdm5b* knockdown resulted in spontaneous seizures, hyperactivity and hippocampus-dependent long-term memory and long-term potentiation deficits. These findings identify KDM5B as a critical regulator of gene expression and synaptic plasticity in the adult hippocampus and suggest that at least some of the cognitive phenotypes associated with *KDM5B* gene variants are caused by direct effects on memory consolidation mechanisms.

Significance statement

The histone lysine demethylase KDM5B has been implicated in cognitive performance and intellectual disability conditions in the human population. In the present manuscript we show that mice expressing a demethylase-deficient KDM5B and mice with a specific knockdown of KDM5B in the adult hippocampus exhibit hippocampus-dependent learning and memory phenotypes. Molecular analyses suggest a key role for KDM5B in regulating the dynamic expression of activity-regulated genes during memory consolidation. Deficits in LTP are present in mice with KDM5B knockdown. Together, these findings provide the first evidence for a direct function for KDM5B in memory consolidation in the hippocampus.

Introduction

Genome sequencing studies have implicated genes encoding chromatin modifying factors in intellectual disability and autism spectrum disorders (ID and ASD). These include the gene KDM5B (Lysine demethylase 5B), recessive mutations of which are linked to a rare ID syndrome (Faundes et al., 2018; Martin et al., 2018). Intriguingly, *KDM5B* is tolerant to loss-of-function mutations, and a significant proportion of such heterozygous *KDM5B* variants are incompletely penetrant and inherited (Wang et al., 2022; Zhou et al., 2022). A recent study reported that rare protein truncating variants in *KDM5B* are associated with large effects on cognitive function in the population (Chen et al., 2023). Specifically, protein truncating variants in *KDM5B* negatively correlated with educational attainment, reaction time and verbal-numerical reasoning. This study also reported an association of protein truncating variants of *KDM5B* with neurodevelopmental and psychiatric disorders and epilepsy. The same study found initial evidence that a heterozygous and homozygous deletion of *Kdm5b* exon 7 in mice was associated with dose-dependent cognitive deficits and increased anxiety (Chen et al., 2023). Previous studies have reported significant embryonic or perinatal lethality of homozygous *Kdm5b* loss-of-function mutations in mice. The deletion of exon 1 was associated with lethality by embryonic day 7.5 (Catchpole et al., 2011), whereas either exon 6 (Albert et al., 2013) or exon 7 (Martin et al., 2018) deletion was associated with 36-44% postnatal viability, suggesting that the deletion of exons 6 or 7 produces hypomorphic alleles with partially penetrant effects.

KDM5B, together with KDM5A and KDM5C, are the only histone lysine demethylases known to demethylate trimethylated lysine 4 on histone 3 (H3K4me3), a post-translational histone modification typically present at active gene promoters. H3K4me3 can recruit components of transcriptional initiator (Vermeulen and Timmers, 2010; Lauberth et al., 2013), and integrator complexes (Wang et al., 2023). The latter is associated with the regulation of transcriptional output by stimulating transcriptional elongation (Wang et al., 2023). As KDM5B specifically

removes H3K4me3 to counteract this process, the lack of KDM5B demethylase activity is predicted to lead to transcriptional de-repression of direct target genes. H3K4me3 is also enriched at non-methylated CpG island promoters, where it is thought to counteract DNA methylation (Hughes et al., 2020).

The *KDM5A-D* genes in mammals are paralogues of the ancestral *KDM5* gene in *Drosophila*. *KDM5* mutation is also associated with lethality (Gildea et al., 2000). *Drosophila KDM5* can regulate gene expression with multiple mechanisms, including a mechanism that requires the PHD domain of the protein, and appear to function independently from the demethylase activity of the protein (Liu and Secombe, 2015). Indeed, *Drosophila* KDM5 and mammalian KDM5 proteins interact with other chromatin factors and complexes that function as transcriptional repressors, including components of Sin3/HDAC1 and NuRD complexes (Yheskel et al., 2023). Thus, some KDM5B-mediated transcriptional repressionmay be mediated by demethylase-independent mechanisms. Flies with mutations in the demethylase domains of the protein survive, consistent with demethylase-independent functions, but exhibit specific learning phenotypes (Zamurrad et al., 2018; Belalcazar et al., 2021).

Mutations in the lysine methyltransferases responsible for H3K4me3, KMT2A and KMT2B are also associated with autosomal dominant ID syndromes (Jones et al., 2012; Faundes et al., 2018). Animal experiments have shown that a learning stimulus results in a rapid and transient increase in H3K4me3 levels in the hippocampus, similar to immediate early gene induction (Gupta et al., 2010). Consistent with a functional role for H3K4me3, the lysine methyltransferases, KMT2A and KMT2B are necessary for normal hippocampus-dependent learning and memory in mice (Kerimoglu et al., 2013; Kerimoglu et al., 2017).

Together, these studies suggest that dysregulation of H3K4me3, either by loss of function of methyltransferases or demethylases, can result in ID. Conditional deletion of *Kmt2a* and *Kmt2b* genes in post-mitotic neurons in the mouse, was sufficient to cause learning and memory phenotypes, suggesting that defects in the H3K4me3 machinery directly impacts memory consolidation mechanisms in the hippocampus (Kerimoglu et al., 2013; Kerimoglu et al., 2017). Thus, to understand how KDM5B loss-of-function affects learning and memory, we set out to determine if KDM5B has a direct function in memory consolidation, as opposed to having exclusively developmental functions. We focused on the hippocampus as the function of this brain region in learning and memory is well-established, and hippocampus-dependent memory tasks and electrophysiological correlates such as long-term potentiation (LTP) are robust in mice.

Materials and Methods

Animals

Experiments with mice carrying the *Kdm5b*^{tm1Jtpu}, referred to as the *Kdm5b*$^{\Delta ARID}$ allele (Catchpole et al., 2011) were performed at King's College London. *Kdm5b*$^{\Delta ARID/\Delta ARID}$ males on a C57BL/6J background were crossed with 129S2/Sv females to produce heterozygous F1 mice, that were then inter-crossed to produce WT and homozygous *Kdm5b*$^{\Delta/\Delta}$ F2 mice for experiments. Experimental cohorts of mice were derived from 5 (studies with naïve mice) to 15 (for behavioural analyses) litters. Animals were housed in ventilated cages (37x20x16cm) with *ad libitum* access to water and food (Labdiet Picolab rodent irradiated diet, #5R53) and kept at 19–22°C and 40–60% humidity, under a 12:12-h light/dark cycle. The cages contained bedding (Lignocel wood fiber) and nesting. A maximum of five animals were housed in the same cage. All animal procedures were approved by the King's College London AWERB and the UK Home Office.

Genotyping of mice

Genotyping was performed by extracting genomic DNA from ear notches as previously described (Hurley et al., 2021). The following primer pairs to detect the WT and the mutant alleles were used: WT forward 5'- CCTTAGACGCAGACAGCACA-3', WT reverse 5'-CGTGTTTGGGCCTAAATGTC-3', Kdm5b-ΔARID forward 5'-TGCTCCTGCCGAGAAAGTATCC-3' and Kdm5b-ΔARID reverse 5'-CCACCCCCCAGAATAGAATGA-3'. Thermal cycles for the genotyping reactions were as follows: 95°C, 2 min; 35x (95°C, 15 sec; 64°C, 15 sec; 72°C, 15 sec); 72°C, 12min.

Kdm5b knock-down

Adult (8-12 week old) female C57BL/6J mice received 1 μl ($1x10^{10}$ viral particles) of either AAV1-CMV-GFP-U6-mKdm5b-shRNA, Vector Biolabs #shAAV-262769) or control AAV1-CMV-GFP-U6-scrambled shRNA, #7040) in both dorsal hippocampi as described (Kwapis et

al., 2018). These experiments were approved by the UCI IACUC and KCL AWERB and UK Home office.

Western blot

Brain cortices were dissected from adult mice and whole cell protein prepared by lysing in 8M urea, 1% CHAPS, 50mM Tris (pH 7.9) lysis buffer containing protease inhibitors. Samples were rotated for 30 min at 4°C and then centrifuged for 50 min to remove DNA. Supernatant was stored at -80°C. All reagents and machinery were obtained from BioRad unless stated otherwise. Samples were prepared with Laemmli buffer containing 10% β-mercaptoethanol and resolved with 7.5% Mini-PROTEAN pre-cast polyacrylamide gels and Tris/Glycine/SDS buffer. 60µg of protein were prepared to detect KDM5B protein in brain lysates. Proteins were transferred to a nitrocellulose membrane with the TransBlot turbo system (High molecular weight transfer program). Membranes were blocked with 5% BSA (Sigma) diluted in tris-buffered saline containing 0.1% Tween-20 (TBS-T). Primary antibodies were diluted in TBS-T containing 5% BSA. Primary antibodies were incubated overnight at 4°C in 5% BSA in TBS-T. Membranes were incubated with secondary antibodies diluted in TBS-T containing 5% BSA for 1h at room temperature. Proteins were detected with Clarity ECL reagent and membranes were imaged using the Chemidoc system. Densitometric analyses were performed with ImageJ software (NIH). The following antibodies were used: anti-KDM5B (Abcam, ab181089, 1:1000), αTubulin (Upstate, 05-829), goat anti-rabbit and anti-mouse HRP secondary antibodies (Thermo Fisher Scientific, #31460 and Proteintech, #SA00001-1, respectively, 1:5000). Uncropped full scans of the blots shown in Figure 1B are shown in Extended Data Figure 1-1.

Immunofluorescence

Animals were perfused with either 1x PBS or 1x PBS followed by 4% paraformaldehyde (PFA). Brains were removed and fixed overnight in 4% PFA. Samples were cryopreserved with a sucrose gradient (5%, 15%, 30%, one day each). Free-floating coronal brain sections

(20-30µm) were obtained with a cryostat. Sections were first washed twice in PBS-T (1x PBS 0.3% Triton X-100) and blocked for 1h in blocking buffer (BB: PBS-T 3% NGS 3% BSA). Following blocking, primary antibodies were incubated overnight in BB at 4°C. The following day, slices were washed twice with PBS-T and secondary antibodies were incubated in BB for 2h. Samples were washed three times with PBS-T before nuclei staining with Hoechst3332 (1:5000). Two final washes with 1x PBS were performed before mounting with anti-fade mounting media (Abcam, ab104135). The following antibodies were used (1:200 dilution): Npas4 (Activity signaling, NP41-2), GFP (Abcam, ab13970), H3K4me3 (Cell Signaling Technologies, 9751S), goat anti-rabbit 488 (Thermo Fisher Scientific, A11031), and goat anti-chicken 488 (Thermo Fisher Scientific, A11039. Images were acquired with a Zeiss Axio Observer7 microscope (20x magnification) and Zen Blue software. Image acquisition and analysis were performed blindly. The number of Npas4-positive nuclei in the hippocampal layers CA3 and DG was quantified manually with the Cell Counter plug-in on ImageJ-FIFI (NIH). The number/density and mean staining intensity/cell of H3K4me3 were analysed in CA1/2, CA3 and dentate gyrus with QuPath (Bankhead et al., 2017). The following settings were used to detect the positive cells: Requested pixel size: 0.5; Background radius: 8; Median filter radius: 0; Sigma: 1.5; Cell expansion: 2 (all in µm); Minimum area: 10; Maximum area: 400 (in μm^2); Threshold: 10.

Golgi staining and spine analysis

Fresh brain hemispheres were processed with the FD Rapid GolgiStain™ kit (FD Neurotechnologies) following manufacturer's instructions. Briefly, 100 µm sections were obtained with a vibratome and mounted on gelatine-coated superfrost slides. Following the staining procedure, brightfield images of impregnated dendrites from dorsal hippocampal neurons were captured with a Zeiss Axio Observer7 microscope (63x magnification) and Zen Blue software. Stacks were taken every 0.2 µm and analysed manually with FIJI. Spine density was calculated in 10 proximal dendrites per area and animal, starting 5 µm apart from the ramification. Spine density values are shown as average for each area and animal.

Behaviour

Different cohorts of control and *Kdm5b*-mutant mice were used for the experiments in this study: 1) battery of behavioural tests (Fig. 3), 2) confirmation of the anxiety-related phenotype, 3) RNAseq with naïve animals (Fig. 4A,B), 4) confirmation of the RNAseq results by immunofluorescence and RT-qPCR (Fig. 4C-E), and 5) RNAseq to study gene expression changes upon learning (Fig. 5). For the *Kdm5b* knock-down experiment (Fig. 6), a cohort of mice was used for the behavioural and the electrophysiology analyses, and extra cohorts were used to confirm the *Kdm5b* knock-down one, three, and seven weeks after the surgery. A final cohort was dedicated to study the effect of the knock-down on early gene expression 11 days after the surgery.

Behavioural assessments of F2 mice (cohort 1) started at 7-8 weeks of age, consisting of the following: handling, open field test, object location memory test, elevated plus maze, spontaneous alternation in a Y-maze test, Morris water maze, fear conditioning test and measurement of grip strength. Mice were left to rest for at least 2 days in between tests. Mice in cohorts 3, 4 and 5 were 7-8 weeks old when samples were collected.

Behavioural experiments were conducted between 8:00 and 18:30 under standard room lighting conditions unless stated otherwise. Cages were changed every two weeks but never less than 48h before the day of testing. Behaviours were tracked using Ethovision (Noldus Information Technologies bv, Wageningen, The Netherlands). After each trial of a specific test, boli and urine were removed, and the test area was cleaned with 1% Anistel® (high level surface disinfectant, Trisel Solution Ltd, Cambridgeshire, UK, for cohort 2) or 10% ethanol (cohort 1) to remove any odours. The experiments were blinded and randomized by blocks of mice. Littermates were used as controls with multiple litters examined per experiment (15 for cohort 1 and 12 for cohort 2). Mice were habituated to the behavioural room conditions for at least 30 minutes before the start of each testing session. Before the start of the experiments, mice were individually handled for 5 consecutive days, 2 minutes each day.

Behavioural assessments of shRNA mice were started 2-3 weeks after stereotactic surgeries. Mice were first tested in the elevated plus maze to determine effects on activity and anxiety, handled for 5 days as described and habituated to the test arena for 6 days before training with two identical objects for 10 minutes, followed by a 5 min test session 24h later with one of the objects moved to a new location (Kwapis et al., 2018). Two weeks later, tissue was collected for acute slice preparations and LTP.

Open field

The circular open field arena was made of clear acrylic with a grey base, with internal dimensions of 40 cm diameter and 40 cm high as described (Whittaker et al., 2017). Light intensity inside the arena was 10 lux. Two virtual areas were drawn on Ethovision: a centre zone, 20cm diameter, and a ring 5 cm thick around the perimeter of the arena was defined as the outer zone. Test mice were placed in the open field facing an outer wall to begin the test. Its locomotor activity was tracked for 5 minutes. Total distance moved (cm) in the outer zone was used as a measure of the level of locomotive activity and time (s) spent in the centre zone used as a measure of anxiety.

Object location memory test

The test was performed as described in (Vogel-Ciernia et al., 2013). The arena (40x40x40 cm) was made of white acrylic and had a black stripe in one of the walls as an internal cue. Light intensity was 40 lux in the centre of the arena. Briefly, mice were habituated to the arena for 6 consecutive days, 5 minutes/day. During the training session, mice were placed for 10 minutes in the arena with the two identical objects (100 ml glass beakers filled with cement to prevent them being moved by the mice) next to the wall with the visual cue. 24 h later, during the test session, one of the two objects was randomly moved to the opposite side of the arena, in a centre position, and mice were left to explore the arena for 5 minutes. Exploration was manually scored and the following criteria was used: nose of the mouse is within 1cm of the object, with their head directly facing it. Rearing, standing on the object,

and digging near it were not considered as exploratory behaviours. Discrimination index (DI) was calculated as: (time exploring new location – time exploring old location) / (total exploration time) * 100. Exclusion criteria included exploration time below 3 seconds for either training or testing sessions, and DI above +/-20 for the training session.

Elevated plus maze (EPM)

The EPM was made of black acrylic and consisted of four arms (30 x 5 cm). The two opposing closed arms were enclosed by 15 cm high walls on each side and ends. The two opposing open arms were open, as well as the centre platform (5 x 5 cm). The maze was elevated 40 cm above ground. Light intensity was 100 lux on the open arms and 10 lux on the closed arms. The number of entries onto, time spent on, and latency to enter the closed and open arms were manually scored. An arm entry is defined when all 4 paws were located inside the arm. Mice were placed in the centre platform of the EPM facing a closed arm to start the 5 minutes test.

Spontaneous alternation in a Y-maze

The Y maze apparatus was made of grey acrylic and consisted of three arms, 120° from each other. All arms were 45cm long, 5 cm wide and enclosed by a 10cm wall. Extramaze visual cues were placed at the end of each arm. One arm was considered as the central arm, and the other two arms were randomly closed during the training session. During the training session, mice were placed in the maze in the central arm and allowed to explore it for 10 minutes, after which they were returned to their homecage. After 1h, the testing session was conducted. Both arms were open, and mice were placed in the central arm and allowed to freely explore the maze for 5 minutes. The first choice to turn either to the familiar arm or the new arm (percentage of alternation rate) was monitored, when all four paws were inside that arm. Arm preference was automatically monitored with Ethovision and the Discrimination index was calculated as (time exploring new arm – time exploring old arm) / (total exploration time of the old and new arms) * 100.

Morris water maze

Spatial learning and memory were assessed using a Morris water maze (MWM). The maze (1 m diameter) was filled with water, made opaque with a non-toxic white aqueous emulsion. Water temperature was 23°C, and light intensity was 100lux. A set of extra-maze visual cues were suspended around the pool. Four alternative start positions were nominated to provide the virtual division of the tank into four quadrants. Mice were trained to find a hidden platform (10 cm diameter), submerged 2 cm below the water surface, for 8 consecutive days, four trials a day. Once they found the platform, mice were left on it for 15 seconds before returning them to their homecage. Mice that failed to locate the hidden platform after 60 seconds were placed on the platform for 15 seconds. Animals that failed to stay on the platform following two days of training were excluded from the study. On probe trial the platform was removed and mice were placed into the pool for 60 seconds so they could freely explore the tank. To check for deficiencies in vision or locomotion, mice were placed in the pool with a visible platform (1cm above the surface, with a flag attached to it), one day, four trials. Mouse movement was tracked with Ethovision to calculate mean speed, total distance travelled in the pool and in each quadrant, and the number of platform crossings during the probe trial.

Fear conditioning test

Animals were placed in a soundproof fear conditioning apparatus with stainless steel metal grid floor, containing a camera (Med Associates Inc.). To provide an olfactory cue, an ethanol-soaked tissue was placed under the grid in both training and testing sessions. Mouse behaviour was recorded with VideoFreeze software. For conditioning, mice were placed inside the chamber and left to freely explore it. After 148 seconds, three electric shocks (0.7mA, 2 sec each, 30 seconds apart) were administered. The animals were then removed from the testing chamber after 30 additional seconds. 24 h later, mice were placed in the same chamber for 5 minutes. Freezing behaviour was manually scored from recorded

videos during training and testing sessions. Freezing behaviour was defined as the complete lack of movement during the first 2 seconds of each 5-second window.

Grip strength measurements

To assess the neuromuscular ability of the animals, fore- and hindlimb grip strength was measured with a Linton Grip Strength Meter (MJS Technology, Ltd). Mice were pulled across the meter from left to right measuring forelimb and then hindlimb strength. The average of three measurements per limb and mouse was taken.

RNA extraction

Total RNA was extracted from hippocampal samples with 1ml TRIzol (Thermo Fisher Scientific) and further purified with the Monarch Total RNA miniprep kit (New England Biolabs) following the manufacturer's recommendations (including DNAse treatment step).

qRT-PCR analysis

cDNA was synthesised from 200-400ng RNA with UltraScript 2.0 cDNA Synthesis kit (PCR Biosystems) according to the manufacturer's instructions. qRT-PCRs were performed on a BioRad CFX384 using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$method and *Hprt* or *Gapdh* were used as endogenous control genes. For shRNA experiments, the PrimeTime probe-based gene expression system (IDT) was used to quantify *Kdm5b* expression, relative to *Hprt*. Primer sequences: Arc (Fw: 5'-CTCAACTTCCGGGGATGCAG-3', Rv: 5'-CTGGTATGAATCACTGGGGGC-3'), cFos (Fw: 5'-AGAGCGGGAATGGTGAAGAC-3', Rv: 5'-AGTTGATCTGTCTCCGCTTGG-3'), Egr1 (Fw: 5'-TGAGCACCTGACCACAGAGTC-3', Rv: 5'-TAACTCGTCTCCACCATCGC-3'), Egr2 (Fw:5'-GTGCTGCCTGACAGCCTCTA-3', Rv: 5'-TTGATCATGCCATCTCCCGCC-3'), Gapdh (Fw: 5'-AGGTCGGTGTAACGGATTTG-3', Rv: 5'-TGTAGACCATGTAGTTGAGGTCA-3'), Hprt (Fw: 5'-GTCCCAGCGTCGTGATTAGC-3', Rv: 5'-TGGCCTCCCATCTCCTTCAT-3'), Kdm5b (Fw: 5'-AAGCCAAGCTCTGTTCAGCAA-3', Rv: 5'-GAAGGCAATCGTTCTTCTCACT-3'), Npas4

(Fw: 5'-CTGCATCTACACTCGCAAGG-3', Rv: 5'-GCCACAATGTCTTCAAGCTCT-3'), Kdm5b (for Fig. 6, Fw: 5'-CAAGAGCCCACTGAGAAGAAA-3', Rv: 5'-TCCACATAAGAGGCACACATAC-3') and Hprt (for Fig. 6, Fw: 5'-TGCTCGAGATGTCATGAAGG-3', Rv: 5'-CTTTTATGTCCCCCGTTGAC-3').

RNAseq

Total RNA (n=4/genotype, balanced for sex) were sent to Novogene for library preparation and sequencing. After mRNA enrichment, mRNA quality was analysed using Agilent Total RNA 6000 Pico on a Bioanalyser (Agilent, 2100). Pair-end sequencing (150bp read length) was performed on the Illumina Novaseq 6000 platform. Further data analyses were performed using the Galaxy Europe server (https://usegalaxy.eu) (https://academic.oup.com/nar/article/50/W1/W345/6572001). Quality of the raw data was checked using FastQC (v0.11.9). Reads were aligned to the mouse genome (mm10) using RNA STAR (v2.7.8a), and aligned reads were counted using FeatureCounts (v2.0.1). Differential expression analyses were performed using DESeq2 (v2.11.40.7). Multiple comparisons were controlled for using an FDR < 0.05. Exact p values and adjusted p-values for all differentially expressed genes are listed in Extended data Table 4-1 and Table 5-1. Genes with adjusted p-value <0.05 were considered as differentially expressed genes (DEGs). Heatmaps were generated with the R package pheatmap. Volcano plots were generated in GraphPad Prism 9.4.1. Differentially expressed genes in at least one comparison (any timepoint between genotypes, or 1h/0h, 3h/0h and 3h/1h within the same genotype) were clusterised with k-means clustering and selecting the optimal cluster number for the dataset (k=4). Activity-induced genes were obtained from (Chen et al., 2017), with only differentially upregulated transcripts detected by both RNAseq and TRAPseq included. Gene ontology analyses were conducted using g:Profiler (https://biit.cs.ut.ee/gprofiler/gost), where GO molecular function and GO biological process of size 0-1500 were checked. The applied threshold was "Benjamini Hochberg FDR < 0.05".

Electrophysiology

Female mice (n=4/treatment, 8 total) were sacrificed for electrophysiology and hippocampal slices prepared as described previously (Vogel-Ciernia et al., 2013). Mice were anesthetized, decapitated, and the brains rapidly removed into ice-cold, oxygenated dissection medium containing (in mM): 124 NaCl, 3 KCl, 1.25 KH2PO4, 5 MgSO4, 0 CaCl2, 26 NaHCO3, and 10 glucose. Hippocampal slices (320 µm, coronal) were cut from a vibratome (Leica, Model:VT1000S) before transferred to an interface recording containing prewarmed (31 ± 1^0 C) artificial cerebrospinal fluid (aCSF) composed of (in mM): 124 NaCl, 3 KCl, 1.25 KH2PO4, 1.5 MgSO4, 2.5 CaCl2, 26 NaHCO3, and 10 glucose. Slices were perfused continuously at a rate of 1.75-2 ml/min while the surface of the slices were exposed to warm, humidified 95% O2/5% CO2. Recordings began following at least 2h of incubation.

Field excitatory postsynaptic potentials (fEPSPs) were recorded from CA1b stratum radiatum apical dendrites using a glass pipette filled with 2M NaCl (2-3 MΩ) in response to orthodromic stimulation (twisted nichrome wire, 65 µm diameter) of Schaffer collateral-commissural projections in CA1c stratum radiatum. Pulses were administered 0.05 Hz using a current that elicited a 50% maximal spike-free response. After maintaining a stable baseline (20 min), long-term potentiation (LTP) was induced by delivering 5 'theta' bursts, with each burst consisting of four pulses at 100 Hz separated by 200 msec (i.e., theta burst stimulation or TBS). The stimulation intensity was not increased during TBS. Data were collected and digitized by NAC 3.0 (Neurodata Acquisition System, Theta Burst Corp., Irvine, CA) and stored on a disk.

Data in the text are presented as means ± SD, while in the figures as mean ± SEM. The fEPSP slope was measured at 10–90% fall of the slope and data in figures on LTP were normalized to the last 20 min of baseline.

Statistics

Data are reported as mean ± SEM. Graphs show individual data points. Normal distribution was tested with d'Agostino and Pearson omnibus, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. If the test was passed, statistical analysis was performed using parametric statistical analyses. Before pairs of comparisons, we performed the F test to compare variances. In experiments with normal distribution statistical analyses were performed using the unpaired two-sided Student's t test. T-test with Welch's correction was applied when variances were unequal. Two- and Three-way ANOVA with the appropriate post hoc tests were also performed as indicated in the figure legends. Significant p values ($p<0.05$) are reported in the results section and/or figure legends provide details of relevant statistical parameters, including group sizes. Statistical analyses were performed with GraphPad Prism (version 9.4.1). Experiments in this study were blinded and animals randomised for in vivo studies.

Results

KDM5B demethylase-deficient mice exhibit megalencephaly

Kdm5b$^{\Delta ARID/\Delta ARID}$ (*Kdm5b*$^{\Delta/\Delta}$) mice were generated to circumvent the embryonic and early postnatal lethality of other loss-of-function models. The deletion of exons 2 to 4 leads to a partial truncation of the JmjN domain and the deletion of the ARID domain of the KDM5B protein (Fig. 1A), that disrupts its H3K4me3 demethylase activity (Jamshidi et al., 2021). We confirmed the loss of full-length KDM5B protein and the appearance of a shorter KDM5B-ΔARID protein in the brain of adult *Kdm5b*$^{\Delta/\Delta}$ mice by Western blot (Fig. 1B,C). The mutant KDM5B-ΔARID protein appears to be either unstable or not as well detected by KDM5B-specific antiserum in western blot, leading to an apparent reduction in total KDM5B protein levels in the homozygous mutants, compared to wildtype mice (Fig. 1D). We did not observe differences between male and female animals.

444 To assess the impact on H3K4me3, hippocampal sections from *Kdm5b*$^{\Delta/\Delta}$ and control mice
445 were immunostained with an antibody to H3K4me3. We observed a clear increase in the
446 number of H3K4me3-positive cells in the hippocampus (Fig. 1E,F). This increase was
447 observed in both sexes (Two-way ANOVA sex effect: $F_{1,4}$=0.1824, p=0.6913). Moreover, the
448 intensity of H3K4me3 across the population of cells in the hippocampus shifted towards
449 increased H3K4me3 in the mutants in all hippocampal subregions, cornu ammonis 1 (CA1)
450 (Fig. 1G,H), cornu ammonis 3 (CA3) (Fig. 1I,J) and dentate gyrus (DG) (Fig. 1K,L).

451 In an attempt to further reduce any developmental abnormalities associated with KDM5B
452 deficiency, all animals used in this study were on a C57BL/6Jx129S2/Sv F2 genetic
453 background (Zou et al., 2014, and data from the International Mouse phenotyping
454 Consortium (IMPC): http://www.mousephenotype.org/). Homozygous *Kdm5b*$^{\Delta/\Delta}$ mice
455 generated on this genetic background generated from heterozygous intercrosses were
456 present at expected ratios at P21 (Fig. 2A). Postnatal *Kdm5b*$^{\Delta/\Delta}$ mice displayed a growth
457 deficit and reduced body weights (Fig. 2B). Homozygous mutants exhibited increased brain-
458 to-body weight ratios at postnatal day 21, while the liver-to-body weight ratios did not differ
459 between genotypes, suggesting specific neurodevelopmental alterations (Fig. 2C-G).
460 Histological assessments did not reveal any gross abnormalities in cortical architecture (Fig.
461 2H,I) or spine density in proximal dendrites of CA1) pyramidal neurons and of DG granule
462 cells in the hippocampus (Fig. 2J).

463

464 **Hippocampus-dependent learning and memory deficits in *Kdm5b*$^{\Delta/\Delta}$ mice**

465 To determine whether KDM5B loss of function affects learning and memory, we tested these
466 mice in hippocampus-dependent tasks. Data from males and females were combined,
467 unless mice of different sexes exhibited differences in specific tests, where the data from
468 both sexes are shown. During the handling sessions we observed that the reduced body
469 weight observed at pre-weaning stages was still evident at 2 months of age (Fig. 3A).

Kdm5b$^{\Delta/\Delta}$ mice exhibited hyperactivity in the open field (Fig. 3B). They also spent less time in the inner area of the arena, suggesting an anxiety-like phenotype (Fig. 3C). However, this phenotype was not observed in the elevated plus maze (EPM) in the same cohort of mice (Fig. 3D) and was not consistent in a second cohort (for that cohort: distance travelled in the open field genotype effect: p=0.9966; time (%) spent in the open arms in the EPM genotype effect: *p=0.0385), suggesting that the anxiety-like phenotype is variable and potentially secondary to hyperactivity. Next, we performed the novel object location memory test (OLM) to study long-term spatial memory. A significant deficit in long-term spatial memory was observed in the mutant mice 24 h after training (Fig. 3E). Exploration times were equal between genotypes in both training and testing sessions (Fig. 3F), indicating a specific deficit in object location memory. In contrast, short term spatial memory and cognitive flexibility was preserved in *Kdm5b*$^{\Delta/\Delta}$ mice, as judged, respectively, by the discrimination index and spontaneous alternation percentage in the spontaneous alternation test in a Y-maze (Y-SAT) (Fig. 3G,H). Next, we examined long-term spatial learning and reference memory using the Morris water maze (MWM). During the training sessions with the hidden platform version of the test, performance improved significantly in both groups, although escape latency in mutant mice remained significantly higher compared to WT littermates (Fig. 3I). During the probe trial on day 8, *Kdm5b*$^{\Delta/\Delta}$ mice displayed a reduced number of platform crossings (Fig. 3J), indicating impaired spatial memory. All groups of mice explored the target quadrant significantly more than other quadrants during the probe trial, indicating that spatial memory had been acquired, but homozygous females showed significantly reduced exploration time in the target quadrant compared to wildtype females (Fig. 3K), consistent with a deficit in spatial learning. There was no effect on swim speed during the Morris water maze test, excluding a reduction in the ability of mice to explore the water maze as a potential explanation for the reduced performance of the mutants (Fig. 3L). No significant differences were observed between genotypes in the visible platform task in male mice, excluding poor vision, altered motivation or sensorimotor alterations as a cause for the deficit in mutant male mice. However, an increased latency to find the visible platform was observed in

females (Fig. 3M), which might be indicative of visual or motivation changes, or subtle anxiety differences that could be contributing to the deficits observed during the training and testing sessions. Grip strength analysis revealed reduced front- and hindlimb strength in mutant mice (Fig. 3N,O). Finally, we performed the contextual fear conditioning test to assess associative long-term memory. During the training session, both groups of mice increased their freezing behaviour following three unconditioned stimuli (US) of 0.7 mA, 2 s foot shocks) (Fig. 3P). However, freezing behaviour was significantly reduced in *Kdm5b*$^{\Delta/\Delta}$ mice compared to their wildtype littermates. In the testing session 24 h later, reduced freezing was observed on the mutant animals (Fig. 3Q). The increased movement during the training session was also observed when tracking the animals' movement, but their mean speed was similar during the shock administration, excluding differences in the ability of mutants to discern or respond to the shock (Fig. 3R). Nevertheless, the hyperactive phenotype in these mice (see Fig. 3B) should be considered as an inevitable confounding factor on this test. In summary, although the hyperactivity of the mutants complicates the interpretation of the contextual fear conditioning test, and confounds exist for the Morris water maze, *Kdm5b*$^{\Delta/\Delta}$ mice exhibited clear deficits in the hippocampus-dependent object location task, with no confounds apparent, leading us to conclude that these mice exhibit hippocampus-dependent learning and memory deficits

Altered baseline and learning-induced hippocampal gene expression in *Kdm5b*$^{\Delta/\Delta}$ mice

As KDM5B regulates gene transcription, we next asked if reduced KDM5B demethylase activity affected the transcriptional landscape in the mouse hippocampus. We performed RNA sequencing of adult wild-type and homozygous hippocampi. We detected 20 differentially expressed genes (DEGs), of which 14 were up- and 6 were down-regulated (Fig. 4A, B and Extended data Table 4-1). Intriguingly, two key early response genes associated with learning and memory, *Egr1* and *Npas4*, were amongst the downregulated

genes (Fig. 4B). To validate this finding, we quantified transcripts for several immediate early genes (*Egr1*, *Npas4*, *cFos*, *Egr2* and *Arc*) by quantitative PCRs (qRT-PCR) from a separate set of hippocampal samples. Consistent with the RNA-seq data, the expression of *Egr1*, *Npas4* and *cFos* were significantly downregulated in the mutants (Fig. 4B). We next asked if these results translated into alterations in protein levels. As Npas4 is specifically and selectively induced by neuronal activity in the CA3 and DG regions of the hippocampus (Ramamoorthi et al., 2011), Npas4-positive cells were quantified by immunostaining. The number of Npas4-positive cells was reduced in *Kdm5b*$^{\Delta/\Delta}$ mice compared to their wildtype littermates (Fig. 4D,E).

Activity-dependent transcription factors such as Npas4 are crucial for memory formation (Weng et al., 2018; Sun et al., 2020). Thus, we decided to test if KDM5B deficiency is associated with abnormal activity-dependent gene regulation during hippocampus-dependent learning. We compared the hippocampal transcriptomes of three groups of mice with RNA-seq: naïve animals, and mice that were sacrificed 1h or 3h following training in the contextual fear conditioning (CFC) paradigm (see Extended data Fig. 5-1 and Table 5-1). As validation, we were again able to confirm the reduction of immediate early gene expression by qRT-PCR in these samples (Extended data Fig. 5-1A). To understand the differences in activity-induced transcriptional responses between wildtype and mutant mice, we clustered the differentially expressed genes by k-means clustering according to their trajectories (Extended data Table 5-2). Four different clusters were identified, referred to as Clusters 1-4 below. Cluster 1 exemplified a typical immediate early gene trajectory of rapid, transient induction at 1h (Fig. 5A). These genes were functionally enriched for transcriptional regulation (Extended data Fig. 5-1G), consistent with the fact that the majority of these genes encode transcriptional factors like c-Fos, Fosb, Egr1, Egr2 and Npas4. In agreement with our previous experiments (Fig. 4), this analysis revealed that Cluster 1 genes were downregulated in naïve, control mutant animals, compared to wildtypes (Fig. 5A). These genes were rapidly induced in mutants, to a larger extent than in wildtypes, such that they

were expressed at the same level as wildtype controls at both 1h and 3h post-CFC (Fig. 5A). This observation was confirmed by qRT-PCR for the immediate early genes: *Egr2*, *cFos, Egr1, Npas4* and *Arc* (Extended data Fig. 5-1B-F).

We detected three other clusters with different trajectories (Fig. 5B-D). Cluster 2 genes showed an induction that gradually increased over time after the learning stimulus (Fig. 5B). These genes included late response genes, some with known functions in neurogenesis and synaptic plasticity e.g. *Fgfr1* (Zhao et al., 2007). Similar to Cluster 1, Cluster 2 genes were downregulated in mutants at baseline, and reached similar expression levels to wildtype controls by 3h (Fig. 5B). Cluster 3 included genes that were constitutively overexpressed in homozygous mutants (Fig. 5C). This cluster was enriched for transcriptional regulation and cation binding proteins and included genes encoding transcription factors such as Stat4 and Stat6, as well as Zn finger proteins. These genes included *Cacna1i*, a gene encoding a calcium channel subunit. Gain-of-function mutations in this gene associated with neurodevelopmental disorders and epilepsy (El Ghaleb et al., 2021). Cluster 3 also included *C1ql2* and *C1ql3*, encoding complement proteins strongly expressed in mossy fibres with a crucial role in the control of synapse stability and number (Matsuda et al., 2016). Cluster 4 genes represented genes that were downregulated in response to a learning stimulus in both wildtype and mutant hippocampi, with the downregulation more pronounced in mutants (Fig. 5D). This cluster was enriched for transcriptional and signalling regulators (Extended data Fig. 5-1G). Interestingly, these included the *Pde10a* gene, known to enhance fear memories in certain contexts e.g. (Guo et al., 2016), but also genes like *Hgf* known to promote learning and memory, perhaps providing an explanation of learning deficits in our KDM5B mutant mice (Kato et al., 2012). Cluster 4 also includes *Top1*, whose inhibition leads to reduced transcription of genes associated with ASD and neurotransmission regulation (Mabb et al., 2014), and *Arid1b*, which is also implicated in ASD and ID (Moffat et al., 2022).

To better understand how gene expression varied between genotypes at the different time points, we visualised genes differentially expressed between WT and mutant samples at baseline (home cage test-naive, 0h), 1h and 3h after fear conditioning stimulus. Naive mice showed a small number of DEGs between genotypes (Fig. 5E, 12 DEGs). More DEGs were observed between genotypes in activity-induced transcriptional responses 1h (Fig. 5F, 106 DEGs) and 3h (Fig. 5G, 32 DEGs) after training. The majority of DEGs in all conditions were upregulated in the mutant animals, in both female and male mice (Fig. 5E-G), consistent with KDM5B acting as a transcriptional repressor.

To understand how the activity-induced transcriptional responses differed between wildtype and mutants, we compared the hippocampal transcriptomes of homecage control mice with mice 1h and 3h post-US. Wildtype animals showed the expected increase in expression of activity-dependent immediate early genes such as *Fosb*, *Npas4* and *Egr3* 1h after training (Fig. 5H, 1h). Three hours after training, gene expression levels of activity genes were back to baseline (Fig. 5H, 3h), consistent with previous studies (Deliu et al., 2018). In addition to the immediate early genes observed in wildtype mice, *Kdm5b*$^{\Delta/\Delta}$ mice displayed a marked upregulation of many more genes (Fig. 5I, 1h; 252 DEGs). Strikingly, three hours after training, >200 genes were still differentially expressed, including immediate early genes like *Fosb*, *Nr4a2* and *Egr3* (Fig. 5I, 3h; 240 DEGs and Extended data Fig. 5-1H-J). Together, these data shows that reduced KDM5B demethylase activity alters the transcriptional landscape in the hippocampus in a number of different ways. These include downregulated baseline levels of immediate early genes, and more pronounced and sustained activity-dependent upregulation following learning relative to these baseline levels. In conclusion, the abnormal expression of several classes of genes implicated in learning and memory in the hippocampus are likely to contribute to the hippocampus-dependent learning deficits in these mice.

KDM5B directly regulates learning and memory in the adult hippocampus

Our data thus far suggested that KDM5B has a direct role in learning and memory. However, as KDM5B is known to play important roles during development (Catchpole et al., 2011; Albert et al., 2013), the phenotypes observed in *Kdm5b*$^{\Delta/\Delta}$ mice and other *Kdm5b*-deficient mouse models (Martin et al., 2018; Chen et al., 2023), might all be secondary to abnormal brain development and maturation. To determine if KDM5B has a direct role in learning and memory in the adult brain, we knocked down *Kdm5b* expression in the dorsal hippocampus (CA1) of adult mice by AAV-mediated delivery of a *Kdm5b*-specific shRNA (Fig. 6A,B).

A quantification of *Kdm5b* mRNA over time by qRT-PCR, showed a significant knockdown of *Kdm5b* by 3 weeks after viral delivery, and beginning to return to normal levels by 7 weeks (Fig. 6C). Importantly, we observed a shKDM5B-dependent downregulation of immediate early genes in naïve animals compared to controls that received AAV with a scrambled shRNA (Fig. 6D). This observation suggests that the reduced expression of these immediate early genes in *Kdm5b*$^{\Delta/\Delta}$ mice (see Fig. 4) are not secondary to developmental abnormalities. *Kdm5b* knockdown (shRNA) mice showed a significant hyperactivity phenotype in the open field versus controls (Fig. 6E), recapitulating observations with *Kdm5b*$^{\Delta/\Delta}$ mice (see Fig. 3B). Importantly, *Kdm5b* knockdown resulted in a substantial deficit in hippocampus-dependent learning and memory in the OLM task (Fig. 6F), similar to those in *Kdm5b*$^{\Delta/\Delta}$ mice, indicating that *Kdm5b* is essential for normal long-term memory consolidation in the hippocampus. Exploration time did not show differences between groups in neither training nor testing sessions (Student's t-test: p=0.4940 for training, and p=0.1242 for testing). Furthermore, the shRNA mice exhibited a striking incidence of spontaneous seizures (Fig. 6G), suggesting that *Kdm5b* knockdown altered neuronal activation and/or circuitry in the hippocampus.

Kdm5b regulates synaptic plasticity

To determine if *Kdm5b* knock-down affected synaptic plasticity, we examined short- and long-term potentiation (LTP) in acute hippocampal slices from these mice. We measured LTP in the dorsal CA1b region of the hippocampus, which we have found to be critically important for the hippocampus-dependent object location memory task in previous studies (Barrett et al., 2011; Vogel-Ciernia et al., 2013). Theta burst stimulation produced an immediate increase in potentiation in slices from control mice that gradually decayed to a plateau level that was approximately 50% above pre-TBS baseline (Fig. 6H). Hippocampal slices prepared from *Kdm5b* shRNA mice had similar short-term potentiation to control slices, however, the LTP was significantly less stable as the potentiation fell below control levels 1h post-theta burst stimulation. The mean potentiation for the last 10 min of 1h post-theta burst stimulation was 31 $\pm$ 11% for *Kdm5b* shRNA slices and 47 $\pm$ 6% for control slices (Fig. 6I), suggesting that *Kdm5b* is necessary for the consolidation of LTP in area CA1 of the hippocampus.

We tested whether synaptic events leading to induction of LTP are negatively affected by *Kdm5b* knock-down by measuring short-term plasticity changes including input/output curves (i/o) and paired-pulse facilitation. The input/output curves were comparable for controls and *Kdm5b* shRNA mice (Fig. 6J). The slope of the io curves was not significantly different between groups ($p<0.90$, data not shown). Paired-pulse facilitation (Fig. 6K), a measure of transmitter release kinetics, also did not show any significant difference between groups and stimulus intervals (2 way-ANOVA; $F_{(2,28)} = 2.28$, $p<0.12$). Thus, it appears that the lack of stability in LTP upon *Kdm5b* knockdown involves cellular events that are set in motion after induction and expression of LTP, an interpretation that is consistent with the observation that short-term potentiation was unaffected in both groups of mice.

Discussion

Rare coding variants in *KDM5B* are associated with cognitive function in adults and *KDM5B* is associated with a recessive intellectual disability disorder (Faundes et al., 2018; Martin et al., 2018; Chen et al., 2023). In this manuscript, we show that mice homozygous for a hypomorphic allele of *Kdm5b* that lacks H3K4me3 demethylase activity exhibited learning and memory deficits. *Kdm5b* deficiency resulted in alterations in the expression of genes implicated in learning and memory. Acute knockdown experiments showed that *Kdm5b* is critical for normal function of the hippocampus after development has been completed. Notably, two individuals carrying *KDM5B* variants show epileptic spasms and/or generalized seizures (Martin et al., 2018; Mangano et al., 2022), although the latter is more likely associated with a deficit in Nav1. Following *Kdm5b* knockdown in the dorsal hippocampus of adult mice, mice exhibited seizures, consistent with an essential role for *Kdm5b* in maintaining normal circuit homeostasis. These mice had deficits in hippocampus-dependent learning and memory and LTP. Together, this study provides a significant advance in our understanding of the role of KDM5B in learning and memory. Our findings show that KDM5B functions in the adult hippocampus to control learning and memory and imply that at least some of the cognitive deficits associated with KDM5B deficiency could be amenable to treatment in adults.

Learning and memory genes regulated by KDM5B

Our gene expression analyses revealed several potential ways in which KDM5B deficiency might disrupt memory and learning. First, the expression of activity-dependent, immediate early genes was reduced in *Kdm5b*$^{\Delta/\Delta}$ mice. The deletion of several of these genes have been shown to affect long-term memory formation, whilst leaving short-term memory intact (Jones et al., 2001; Fleischmann et al., 2003; Ramamoorthi et al., 2011), similar to our findings with *Kdm5b*$^{\Delta/\Delta}$ mice. Second, we found that these immediate early genes were

684 induced by neuronal activation in the hippocampus of *Kdm5b* mutants up to levels equivalent
685 to those in wildtype mice, and therefore hyper-activated relative to baseline levels.
686 Furthermore, these genes were still expressed 3h after neuronal activation in the mutants, at
687 a time point when their expression has returned to baseline in wildtype mice. This inability of
688 gene expression to return to baseline levels is consistent with the H3K4me3 demethylase
689 function of KDM5B. One might speculate that this reduced expression at baseline and
690 apparent over-activation and altered dynamics of IEGs might disrupt normal memory
691 allocation and consolidation mechanisms in *Kdm5b* mutants, theories that need to be tested
692 for *Kdm5b* and other related disorders (Han et al., 2007; Han et al., 2008). For instance,
693 Deliu et al. reported altered trajectories of gene expression in response to conditioning in
694 *Setd5* mutant mice, albeit that baseline gene expression was not markedly different in these
695 mutants compared to wildtype controls (Deliu et al., 2018). Third, *Kdm5b* mutant animals
696 also showed an abnormal overexpression of a cluster of genes that was not affected by
697 neural activity. This cluster, which included proteins with a regulatory role over synaptic
698 function and transcription regulation factors include those genes constitutively affected by
699 the lack of KDM5B demethylase activity. Our results also implicate KDM5B in the regulation
700 of gene transcription through a number of zinc finger proteins, e.g. Zfhx2, Znfx1, Zbtb22 and
701 Zfp810, whose expression is either constitutively affected or abnormally downregulated upon
702 learning in mutant mice (Komine et al., 2012).

703

704 Our GOterms analyses also revealed that WT1 is one of the top transcription factors
705 modulating the expression of genes that are downregulated upon a learning stimulus
706 (Cluster 4, see Fig. 5D). A recent study showed that WT1 regulates neuronal excitability,
707 LTP and long-term memory formation. WT1 limits memory strength, thus enabling the
708 memory flexibility required for normal memory formation (Mariottini et al., 2019). The
709 possible relationship between KDM5B and WT1 warrants further investigation.

710

Together, these data showed that the expression of several different types and classes of genes that are regulated by neuronal activation and necessary for learning and memory are affected by *Kdm5b* deficiency.

H3K4me3 dysregulation in learning and memory

The finding that mice with *Kdm5b* knockdown in the adult hippocampus exhibit long-term memory deficits, suggests that KDM5B has a direct role in learning and memory, independent from any developmental functions. The most likely mechanism is that a deficiency in KDM5B demethylase activity impacts H3K4me3 dynamics and transcriptional output of key learning-associated genes (Gupta et al., 2010; Collins et al., 2019b; Collins et al., 2019a). Abnormalities may be present in different brain regions and cell types and during distinct stages of memory formation, such as allocation (Han et al., 2007; Han et al., 2008), consolidation (Gupta et al., 2010) and retrieval (Webb et al., 2017). Thus, future studies may need to assess H3K4me3 dynamics at specific gene promoters in different cell types. The conditional deletion of *Kmt2a* and *Kmt2b* in post-mitotic neurons using CamKIIcre was sufficient to cause hippocampus-dependent learning and memory deficits, suggesting a direct role for these genes in excitatory neurons (Kerimoglu et al., 2017). Together, these findings further support the notion that H3K4me3 regulation is important for learning and memory and suggest that dysregulation of H3K4me3, in either direction, can lead to memory deficits. However, as chromatin regulators typically function in large, multi-molecular complexes, other mechanistic explanations cannot be ruled out. Furthermore, a direct comparison between genes regulated by *Kmt2a* and *Kmt2b* has shown that these factors regulate different genes that likely impact neuronal plasticity in distinct ways (Kerimoglu et al., 2017). The two other H3K4me3-specific demethylases, KDM5A and KDM5C have also been implicated in learning and memory. A *Kdm5a*$^{-/-}$ mouse model was shown to exhibit deficits in the Morris water maze and *Kdm5c*$^{-/y}$ mice have deficits in contextual fear memory and novel object recognition (El Hayek et al., 2020; Vallianatos et al., 2020). Aguilar-Valles *et al.* have shown that *Kdm5c* knock-down in the nucleus accumbens in adult mice results in

increased H3K4me3 at gene promoters and deficits in methamphetamine-associated memory formation (Aguilar-Valles et al., 2014). To the best of our knowledge, *Kdm5a* has not yet been deleted or knocked down in post-mitotic neurons in the adult brain, so direct functions for this factor in learning and memory still need to be formally proven. Intriguingly, the contextual fear memory deficits in *Kmt2a*$^{+/-}$ and *Kdm5c*$^{-/y}$ mice were rescued in *Kmt2a*$^{+/-}$;*Kdm5c*$^{-/y}$ double heterozygous mice, suggesting that these factors regulated the same or similar processes, but in opposite directions (Vallianatos et al., 2020).

Cellular/synaptic functions of KDM5B

Mouse models for other H3K4me regulators such as *Kmt2a*, *Kdm5c*, *Kdm5a* or *Set1a* display both memory deficits and a decrease in spine density in the hippocampus or cortex (Mukai et al., 2019; El Hayek et al., 2020; Vallianatos et al., 2020). We did not detect significant changes in spine number in the hippocampal stratum oriens and radiatum, or stratum moleculare in the *Kdm5b* mutant mice, suggesting that overt changes in excitatory spine density might not account for the memory deficits in these mutants. The LTP deficit appears to be due to postsynaptic abnormalities, because we did not find any alternations in presynaptic transmitter release. Future studies will need to examine postsynaptic membrane proteins that are known to be involved in LTP and their potential regulation by KDM5B-dependent mechanisms. The exact cell types affected by *Kdm5b* deficiency will also need to be determined.

We recognise certain limitations in our study. We set out to test if KDM5B demethylase activity have a role in long-term memory consolidation. Although our results are consistent with this hypothesis, KDM5B protein levels were also downregulated in *Kdm5b*$^{\Delta/\Delta}$ mice, limiting our ability to conclusively ascertain the contribution of demethylase deficiency vs. other KDM5B functions. However, it is also intriguing to note that mutations affecting the

765 demethylase activity of *Drosophila* KDM5 leads to memory deficits and decreases
766 neurotransmission (Zamurrad et al., 2018; Belalcazar et al., 2021).

767

768 The relevance of the increased seizures in *Kdm5b* knock-down mice to clinical phenotypes
769 associated with KDM5B deficiency is still unclear. Chen *et al.* recently reported protein
770 truncating variants of *KDM5B* in individuals with epilepsy, but so far, the clinical description
771 of the small number of individuals with recessive *KDM5B* intellectual disability syndrome has
772 not revealed a significant association (Faundes et al., 2018; Martin et al., 2018). We have
773 not observed an obvious increase in spontaneous seizures in our homozygous mouse
774 model. One possibility is that KDM5B deficiency throughout development allows for sufficient
775 homeostatic compensatory mechanisms to ameliorate an overt excitatory/inhibitory
776 imbalance that might lead to spontaneous seizures in some individuals.

777

778 Mutations in chromatin modifying and remodelling factors represent a significant proportion
779 of mutations associated with neurodevelopmental disorders and intellectual disability
780 (Valencia et al., 2023). The functions of most of these factors in the developing and adult
781 brain are still unknown. As these factors are pleiotropic and likely regulates multiple
782 developmental processes and functions in the brain, identifying their salient functions and
783 mechanisms of action remain a significant challenge. This study, together with other recent
784 reports on other chromatin remodelling factors (Kerimoglu et al., 2013; Vogel-Ciernia et al.,
785 2013; Kerimoglu et al., 2017; Chen et al., 2023), suggest that these factors play central roles
786 in the regulation of genes necessary for learning and memory and that intellectual disability
787 disorders and cognitive deficits caused by mutation of these factors may to some extent be
788 caused by direct roles in postmitotic neurons. Reduced function of these factors appears to
789 be associated with altered trajectories of activity-dependent gene expression and abnormal
790 engagement of downstream synaptic plasticity mechanisms (Deliu et al., 2018).

Understanding these pathological mechanisms may lead to the development of targeted and improved therapies for intellectual disability disorders.

Study approval

All animal work were approved by local ethical review panels (AWERB, King's College London) and IACUC (UCI 20-095) and work conducted in the UK approved by a Home Office Project licence (PP6246123).

Data availability

RNAseq data (fastq files) were deposited at the Gene Expression Omnibus (GEO) archive under the accession number GSE240887 and made freely available upon publication.

Author contributions

MAB, KPG, MAW and LPS conceived the study and designed the experiments and LCA, CF, MAW, KPG and MAB supervised the research. LPS, SB, RM, TG and MAB performed behavioural studies, EK performed LTP experiments, LPS, AG, JAC and AMP performed bioinformatic analyses, FGG, AK and MAB performed surgeries for shRNA knockdown and DPM performed qRT-PCR experiments. LPS, EK and MAB wrote the manuscript with input from co-authors.

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Figures

Figure 1. *Kdm5b*$^{\Delta/\Delta}$ mice express reduced total KDM5B levels and show increased number of H3K4me3-positive cells in the hippocampus

A) Schematics of wildtype (WT) and KDM5B$^{\Delta ARID}$ protein domains. The mutant allele results in a truncated carboxyl end of the JmJN domain (JmjN-T) together with a deletion of the entire ARID domain. **B)** Representative western blot images from WT (+/+) and homozygous (Δ/Δ) mutant mice postnatal day 5 (PND5) hippocampal samples showing the lack of full-length KDM5B protein in cortical samples from Δ/Δ mice. αTubulin was used as loading control. Full-length KDM5B (arrowhead) and truncated ΔARID (asterisk) protein bands are indicated. See Extended data Fig. 1-1 for uncropped blot. Molecular weight (M.W.) markers in kDa are shown on the left. Female (squares) and male (diamonds) samples are included, although no sex differences were observed. **C, D)** Quantification of full-length and total (full-length and truncated) KDM5B protein from B. **E, F)** Quantification of the density of H3K4me3+ (cyan) cells in the hippocampus of 8 week-old wild-type and mutant mice. Nuclei were counterstained with DAPI (grey). Scale bar, 50µm. Female (squares) and male (diamonds) samples are included, although no sex effect was observed. **G, I, K)** Cumulative frequencies of H3K4me3-positive cells as a function of their staining intensity. Cumulative probability was calculated including all the detected cells (CA1: 406-903 cells, CA3: 236-925 cells, DG: 925-2491 cells; 4 animals/genotype). **H, J, L)** Representative H3K4me3 (cyan) immunostaining of hippocampal sections in 8 weeks-old control and mutant mice. Sections were counterstained with DAPI (grey). Scale bar, 200µm. Data in C, D and F is shown as mean ±SEM and was analysed with Student's t-test. *p<0.05, **p<0.01.

Figure 2. *Kdm5b*$^{\Delta/\Delta}$ homozygous mice are viable but exhibit growth retardation and increased brain:body weight.

A) Homozygous mutants are viable and present at expected Mendelian ratios at P21; n=128, Chi^2, p=0.9207. Similar Mendelian rates are observed when analysing males and

females separately (Males: 25.38% +/+, 51.52% Δ/Δ, 23.11% Δ/Δ; Females: 27.96% +/+, 47.67% Δ/Δ, 24.37% Δ/Δ). **B)** Pre-weaning body weight (g) measurements show decreased body weight in mutant animals. **C-F)** Body and brain weight, and brain:body and liver:body weight ratios are shown for +/+ (n=11) and Δ/Δ (n=13) P21 mice. **G)** Representative images of brains from wildtype and homozygous mutant mice indicating similar sizes. **H)** General cortical brain architecture is not affected in homozygous animals. Representative images of cortical brain sections, where nuclei were visualized with Hoechst3332 are shown. Scale bar, 50 µm; n=13 +/+ and n=14 Δ/Δ animals. **I)** Layer thickness was measured in the somatosensory cortex. **J)** Golgi-cox-stained proximal basal and apical dendrites of CA1 pyramidal neurons and apical dendrites of dentate gyrus granule cells in the dorsal hippocampus were analysed. Graphs depict the average spine density per animal (10-20 dendrites/hippocampal region). Images show representative Golgi-Cox-stained dendrites. Scale bar, 2.5µm. N=6 mice/genotype. Data is shown as mean ±SEM, including female and male mice. Data was analysed with two-way ANOVA (B, I) or Student's t-test or Mann-Whitney test when appropriate(C-F, J). *p<0.05, **p<0.01.

Figure 3. *Kdm5b*$^{\Delta/\Delta}$ homozygous mice exhibit hyperlocomotion and learning deficits.

A) Body weight differences in 2 month old mice before the start of the behavioural tests. Two-way ANOVA genotype effect: ****p<0.0001; sex effect: ***p<0.001. **B)** Distance moved (cm) in the outer zone of the open field arena. Two-way ANOVA genotype effect: ***p=0.0005. Three-way ANOVA sex effect: p=0.33. **C)** The amount of time (s) in the inner zone of the open field is shown. Two-way ANOVA genotype effect: **p=0.0087; sex effect: *p=0.0107. **D)** Time (%) spent in the open arms in the **e**levated plus maze (EPM), indicative of reduced anxiety. Two-way ANOVA genotype effect: p=0.8675, sex effect: p=0.4664. **E)** Discrimination index (DI) during the training and test (24h) phases in the object location memory (OLM) test to analyse long term spatial memory. Note significant learning in wild-type mice compared to training, but no significant learning in *Kdm5b*$^{\Delta/\Delta}$ mutants, and the reduced DI on test session between the two genotypes. Two-way ANOVA interaction effect:

p=0.0057. Two-way ANOVA sex effect: p=0.6317 (training) and p=0.9086 (testing) **F) There are no genotype differences in total exploration time (s) during training nor testing session in the OLM test. Two-way ANOVA genotype effect: p=0.2105 (training), p=0.5919 (testing) and sex effect: p=7506 (training) and p=0.2431 (testing). **G)** DI during the testing session of the spontaneous alternation in a Y maze (Y-SAT), performed 1 h after training to assess short-term spatial memory. Two-way ANOVA sex effect: p=0.0768. **H)** Spontaneous alternation rate in the Y-SAT shows no differences between genotypes. **I)** Latency (s) to reach the hidden platform during the training phase of the Morris water maze (MWM). Data represents the mean of the 4 trials/day. Two-way ANOVA genotype effect: ***p=0.0001. Three-way ANOVA sex effect: p=0.0019. **J, K)** Graphs show the number of platform crossings (J) and time spent (%) in the different quadrants (K) during the probe trial performed on day 8. Chance, 25%, is depicted with a dashed line. Two-way ANOVA sex effect in J: p=0.3413. **L)** Swimming speed (cm/s) was similar between genotypes during the training phase of the MWM. Three-way ANOVA sex effect: p=0.0757, genotype effect: p=0.1008. **M)** Latency (s) to climb the platform during the visible phase of the MWM. Three-way ANOVA genotype effect: *p=0.0220; sex effect p=0.1982. **N, O)** Front- and hindlimb grip strength (gm) was tested three times and the average is shown. Two-way ANOVA sex effect: p=0.1118 (N) and p=0.1924 (O). **P)** Freezing behaviour was assessed during contextual fear conditioning training, when three shocks were administered. Three-way ANOVA sex effect: p=0.07, genotype effect: ****p<0.0001. **Q)** Freezing percentages in the context testing session, 24 h later. Two-way ANOVA sex effect: p=0.1489. **R)** Mean speed was higher in mutant mice, in line with their reduced freezing behaviour during training (two-way ANOVA genotype effect: **p=0.0053). However, no differences were observed between genotypes during the 2-sec long foot shocks (Tukey's post hoc test p=0.9943 (shock 1), p>0.9999 (shock 2), p=0.9998 (shock 3), thus discarding differences in sensitivity and responses to shocks as a contributing factor. Data was analysed with two-way ANOVA (A, E, F) or repeated-measures two-way ANOVA (B, I, L, M, P, R) followed by Tukey's post-hoc test. Data in C, J, K, N, O and Q was analysed with Student's t-test, or Mann-Whitney test

when appropriate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. A one-sample t-test was used (K) to analyse whether time spent in the target quadrant was above chance. ##p<0.01, ###p<0.001, ####p<0.0001. For all experiments, n=17 +/+ male, n=14 +/+ female, n=17 Δ/Δ male and n=13 Δ/Δ female mice. Female (squares) and male (diamonds) samples are included.

Figure 4. Altered gene expression in *Kdm5b*$^{\Delta/\Delta}$ mice.

Hippocampi were dissected from naïve animals and RNAseq analyses were performed (n=4 animals/genotype). **A)** Heatmaps show differentially expressed genes in wildtype control (+/+) and mutant (Δ/Δ) mice. Yellow=upregulated and blue=downregulated in mutants. See Extended data Table 4-1 for detailed RNA-sequencing data information. **B)** Volcano plot displaying baseline gene expression changes between wild-type and *Kdm5b*$^{\Delta/\Delta}$ homozygous mutant mice. Red and blue dots depict differentially up- and downregulated genes, respectively. Differentially expressed immediate early genes are labelled. **C)** Immediate early gene expression in the dorsal hippocampus from WT and *Kdm5b*$^{\Delta/\Delta}$ mice was analysed by qPCR; n=9 animals/genotype. Two-way ANOVA genotype effect ***p=0.0001. **D)** Immunostaining of sections from DG and CA3 with an Npas4-specific antibody (magenta), counterstained with DAPI (grey) are shown. White arrowheads indicate positive nuclei. **E)** The number of Npas4-positive cells (arrowheads) was quantified in mm^2 areas of the CA3 and DG. Scale bar, 200µm, 100 µm for higher magnification images; n=10 mice/genotype. Data is shown as mean ±SEM, including both female and male mice. Data was analysed with Student's t-test (C, E): *p<0.05, **p<0.01.

Figure 5. Learning-associated gene expression changes in *Kdm5b*$^{\Delta/\Delta}$ mice.

Wildtype (+/+) and *Kdm5b*$^{\Delta/\Delta}$ (Δ/Δ) mice were trained in the fear conditioning chamber and culled 1h or 3h later. Homecage, test-naive mice were used as controls. Dorsal hippocampus was dissected and RNAseq analyses were performed. n=3 males and 3

females per genotype and timepoint. **A-D)** Differentially expressed genes were clustered by their expression trajectories with k means clustering (k=4). Selected genes within each cluster are shown on the right. **E-G)** Heatmaps show differentially expressed genes in control and mutant mice at baseline levels (E) or 1h (F) and 3h (G) after fear conditioning. Yellow = upregulated and blue=downregulated. **H,I)** Volcano plots display gene expression changes after conditioning. Red and blue dots depict differentially up- and downregulated genes, respectively, at 1h or 3h compared to control animals for each genotype. Differentially expressed activity-regulated genes are labelled in orange. Data is shown as mean ±SEM, including female and male mice. Data was analysed with repeated measures two-way ANOVA (A-D): ***p<0.001, ****p<0.0001. See Fig. 5-1, Table 5-1 and Table 5-2 for extended data.

Figure 6. *Kdm5b* knockdown in the dorsal hippocampus (CA1) of adult mice abrogates hippocampus-dependent memory consolidation and diminishes long-term potentiation.

A) Diagram of experimental work-flow. Approximately 3 weeks after stereotactic viral delivery into CA1, mice were habituated and tested in the object location memory task. Upon completion of behavioural tests, synaptic plasticity was assessed in acute brain slices from these mice. **B)** Representative immunostaining of dorsal hippocampus transduced with GFP-expressing AAV1, 11 days after surgery. Scale bar, 200µm. Panels display representative CA1, CA3 and DG inset images with GFP (green) and DAPI (blue) labelling. Scale bar, 50µm. **C)** qRT-PCR analysis of *Kdm5b* expression, relative to *Hprt*, from total RNA extracted from the dorsal hippocampus at indicated times (3 and 7 weeks (wks) after viral delivery). N=3-5 mice/group. **D)** qRT-PCR analysis of *Kdm5b* and immediate early gene expression, relative to *Hprt*, from total RNA extracted from the dorsal hippocampus, 11 days post-transduction. Two-way ANOVA shRNA effect for IEGs: **p=0.0016. N=3 mice/group. **E**) Distance moved (cm) in the test arena (open field) on the first day of habituation as a

measure of general activity (n=10 control and n=10 shRNA females). **F)** Discrimination index in the OLM task for control and shRNA mice during training and 24h long-term memory tests are shown. Note significant learning in control mice compared to training, but no significant learning in shRNA mice compared to controls (n=9 control and n=9 shRNA female mice). Two-way ANOVA interaction effect: *p=0.0257. **G)** Number of mice of each group (n=10 each) showing spontaneous seizures during handling (pink), compared to no seizures (grey). Fisher's exact test: **p=0.0031. **H)** Short- and long-term plasticity changes measured from hippocampal area CA1b apical dendrites in acute hippocampal slices (n=8 slices from each group; n=4 mice/group). Following a 20 min stable baseline recording, theta burst stimulation (TBS, arrow) was delivered to induce LTP and recordings were followed for an additional 1h. The fEPSP slope measured from *Kdm5b* shRNA slices was noticeably lower relative to controls by the end of the recording period. Inset: representative traces collected during baseline (black line) and 60 min post-TBS (red line). Scale: 1 mV/5 ms. **I)** The mean potentiation 50-60 min post-TBS was significantly reduced in slices from *Kdm5b* shRNA mice relative to controls (**p = 0.0056, n=8 each). **J, K)** Short-term plasticity measures in slices from *Kdm5b* shRNA mice including (J) input/output curve and (K) paired-pulse facilitation did not reveal any significant differences from shScramble controls. Data is shown as mean ±SEM, analysed with Student's T-test (C, D, E, I), two-way ANOVA (F) and Fisher's exact test (G). *p<0.05, **p<0.01, ***p<0.001. Control, shScramble; shRNA, shKdm5b.