

Associations of the Intellectual Disability Gene *MYTIL* with Helix–Loop–Helix Gene Expression, Hippocampus Volume and Hippocampus Activation During Memory Retrieval

Agnieszka Kepa^{1,2,18}, Lourdes Martinez Medina^{1,2,18}, Susanne Erk³, Deepak P Srivastava^{1,4}, Alinda Fernandes¹, Roberto Toro^{5,6}, Sabine Lévi^{7,8,9}, Barbara Ruggeri^{1,2}, Cathy Fernandes^{1,2}, Franziska Degenhardt¹⁰, Stephanie H Witt¹¹, Andreas Meyer-Lindenberg¹², Jean-Christophe Poncer^{7,8,9}, Jean-Luc Martinot^{13,14,15}, Marie-Laure Paillère Martinot^{13,14,15,16}, Christian P Müller¹⁷, Andreas Heinz³, Henrik Walter³, Gunter Schumann^{1,2} and Sylvane Desrivières^{*,1,2}

¹Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK; ²Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK; ³Department of Psychiatry and Psychotherapy, Campus Charité Mitte, Charité, Universitätsmedizin Berlin, Berlin, Germany; ⁴Department of Basic and Clinical Neuroscience, Maurice Wohl Clinical Neurosciences Institute, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK; ⁵Human Genetics and Cognitive Functions, Institut Pasteur, Paris, France; ⁶CNRS URA 2182, Genes, synapses and cognition, Institut Pasteur, Paris, France; ⁷INSERM UMR-S 839, Paris, France; ⁸Université Pierre et Marie Curie, Paris, France; ⁹Institut du Fer à Moulin, Paris, France; ¹⁰Department of Genomics, Life and Brain Center, and Institute of Human Genetics, University of Bonn, Bonn, Germany; ¹¹Department of Genetic Epidemiology in Psychiatry, Central Institute of Mental Health, University of Heidelberg, Mannheim, Germany; ¹²Department of Psychiatry, Central Institute of Mental Health, Mannheim, Germany; ¹³Institut National de la Santé et de la Recherche Médicale, INSERM CEA Unit I 000, "Imaging & Psychiatry", IFR49, CEA, DSV, IBM-Service Hospitalier Frédéric Joliot, Orsay, France; ¹⁴University Paris Sud, Orsay, France; ¹⁵Université Paris Descartes, Sorbonne Paris Cité, Paris, France; ¹⁶AP-HP Department of Adolescent Psychopathology and Medicine, Maison de Solenn, University Paris Descartes, Paris, France; ¹⁷Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany

The fundamental role of the brain-specific myelin transcription factor I-like (*MYTIL*) gene in cases of intellectual disability and in the etiology of neurodevelopmental disorders is increasingly recognized. Yet, its function remains under-investigated. Here, we identify a network of helix–loop–helix (HLH) transcriptional regulators controlled by *MYTIL*, as indicated by our analyses in human neural stem cells and in the human brain. Using cell-based knockdown approaches and microarray analyses we found that (1) *MYTIL* is required for neuronal differentiation and identified *ID1*, a HLH inhibitor of premature neurogenesis, as a target. (2) Although *MYTIL* prevented expression of *ID1*, it induced expression of a large number of terminal differentiation genes. (3) Consistently, expression of *MYTIL* in the human brain coincided with neuronal maturation and inversely correlated with that of *ID1* and *ID3* throughout the lifespan. (4) Genetic polymorphisms that reduced expression of *MYTIL* in the hippocampus resulted in increased expression of *ID1* and *ID3*, decreased levels of the proneural basic HLH (bHLH) transcriptional regulators *TCF4* and *NEUROD6* and decreased expression of genes involved in long-term potentiation and synaptic transmission, cancer and neurodegeneration. Furthermore, our neuroimaging analyses indicated that *MYTIL* expression associated with hippocampal volume and activation during episodic memory recall, as measured by blood-oxygen-level-dependent (BOLD) signals. Overall, our findings suggest that *MYTIL* influences memory-related processes by controlling a neuronal proliferation/differentiation switch of ID-bHLH factors.

Neuropsychopharmacology (2017) **42**, 2516–2526; doi:10.1038/npp.2017.91; published online 7 June 2017

*Correspondence: Dr S Desrivières, Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology & Neuroscience, King's College London, 16 De Crespigny Park, Denmark Hill, London SE5 8AF, UK, Tel: +44(0)20 7848 0528, Fax: +44(0)20 7848 0866, E-mail: sylvane.desrivieres@kcl.ac.uk

¹⁸These authors contributed equally to this work.

Received 5 August 2016; revised 27 December 2016; accepted 17 January 2017; accepted article preview online 4 May 2017

INTRODUCTION

The generation of post-mitotic neurons from proliferating progenitors, their differentiation and migration to their final positions in the brain, the axonal outgrowth and the generation of functional synapses are key events required for normal brain development throughout life. Any disturbance in these co-ordinated steps can result in intellectual

inability and serious developmental and neurodegenerative disorders (Vaillend *et al*, 2008).

The co-ordinated actions of transcription factors that drive tissue- and developmental stage-specific programmes of gene expression tightly regulate cell fate specification and differentiation. Progression through neurogenesis is partly determined by the sequential activities of evolutionarily conserved basic helix–loop–helix (bHLH) transcription factors. Generally, the determination genes *ATOH1*, *NEUROD4*, *ASCL1*, *NEUROG1*, and *NEUROG2* initiate neurogenesis and induce the expression of the differentiation effectors *NEUROD1*, *NEUROD2*, and *NEUROD6* (Sugimori *et al*, 2007). Given the complexity of cell fate determination processes, it is not surprising that the activity of proneural bHLH needs to be tightly regulated. To become transcriptionally active, these powerful tissue-specific transcription factors must form heterodimers with more broadly expressed bHLH proteins, or E-proteins, encoded by the *TCF4*, *TCF3*, and *TCF12* genes in humans. Conversely, their function is counteracted by another class of HLH proteins, the inhibitor of differentiation (ID). These dominant negative ID proteins control cell fate determination and inhibit differentiation during development and in adult life by sequestering bHLH proteins (For a recent review, see (Wang and Baker, 2015)). *ID* genes have various patterns of expression, with high *Id1* expression a characteristic of neural stem cells. Deletion of both *Id1* and *Id3* in mice results in severe phenotypes, including embryonic lethality with cardiac defects, premature withdrawal of neuroblasts from the cell cycle and premature neuronal differentiation (Lyden *et al*, 1999). These phenotypes of *Id1*^{-/-}/*Id3*^{-/-} mice, not seen in the single mutants, illustrate substantial functional overlap between these two genes.

Another transcription factor that may regulate tissue- and developmental stage-specific programmes of gene expression is myelin transcription factor 1-like (*MYT1L*), a brain-specific transcription factor that may be required for the expression of neuronal genes. This is suggested by the findings that the encoded protein is sufficient to convert mouse embryonic fibroblasts into functional neurons when overexpressed together with two other transcription factors, *Brn2* and *Ascl1* cells (Vierbuchen *et al*, 2010). While in this report *Myt1l* did not seem to be necessary for reprogramming process *per se*, it enhanced the differentiated phenotypes of neurons, suggesting a role for this gene in neuronal maturation. The formation of such induced neuronal cells appears to be more complicated in humans. Although overexpression of *MYT1L* together with *BRN2* and *ASCL1* could generate functional neurons from human pluripotent stem cells, further addition of *NEUROD1* is necessary to convert human fibroblasts into neuronal cells (Pang *et al*, 2011). This highlights cell-type and species-specific effects of *MYT1L* and other lineage-determining transcription factors. A role for *MYT1L* in neural development is further supported by the identification of *MYT1L* mutations in patients diagnosed with various neurodevelopmental disorders. The identification of mutations on chromosome band 2p25.3 disrupting the *MYT1L* gene in patients with mild to moderate intellectual disability and speech delay (Bonaglia *et al*, 2014; de Ligt *et al*, 2012; De Rocker *et al*, 2015; Mayo *et al*, 2015; Stevens *et al*, 2011) provided the first links between haploinsufficiency of *MYT1L* and intellectual disability (De Rocker *et al*, 2015). *MYT1L* mutations have

also been associated with diseases, such as schizophrenia (Lee *et al*, 2012; Vrijenhoek *et al*, 2008), autism (De Rubeis *et al*, 2014; Meyer *et al*, 2012), and major depressive disorder (Wang *et al*, 2010).

Despite such links with disease, there has been little research to date on the mechanisms by which *MYT1L* contributes to neuronal differentiation and its associations with relevant human brain phenotypes. To address this, we investigated the consequences *MYT1L* depletion on gene expression and neural differentiation in human neural stem cells in culture and in the human brain. We also measured associations between *MYT1L* genotypes with hippocampal structure and function using structural and functional neuroimaging. These analyses highlight a previously unrecognized *MYT1L*-dependant transcriptional network that offers a unique opportunity to understand the pleiotropic roles of *MYT1L* in human disease.

MATERIALS AND METHODS

Knocking-Down *MYT1L* in Cultured Cells

To inhibit *MYT1L* function in human neural stem cells, we transduced the SPC04 cell line (Desrivieres *et al*, 2015) with lentiviruses encoding either a control short hairpin RNA (shRNA) or a shRNA against *MYT1L* and measured the consequences on gene expression as indicated below.

Lentiviral production and transduction of neural stem cell cultures. eGFP-containing pGIPZ lentiviral vector with the shRNAmir sequence targeting *MYT1L* (V2LMM_32678) or the non-silencing shRNAmir (RHS4346) were obtained from the Open Biosystem shRNAmir library (ThermoFisher Scientific Open Biosystems, UK). The sense target sequences for these shRNAs were: *MYT1L*: 5'-CCGTGACTACTTTGACGGAAAT-3'; non-silencing control: 5'-ATCTCGCTTGGGCGAGAGTAAG-3'. Replication incompetent lentiviruses containing the *MYT1L* or non-silencing shRNAmir were obtained by transfecting HEK 293T cells using the Trans-lentiviral GIPZ Packaging System (ThermoFisher Scientific Open Biosystems) following the manufacturer's instructions. Supernatants containing lentiviruses were collected 48 h and 72 h post transfection, pooled, cleared by centrifugation at 1600 g at 4 °C for 10 min, filtered and ultracentrifuged at 25 000 r.p.m. for 1.5 h at 4 °C. Supernatants were carefully removed and lentiviral pellets resuspended in 30 µl of phosphate-buffered saline and aliquoted for storage at -80 °C until use. One day before transduction, SPC04 cells were seeded in 6-well laminin-coated plates at 20 000 cells/cm². The following day, the growth media was replaced by the pre-differentiation medium in the presence of 3.5 × 10⁵ TU/ml of lentivirus and the cells were incubated for 48 h, after which differentiation was induced. Transduction efficiency was monitored by a fluorescent microscope (Leica DMIL) with a Leica camera DFC420C (×10 objective).

Gene expression analyses. Effects of shRNAs on *MYT1L* mRNA levels and on global gene expression were assessed by quantitative PCR (qPCR) and microarray analyses.

qPCR analyses. cDNAs obtained by reverse-transcription using the SuperScript III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions, were

amplified by PCR. RNA expression levels were assessed with *RPL18* (60 S Ribosomal Protein L18) as internal controls for normalization, using the following forward and reverse primers: *RPL18* forward 5'-GAGAGGTGTACCGGCAT TTC-3', reverse 5'-CTCTGGCACGCTCGAACT-3'; *MYT1L* forward 5'-TGGAGAGCAACCTGAAGACC-3', reverse 5'-ATTCCTCTCACAGCCTGCTT-3'. PCRs were run in triplicate in the Applied Biosystems real-time PCR device (7900HT Fast Real Time PCR system) in 20 µl reactions containing 4 µl cDNA, 0.5 µM of each forward and reverse primers and 1× Power SYBR Green Mix (Applied Biosystems) using the following cycles: 95 °C for 15 min and 40 cycles at 95 °C for 30 s and 59 °C for 30 s. The PCR reaction products were evaluated by a melting curve analysis and the PCR products quantified using the SDS software (Applied Biosystems) comparing threshold cycles (Ct). Statistical analysis was performed on the delta Ct values (ie, experimental Ct - control Ct) by ANOVA, followed by Bonferroni- or Dunnett- based *post hoc* analyses ($\alpha = 0.05$, two-sided) using the SPSS software.

Microarray analyses. Total RNA samples were processed using the TargetAmp-Nano Labeling Kit (Cambio Ltd.) and hybridized to Illumina HumanHT-12 v4 Expression Bead-Chips according to the manufacturers' instructions at the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford. Raw data extracted by the Illumina BeadStudio software were further processed in R statistical environment (<http://www.r-project.org>). Raw expression data were pre-processed by variance stabilizing transformation and quantile normalization using the *lumi* package. Non-detected probes and probes that did not vary, based on median coefficient of variation across arrays, were filtered out. Differential expression between control vs knockdown conditions was assessed using the *limma* package. *P*-values were adjusted for multiple testing according to the false discovery rate (FDR) procedure of Benjamini and Hochberg and differentially expressed genes were selected at $FDR < 5\%$. The functional annotation clustering tool, part of the Database for Annotation, Visualisation and Integrated Discovery (DAVID) web server (Huang et al, 2009) was used to determine enrichment of functional groups in set of genes mapped by differentially expressed probes (at $FDR \leq 10\%$). Clustered GO terms with $P_{EASE} \leq 0.01$ (ie, enrichment scores > 2) were considered specifically enriched.

Gene Expression Analyses in Post-Mortem Human Brain Samples

We analyzed publicly available data sets to follow spatio-temporal gene expression patterns in the human brain and identify expression quantitative trait loci (eQTL) in *MYT1L* (ie, single-nucleotide polymorphisms (SNPs) associated with differences in gene expression). These are detailed in the Supplementary Methods.

Magnetic Resonance Imaging Study of the Human Hippocampus

To investigate the impact of *MYT1L* SNPs on hippocampus structure and function in the living human brain, we used structural magnetic resonance imaging (MRI) and an

established reliable functional MRI (fMRI) neuroimaging task (Erk et al, 2010).

Human structural MRI analyses

Subjects. A sample of $N = 1583$ adolescents (age = 14.57 ± 0.45), participants of the IMAGEN project (Schumann et al, 2010) were analyzed in this study. Written informed consent was obtained from all participants and their legal guardians.

MRI. Details of the MRI protocols and quality checks are in the Supplementary Methods. Out of 1909 images, 1583 passed these quality control checks and were used in association analyses with *MYT1L* genotypes.

SNP genotyping and quality controls and selection of *MYT1L* genotypes. DNA purification and genotyping procedures have been described previously (Desrivieres et al, 2015). Ninety five SNPs contained within the *MYT1L* locus and its surrounding ± 10 kb region were selected and the corresponding genotypes extracted from genome-wide information collected at 502 160 SNPs for 1837 subjects (900 males, 937 females) using PLINK (<http://zzz.bwh.harvard.edu/plink/>). This data set had been filtered to remove SNPs with call rates of $< 98\%$, minor allele frequency $< 1\%$ or deviation from the Hardy-Weinberg equilibrium ($P \leq 1 \times 10^{-4}$), and individuals with $> 2\%$ missing genotypes or of unspecified sex.

Genetic association analyses. Linkage disequilibrium (LD)-based set-test association analyses were performed, testing for the additive dosage of each SNP on the volumes of the right and left hippocampus using the set-test function of PLINK, with the following parameters: LD threshold = 0.5, SNP *p*-value threshold = 0.01; maximum number of independent SNPs = 5. Empirical *p*-value for the *MYT1L* set was obtained after 10 000 permutations. Gender, intracranial volume and dummy variables for scanning site were used as covariates of no interest in the analyses. Handedness and age did not influence the above associations and were not included as a covariate in our analyses. For the single SNP associations of rs17338519 with hippocampal volume, linear regression analyses were performed using the same covariates as above. Results presented for these analyses included from 1,398 individuals with non-missing status (900 males, 937 females; and 1227 right handed).

Human fMRI study of episodic memory recall

Subjects. 285 healthy adults (mean age 34.0 ± 9.8) German volunteers with grandparents of European ancestry were recruited at Bonn, Mannheim and Berlin. Participants had no history of any neurological disorder or current psychiatric Axis I disorder including drug or alcohol dependence, as verified by SCID-I. The study was approved by the local ethics committees of the Universities of Bonn, Heidelberg and Berlin and all subjects gave written informed consent to the study. $N = 3$ subjects were rs17338519 AA homozygotes, $n = 42$ AC heterozygotes and $n = 240$ CC homozygotes. The allele frequencies were in Hardy-Weinberg equilibrium. Genotype distributions did not differ between sites. Gender, age, handedness and level of education, did not differ significantly between genotype groups.

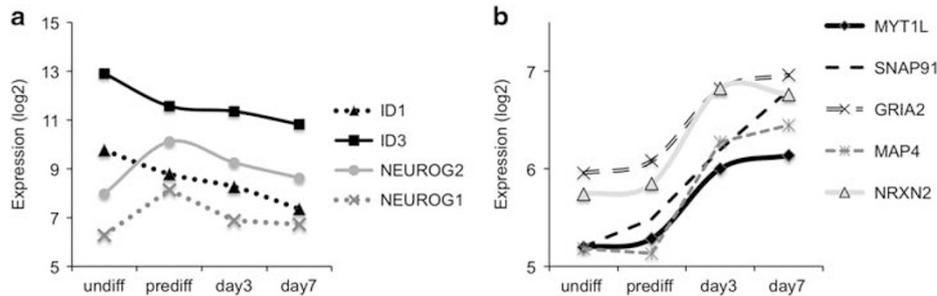


Figure 1 Changes in gene expression patterns during differentiation of the human neural stem cell line SPC04. Changes in HLH (*ID1* and *ID3*) and bHLH (*NEUROG1* and *NEUROG2*) mRNA levels (a), and induction of a subset of terminal differentiation genes co-expressed with *MYT1L* (b) were accessed by microarray analyses. Results show variations in expression in various stages of differentiation (pre-differentiation, and 3 or 7 days after induction of differentiation), relative to that of undifferentiated cells and represent mean \pm SEM of $n = 3$ independent experiments.

DNA-extraction and genotyping. Genotype data for rs17338519 were extracted from a genome-wide data set generated at the Department of Genomics, Life & Brain Center, University of Bonn using Human610-Quad Bead Arrays (Illumina, San Diego, CA, USA).

Functional imaging task. During fMRI scanning, subjects completed an episodic memory task that was part of three consecutive memory tasks, ie, encoding, recall and recognition of face-profession pairs (for a detailed description see Erk *et al* (2010)). During recall, the task in focus here, faces were presented together with the question whether the depicted person had to complete apprenticeship or academic studies to qualify for the respective profession that had been learned during encoding. Subjects had to indicate by button press, which qualification was correct. In the control condition, subjects had to assess whether the left or the right ear of different head contours was larger. The task consisted of 4 blocks of 4 faces (a 6 s) and 4 blocks of 6 head contours (a 4 s) each. Each block lasted 24 s. Task parameters and image processing are detailed in the Supplementary Methods.

For each subject, statistical contrast images of memory *vs* control were obtained and analyzed using the general linear model in a second-level random effects analysis (two-sample *t*-test) with site, age, gender and hippocampal volume as covariates. Given the small number of AA individuals, we pooled individuals with either one or two copies of the A allele (AA,AC). We used a region of interest (ROI) approach to investigate BOLD signals in the hippocampus, using anatomical labels provided by the Wake Forest University PickAtlas (www.fmri.wfubmc.edu/downloads).

Statistical inference. The significance threshold was set to $p < 0.05$, corrected for multiple comparisons within our *a priori* defined anatomical ROI, the hippocampus. For all analyses, we employed conservative analysis statistics by using family wise error rate (FWE), exerting strong control of type I error over multiple comparisons.

RESULTS

Induction of *MYT1L* follows Neural Commitment, Correlating with the Expression of Terminal Differentiation Genes

To investigate the role of *MYT1L* in neural differentiation, we followed its expression in SPC04, a neural progenitor cell

line that efficiently differentiates under defined culture conditions (Supplementary Methods). These cells express markers of differentiated neurons and produce neurite outgrowth starting 3 days after induction of differentiation (Desrivières *et al*, 2015). Microarray analyses indicated that proliferating, undifferentiated SPC04 cells express high levels of the stem cells markers *ID1* and *ID3*. The loss of proliferation correlated with drop in expression of these genes. Conversely, expression of *NEUROG1* and *NEUROG2* increased when that of *ID1* and *ID2* decreased, being maximal at the stage preceding induction of differentiation (Figure 1a). These patterns of expression contrasted sharply with that of *MYT1L*, whose expression was induced with differentiation, correlating with that of markers of terminal differentiation (Figure 1b). Functional annotation clustering confirmed that expression of *MYT1L* positively correlated with transcripts ($r > 0.85$; Supplementary Table 1) involved in neurite development and synaptic transmission.

Knockdown of *MYT1L* Enhances Expression of *ID1* and *ID3* and Inhibits Neuronal Differentiation

We next studied the consequences of *MYT1L* loss of function on neuronal differentiation and gene expression. We infected SPC04 cells with lentiviruses encoding either a shRNA against *MYT1L* or a control shRNA and allowed them to differentiate for 7 or 14 days. As shown in Figure 2a, treatment with *shMYT1L* for 7 or 14 days, resulted in significant reduction of *MYT1L* mRNA levels ($F(1,9) = 29.01$, $p = 0.002$). Morphological examination of the infected cells indicated that while infection with control lentiviruses generated cells with apparent neuronal morphology with extended networks of neurites visible 7 days after induction of differentiation, infection with *shMYT1L*-expressing lentiviruses prevented these morphological changes, resulting in larger cell bodies and less elaborated neurite outgrowth (Figure 2b and c; see Supplementary Methods for quantification of neurite outgrowth). We then conducted microarray analyses to identify gene networks affected by *MYT1L* knockdown. These analyses indicated that most transcripts that were differentially expressed upon *shMYT1L* treatment were downregulated (Supplementary Table 2 and Figure 2d), indicating that *MYT1L* acts mainly as a transcriptional activator. Functional clustering analyses indicated that genes downregulated by *shMYT1L* were enriched for genes involved in neurite outgrowth, axonal development, synaptic

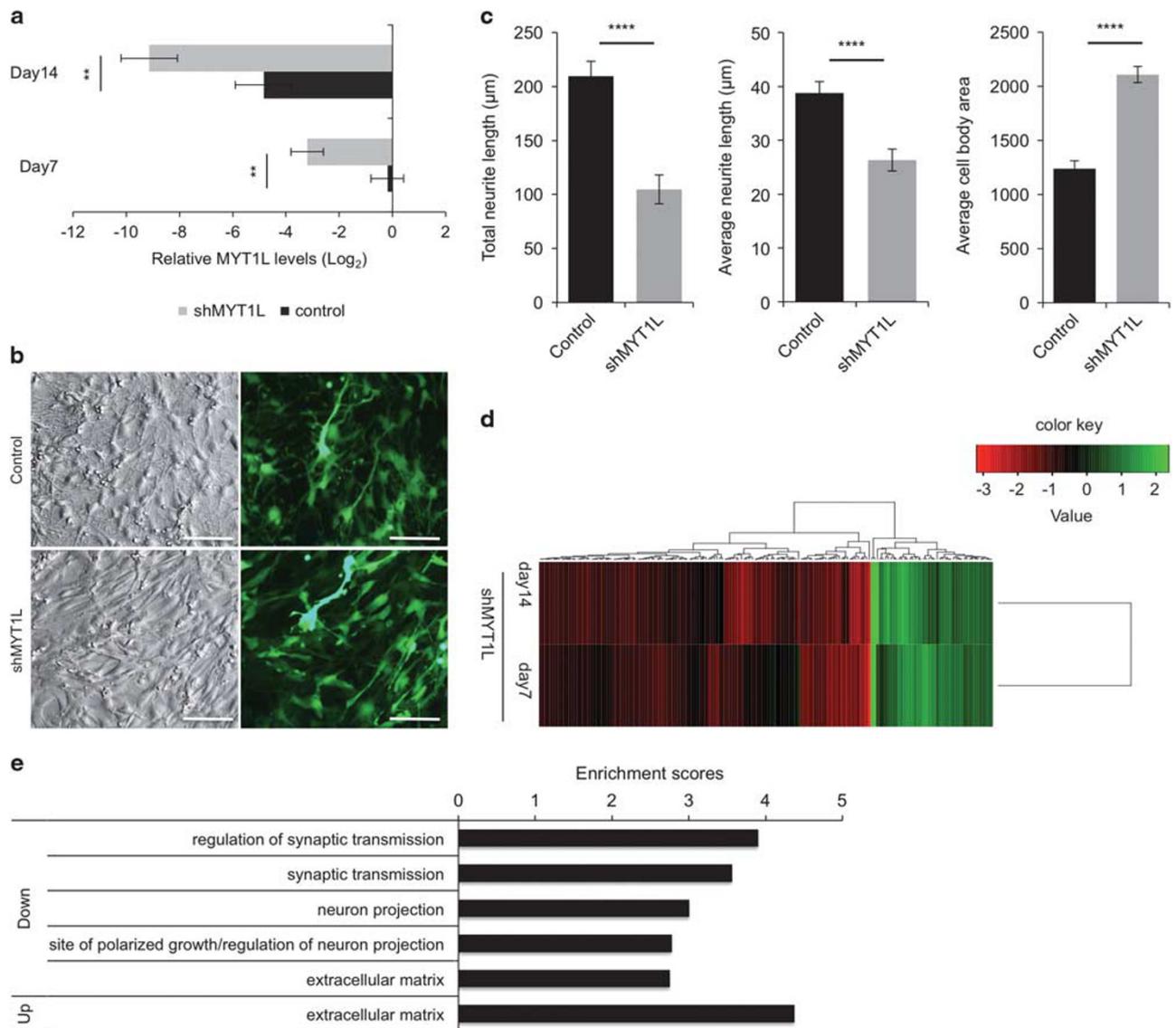


Figure 2 Effects of *MYT1L* knockdown on neural differentiation and global gene expression in SP04 cells. Cells were transduced with lentiviral vectors encoding *shMYT1L* or a non-silencing shRNA control and induced to differentiate for 7 or 14 days. (a) Efficiency of *MYT1L* knockdown accessed by PCR 7 and 14 days after induction of differentiation. Effects of treatment with *shMYT1L* on *MYT1L* and mRNA levels, split according to the length of differentiation. Results represent mean \pm SEM of $n = 3$ independent experiments; $**p < 0.01$. (b) Effects of *shMYT1L* on morphology of SPC04 cells, 7 days after induction of differentiation. Changes in morphology are evident when comparing phase contrast pictures of cells transduced with control, non-silencing lentiviruses with that of cells transduced with *shMYT1L*-encoding lentiviruses: control cells showed enhanced neuronal features such as increased neurite elongation and retracted cell bodies. In contrast, *shMYT1L* prevented these morphological changes, resulting in a flat, more adherent morphology. The fluorescence pictures reveal the high efficiency of transduction by the lentiviruses that expressed the enhanced green fluorescent protein as marker. Scale bar = 100 μ m. (c) Quantification of the morphological changes described in (b) were obtained using the neurite outgrowth plugin in MetaMorph; total neurite length and average length of neurite per cell and cell soma area were recorded in a total of 473 cells (number of cells per group: control *MYT1L*, $n = 298$; *shMYT1L*, $n = 175$). Unpaired *t*-tests were used to compare means in each group: $****p < 0.0001$. (d) Microarray mRNA profiling analysis reveals target genes differentially affected by *MYT1L*. Probe sets differentially expressed following *shMYT1L* treatment were clustered together based on euclidean distance, and their expression levels (each line represents a single probe set) are displayed as fold change compared to control treatment. (e) Functional clustering of differentially expressed genes showing enrichment for genes involved in neuronal maturation and the extracellular matrix composition.

transmission and extracellular matrix composition (Figure 2e). Interestingly, the HLH *ID1* and *ID3* were among the few genes whose expression was induced upon *MYT1L* knockdown (Supplementary Table 2). These results suggested that *MYT1L* facilitates withdrawal from the cell cycle and commitment to neural differentiation by preventing expression of *ID1* and *ID3*.

Expression of *MYT1L* is Inversely Correlated with *ID1* and *ID3* Levels in the Human Brain Throughout the Lifespan

To investigate the relevance of these findings to human brain development, we analyzed publicly available data sets of human post-mortem brain samples (Supplementary Methods), comparing the expression patterns of *MYT1L*,

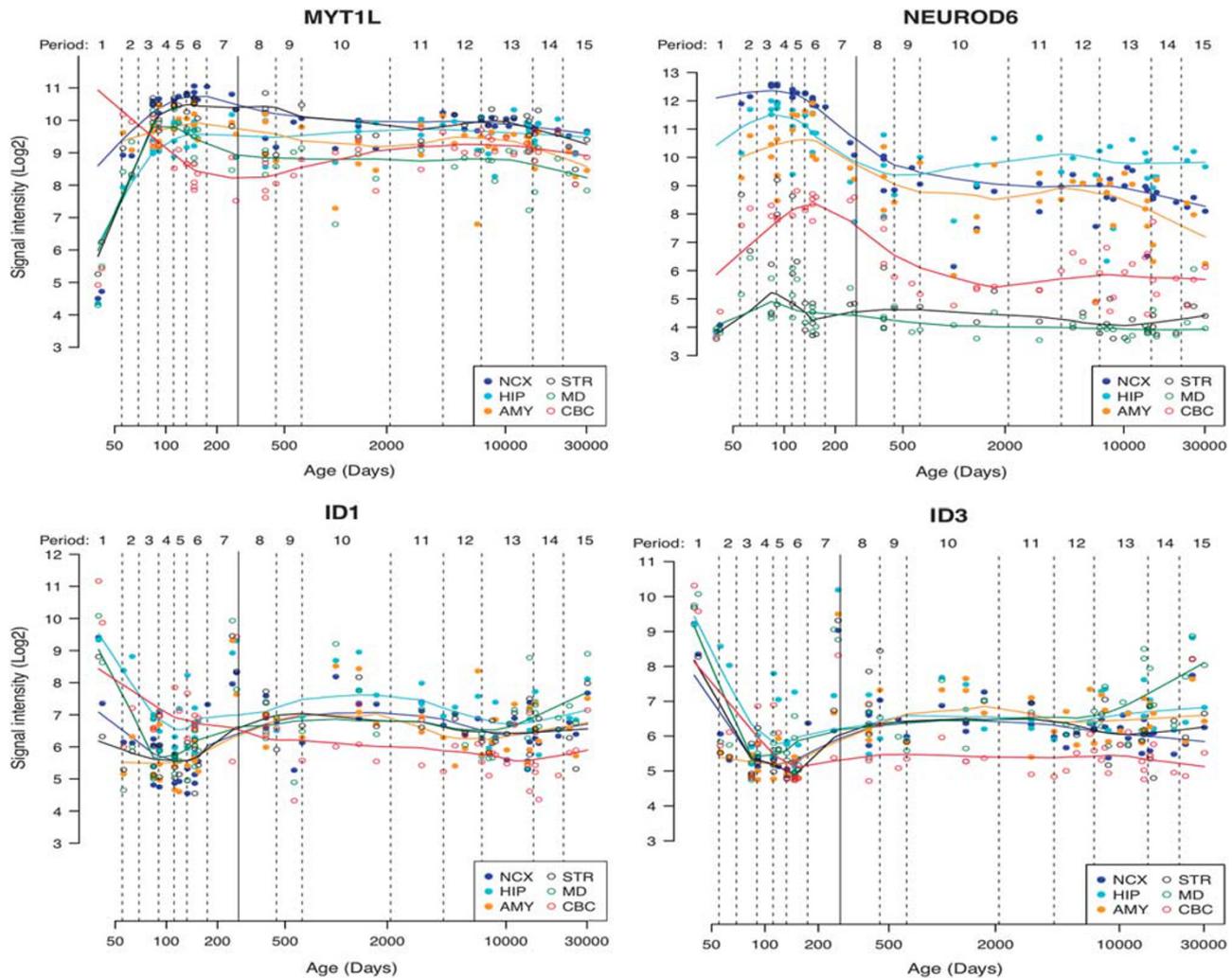


Figure 3 Analyses of gene expression in the human brain across the lifetime reveals patterns of *MYT1L* mRNA expression that correlates highly with that of *NEUROD6*, notably in the neocortex, the hippocampus and the amygdala. Conversely, *MYT1L* expression correlates inversely with that of *ID1* and *ID3* throughout the life span.

ID1 and *ID3* in the human brain across the lifespan (Figure 3). *MYT1L* is expressed throughout the human brain. Its expression increases sharply during prenatal development to reach a maximum during mid and late stages of fetal development. Thereafter, expression of *MYT1L* drops, remaining moderate throughout childhood and adolescence and decreasing further in adult life. Such expression profile coincides with the expression of neuronal differentiation markers throughout the lifetime (data not shown but available upon request), overlapping with that of tissue- and region-specific bHLHs, such as *NEUROD6*. Conversely, expression of *ID1* and *ID3* follows reversed patterns, being highest when *MYT1L* expression is lowest (ie, during embryonic development and childhood) and decreasing when *MYT1L* expression increased. These opposite expression patterns further support a functional antagonism between *MYT1L* and *ID1/ID3*.

Mutations Decreasing *MYT1L* Expression in the Human Hippocampus Associate with Induction of *ID1* and *ID3* and Repression of Neural Differentiation Genes

If, as suggested above, *MYT1L* represses expression of *ID1* and *ID3* in the human brain, mutations altering *MYT1L* functions should affect *ID1* and *ID3* levels such that their expression is induced when *MYT1L* is inhibited, or *vice versa*. To test this, we searched the UKBEC data set (Trabzuni *et al*, 2012) for potential *cis*-acting eQTLs, that are SNPs within the *MYT1L* locus that correlate with altered *MYT1L* mRNA levels (Supplementary Methods). We identified several such eQTLs, clustered around a conserved tissue-specific transcriptional enhancer upstream of *MYT1L* (Figure 4a; <http://enhancer.lbl.gov/>; (Visel *et al*, 2007)). The presence of minor alleles at these loci was associated with decreased expression of *MYT1L* specifically in the hippocampus. This is illustrated in Supplementary Figure 1 for rs55800610, the most significant of these eQTLs. Individuals carrying the minor A-allele at this locus had significantly

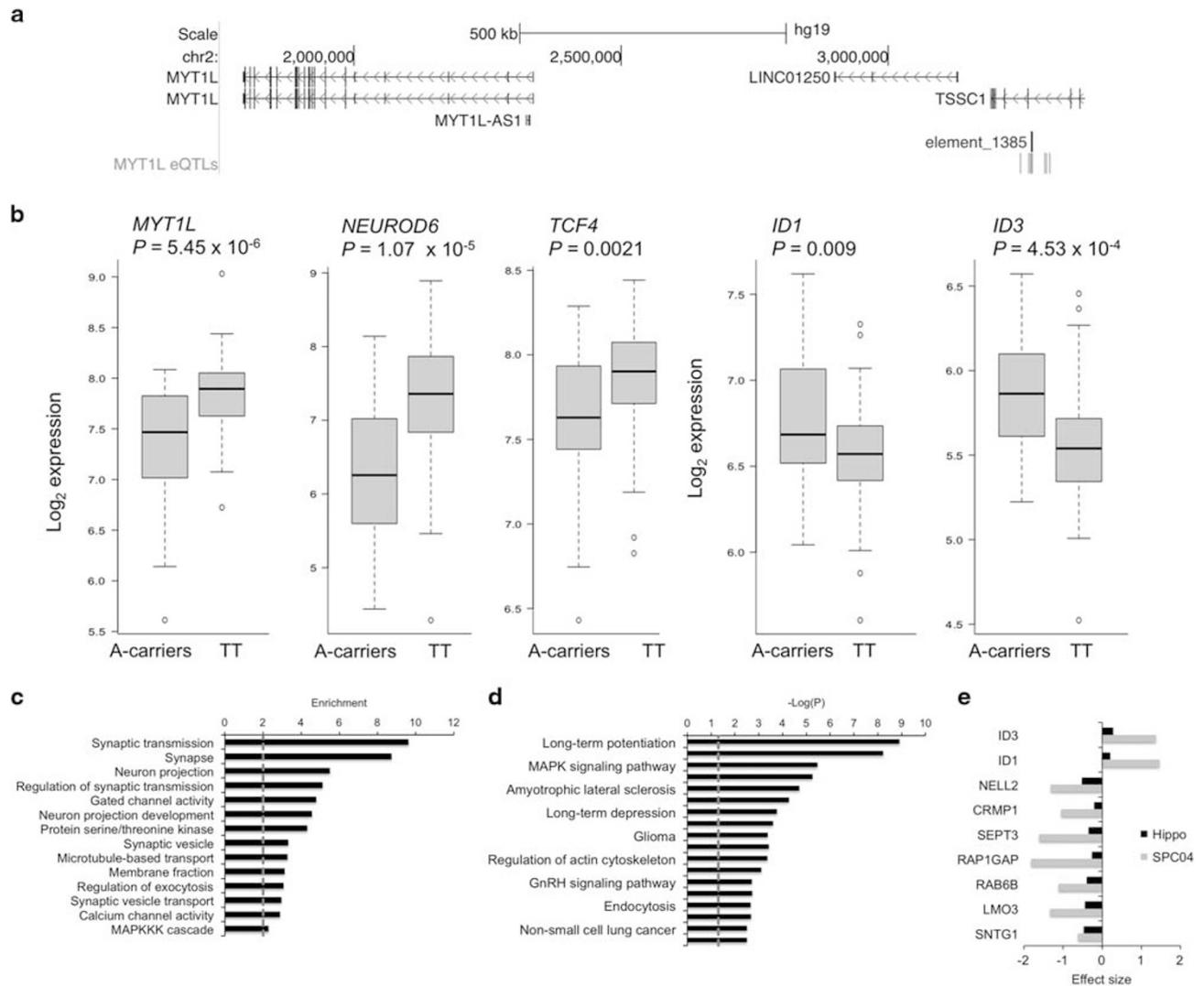


Figure 4 Effects of a *MYT1L*-associated eQTL on gene expression in the hippocampus. (a) Genomic view of the *MYT1L* locus, indicating SNPs acting in cis to influence *MYT1L* expression (*MYT1L* eQTLs, red bars). These SNPs are all in high linkage disequilibrium ($r^2 > 0.8$), surrounding a conserved distant-acting and tissue-specific transcriptional enhancer (ie, human element hsl385, labeled as element_1385) from the VISTA Enhancer database, (Pennacchio et al, 2006). Genomic co-ordinates are based on the hg19 genome assembly. (b) The minor alleles at these eQTLs decrease mRNA levels of *MYT1L* (cis-effects) in the hippocampus, as illustrated for the rs55800610 gene variant. The minor A-allele at this SNP also associates with reduced *NEUROD6* and *TCF4* mRNA levels and increased mRNA levels of *ID1* and *ID3* in the hippocampus (trans-effects). Because of the low minor allele frequency (MAF = 0.07), heterozygotes and individuals homozygotes for the minor allele were grouped for analyses. Genotypes count: A-carriers (AA+AT), $n = 20$; TT, $n = 114$. (c and d) Gene set enrichment analyses of all genes downregulated in individuals carriers of the rs55800610 A-allele indicating their clustering into specific biological functions (c) and belonging to a known biological pathway (d). The dashed vertical line represents the significance threshold ($P_{adj} < 0.05$). (e) Genes whose expression is affected by *MYT1L* decrease both in SPC04 cells and in the human hippocampus, and the measured effect sizes (negative = down-regulation, positive = up-regulation).

lower *MYT1L* mRNA levels in the hippocampus ($P = 4.4 \times 10^{-6}$). A similar, nominally significant decrease was also observed in the thalamus ($P = 0.042$) but not in eight other brain regions. As hypothesized, the reduction of *MYT1L* levels in the hippocampus of carriers of the rs55800610 A-allele was accompanied with significant increased levels of *ID1* and *ID3* (Figure 4b).

This SNP also affected expression of a large number of genes *in trans* ($N = 850$, $FDR \leq 0.05$), most of which ($> 90\%$), such as the proneural genes *NEUROD6* and *TCF4* (Figure 4b and Supplementary Table 3), were downregulated in carriers of the rs55800610 minor A-allele. The affected genes contributed largely to the regulation of synaptic

transmission and long-term potentiation. Also noteworthy are significant enrichments for genes involved in intracellular signaling (calcium- and MAP kinase-related signaling pathways), in cancer (glioma, non-small cell lung cancer) and in neurodegenerative diseases like amyotrophic lateral sclerosis and Alzheimer's disease (Figure 4c and d).

Mutation Linked to Decreased *MYT1L* Expression Associates with Hippocampal Volume and Hippocampal Activation During Memory Retrieval

The results above suggested that *MYT1L* may play a role in development and function of the hippocampus. We

investigated this further by analyzing associations between *MYT1L* SNPs and structural and fMRI in the human hippocampus. We first analyzed possible associations between *MYT1L* and inter-individual variations of hippocampal volume in IMAGEN, a cohort of $N=1,398$ healthy adolescents, whose characteristics are listed in Supplementary Table 4. As rs55800610 was not directly genotyped in IMAGEN, we performed gene set-based analyses with SNPs genotyped in this sample to identify independent SNPs associating with hippocampal volume. For this, 95 SNPs contained within the *MYT1L* locus and its surrounding ± 10 kb region were tested for association by linear regression analyses taking LD between SNPs into account. Five SNPs in high LD were associated with right hippocampal volume below a p -value of 0.01. Only 1 of these, rs17338519, was independently significant based on an r^2 threshold of 0.5, remaining significant after 10 000 permutations (empirical p -value = 0.0098). For the left hippocampus, two SNPs in high LD ($r^2 > 0.5$), including rs17338519, were associated with hippocampal volume below a p -value threshold of 0.01. However, this association did not remain significant after 10 000 permutations (empirical p -value = 0.3598). Linear regression analyses revealed significant association between genotypes at rs17338519 and right hippocampal volume. As shown in Figure 5a, the volume of the right hippocampus significantly decreased with the number of minor T-alleles at this locus ($\beta = -75.96$, $p = 4.40 \times 10^{-4}$, Figure 5a). Although no SNP passed the threshold of significance for analyses on the left hippocampus, we noted that the effects of rs17338519 on the right and left hippocampus were in the same direction ($\beta = -55.55$, $p = 0.0079$, for effects of rs17338519 on the left hippocampus). We also used the UKBEC data set to test whether genotypes at rs17338519 were related to *MYT1L* expression levels in the human brain. Regression analyses indicated that the presence of the T-allele associated with decreased *MYT1L* mRNA levels in the hippocampus and the cortex ($\beta = -0.22$, $p = 0.034$; $\beta = -0.22$, $p = 0.0035$; Figure 5b).

To investigate a possible link between *MYT1L* and hippocampal function, we used an established reliable neuroimaging task (Erk et al, 2010), testing for the effects of rs17338519 on hippocampus activation during episodic memory recall in $N = 285$ healthy individuals. We observed increased activation of the right hippocampus ($x = 16$, $y = -6$, $z = -12$; $Z = 3.65$, $p = 0.022$ FWE corrected for ROI) during memory recall in carriers of the T-allele (Figure 5c). Genotype groups in this sample did not differ significantly for memory encoding or recognition. These data suggest that *MYT1L* maintains hippocampal structural integrity and its activation during learning and memory.

DISCUSSION

We have shown for the first time that the *MYT1L* transcription factor regulates neurogenesis by repressing ID genes *ID1* and *ID3*, and inducing proneural bHLH genes such as *TCF4*, *NEUROD2*, and *NEUROD6*. Loss-of-function experiments *in vitro* and the identification of deleterious mutations in the human brain indicated that this transcriptional regulator is necessary for the expression of genes implicated several aspects of neural development such as neurite outgrowth, axonal development and the regulation of

synaptic transmission, as well as extracellular matrix composition. In line with a role for ID proteins in cell cycle and senescence (Ruzinova and Benezra, 2003), *MYT1L*-regulated genes were also enriched for genes involved in cancer and the neurodegenerative diseases amyotrophic lateral sclerosis and Alzheimer's disease. Neuroimaging analyses indicated that expression of *MYT1L* in the hippocampus associates with hippocampus volume and activation during episodic memory recall. Thus, we propose that *MYT1L* controls neural development and influences cognitive capacity by displacing the balance of bHLH and ID factors such that bHLH predominate.

The links that we identified between *MYT1L* and *ID* gene expression are interesting. ID proteins control proliferation and differentiation of a variety of tissue-specific stem cells in response to signaling pathways triggered by their extracellular microenvironment (Chaudhary et al, 2001; Jankiewicz et al, 2006; Niola et al, 2012; Ying et al, 2003; Yokota et al, 1999). In this context, one notable finding is the link that we established between *MYT1L* and extracellular matrix gene expression. Interestingly, *RAP1GAP*, a gene downregulated by loss of function of *MYT1L*, both in SPC04 cells and in the hippocampus (Figure 4e and Supplementary Tables 2 and 3), is known as a direct target of the bHLH—ID transcriptional pathway that encodes a key regulator of cell adhesion, as determined by investigations in the developing mouse brain. ID-mediated repression of *Rap1GAP* has been found to enhance adhesion of neural stem cells to their extracellular microenvironment, thereby synchronizing neural stem cell functions to residency in the specialized stem cell niche (Niola et al, 2012). Therefore, *MYT1L*-mediated negative control of *ID* expression may activate a transcriptional program to synchronize the committed differentiation of neural stem cell progenitors and their detachment from the stem cell niche. Consistent with this role in regulating stem cell anchorage to the niche and neural differentiation, *ID1/RAP1GAP* has a central role in tumor formation, particularly in the formation of highly aggressive glioblastomas (Niola et al, 2013; Soroceanu et al, 2013). Our results also implicate *MYT1L* in glioma formation, through a process that besides *RAP1GAP*, may require MAP kinases and calcium-dependent signaling mechanisms (Ren et al, 2007). Indeed, the *MYT1L* gene was found to be deleted in $\sim 5\%$ of glioblastoma multiforme samples and the protein absent or downregulated in $>80\%$ of these samples (Hu et al, 2013), leading to the suggestion that gliomagenesis requires neutralization of terminal differentiation.

Importance of the *MYT1L-ID-HLH* network for normal brain development and function is reflected by the severe developmental delays and complex phenotypes associated with disruptions in some of its core components. Although *MYT1L* mutations have been associated with intellectual disability with early onset obesity (Doco-Fenzy et al, 2014; Stevens et al, 2011), autism (Meyer et al, 2012; Rio et al, 2013), and schizophrenia (Lee et al, 2012; Vrijenhoek et al, 2008), *TCF4* mutations cause Fuchs' corneal endothelial dystrophy, Pitt-Hopkins syndrome, a disorder that is part of the clinical spectrum of Rett-like syndromes characterized by intellectual disability and developmental delay (Whalen et al, 2012), and have been described in patients with schizophrenia, autism, and other neurodevelopmental disorders

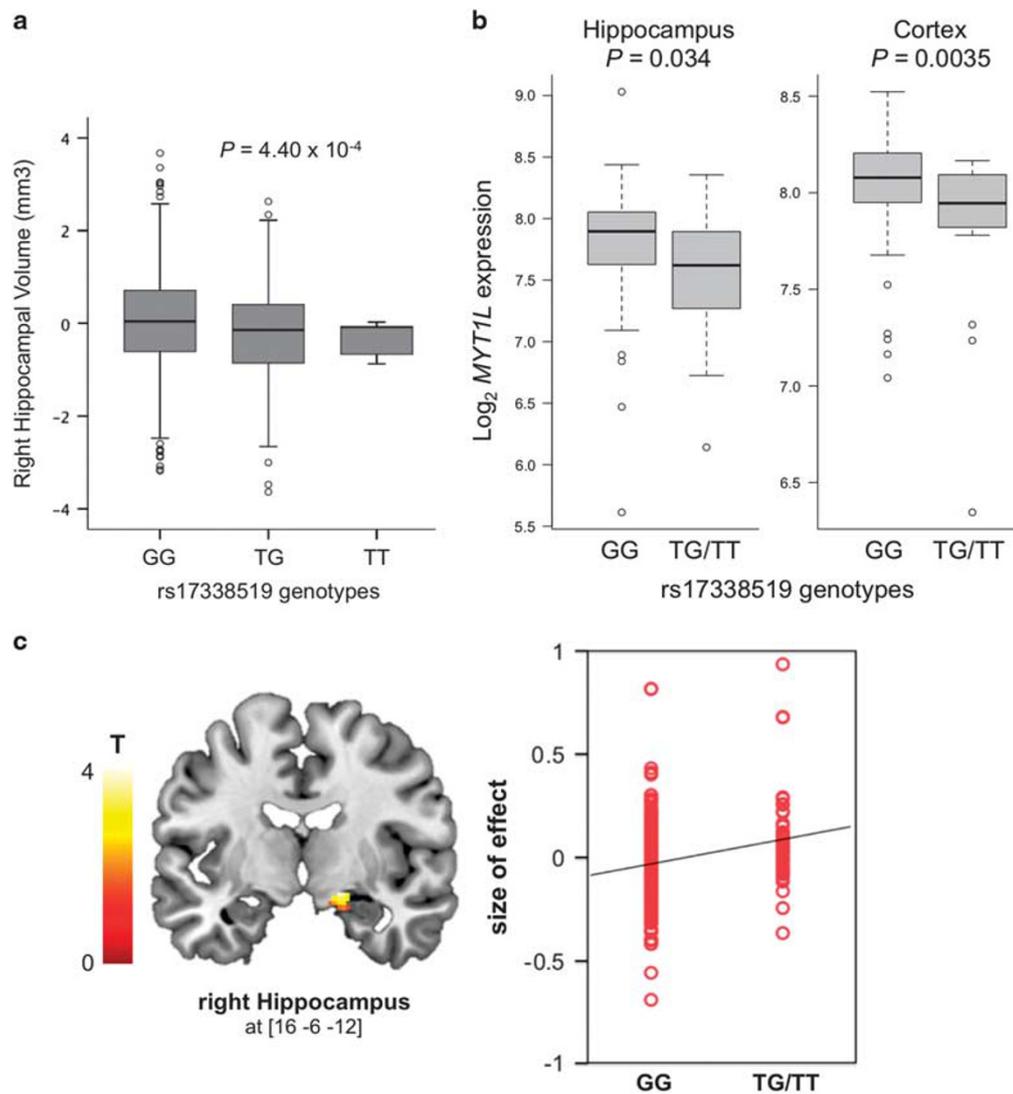


Figure 5 Associations between *MYTIL* expression and hippocampus volume and hippocampus activation during episodic memory recall. (a) Association of rs17338519 with hippocampal volume in a sample of $N = 1,398$ adolescents. Linear regression analyses indicated that the number of minor T-alleles associated with lower volume of the right hippocampus ($P = 4.40 \times 10^{-4}$). (b) Expression of *MYTIL* stratified by genotypes at rs17338519, in the hippocampus and cerebral cortex of 134 post-mortem human brains. For the cortex, expressions of *MYTIL* in the occipital, temporal, and frontal cortices were averaged. Regression analyses indicated that the presence of the T-allele at rs17338519 associated with decreased *MYTIL* mRNA levels in the hippocampus ($P = 0.034$) and the cortex ($P = 0.0035$). Genotypes count: GG = 110; T-carriers = 24. (c) Association of rs17338519 with hippocampal activation during an fMRI task of episodic memory recall in 285 healthy adults. The left panel shows the ROI-derived brain activation map within the hippocampus, with increased activation of the right hippocampus ($x = 16, y = -6, z = -12$) during episodic memory recall in carriers of the T-allele ($N = 285$; $p = 0.02$ FWE corrected for multiple testing across ROI). The right panel shows a quantification of rs17338519 genotypes effects on the activation of the right hippocampus. Each dot represents size of effect in one subject.

(Forrest *et al*, 2014). Strikingly, expression of *ID* genes is also linked to Rett syndrome (Wang and Baker, 2015). These genes are repressed by *MeCP2*, a gene required for maturation of neurons and whose defect causes Rett syndrome, mental retardation, encephalopathy, and autism susceptibility (Gao *et al*, 2015; Peddada *et al*, 2006). Such results indicate that differential regulation of the *ID-HLH* pathway by *MYTIL* may underlie the phenotypic overlap of several neurodevelopmental disorders.

The identification of genetic variants affecting *MYTIL* expression in the hippocampus had guided our MRI studies on the hippocampus. The location of the rs55800610 variant within a highly conserved tissue-specific enhancer that

directs spatially restricted gene expression in the developing brain might underlie the restricted effect of this SNP in the hippocampus, as rs17338519 that is not located in such regulatory region has broader effects in the brain. Finding such functions for *MYTIL* in the hippocampus is in agreement with this region being one of the few in the mammalian brain that retains its ability to produce functional neurons throughout life. Besides its major role in postnatal neurogenesis (Schwarz *et al*, 2012) the hippocampus is crucial for episodic memory (Bliss and Collingridge, 1993) and changes in hippocampal volumes are hallmarks of several common neurological and psychiatric disorders including mental retardation (Nadel, 2003). Yet,

the hippocampus is not the only key brain area for memory and more generally, cognitive ability. Structural and functional neuroimaging have revealed a network of cortical brain areas involved in general cognitive functions (Deary et al, 2010). Our findings that the minor allele at rs17338519, that affects hippocampal activation during memory recall, is linked with decreased *MYT1L* expression in both hippocampus and cortex are in agreement with this.

It is remarkable that the learning and memory deficits of subjects with intellectual disability are associated with decreased size of the hippocampus and impairments of the prefrontal cortex and hippocampal system. These deficits become considerably more noticeable during late childhood and adolescence (Nadel, 2003), possibly as genetic factors underlying inter-individual variability in these brain systems become effective at this stage of development (Haworth et al, 2010). Our data suggest that *MYT1L* is one of these factors underlying inter-individual differences in hippocampal volume. The increased hippocampal activation observed in our fMRI study also suggests that decreased *MYT1L* levels may lessen neuronal efficiency during cognitive task performance. However, we acknowledge that the use of two distinct cohorts of different ages for our MRI analyses has limitations. Future longitudinal studies of cohorts with structural MRI and suitable fMRI assessments are required to determine if genetic alterations in *MYT1L* predispose to changes in brain structure and function across the lifetime. Nonetheless, the discoveries described here provide etiologically relevant mechanistic explanations for a role of *MYT1L* in neural development.

Data Access

The microarray data generated for this publication have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE65945 and GSE65857.

FUNDING AND DISCLOSURE

This work was funded by Wellcome Trust grant reference 090532/Z/09/Z and MRC Hub grant G0900747 91070. This work received further support from the European Union-funded FP6 Integrated Project IMAGEN (reinforcement-related behavior in normal brain function and psychopathology; LSHM-CT- 2007-037286). S.D. and G.S. are also supported in part by the NIH BD2K award, U54EB020403, the FP7 projects IMAGEMEND (602450; IMAGING GENetics for MENTAL Disorders) and MATRICS (603016), the Innovative Medicine Initiative Project EU-AIMS (115300-2), a Medical Research Council Programme Grant 'Developmental pathways into adolescent substance abuse' (93558), the Swedish funding agency FORMAS, the Medical Research Council and the Wellcome Trust (Behavioural and Clinical Neuroscience Institute, University of Cambridge), the National Institute for Health Research (NIHR) Biomedical Research Centre at South London and Maudsley NHS Foundation Trust and King's College London, the Bundesministerium für Bildung und Forschung (BMBF grants 01GS08152; 01EV0711; eMED

SysAlc01ZX1311A; Forschungsnetze AERIAL and BipoLife) and the Deutsche Forschungsgemeinschaft (DFG grants FOR 1617, SFB 940 and SM 80/5-2). The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics for the generation of the Gene Expression data. A.K. and L.M.M were recipients of a studentship from the Medical Research Council, UK and Consejo Nacional de Ciencia y Tecnología (CONACyT; México), respectively.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)