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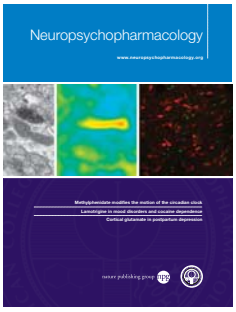


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# Associations of the intellectual disability gene *MYT1L* with helix-loop-helix gene expression, hippocampus volume and hippocampus activation during memory retrieval

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**ABSTRACT**

The fundamental role of the brain-specific myelin transcription factor 1-like (*MYT1L)* gene in cases of intellectual disability and in the aetiology of neurodevelopmental disorders is increasingly recognised. Yet, its function remains under-investigated. Here, we identify a network of helix-loop-helix (HLH) transcriptional regulators controlled by *MYT1L*, 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 *(i) MYT1L* is required for neuronal differentiation and identified *ID1,* a HLH inhibitor of premature neurogenesis, as a target. *(2)* While *MYT1L* prevented expression of *ID1*, it induced expression of a large number of terminal differentiation genes. *(3)* Consistently, expression of *MYT1L* 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 *MYT1L* 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 *MYT1L* 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 *MYT1L* influences memory-related processes by controlling a neuronal proliferation/differentiation switch of ID-bHLH factors.

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## 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 coordinated steps can result in intellectual inability and serious developmental and neurodegenerative disorders (Vaillend *et al*, 2008).

The coordinated actions of transcriptions 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

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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. While 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

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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.

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## MATERIALS AND METHODS

### Knocking-down *MYT1L* in cultured cells

To inhibit *MYT1L* function in human neural stem cells, we transduced the SPC04 cell line (Desrivières *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, UK) following the manufacturer's instructions. Supernatants containing lentiviruses were collected 48h and 72h post transfection, pooled, cleared by centrifugation at 1600 x g at 4°C for 10 minutes, filtered and ultracentrifuged at 25,000 rpm for 1.5 hours 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, SPC-04 cells were seeded in 6-well laminin-coated plates at 20,000 cells/cm$^2$. The following day, the growth media was replaced by the pre-differentiation medium in the presence of 3.5x10$^5$ TU/ml of


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lentivirus and the cells were incubated for 48h, after which differentiation was induced. Transduction efficiency was monitored by a fluorescent microscope (Leica DMIL) with a Leica camera DFC420C (x10 objective).

*Gene expression analyses:* Effects of shRNAs on *MYT1L* mRNA levels and on global gene expression were assessed by q*uantitative 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* (60S Ribosomal Protein L18) as internal controls for normalisation, using the following forward and reverse primers: *RPL18* forward 5'–GAGAGGTGTACCGGCATTTC–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 20µl reactions containing 4µl cDNA, 0.5µM of each forward and reverse primers and 1x Power SYBR Green Mix (Applied Biosystems) using the following cycles: 95°C for 15 minutes and 40 cycles at 95°C for 30 seconds and 59°C for 30 seconds. 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 (i.e., experimental Ct - control Ct) by ANOVA, followed by Bonferroni- or Dunnett- based post-hoc analyses ($\alpha$ = 0.05, 2-sided) using the SPSS® software.

*Microarray analyses:* Total RNA samples were processed using the TargetAmp™-


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Nano Labeling Kit (Cambio Ltd.) and hybridized to Illumina HumanHT-12 v4 Expression BeadChips 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 versus 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 ≤ 10%). Clustered GO terms with $P_{EASE} \leq 0.01$ (i.e., enrichment scores > 2) were considered specifically enriched.

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**Gene expression analyses in post-mortem human brain samples**

We analysed publicly available datasets to follow spatiotemporal gene expression patterns in the human brain and identify expression quantitative trait loci (eQTL) in *MYT1L* (i.e., single nucleotide polymorphisms (SNPs) associated with differences in gene expression). These are detailed in the Supplementary Methods.


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**Magnetic Resonance Imaging (MRI) 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 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 analysed in this study. Written informed consent was obtained from all participants and their legal guardians.

*Magnetic Resonance Imaging.* Details of the MRI protocols and quality checks are in the Supplementary Methods. Out of 1,909 images, 1,583 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 (Desrivières *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 1,837 subjects (900 males, 937 females) using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). This dataset had been filtered to remove SNPs with call rates of <98%, minor allele frequency <1% or deviation from the Hardy-Weinberg equilibrium (P ≤ 1×10-4), 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,


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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; 1,227 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.

*DNA-extraction and genotyping.* Genotype data for rs17338519 were extracted from a genome-wide data set generated at the Department of Genomics, Life & Brain

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Center, University of Bonn using Human610-Quad Bead Arrays (Illumina, San Diego, CA, USA).

*Functional imaging task.* During functional MRI scanning, subjects completed an episodic memory task that was part of three consecutive memory tasks, i.e. 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 in order 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 6s) and 4 blocks of 6 head contours (a 4s) each. Each block lasted 24s. Task parameters and image processing are detailed in the Supplementary Methods.

For each subject, statistical contrast images of memory versus control were obtained and analysed 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).

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*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.

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## 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 *ID* 1 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.

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### 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


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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 (Figures 2B and 2C; 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 sh*MYT1L* treatment were down-regulated (Supplementary Table 2 and Figure 2D), indicating that *MYT1L* acts mainly as a transcriptional activator. Functional clustering analyses indicated that genes down-regulated by *shMYT1L* were enriched for genes involved in neurite outgrowth, axonal development, synaptic 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*.

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**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 analysed publicly available datasets of human post-mortem brain samples (Supplementary Methods), comparing the expression patterns of *MYT1L*, *ID1* and *ID3*


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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 (i.e., 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 *ID*1 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 dataset (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


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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 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 hypothesised, 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 ≤ 0.05), most of which (> 90%), such as the proneural genes *NEUROD6* and *TCF4* (Figure 4B and Supplementary Table 3), were down-regulated 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 signalling (calcium- and MAP kinase-related signalling pathways), in cancer (glioma, non-small cell lung cancer) and in neurodegenerative diseases like amyotrophic lateral sclerosis and Alzheimer's disease (Figures 4C and 4D).

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**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 analysing associations between *MYT1L* SNPs and structural and functional MRI in the human hippocampus. We first analysed possible associations between *MYT1L* and inter-individual


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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-squared threshold of 0.5, remaining significant after 10,000 permutations (empirical p-value = 0.0098). For the left hippocampus, two SNPs in high LD (r2>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 x $10^{-4}$, 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 dataset 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


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= -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 family wise error corrected for region of interest) 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.

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**DISCUSSION**

We have shown for the first time that the MYT1L transcription factor regulates neurogenesis by repressing inhibitor of differentiation 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 signalling 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


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SPC04 cells and in the hippocampus (Figure 4E and Supplementary Tables 2 & 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 tumour 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 signalling mechanisms (Ren *et al*, 2007). Indeed, the *MYT1L* gene was found to be deleted in about 5% of glioblastoma multiforme samples and the protein absent or down-regulated in more than 80% of these samples (Hu *et al*, 2013), leading to the suggestion that gliomagenesis requires neutralization of terminal differentiation.

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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. While *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)


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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 (Forrest *et al*, 2014). Strinkingly, expression of *ID* genes is also linked to Rett syndrome (Wang *et al*, 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 *MYT1L* may underlie the phenotypic overlap of several neurodevelopmental disorders.

The identification of genetic variants affecting *MYT1L* 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 *MYT1L* 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,

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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-individuals 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 aetiologically relevant mechanistic explanations for a role of *MYT1L* in neural development.

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The authors declare no competing financial interests.

**Data access**

The microarray data generated for this publication have been submitted to the NCBI



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Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE65945 and GSE65857.

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**FIGURE LEGENDS**

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 ± SEM of n=3 independent experiments.

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 ± 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

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that expressed the enhanced green fluorescent protein as marker. Scale bar = 100μm. 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 differently expressed genes showing enrichment for genes involved in neuronal maturation and the extracellular matrix composition.

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.

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


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tissue-specific transcriptional enhancer (i.e., human element hs1385, labeled as element_1385) from the VISTA Enhancer database, (Pennacchio *et al*, 2006)). Genomic coordinates 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-D) Gene set enrichment analyses of all genes down-regulated 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).

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Figure 5: Associations between *MYT1L* 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 x $10^{-4}$). B) Expression of *MYT1L* stratified by genotypes at rs17338519, in the hippocampus and cerebral cortex of 134 post-


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mortem human brains. For the cortex, expressions of *MYT1L* in the occipital, temporal and frontal cortices were averaged. Regression analyses indicated that the presence of the T-allele at rs17338519 associated with decreased *MYT1L* 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 family wise error (FWE) corrected for multiple testing across region of interest). 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.

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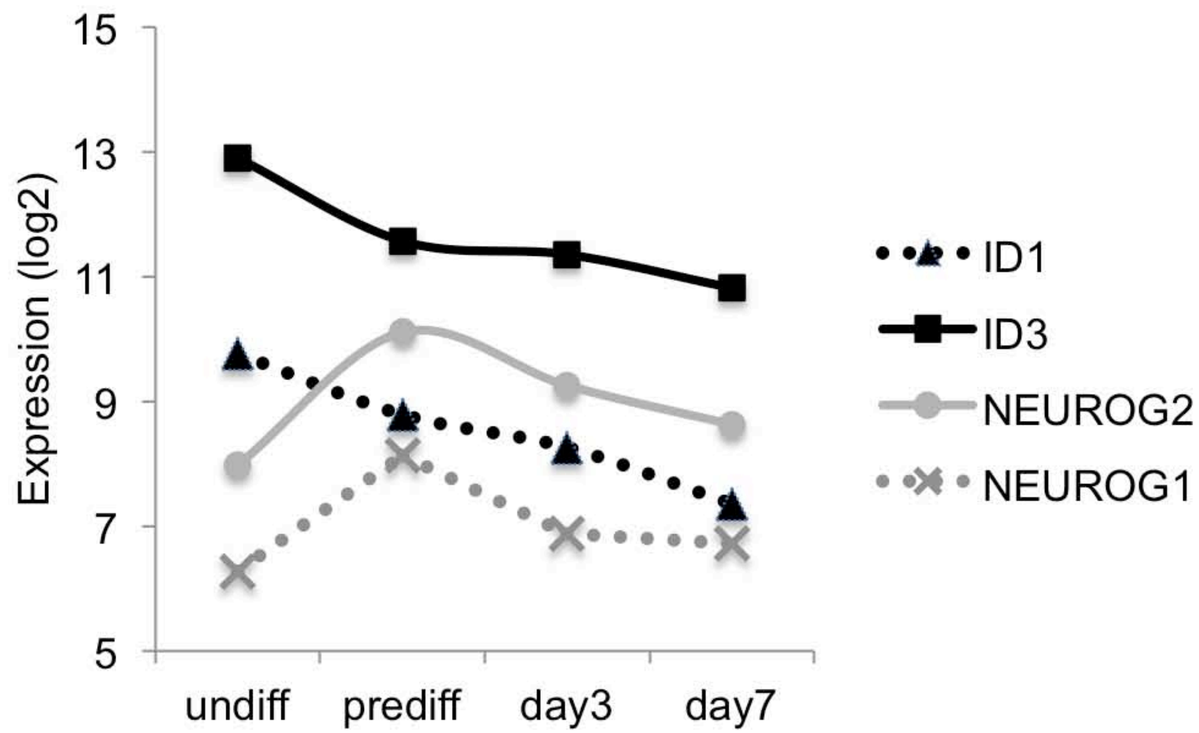
Kepa et al., Figure 1A

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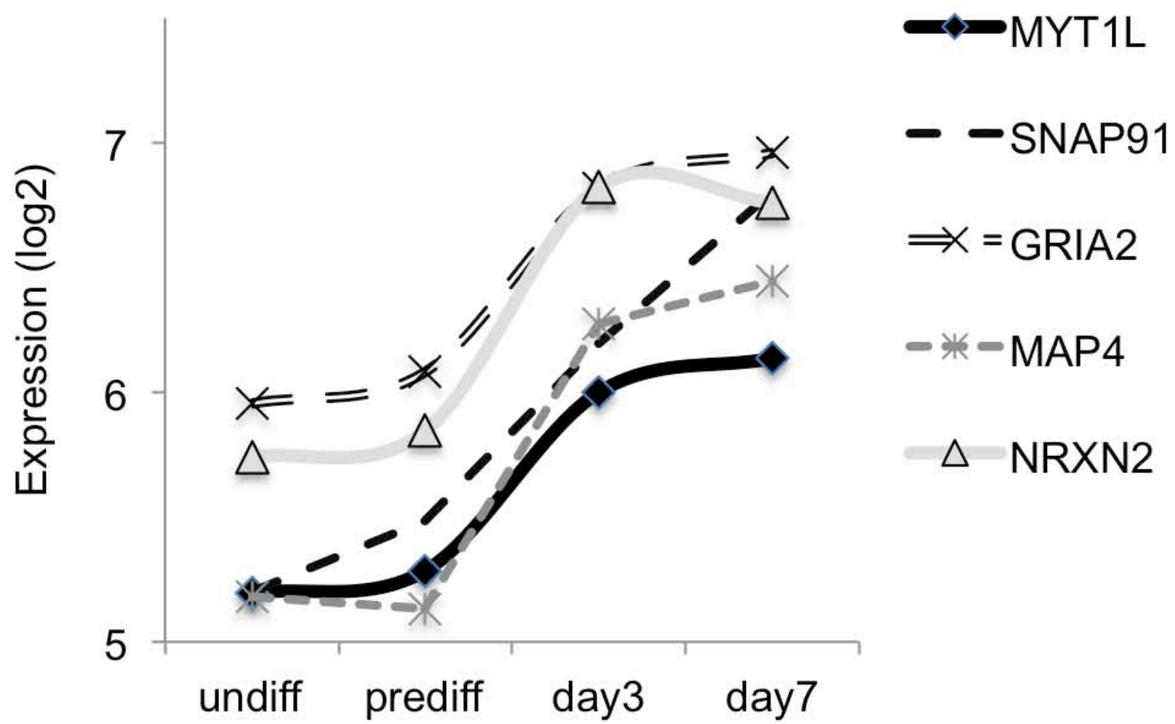
Kepa et al., Figure 1B

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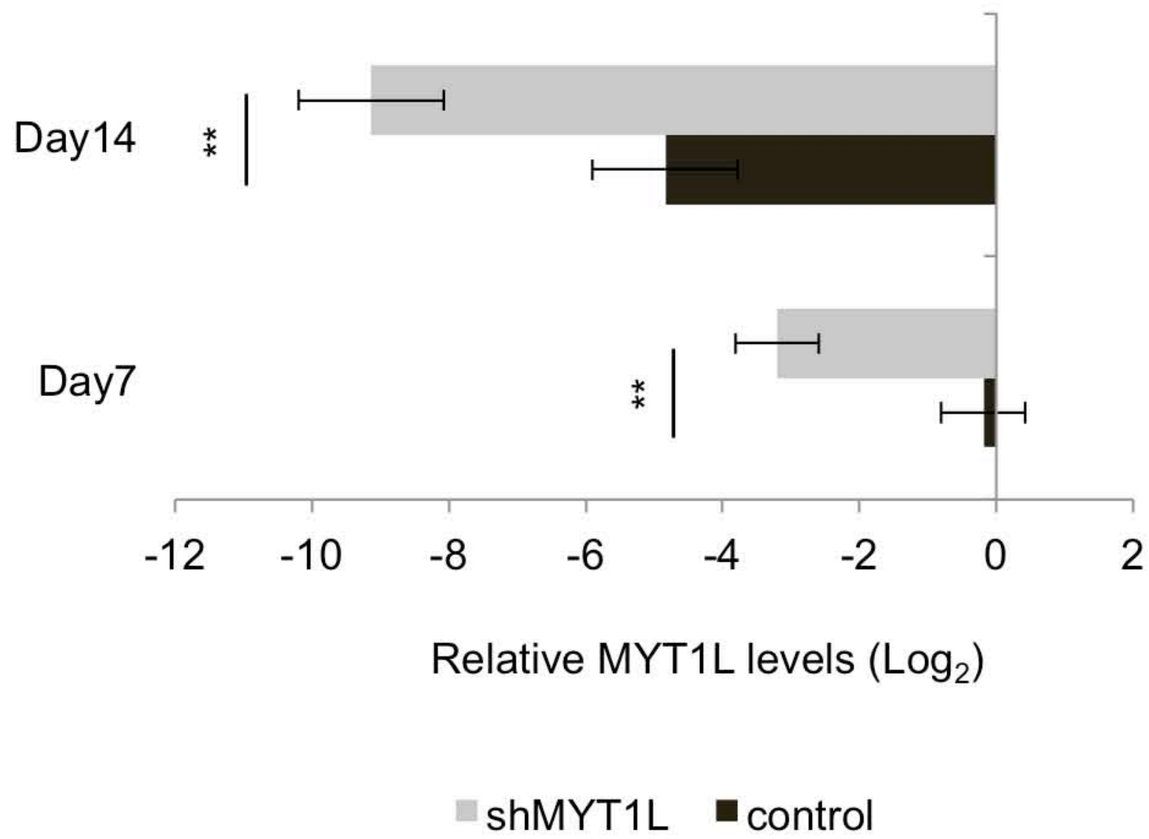
Kepa et al., Figure 2A

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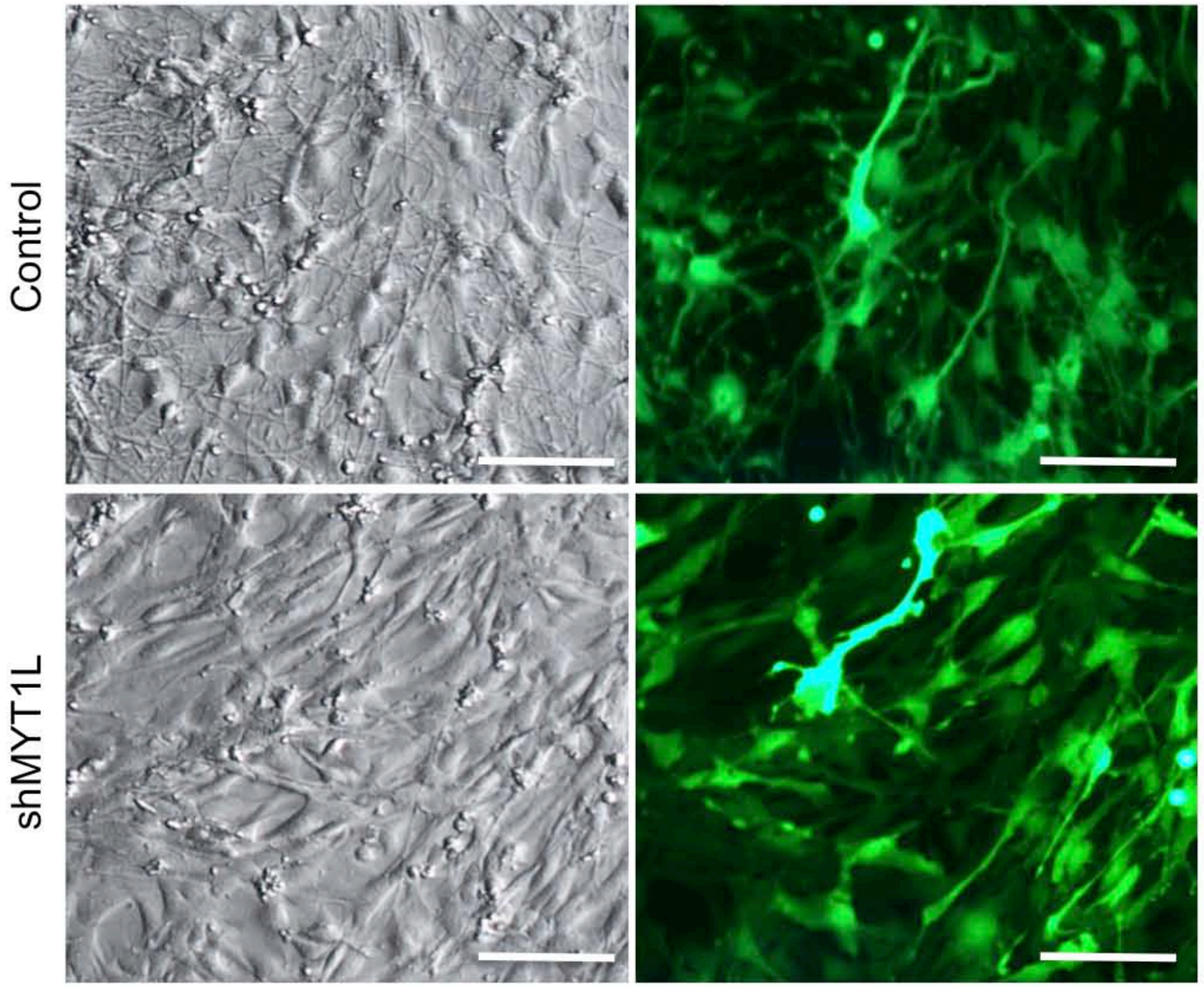
Kepa et al., Figure 2B

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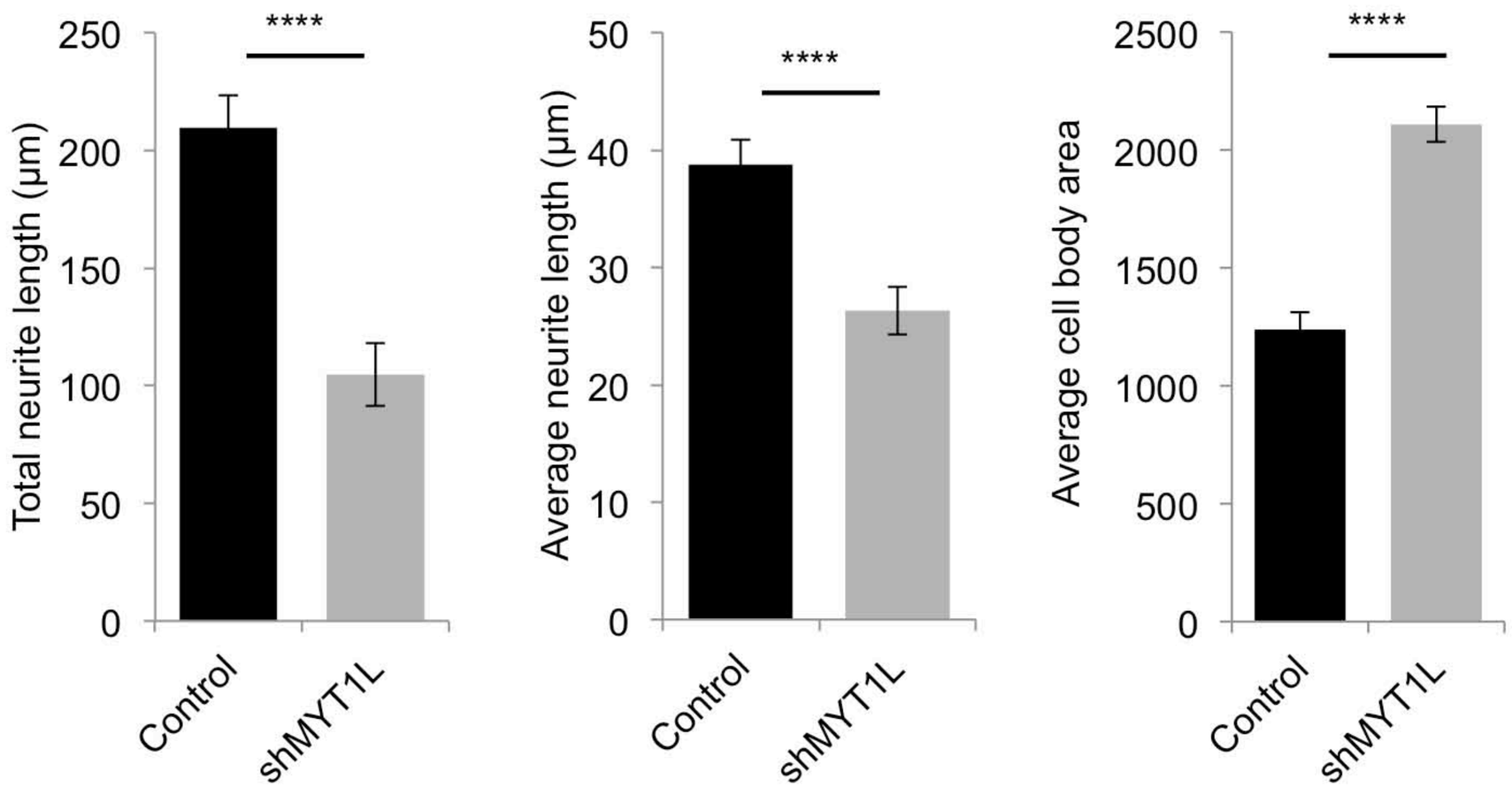
Kepa et al., Figure 2C

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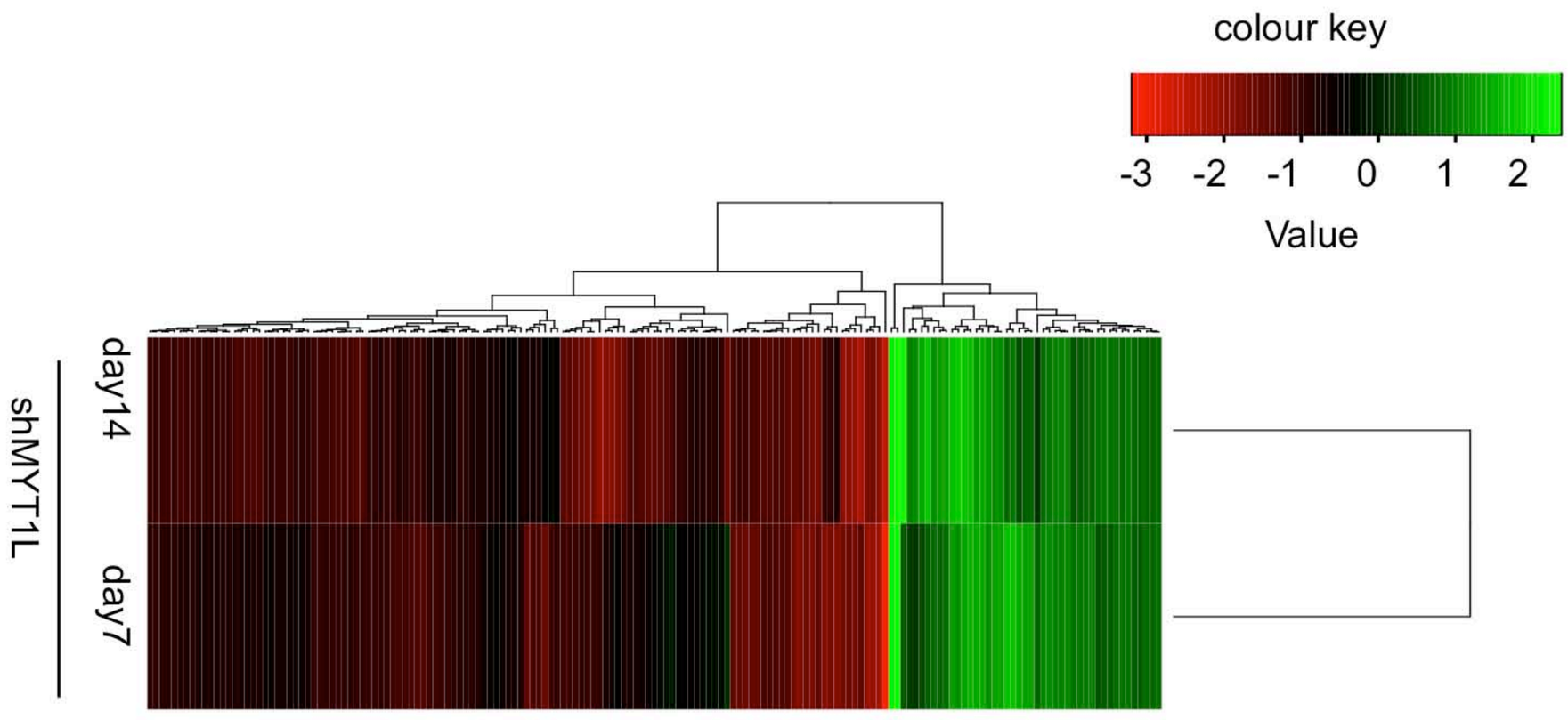
Kepa et al., Figure 2D

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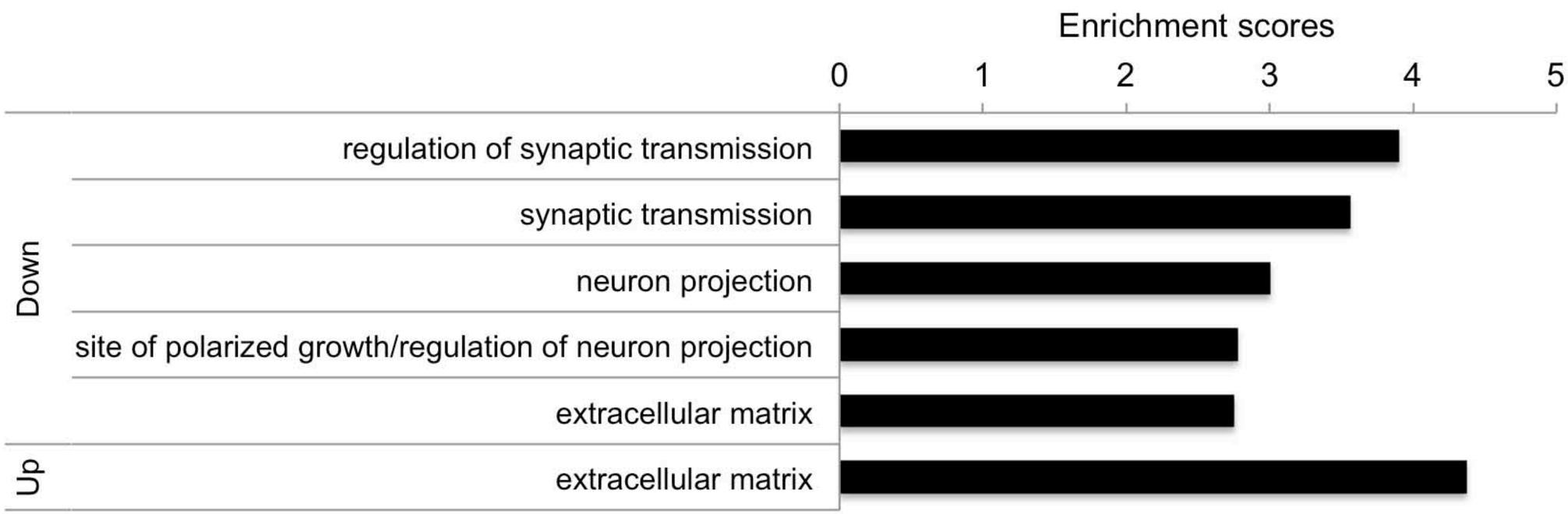
Kepa et al., Figure 2E

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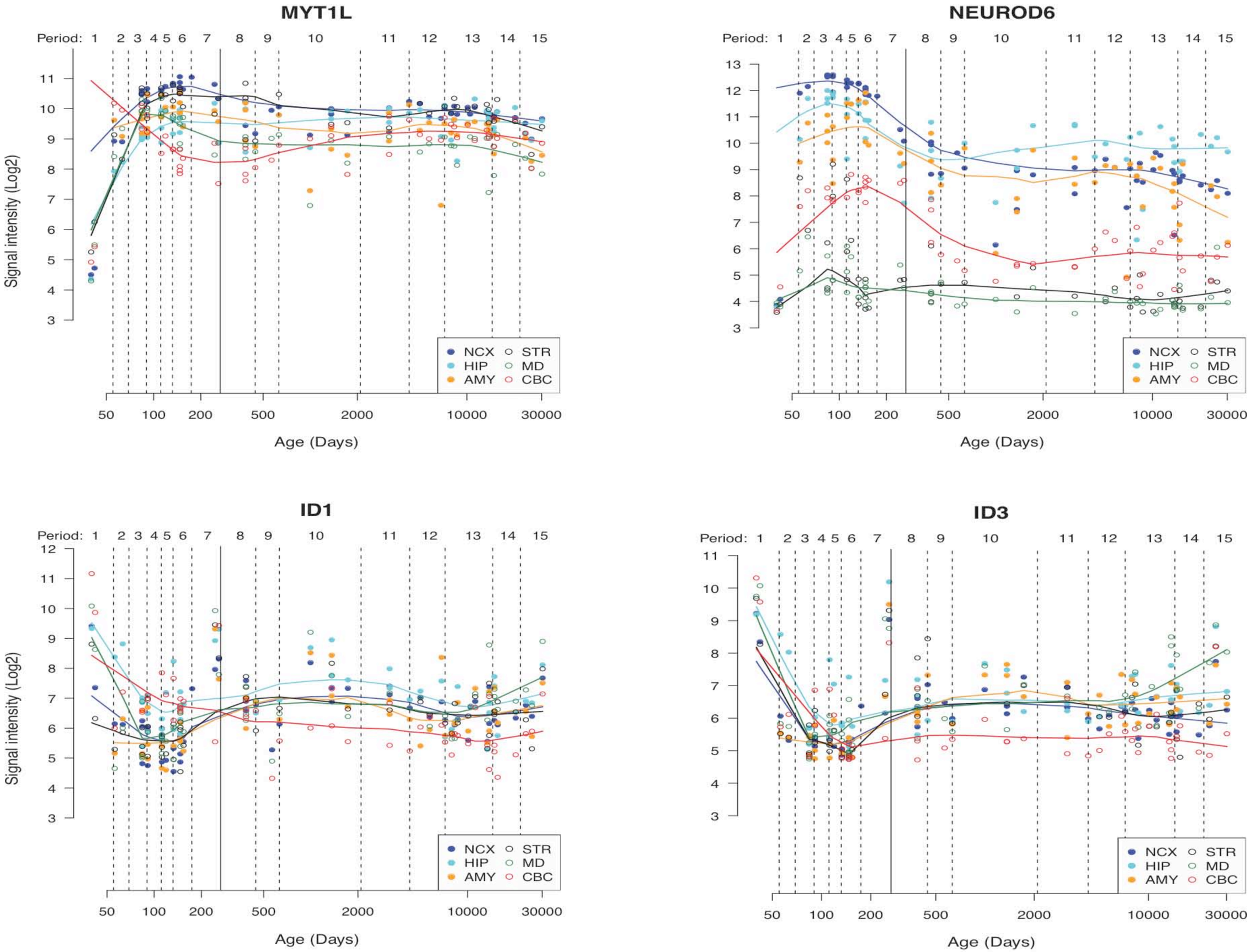
Kepa et al., Figure 3

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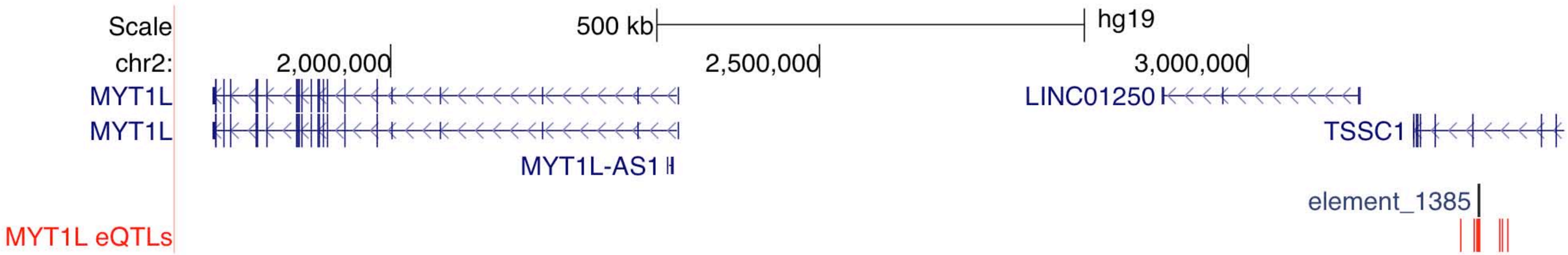
Kepa et al., Figure 4A

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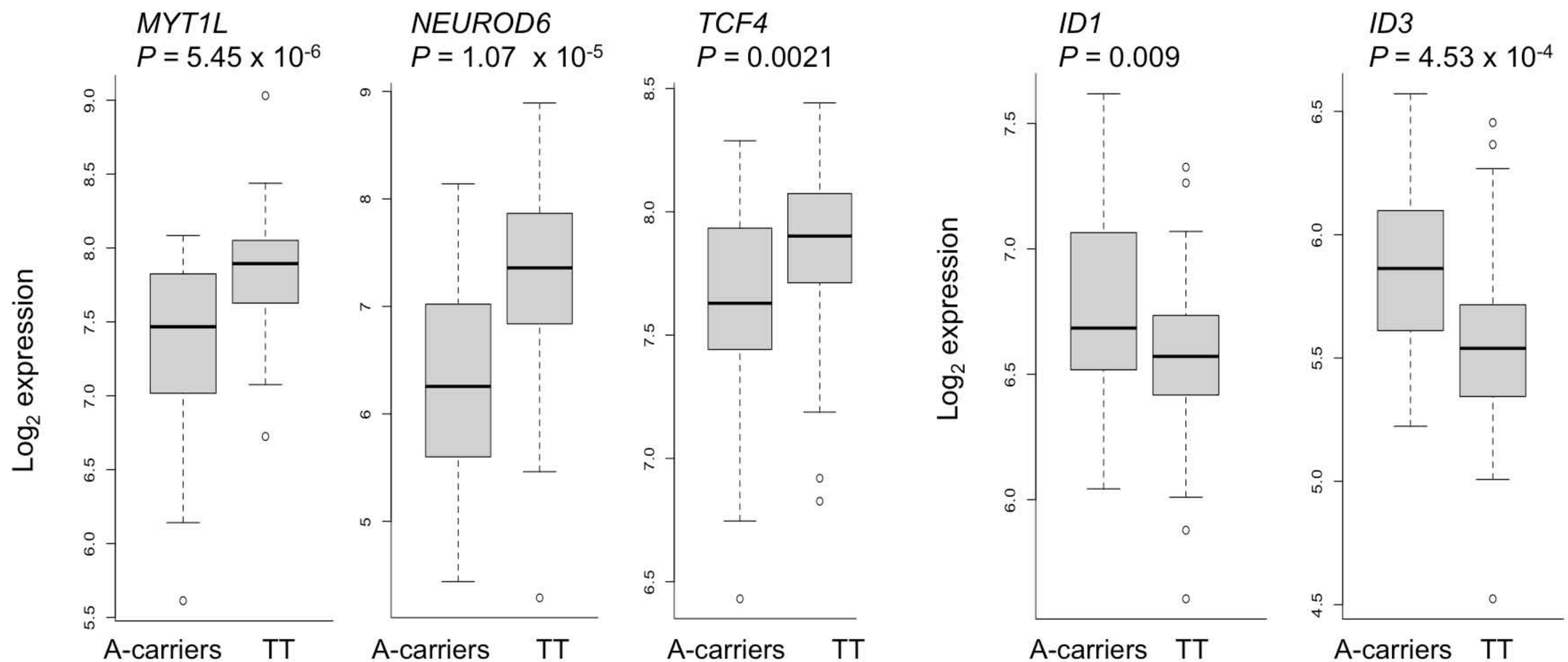
Kepa et al., Figure 4B

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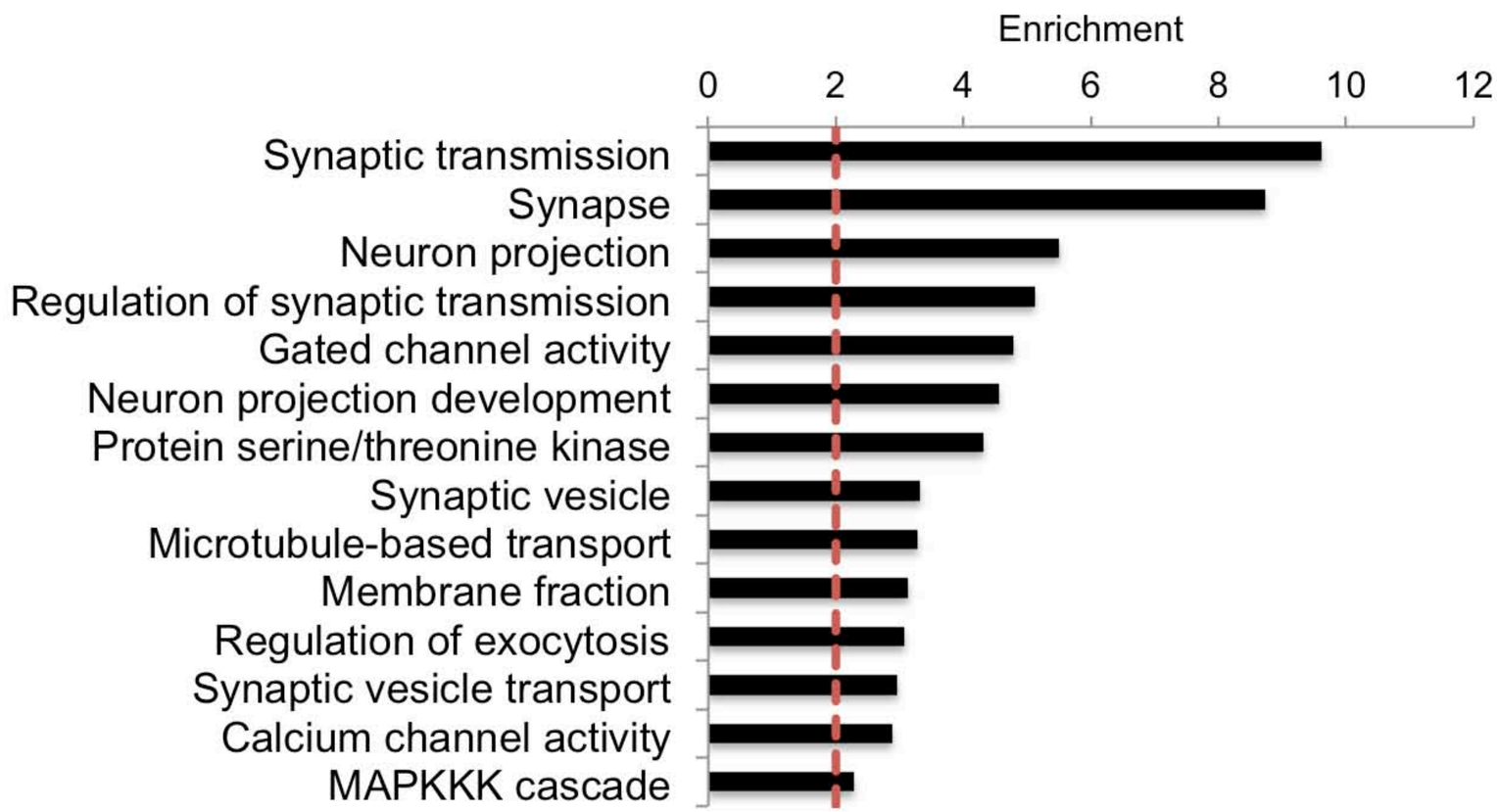
Kepa et al., Figure 4C

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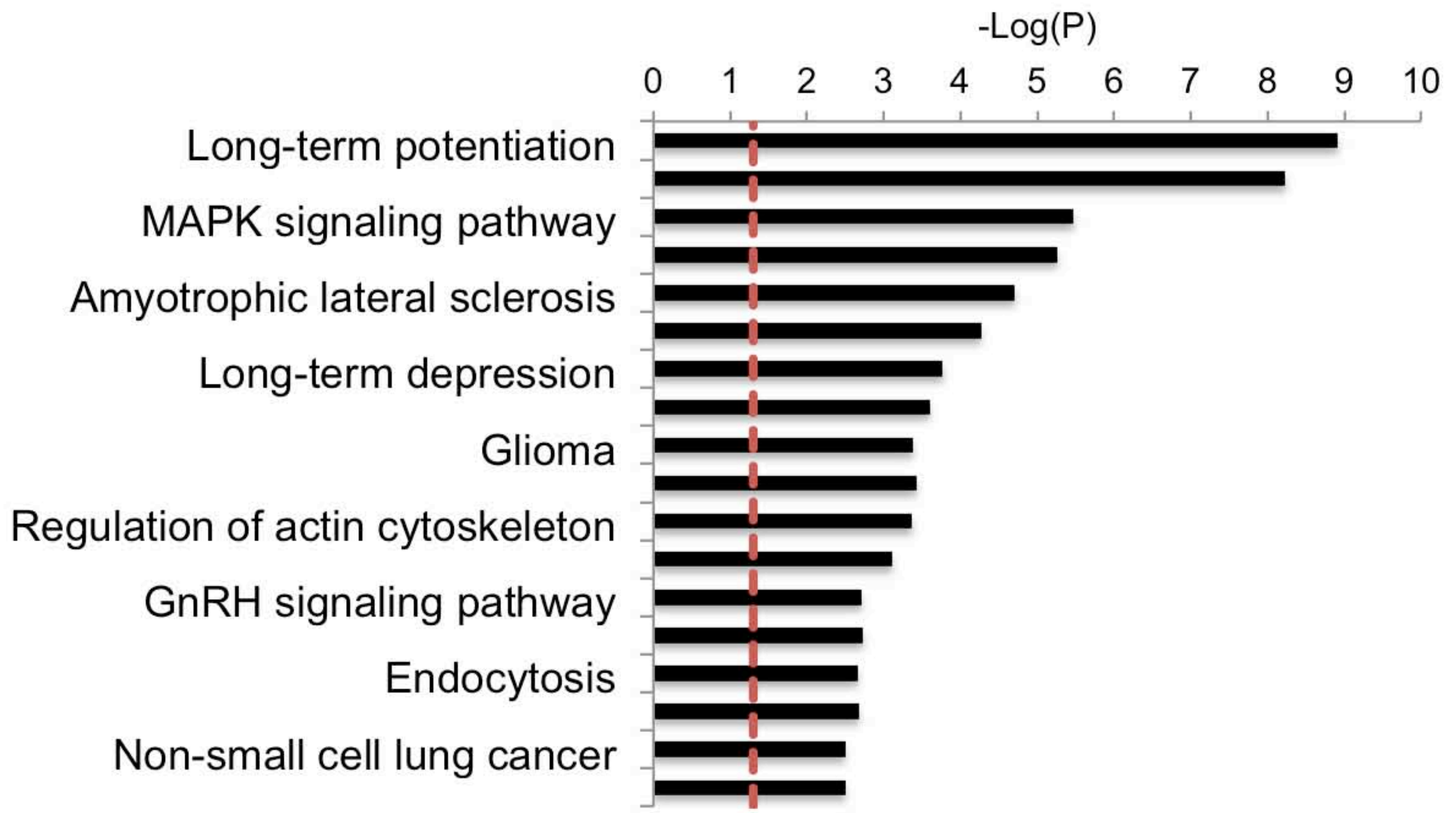
Kepa et al., Figure 4D

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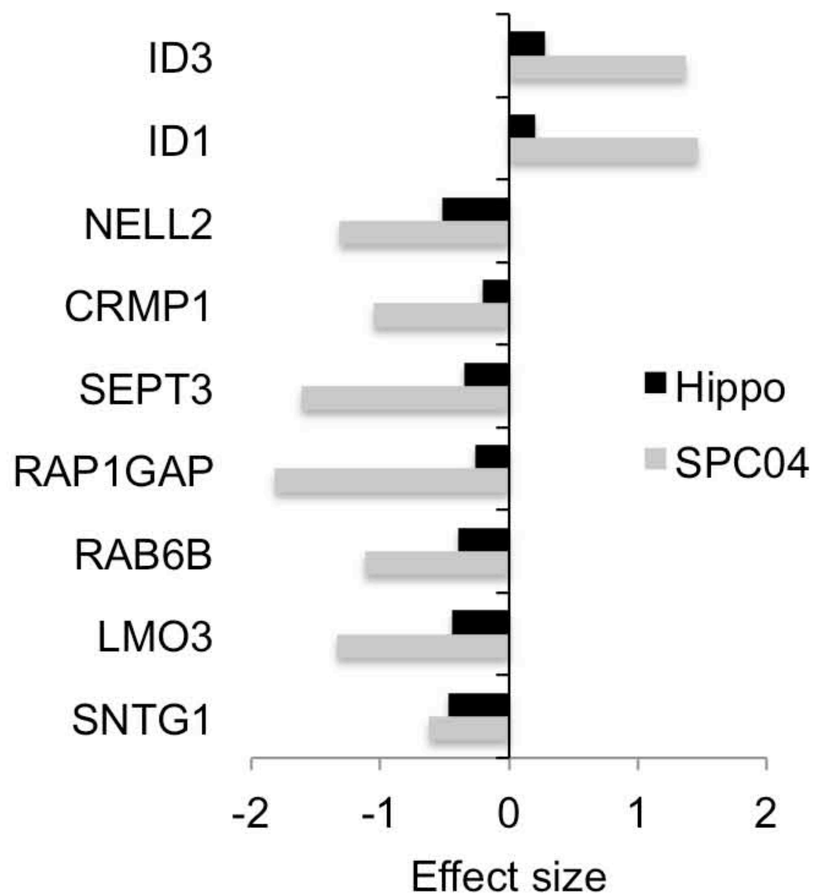
Kepa et al., Figure 4E

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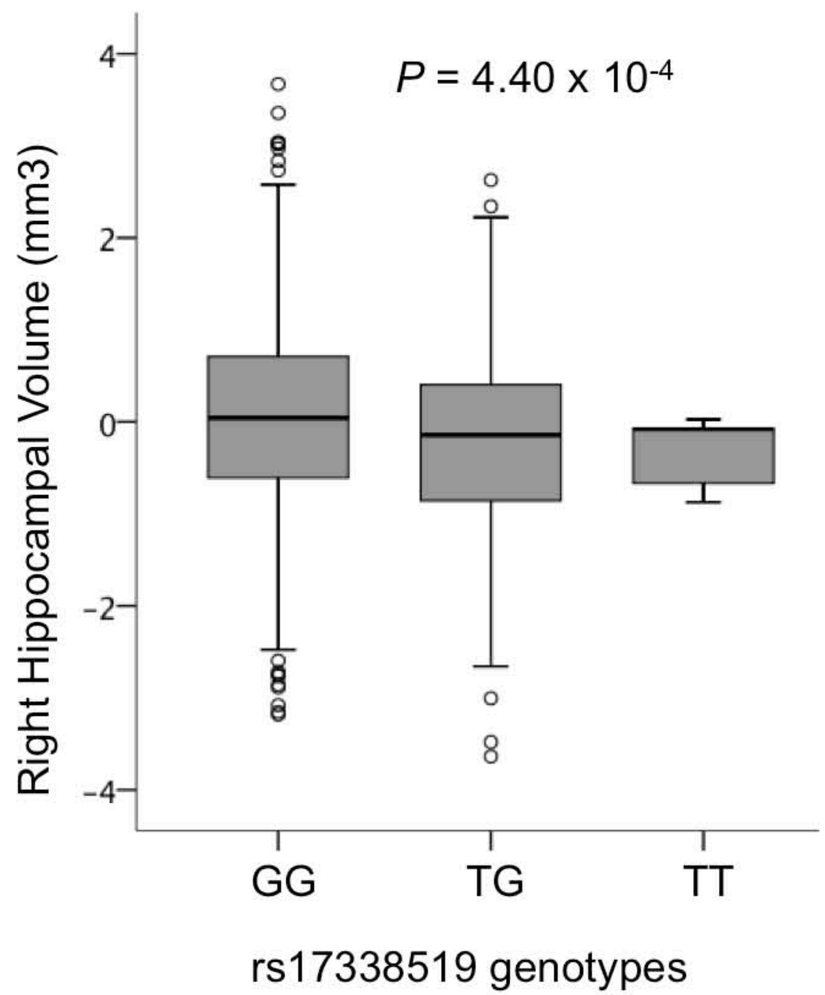
Kepa et al., Figure 5A

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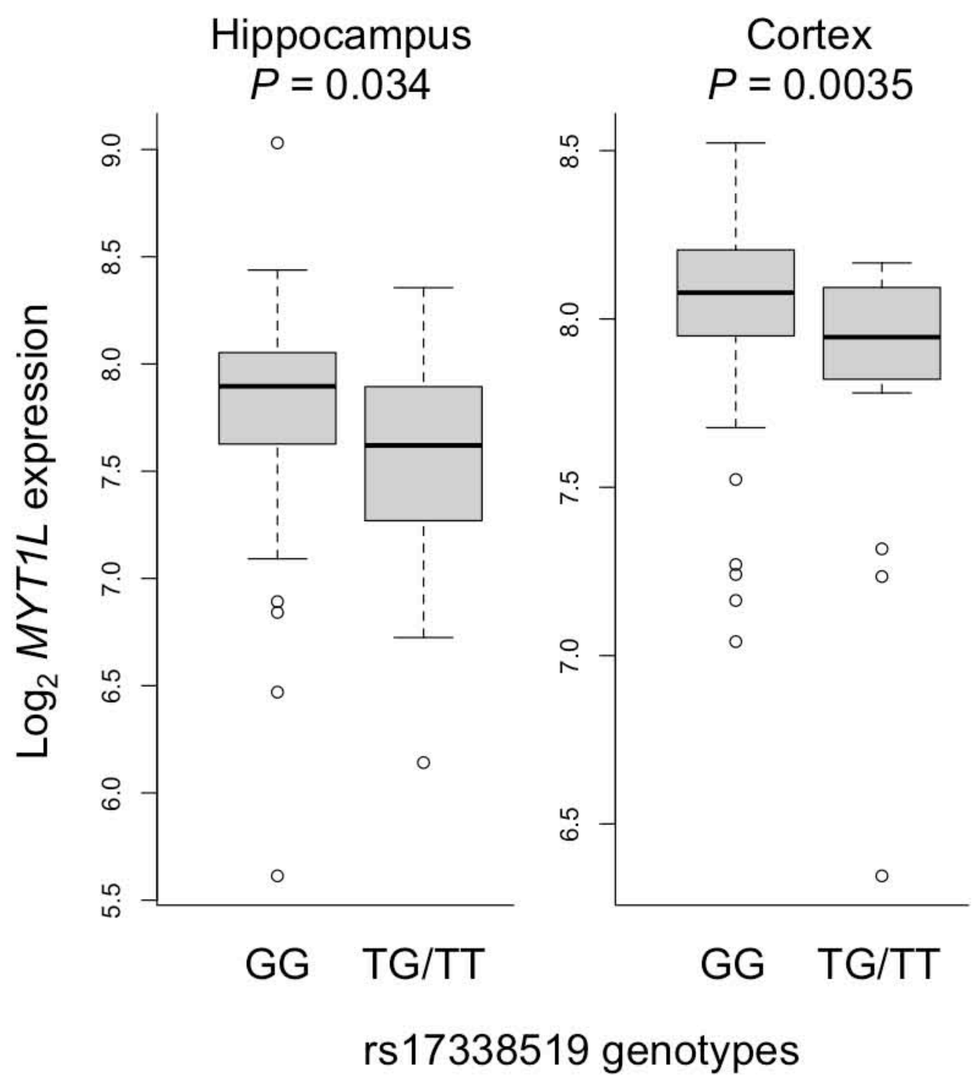
Kepa et al., Figure 5B

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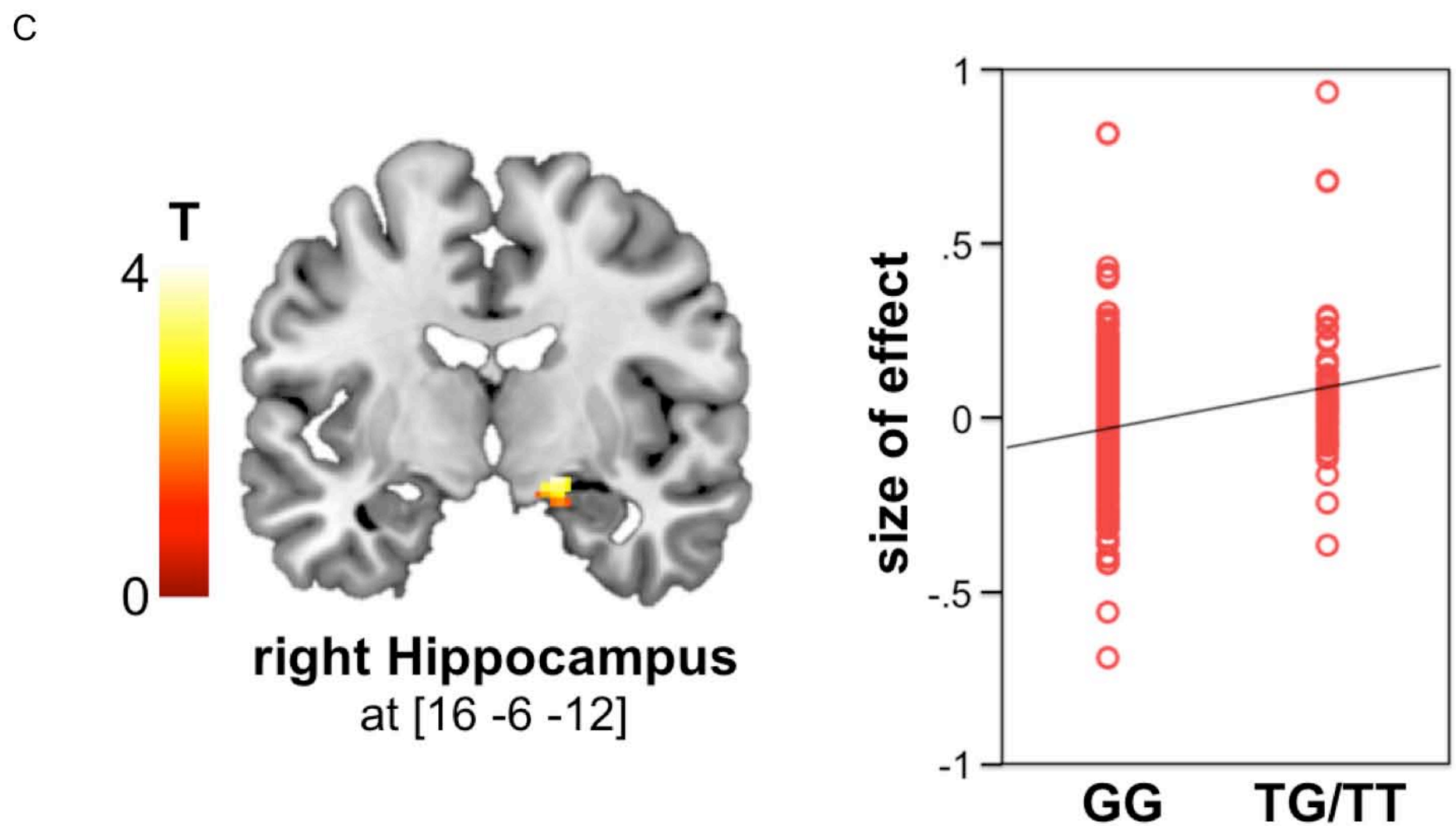
Kepa et al., Figure 5C

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