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ARTICLE

Regulation of impulsive and aggressive behaviours by a novel lncRNA

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Abstract

High impulsive and aggressive traits associate with poor behavioural self-control. Despite their importance in predicting behavioural negative outcomes including suicide, the molecular mechanisms underlying the expression of impulsive and aggressive traits remain poorly understood. Here, we identified and characterized a novel long noncoding RNA (lncRNA), acting as a regulator of the monoamine oxidase A (MAOA) gene in the brain, and named it MAOA-associated lncRNA (MAALIN). Our results show that in the brain of suicide completers, MAALIN is regulated by a combination of epigenetic mechanisms including DNA methylation and chromatin modifications. Elevated MAALIN in the dentate gyrus of impulsive-aggressive suicides was associated with lower MAOA expression. Viral overexpression of MAALIN in neuroprogenitor cells decreased MAOA expression while CRISPR-mediated knock out resulted in elevated MAOA expression. Using viral-mediated gene transfer, we confirmed that MAALIN in the hippocampus significantly decreases MAOA expression and exacerbates the expression of impulsive-aggressive behavioural traits in CD1 aggressive mice. Overall, our findings suggest that variations in DNA methylation mediate the differential expression of a novel lncRNA that acts on MAOA expression to regulate impulsive-aggressive behaviours.

Introduction

High levels of impulsive and aggressive traits associate with poor behavioural self-control and with the development of psychopathology characterized by disruptive and addictive behaviours, such as substance disorders and antisocial personality disorder (APSD) [1–7]. Impulsive-aggressive behaviours also strongly predict lifetime suicidal behaviour [4, 8, 9] and act as suicide endophenotypes. Accordingly, individuals who die by suicide have higher levels of impulsive and aggressive behaviours even after controlling for psychopathology [10], and relatives of suicide completers have increased levels of impulsive-aggressive traits,

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which in turn explain familial aggregation of suicidal behaviours [11, 12]. Epigenetic mechanisms have been implicated in the regulation of complex behavioural phenotypes. For instance, regulation of stress-related traits occurs through differential methylation of genes coding for components of the hypothalamus–pituitary–adrenal axis, both in rodents [13–15] and humans [16, 17]. Interestingly, individuals who die by suicide show evidence of altered DNA methylation patterns in genes involved in neuroplasticity, learning and behavioural regulation in the hippocampus, which associate with changes in gene expression [18, 19].

The monoamine oxidase (MAO) genes associate with impulsive-aggressive behaviours in both humans and animals. For instance, extreme aggression and behavioural disinhibition has been reported in Dutch carriers of a rare nonsense mutation in the MAOA gene that results in a MAOA knock down [20]. Similarly, a naturally occurring nonsense mutation in the mouse *MaoA* gene causes heightened levels of reactive aggression [21, 22]. Moreover, in humans, the low functioning MAOA variable number tandem repeat allele (MAOA-L) associates with higher risk of substance abuse [1, 6], ASPD [5, 7] and impaired performance in a vast array of behavioural assays indexing components of impulsive and aggressive behaviours [2, 3]. A growing body of evidence now suggests that DNA methylation in regulatory regions outside promoter regions may induce variable effects on gene regulation [23]. For instance, early-life stress induces stable DNA methylation changes in the intergenic region (IGR) between the arginine vasopressin (AVP) and oxytocin genes, and associates with variation in AVP expression in mouse brain [24]. Noticeably, the MAOA and MAOB genes follow a very similar genomic organization as the AVP/oxytocin genes. Like AVP and oxytocin, MAOA and MAOB are functionally related, located tail-to-tail and separated by a short IGR. We thus hypothesized that, similarly to AVP/oxytocin, the MAOA/B IGR may contain regulatory sequences of MAOA/B amenable to epigenetic regulation by the environment. Here, investigating individuals with high levels of impulsive-aggressive traits, we identified a novel and previously unannotated long non-coding RNA (lncRNA) that maps to the MAOA/B IGR, characterized its epigenetic regulation, defined its regulatory role on MAOA expression in human brain and confirmed its involvement in the regulation of aggressive and impulsive behaviour in mice.

Materials/subjects and methods

Detailed methods are described in the Supplementary Information, available at MP's website.

DNA CH3 discovery and validation

Custom-designed CH3 microarray: whole post-mortem tissue (discovery cohort)

Human post-mortem brain samples were obtained from the Douglas-Bell Canada Brain Bank, and hippocampal sections were dissected from brains of individuals who died by suicide with high impulsive-aggressive traits (cluster B personality disorders, impulse-control disorders, and substance dependence; $N = 35$) or from age- and sex-matched psychiatrically healthy controls ($N = 16$).

Methylated DNA was extracted from whole-tissue homogenates using methylated DNA immunoprecipitation, as previously described [25]. Levels of methylation were quantified from 35 suicide and 16 control samples using custom-designed microarrays (Agilent Technologies) covering 50 kb upstream MAOA gene, including the entire MAOA gene, the MAOA/B IGR and a significant portion of the MAOB gene. Differential methylation was assessed using dye-labelled probes and differential methylation had to pass biostatistics analysis.

Isolation of DNA from neuronal and non-neuronal nuclear populations

Neuronal and non-neuronal nuclei were isolated from dounced brain tissue using fluorescence-assisted cell sorting (FACS; FACSVantage SE system (BD Bioscience, San Jose, CA), as previously described [18, 19, 26]. Monoclonal anti-NeuN antibodies were used to identify neuronal fractions and detected by Alexa488-labelled anti-mouse antibodies.

DNA was extracted from sorted nuclei using QIAGEN DNeasy extraction kits (QIAGEN). Extracted DNA from FACS-sorted nuclei was treated with sodium bisulfite and analysed using Epityper to quantify CpG-specific levels of DNA methylation [27].

Quantification of gene expression

Total RNA was extracted using QIAGEN RNeasy extraction kits (QIAGEN) and MAOA and MAOB expressions were quantified using quantitative RT-PCR in an ABI-7900HT (Applied Biosystems, Foster City, CA, USA) using commercially available probes for MAOA (Taqman) and MAOB (Life Technologies).

Molecular characterization of MAOA-associated lncRNA (MAALIN)

Identification of MAALIN

MAALIN was initially detected by PCR using a series of overlapping primers to evaluate the length of the lncRNA

fragment (Supplementary Table 1; Supplementary Fig. 1). Using the approach described in [28], the pCMV-eGFP-SV40pA-FRT-GalK-FRT cassette was recombineered into WI2-661I23, a CHORI fosmid clone containing the MAOA–MAOB IGR, to create the pVY0003 plasmid.

5′ and 3′ rapid amplification of cDNA ends (RACE)

Identification of 5′ and 3′ ends of MAALIN The 5′ and 3′ extremities of MAALIN were characterized after over-expression (OE) of the pVY0003-MAALIN vector in HEK293 cells. RNA was extracted using the RNeasy mini kit (QIAGEN) and treated with DNAse I according to the manufacturer's instructions.

Primers were designed every 200–500 bp to cover ~9 kb of the IGR between the MAOA and MAOB genes. For both 5′ and 3′ RACE PCRs, cDNA synthesis was performed using the SMARter PCR cDNA synthesis kit (Clontech). The 5′ RACE PCR was performed using the universal primers (UPM) provided by the manufacturer used as forward primer and the primer PCR nested as reverse. The 3′ RACE PCR was performed using the forward primers PCR14–PCR16 (Supplementary Table 2) and, as reverse primer, the UPM primer provided by the manufacturer. The validity of the 3′ and 5′ RACE was confirmed by reproducing our results a second time with no RT negative controls for every amplification using the same experimental conditions described above (Supplementary Fig. 2).

RNAscope in situ hybridization Frozen fixed hippocampal tissue was probed with a MAALIN-specific probe and detected using RNAscope 2.5 HD Detection Reagent kit (Advanced Cell Diagnostics). Sections were counter-stained using hematoxyline-eosine (Sigma) and visualized using Leica DM 5000B microscope at 40×. Visual counts were used to determine the percentage of MAALIN-expressing cells.

Quantification of MAALIN expression in the HPC MAALIN expression was quantified in control and impulsive-aggressive suicide cases using RT-qPCR in an ABI-7900HT (Applied Biosystems, Foster City, CA, USA) using custom MAALIN primers (see Detailed Methods, Supplementary Information).

Luciferase assays

Three constructs were created to assess transcriptional activity of different regions of MAALIN promoter. Construct 1, construct 2 and construct 3 were amplified using primers described in Supplementary Table 3. PCR products were ligated into pGL3 basic vector or a pCpG-free plasmid (InvivoGen) for methylation studies. To assess the impact of methylation on transcriptional activity, we artificially methylated constructs using the methyltransferase enzyme M.Sss1 (New England Biolabs). Unmethylated pGL3 constructs were transfected into Be(2)C, HEK293 and neural progenitor cell (NPC) cells, and resulting luciferase activity was measured and normalized using pCMV-luciferase and renilla. Methylated pCpG-free constructs were transfected into NPCs and normalized using pGL3-luciferase. Luciferase activity was assayed to determine relative promoter transcriptional activities.

Chromatin immunoprecipitation (ChIP) in MAALIN promoter

When enough tissue was available, samples used to determine expression levels and DNA methylation levels were also used for ChIP analyses of H3K4me3 and H3K27me3 (Suicide: $N = 28$; Control: $N = 14$). Dounced tissues were digested with micrococcal nuclease and lysed. Immunoprecipitation experiments were conducted using H3K4me3 (Abcam; ab8580) and H3K27me3 (Abcam; ab6002) antibodies. Enrichment of MAALIN promoter in H3K4me3- and H3K27me3-immunoprecipitated fractions was assessed by qPCR using primers listed in Supplementary Table 4.

Functional characterization of MAALIN

MAALIN OE in NPCs

Construction of HSV-MAALIN vector MAALIN-containing GFP-reporter vectors were constructed for transfection of NPC cells. The pVY0009/HSV-MAALIN vector was constructed by inserting a PCR-amplified MAALIN fragment into pHSV-p1005+. NPCs were subsequently transfected with pVY0009 and sorted after 24 h.

Quantification of MAOA expression Transfected NPCs were sorted based on GFP expression using a FACS Aria II. RNA was extracted from GFP+ cells and MAOA and MAOB expression was detected as defined above ("Quantification of Gene Expression" section) and through RNAseq.

Knock-out of MAALIN in NPCs using CRISPR

To determine the effects of abolishing MAALIN expression, an 8217 bp region encompassing MAALIN was deleted from the genome of NPCs using CRISPR. After amplification, NPCs were harvested to confirm MAALIN knock-out by qRT-PCR and to assess the effect of MAALIN knock-out on MAOA expression, which was determined as previously ("Quantification of Gene Expression" section) and RNAseq.

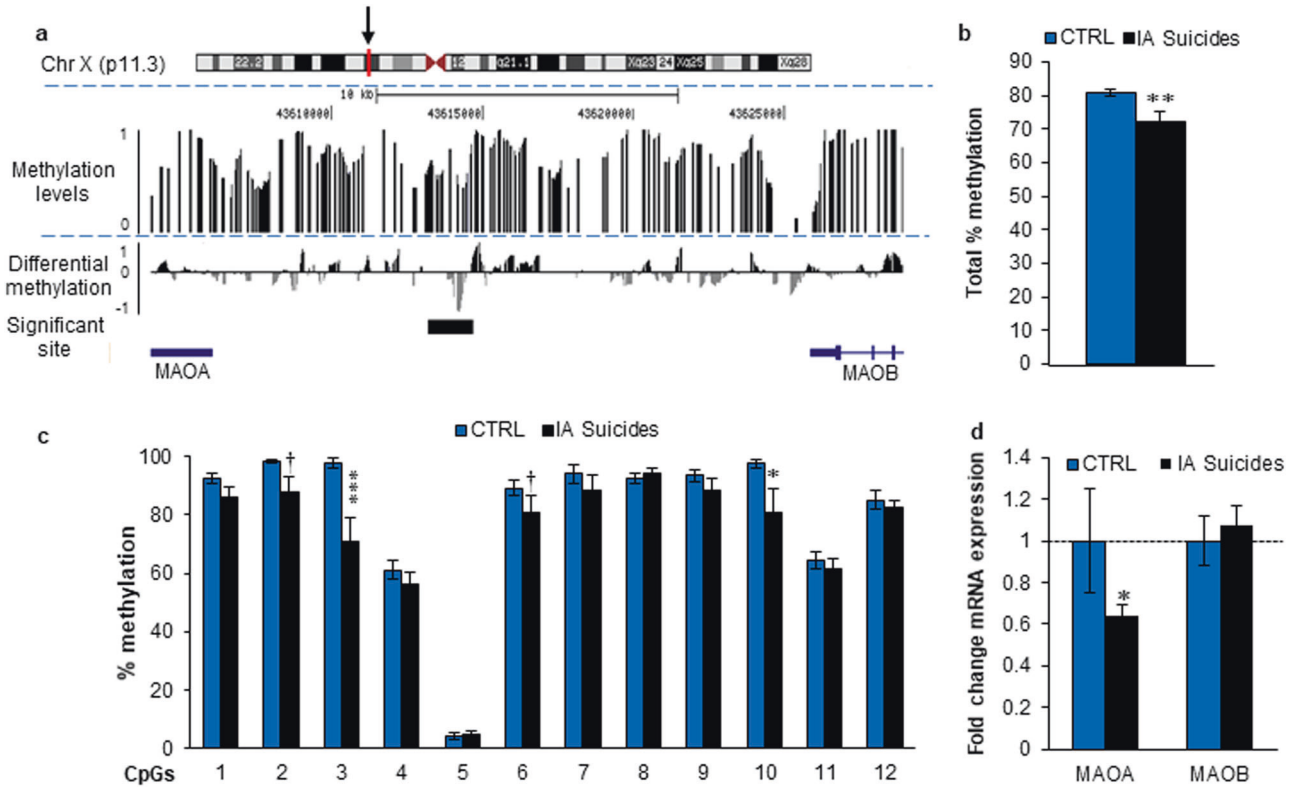

Fig. 1 Methylation of the MAOA–MAOB intergenic region in dentate gyrus. a Overview of DNA methylation patterns in the MAOA/B intergenic region using microarrays. Top, depiction of chromosome X, arrow denotes site of differential DNA methylation within MAOA and MAOB intergenic region. Middle, average CpG methylation observed across MAOA–MAOB intergenic region (1 (maximum level) to 0 (no DNA methylation detected)). Bottom, differential DNA methylation between impulsive-aggressive (IA) suicides and controls; black and grey represent more (maximum 1) and less methylation (maximum −1), respectively. The thick black bar denotes the region of significant hypomethylation in IA suicides compared with controls (CTRL). Thinner blue bars indicate the 3′ ends of MAOA and MAOB genes. Total DNA methylation (**b**) in the MAOA–MAOB intergenic region in neuronal DNA and DNA methylation at individual CpGs (**c**) as quantified by Epityper. Bars represent mean DNA methylation % and error bars represent standard error of the mean (SEM). **d** MAOA mRNA expression is down-regulated in the dentate gyrus of suicide completers relative to controls, while MAOB shows no difference. Data are represented as fold change from control values and error bars represent SEM. Data presented in **a** are from microarray experiments and in **b** and **c** are from Epityper on neuronal populations. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0005$, $^{\dagger}p < 0.1$. Data presented in **a** and **d** were generated from whole-tissue homogenates. Data presented in **b** and **c** were obtained from neuronal nuclei fractions.

A region of 8217 bp, starting 74 bp upstream the 5′ end and ending 110 bp downstream the 3′ end of MAALIN was deleted from the NPC's genome using guide RNAs designed to target specifically this region. In addition, a repair DNA template of 142 bp composed of a complementary sequence to the flanking cut sites was used to fill the deletion site and allow the ligation of DNA.

NPCs were transfected with the vector containing the gRNA-Cas9 and the repair constructs using optimized transfection parameters. 24 h after electroporation, cells were sorted by fluorescence-assisted cell sorting (FACS) based on RFP signal into individual wells of 96-well plates and incubated for about 3 weeks. Clones that survived the FACS sorting were expanded and collected for extraction of genomic DNA and total RNA. Potential knockout (KO) clones were tested using qRT-PCR.

Behavioural validation in mouse

MAALIN viral transduction in adult mice HPC Male CD1 (ICR) mice (35–45 g; Charles River Laboratories) were sexually experienced retired breeders obtained at 4 months of age. Male CD1 were acclimatized to the housing facilities for 1 week before the start of experiments. Stereotaxic surgery was performed to inject HSV-MAALIN-GFP, HSV-scramble-GFP or HSV-GFP into the dentate gyrus (AP −2.0; ML ±1.3; DV −2.1 from bregma; 0° angle). Transduction efficiency was verified by GFP expression in the dentate gyrus, as observed by fluorescent microscopy and qRT-PCR.

Assessment of impulsivity and aggression behaviours Aggression screening was performed using exposure to

intruder mice (C57BL/6J mice; 20–30 g; from The Jackson Laboratory) as previously described [29]. Aggression was screened for both before and after viral infusion to, first, confirm equal aggression levels in MAALIN and control (scramble, GFP) groups, then to assess the impact of MAALIN OE on behavioural indices of aggression.

Ethological analysis of aggressive behaviour was performed using the Noldus Observer software (Noldus). Ethological indices of aggressive behaviour included: (i) latency to initial aggression, (ii) the number of aggressive bouts and (iii) the mean duration of aggressive bouts. Latency to initiate attack was analysed at days 3, 4 and 5. Data from the HSV-MAALIN-GFP group for all other behavioural features were assessed at day 4 and contrasted to those from the HSV-scramble-GFP and HSV-GFP groups.

RNAseq and bioinformatics analyses Alignment was carried out on the processed reads with tophat2 on the GRCh38 version of the human genome [30]. Quantification of the gene expression was carried out with HTseq [31], using the ENSEMBL 88 version of the annotated GRCh38 genome [32]. Differential gene expression and gene count normalization were carried out in R [33] using the DESeq2 package [34]. Genes differentially expressed were defined by contrasting the different cell types, using either Rencells or HSV1005 as reference levels.

Rank–rank hypergeometric overlap (RRHO)

RRHOs were calculated to compare the differential gene expression profiles [35]. All the genes from each of our comparisons were ranked based on their −log10 p values signed according to the direction of their fold change. These lists were submitted to a web-based implementation of the RRHO algorithm available at https://systems.crump.ucla.edu/rankrank/rankrankbatch.php.

Statistical analyses

Described in Detailed Methods, Supplementary Information.

Results

Cell-type-specific DNA methylation profiles in MAOA/MAOB IGR

Using dentate gyrus tissue from suicides with high levels of impulsive-aggressive behaviours ($N = 35$), and psychiatrically healthy controls ($N = 16$), we mapped CpG DNA methylation patterns across the MAOA and MAOB genes, intergenic and regulatory regions, including 50 kb upstream of the MAOA transcription start site (TSS), introns and exons and up to 50 kb downstream of the transcription end site. Our analysis shows a significant hypomethylation of a sequence in the IGR between MAOA and MAOB in impulsive-aggressive suicides compared with healthy controls (Fig. 1a).

Since these methylation differences were observed in tissue homogenates, we next examined neuronal and non-neuronal nuclei isolated from the same dentate gyrus tissue using fluorescence-assisted cell sorting (FACS) [26] and determined what cellular fraction accounted for differential methylation (see Supplementary Fig. 3 for a description of the experimental and methodological design). Our analyses of neuronal and non-neuronal cellular fractions confirmed a significant hypomethylation in the neuronal subpopulation of impulsive-aggressive suicides compared with controls (group main effect: $F_{(1,85)} = 12.66$, $p < 0.001$; Fig. 1b). Post hoc analyses revealed a significant hypomethylation at CpG sites 3 (ChrX: 43 613 593; $p < 0.0005$) and 10 (ChrX: 43 614 769; $p < 0.01$) in cases compared with controls, and a trend toward a significant hypomethylation at CpG sites 2 (ChrX: 43 613 518; $p = 0.099$) and 6 (ChrX: 43 613 656; $p = 0.093$) (Fig. 1c). Conversely, in non-neuronal cellular fractions, while certain CpGs showed site-specific differences, we did not observe a difference in total CpG methylation levels for the entire region (Supplementary Fig. 4a, b). These data suggest that the DNA hypomethylation observed in the MAOA–MAOB IGR of impulsive-aggressive suicides is driven by methylation differences in the neuronal cell fraction.

We investigated the external validity of our findings in two independent cohorts. Using dentate gyrus tissue from a replication cohort composed of impulsive-aggressive suicides ($N = 23$) and psychiatrically healthy male controls ($N = 13$), we found a similar trend toward lower DNA methylation at CpG sites 3 (ChrX: 43 613 635; $p = 0.06$) and 2 (ChrX: 43 613 593; $p = 0.07$) in cases, compared with controls (Supplementary Table 5). We further investigated methylation patterns of this region in DNA isolated from blood samples from inmates with pathologically high levels of impulsive-aggression, defined by a diagnosis of ASPD (ASPD: $N = 86$, Control: $N = 73$; full description of the sample in Detailed Methods, Supplementary Information). We found a similar pattern of hypomethylation in the MAO IGR in the inmate population as compared with psychiatrically healthy controls (group main effect: $F_{(1,522)} = 3.76$, $p < 0.05$; Supplementary Fig. 4c, d).

As similar intergenic differential DNA methylation has previously been reported to associate with changes in the expression of surrounding genes [24, 36], we therefore tested whether MAOA and MAOB expression is changed in the dentate gyrus of cases compared with controls. Quantification of MAOA and MAOB expression in cases relative

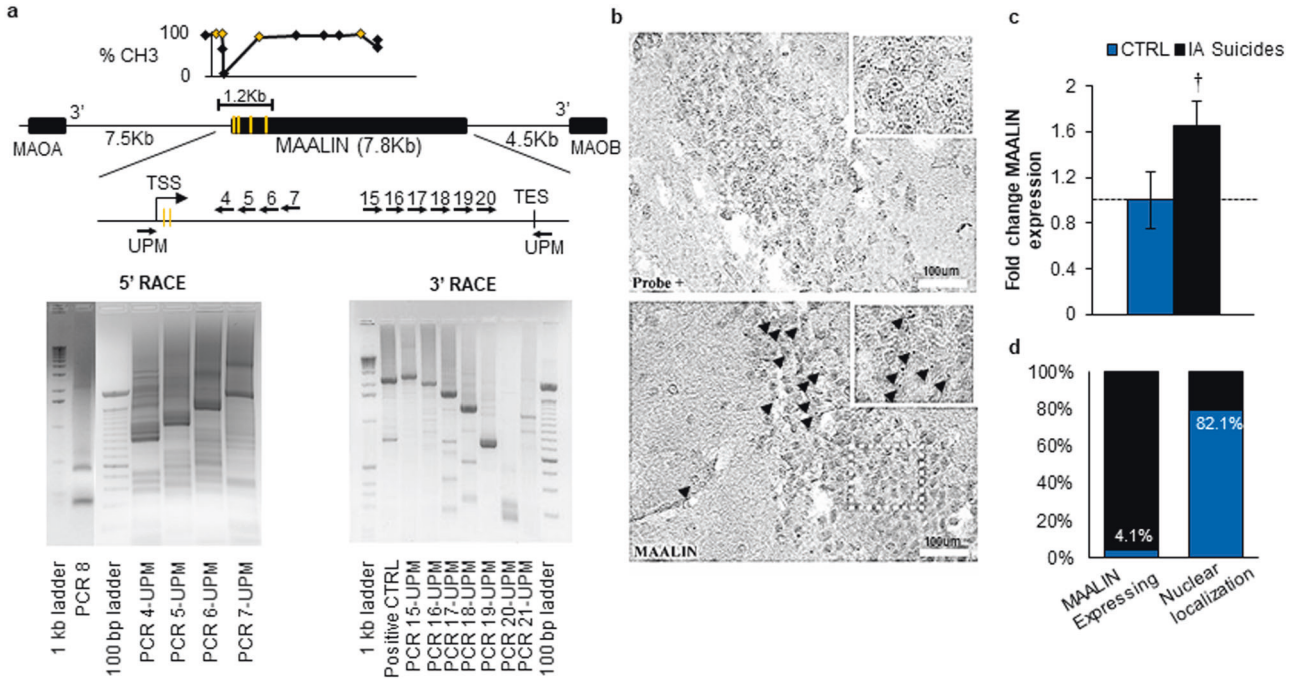

Fig. 2 Characterization of the MAALIN transcript. a Rapid amplification of cDNA ends (RACE) characterization of MAALIN transcript. Top, schematic representation of MAALIN gene within the MAOA/B intergenic region with graph depicting DNA methylation patterns (orange bars indicate CpG sites of significant hypomethylation) within its promoter region and the RACE experimental design, with arrows representing the primers used (3–21) for 5′ and 3′ RACE. Bottom left, 5′ ends of transcripts were identified using nested PCR (using PCR8 primer); increasing fragments were obtained with PCR4 UPM to PCR7 UPM. Bottom right, the 3′ end of transcripts was mapped to the region between PCR19-UPM (750 bp) and PCR20-UPM (no bands). **b** In situ hybridization showing control (top panel) and MAALIN probes in neuronal cells in human hippocampus (bottom panel). MAALIN expression is low and mainly concentrated in the nucleus. **c** MAALIN expression is upregulated in the dentate gyrus of IA suicides relative to controls. Data are shown as fold change relative to controls with error bars representing SEM. **d** Proportion of neurons expressing MAALIN (blue; left bar) and proportion of nuclear (blue) versus cytoplasmic (black) compartmentalization of MAALIN in human hippocampus. $^{\dagger}p < 0.1$.

to controls suggested a significant downregulation of MAOA ($t_{(1,42)} = -2.00$; $p < 0.05$), but not of MAOB ($t_{(1,46)} = 0.47$; $p > 0.1$) (Fig. 1d), suggesting a potential regulatory role of this IGR on MAOA expression.

Characterization of a novel lncRNA in the human brain

We next considered how methylation levels within MAOA and B IGR might regulate MAOA expression. We noticed that while overall DNA methylation levels were high across this entire region, these levels progressively decreased starting at CpG3 (~75%) and CpG4 (~60%) to markedly low levels at CpG5 (~5%; Fig. 1c). Such patterns are similar to those observed in promoter regions, where DNA methylation levels are usually low [37, 38], and suggested that this region might drive the expression of a transcript encoded in the MAO IGR.

Previous studies reported the expression of noncoding RNA transcripts from IGRs [39] acting as enhancers and modulating the expression of genes in surrounding genetic loci [36]. Thus, we hypothesized that this MAO IGR may harbour a novel noncoding RNA transcript regulating the expression of MAOA. Using 5′ and 3′ RACE, we successfully identified and characterized a novel transcript (Fig. 2a; Supplementary Fig. 2a, b). Our analyses revealed a single exon transcript of 7792 nucleotides, starting 7521 bp downstream from MAOA 3′ end (ChrX: 43 613 565) and ending 4501 bp upstream of MAOB 3′ end (ChrX: 43 621 356), thus corresponding to a previously unannotated long intergenic noncoding RNA (lncRNA). We named this lncRNA MAALIN and quantified its expression in brain samples, revealing a significant upregulation in cases ($t_{(1,43)} = 1.63$; $p = 0.05$; Fig. 2c) compared with controls. In addition, we used RNAscope to confirm the expression of MAALIN in neurons from the hippocampus. As expected for lncRNAs, our results showed low levels of MAALIN expression, with only 2.1–5.3% ($N = 4$, average 4.1%) of neuronal cells in the HPC expressing at least one fragment of MAALIN RNA (Fig. 2b, d). Furthermore, our results show that more than 82% of these RNA fragments are found in the nucleus (Fig. 2d). Together, this strongly supports the existence of a novel and previously unannotated lncRNA (MAALIN) in human HPC, the expression of which is upregulated in impulsive-aggressive suicides.

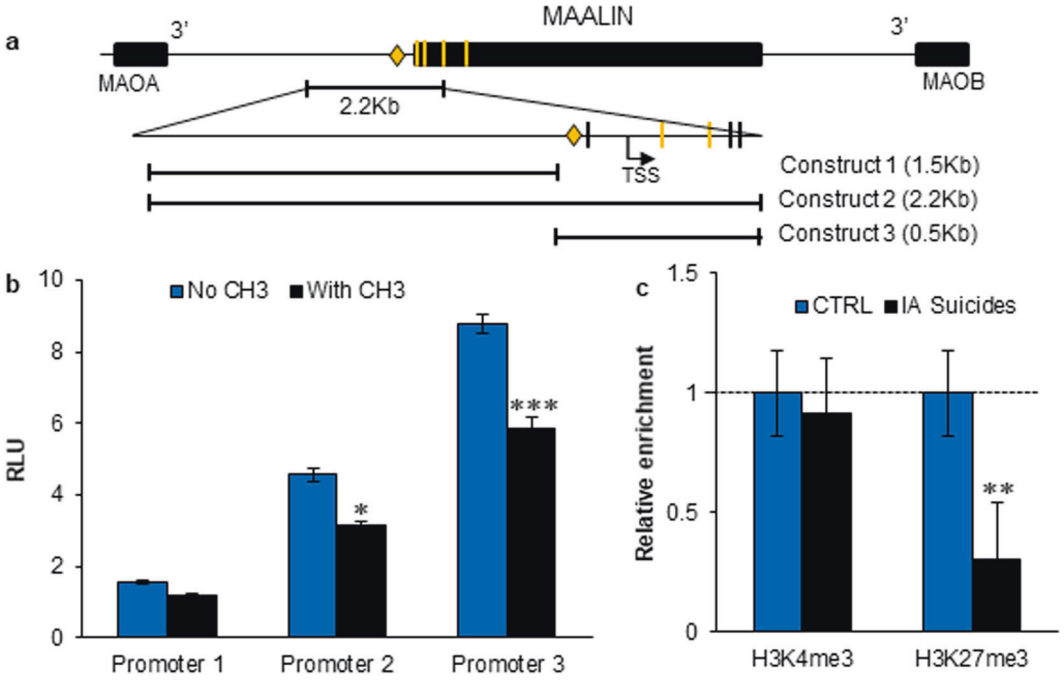

Fig. 3 Effect of methylation on the transcriptional activity of the MAOA–MAOB intergenic region. a-b Construct 1 is a 1.5 kb fragment of DNA upstream of MAALIN, excluding the region differentially methylated in IA suicides. Construct 2 is a 2.2 kb fragment of DNA spanning upstream and start site of MAALIN, including the differentially methylated site. Construct 3 is a 625 bp fragment within the differentially methylated site. Bars represent mean relative light unit (RLU) and error bars represent SEM. **c** H3K27me3, but not H3K4me3, levels decrease in IA suicide completers compared with controls in the 5′ region of the MAALIN gene. Bars represent fold change of chromatin enrichment (IP/INP) compared with controls and error bars show SEM. Orange lozenge on the schematic representation of MAALIN gene indicates site of differential enrichment for H3K27me3 between IA suicides and CTRL. $*p < 1.0 \times 10^{-3}$, $**p < 1.0 \times 10^{-4}$, $***p < 1.0 \times 10^{-8}$.

Epigenetic regulation of MAALIN in human brain

Our data show that the 5′ end of MAALIN is located within the hypomethylated "promoter-like" region that we identified in the dentate gyrus of impulsive-aggressive suicide cases. Thus, we hypothesized that differential methylation in this region may alter the expression level of MAALIN. We created different constructs to assess the transcriptional activity of different regions of the MAALIN promoter and test the effect of methylation on the transcriptional activity of this IGR. These constructs contained different sizes of the MAALIN promoter with or without the sequence identified as being differentially methylated (Fig. 3a). Using luciferase assays, we first confirmed transcriptional activity associated with the sequence where differential methylation was observed. These effects were seen in HEK293 ($F_{(3,17)} = 378.76$; $p < 5 \times 10^{-13}$), neuroblastoma cell lines ($F_{(3,17)} = 47.69$; $p < 5 \times 10^{-7}$) and NPCs ($F_{(3,13)} = 125.4$; $p < 5 \times 10^{-7}$; Supplementary Fig. 5a–c). Importantly, by artificially increasing DNA methylation within the site of hypomethylation, we significantly decreased its transcriptional activity ($F_{(1,30)} = 15.58$, $p = 0.0001$) in both constructs containing this sequence (construct 2: $p < 1 \times 10^{-11}$ and construct 3: $p < 1 \times 10^{-19}$; Fig. 3b). Interestingly, our results suggest that the distal promoter of MAALIN contains a repressive element that suppresses the transcriptional activity of its proximal promoter. In normal conditions, this repressive element may maintain a homoeostatic control of MAALIN expression. However, when DNA methylation levels within MAALIN proximal promoter decrease, this homoeostatic balance may be disrupted leading to elevated MAALIN expression in neuronal cells.

We next investigated promoter- (H3K4me3, H3K27me3) and enhancer- (H3K4me, H3K27Ac) specific epigenetic marks at the MAALIN regulatory region. ChIP performed on dentate gyrus samples from the discovery cohort indicated a significant downregulation of H3K27me3 ($t_{(1,38)} = -4.52$; $p < 1 \times 10^{-4}$), but not H3K4me3 levels ($t_{(1,34)} = -0.33$; $p = 0.74$; Fig. 3c), in cases compared with controls at the locus of hypomethylation. On the other hand, we found no differential enrichment of either H3K4me ($t_{(1,31)} = 0.26$; $p = 0.80$) or H3K27Ac ($t_{(1,32)} = 0.65$; $p = 0.52$) at the region of hypomethylation (data not shown). Thus, our data suggest that the combination of both hypomethylation and open chromatin conformation in MAALIN's regulatory regions promotes its expression at higher levels in the dentate gyrus of impulsive-aggressive suicides.

MAOA regulation via MAALIN expression

Previous studies showed that lncRNAs can regulate the expression of genes found within more or less 300 kb from their site of expression [36], and given that MAOA and MAOB are the only two known genes present in this region (within 600 kb), we hypothesized that MAALIN OE or KO would down- and upregulate, respectively, MAOA expression. Using a recombineering strategy, we first cloned MAALIN into an expression vector, transfected NPCs at developmental day 28 (highest MAALIN expression levels; Supplementary Fig. 6) and used FACS to isolate transfected from non-transfected cells (Fig. 4). Our data show that MAALIN OE in NPCs induces a significant downregulation of MAOA in transfected cells ($F_{(3,19)} = 264.3$, $p < 1 \times 10^{-13}$; Fig. 4g), but not in non-transfected or mock-transfected cells.

Conversely, we used CRISPR to knock-out the MAALIN sequence in NPCs. We first created a cell line in which a region of 8217 bp encompassing the MAALIN sequence and part of its promoter was knocked-out, completely inhibiting MAALIN expression (Fig. 4h; $t_{(1,8)} = 5.31$, $p < 0.0001$). qRT-PCR confirmed that MAALIN KO significantly increases MAOA expression in NPCs ($t_{(1,8)} = 14.74$, $p < 0.0001$; Fig. 4h). Together, these data suggest a regulatory role of MAALIN on the expression of MAOA and given the previous data implicating MAOA in impulsive-aggression, imply a potential effect on behaviour.

Next, we tested whether MAALIN regulates additional genes besides MAOA using RNAseq on NPCs following MAALIN OE and KO. Importantly, our results confirmed the regulatory impact of MAALIN on MAOA. Indeed, the OE of MAALIN (log2 fold change: 10.6; $P_{(adj)} < 5.0 \times 10-^{18}$; Supplementary Table 6) induced a robust downregulation of MAOA (log2 fold change: −1.6; $P_{(adj)} < 0.05$; Supplementary Table 6). On the contrary, MAALIN KO (no detectable copies) significantly increased the expression of MAOA (log2 fold change: 1.7; $P_{(adj)} < 0.005$; Supplementary Table 6). As expected, and commonly observed in this type of assay [40, 41], our analyses show that MAALIN changes also directly and indirectly associate with changes in other genes (Supplementary Table 6). Interestingly, our analysis shows that MAALIN OE and KO induces opposite effects on gene expression (Supplementary Fig. 7). Indeed, RRHO analysis of MAALIN OE and KO showed that both treatments had an opposite impact on gene expression with a significant (maximum Fisher exact test $p < 1.0 \times 10^{-150}$) overlap for genes showing opposite directional changes following MAALIN OE and KO (Supplementary Fig. 7). Overall, our results suggest that MAALIN regulates MAOA expression in NPCs while inducing a reorganisation of transcriptional profiles across the genome.

MAALIN modifies aggressive behaviour

Our findings show that epigenetic modifications in the IGR between MAOA and MAOB, including DNA hypomethylation and reduced H3K27me3, promote higher expression of MAALIN which, in turn, regulates MAOA expression in vitro, and associates with lower expression of MAOA in the dentate gyrus of impulsive-aggressive suicide cases. Since lower levels of MAOA associate with higher levels of impulsive-aggressive behaviour [1–3, 5–7, 20, 22], these data suggest that differences in the expression of MAALIN may explain higher levels of impulsive-aggressive behaviours among suicides. To test this hypothesis, we used HSV-mediated gene transfer to overexpress MAALIN in the hippocampus of CD1 male mice (Fig. 5a), which are inherently aggressive, and assessed aggressive behaviours using a modified version of the resident–intruder test [42, 43] (schematized in Fig. 5b). We first assessed aggression for 3 days before viral infection and confirmed equal levels of aggression in MAALIN and control groups (HSV-GFP and HSV-scramble-GFP; Fig. 5c). OE of MAALIN in the hippocampus of CD1 mice induced a marked reduction in the latency to initiate attack (viral main effect: $F_{(2,138)} = 7.19$, $p < 0.005$; Fig. 5d, e) compared with HSV-scramble-GFP ($p < 0.005$) and HSV-GFP ($p < 0.005$) controls. This effect was more pronounced on day 4 post infection (MAALIN over scramble: $p < 0.05$; MAALIN over GFP: $p < 0.01$) and coincided with peak HSV transgene expression, while the effect was trending at days 3 (MAALIN over scramble: $p = 0.06$; MAALIN over GFP: $p = 0.06$) and 5 (MAALIN over scramble: $p = 0.08$) post infection. These effects were associated with a significant downregulation of MAOA expression in the hippocampus of animals that were infected with HSV-MAALIN ($t_{(1,11)} = 2.60$, $p < 0.05$; Fig. 5f) compared with HSV-GFP control but not HSV-scramble-GFP ($t_{(1,8)} = 0.64$, $p > 0.1$). Interestingly, MAALIN OE did not increase the total number of attacks ($F_{(2,39)} = 0.63$, $p > 0.1$; Fig. 5g), but induced a trend toward a significant increase of the mean duration of attacks ($F_{(2,39)} = 2.22$, $p = 0.06$; post hoc test: MAALIN over scramble $p < 0.05$; MAALIN over GFP $p = 0.06$; Fig. 5h). Thus, these results indicate that MAALIN regulates the expression of impulsive-aggressive behaviours in mice by changing transcriptional programs, including a downregulation of MAOA expression, in the hippocampus.

Discussion

lncRNAs have been increasingly associated with neuropsychiatric disorders (reviewed in [44, 45]). LncRNAs are highly expressed in the brain, in a tissue and cell-type-specific fashion [31, 32], have a direct effect on the

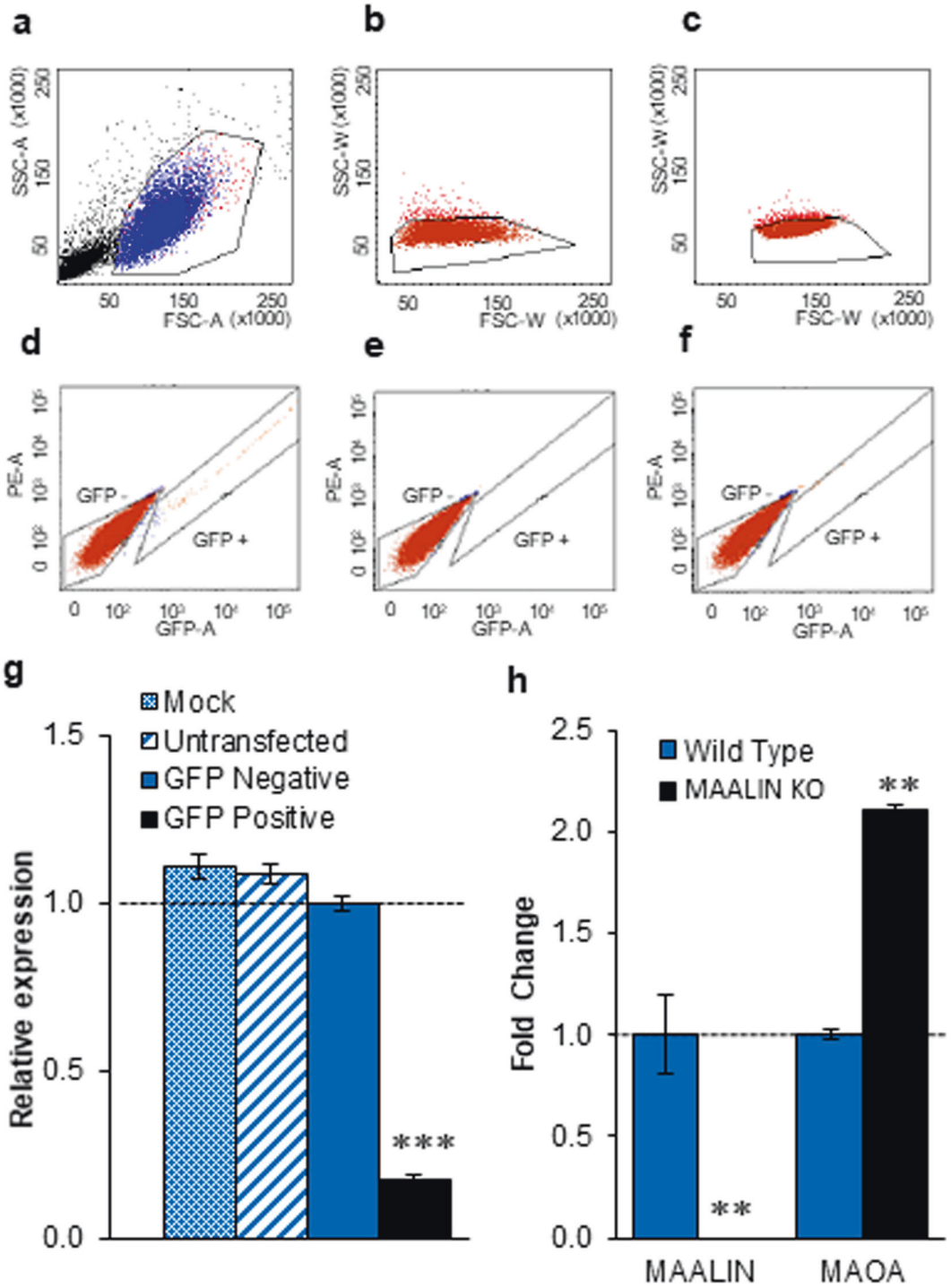

Fig. 4 Effect of MAALIN in vitro overexpression and knock out on MAOA expression in NPCs 35 days post differentiation. a–f Fluorescence-assisted cell sorting (FACS) plots showing NPCs selected on size and internal complexity (**a**–**c**). The population in **c** was sorted independently according to high-intensity GFP expression versus no or low-intensity GFP expression in NPCs transfected with the (**d**) HSV-MAALIN-GFP virus, (**e**) mock transfected, and (**f**) untransfected. Mock-transfected and untransfected cells showed no GFP fluorescence. **g** Relative MAOA expression in FACS-sorted NPCs. **h** Relative MAALIN and MAOA expression in NPCs following MAALIN knock out. Bars represent fold change expression relative to GFP expression levels. Error bars represent SEM. $^{**}p < 1.0 \times 10^{-4}$, $^{***}p < 5.0 \times 10^{-13}$.

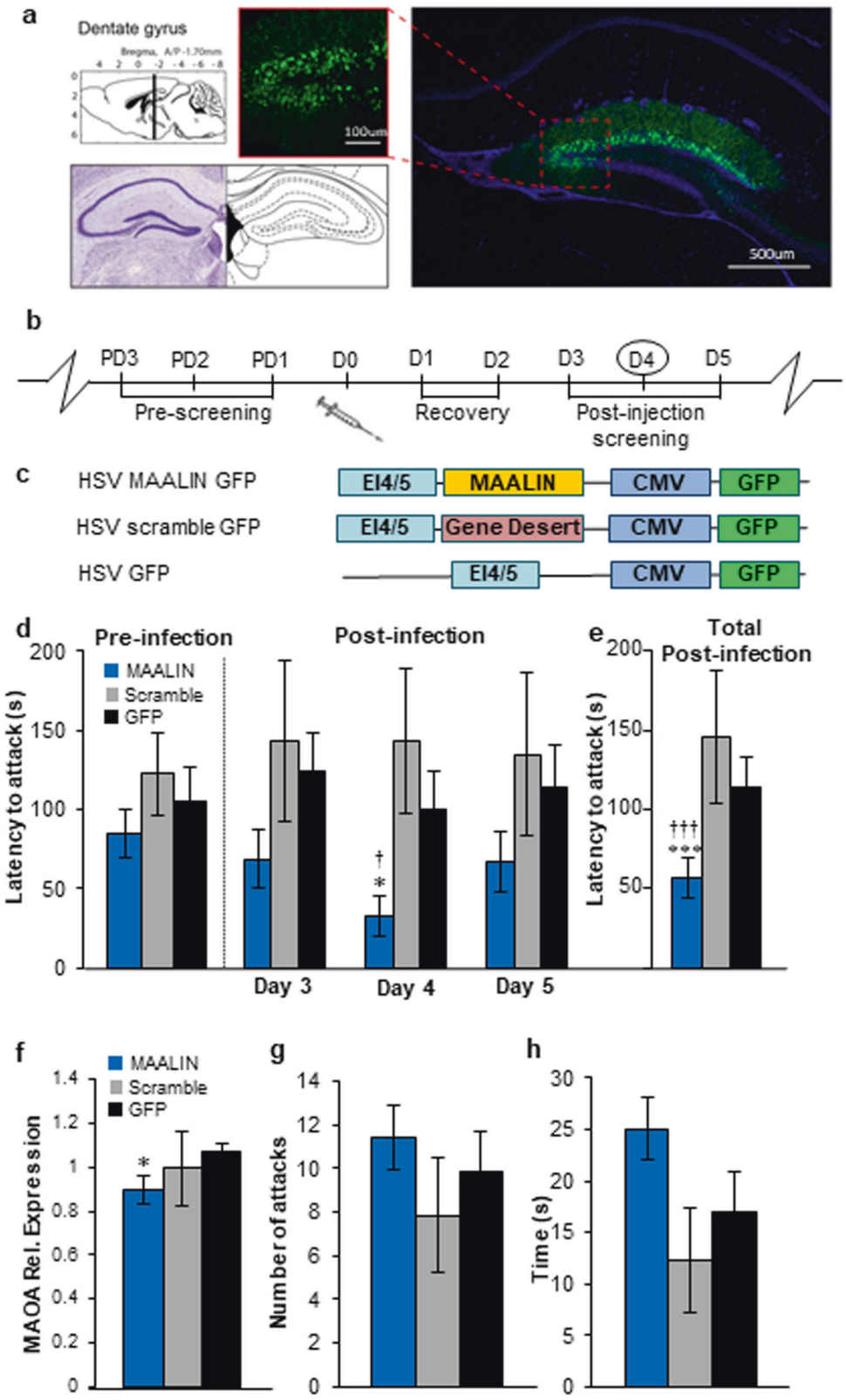

Fig. 5 Behavioural and functional impact of in vivo MAALIN overexpression. a Mouse hippocampal structure and HSV expression. Right, spread of GFP-labelled viral infection in hippocampal neurons. Scale bar, 500 µm. Left, zoomed capture of the dentate gyrus showing strong GFP signal in the cell body of dentate gyrus neurons. Scale bar, 100 µm. **b** CD1 mice were screened for aggressive behaviours 3 days before infection (PD3–PD1). On day 0 (D0), mice were injected with either HSV-MAALIN-GFP, HSV-scramble-GFP (negative CTRL) or HSV-GFP (negative CTRL) into the dentate gyrus and were allowed 2 days of recovery (D1–D2). Aggressive behaviours were screened on days 3–5 (D3–D5) post injection. **c** MAALIN and scramble gene sequences were inserted into a p1005 plasmid following a EI4/5 promoter region; downstream of MAALIN and scramble gene sequences, GFP expression was driven by a CMV promoter. An identical construct, without MAALIN nor scramble gene sequence, was used as a negative control. **d** Latency to attack in mice receiving MAALIN-HSV-GFP was significantly decreased at D4 compared with controls. **e** Mean latency to attack in aggressive CD1 mice (D3–D5) was significantly decreased in mice infected with MAALIN-HSV-GFP. **f** MAALIN dentate gyrus overexpression in mice decreased MAOA expression at D4. The number of attacks (**g**) was unchanged, but the mean attack duration (**h**) was increased at D4. Bars represent mean values ± SEM. $^{***}p < 0.005$ MAALIN OE over GFP CTRL, $^{*}p < 0.05$ MAALIN OE over GFP CTRL, $^{\dagger\dagger\dagger}p < 0.005$ MAALIN OE over scramble GFP CTRL, $^{\dagger}p < 0.05$ MAALIN OE over scramble GFP CTRL.

regulation of gene expression, and fine-tune complex molecular and cellular functions, such as those involved in the behavioural strategies normally used to cope with stress. Here, we identified and characterized a novel lncRNA, which we named MAALIN. We found that it regulates MAOA expression levels and is altered in the dentate gyrus

of individuals with high levels of impulsive and aggressive traits who died by suicide. In addition, we also found that MAALIN expression is controlled by a combination of neuron-specific epigenetic mechanisms involving DNA methylation and histone modifications. Overall, in this study, we highlighted a novel mechanism by which MAOA gene expression is regulated in the human brain and showed that a novel lncRNA is involved in the regulation of aggressive-impulsive behaviours.

Previous studies showed that DNA methylation-sensitive transcription factors such as *oct4* and *nanog* bind the promoters of lncRNAs, modulating their expression to maintain a balance between pluripotency and lineage commitment in mouse embryonic stem cells [46]. Furthermore, genome-wide screenings revealed transcriptional activation at gene enhancers associated with specific chromatin profiles [39]. Consistent with these findings, we showed a depletion of both H3K27me3 and DNA methylation in MAALIN's promoter, and our results highlight the complex interplay taking place between different epigenetic modifications to regulate MAALIN expression. First, lower levels of the repressive mark H3K27me3 in MAALIN promoter suggest that this region is poised for active transcription in the dentate gyrus of impulsive-aggressive suicides. Second, the DNA hypomethylation observed in neuronal populations might allow transcription factors to bind within MAALIN promoter and recruit the transcriptional machinery required for MAALIN's expression. Together, our data suggest that the epigenetic landscape at MAALIN's promoter is modified to allow increased MAALIN expression in the HPC of impulsive-aggressive suicides.

The implication of MAOA in the regulation of impulsive and aggressive behaviours is supported by a large body of evidence. Variations in MAOA levels associate with several dimensions of impulsive-aggressive behaviours as well as with higher risks for the development of mental disorders, including drug and alcohol addiction and ASPD often leading to suicidal behaviours (reviewed in [47]). However, besides the presence of a variable number tandem repeats and differential DNA methylation in MAOA promoter [48, 49], the processes regulating MAOA gene expression in the brain remain poorly understood. In this study, we characterized a new mechanism by which MAOA expression is regulated by a novel lncRNA located in between the MAOA and MAOB genes. This is consistent with previous studies showing that lncRNAs act mainly on neighbouring genes [36]. Indeed, due to its location in the gene desert separating MAOA and MAOB, MAALIN is perfectly positioned to regulate these two genes. However, our data show that MAALIN acts specifically on MAOA and not MAOB. While the precise mechanism by which MAALIN impacts MAOA expression leading to behavioural changes is not clear, lncRNAs have been shown to act through different *trans*- and *cis*-regulatory mechanisms [50] including interactions with chromatin complexes [51, 52], modulators of enzymatic activity [53, 54], interactions with DNA/RNA binding proteins [55–57] and direct DNA interactions [58–60]. Our results using RNAscope show that MAALIN is mainly found in the nucleus, suggesting that it could act through some of these mechanisms in human hippocampal neurons. Indeed, it is a possibility that MAALIN serves as a scaffolding structure bringing chromatin remodelers in the MAOA but not MAOB promoter regions causing the chromatin to switch from an euchromatin to a heterochromatin state and repressing its expression. As of now, it is unclear whether MAALIN could recruit members of repressive complexes to MAOA promoter and more work is required to elucidate the mechanisms by which MAALIN regulates MAOA expression in the brain.

Our results suggest that changes in DNA methylation within the MAALIN region are also found in peripheral tissue from patients suffering from ASPD. Indeed, while different from those observed in neuronal and non-neuronal populations, DNA methylation patterns in the blood of ASPD patients show consistent hypomethylation. Epigenetic mechanisms are cell-type specific [61–63] and the extent to which peripheral methylation patterns can represent those present in the brain has been an issue of important debate in the literature. However, there are a few examples, such as DNA methylation patterns in the promoter of the glucocorticoid receptor gene [64], where there have been consistent methylation patterns found across different tissues. While follow up work will be required to elucidate their clinical relevance, our findings suggest that DNA methylation changes within this region of the genome might be indicative of aberrant control of impulsivity and aggression behaviours in human populations.

The control of impulsive and aggressive behaviour involves a complex circuit linking different brain regions including cortical and subcortical brain regions, the activity of which may be modulated by monoamines. Indeed, altered monoamine levels are strongly associated with impulsive and aggressive behaviours [65]. Interestingly, previous studies showed an upregulation of the immediate early gene Arc expression in the dentate gyrus of mice following aggressive resident–intruder interactions [66]. Other studies showed that impulsive choices are modulated distinctly by pharmacological approaches targeting monoaminergic signalling in several brain regions including the hippocampus (reviewed in [67]). While the hippocampus projects densely to several brain regions, it has been suggested that its functional connection with the amygdala may be directly involved in controlling impulsive/aggressive behaviours [67]. This is consistent with the expression

pattern of MAALIN. Indeed, we showed that MAALIN expression was restricted to a small population of neurons. Thus, its impact may be more pronounced on a subpopulation of neurons projecting specifically to distinct brain regions such as the amygdala or the prefrontal cortex impacting the top-down executive processing of context-induced emotions on decision making.

The impact of MAOA on the expression of impulsive and aggressive behaviours has been well characterized. For instance, total congenital deficiency of MAOA results in Brunner syndrome, characterized by high aggression levels [20]. Low expression of MAOA due to variable number tandem repeats, although not directly associated with higher aggression levels [68, 69], alters emotional and social processing while increasing the risk for ASPD [5–7]. Similarly, mice with a total MAOA deficiency (MAOA-KO) exhibit marked reactive aggression toward conspecifics [21, 22] although this phenotype is absent in hypomorphic MAOA mutant mice still carrying low but detectable MAOA catalytic activity [70]. Together, this suggests that total deficiency in MAOA activity is required for aggressive behaviours to be expressed, which is in contrast with our findings showing that a reduction, but not an ablation of MAOA expression mediated by MAALIN OE in the dentate gyrus, induces aggressive and impulsive behaviours in mice. This discrepancy could be explained by the fact that besides MAOA, MAALIN targets additional genes. It is likely that some of the behavioural effects induced by MAALIN OE in the dentate gyrus may result from a broad reorganization of the transcriptional programs involved in the control of neuronal circuits contributing to the development of pathological and maladaptive personality traits. Thus, through our experiments, we may have identified several new targets involved in the control of complex aggressive and impulsive behaviours, although the contribution of these transcriptional programs on the activity of the neuronal circuitry controlling these complex phenotypes will have to be further investigated. In addition, while MAALIN expression seems to be regulated in an activity-dependent fashion, more work will be required to understand the extent to which its expression and functional activity is influenced by the dynamic environment experienced by the organism. Since lncRNAs are only just beginning to be understood, revealing the mechanisms through which MAALIN regulates gene expression may highlight how certain classes of noncoding RNAs function and fine-tune complex systems involved in cell signalling. Such experiments may also inform us about the role of lncRNAs in mood disorders and ultimately reveal the therapeutic potential of these novel and intriguing molecules in the treatment and prevention of complex behavioural traits.

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Author contributions BL, GT, NM and MJM planned the experiments, wrote the manuscript and supervised the studies. BL conducted and supervised all experiments and analysed all data. KA, TB, GM, VY and JY participated in the experiments. SAG, SJR and EJN provided help and support for the design and interpretation of the animal experiments. LN, DC, CG, JPL, RET and GC contributed to the experiments. RLN provided viral constructs.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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