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Kanarik, Margus

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Original Article

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Corresponding author:

Jaanus Harro; Email: jaanus.harro@ut.ee

Margus Kanarik¹, Kristi Liiver², Marianna Norden^{2,3}, Indrek Teino^{4,5}, Tõnis Org⁶, Karita Laugus¹, Ruth Shimmo², Mati Karelson⁷, Mart Saarma⁴ and Jaanus Harro^{1,4} 

¹Division of Neuropsychopharmacology, Institute of Chemistry, University of Tartu, Tartu, Tartumaa, Estonia; ²School of Natural Sciences and Health, Tallinn University, Tallinn, Harjumaa, Estonia; ³Institute of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia; ⁴Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki, Uusimaa, Finland; ⁵Institute of Molecular and Cell Biology, University of Tartu, Tartu, Tartumaa, Estonia; ⁶Institute of Genomics, University of Tartu, Tartu, Tartumaa, Estonia and ⁷Division of Molecular Technology, Institute of Chemistry, University of Tartu, Tartu, Estonia

Abstract

Modification of mRNA by methylation is involved in post-transcriptional regulation of gene expression by affecting the splicing, transport, stability and translation of mRNA. Methylation of adenosine at N⁶ (m⁶A) is one of the most common and important cellular modification occurring in the mRNA of eukaryotes. Evidence that m⁶A mRNA methylation is involved in regulation of stress response and that its dysregulation may contribute to the pathogenesis of neuropsychiatric disorders is accumulating. We have examined the acute and subchronic (up to 18 days once per day intraperitoneally) effect of the first METTL3/METTL14 activator compound CHMA1004 (methyl-piperazine-2-carboxylate) at two doses (1 and 5 mg/kg) in male and female rats. CHMA1004 had a locomotor activating and anxiolytic-like profile in open field and elevated zero-maze tests. In female rats sucrose consumption and swimming in Porsolt's test were increased. Nevertheless, CHMA1004 did not exhibit strong psychostimulant-like properties: CHMA1004 had no effect on 50-kHz ultrasonic vocalizations except that it reduced the baseline difference between male and female animals, and acute drug treatment had no effect on extracellular dopamine levels in striatum. Subchronic CHMA1004 altered *ex vivo* catecholamine levels in several brain regions. RNA sequencing of female rat striata after subchronic CHMA1004 treatment revealed changes in the expression of a number of genes linked to dopamine neuron viability, neurodegeneration, depression, anxiety and stress response. Conclusively, the first-in-class METTL3/METTL14 activator compound CHMA1004 increased locomotor activity and elicited anxiolytic-like effects after systemic administration, demonstrating that pharmacological activation of RNA m⁶A methylation has potential for neuropsychiatric drug development.

Significant outcomes

- Acute and subchronic systemic administration of the first-in-class METTL3/METTL14 activator drug CHMA1004 (methyl-piperazine-2-carboxylate) was found to exhibit activating and anxiolytic-like properties.
- Behavioural activation by increased m⁶A mRNA methylation was not accompanied by behavioural or neurochemical effects typical for addictive psychostimulants.
- Action on m⁶A mRNA methylation affected catecholamine levels in several brain regions and gene expression related to dopamine neuron viability and stress response.

Limitations

- The number of male and female animals separately in these experiments was small.
- Other types of behavioural tests will be required to fully characterise the psychopharmacological profile of METTL3/METTL14 activators.
- While specific alterations in gene expression were demonstrated, it was not established whether these were directly owing to increased RNA methylation or downstream effects.

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Introduction

Psychiatric disorders mostly develop in gene-environment interactions and have a chronic and relapsing course, likely mediated by multiple epigenetic and downstream regulatory mechanisms. Epigenetic DNA modifications, especially DNA methylation, have garnered much scrutiny in recent years (Jurueña *et al.*, 2021; Panariello *et al.*, 2022; Harvanek *et al.*, 2023; Persaud and Cates, 2023; Pishva *et al.*, 2023). However, limited attention has been paid to modification of mRNA by methylation. Methylation of adenosine at N⁶ (m⁶A) is the most common and most important cellular modification occurring in the mRNA of eukaryotes. m⁶A methylation regulates splicing, transport, stability and translation of mRNA (Zaccara *et al.*, 2019). m⁶A formation is catalysed by a methyltransferase complex containing methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms-tumor-1-associated protein (WTAP) and by a single subunit enzyme methyltransferase-like 16 (METTL16). RNA m⁶A demethylation is catalysed by the fat mass and obesity-associated protein FTO and the RNA demethylase enzyme ALKBH5. These enzymes modify usually only one but sometimes up to three adenosine residues in the mRNA molecules generally located in the 3′ untranslated region. RNA sequences containing m⁶A residues are recognised by a dozen of RNA binding proteins, for example YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3 that regulate the fate of mRNA (Meyer and Jaffrey, 2017). m⁶A modification occurs in all organs but is particularly abundant in mammalian brain. There is increasing evidence on how mRNA m⁶A methylation is involved in regulation of behaviour, suggestive that its dysregulation may contribute to neuropsychiatric disorders (Zaccara *et al.*, 2019; Livneh *et al.*, 2020; Selberg *et al.*, 2021b; Shafik *et al.*, 2022; Su *et al.*, 2023).

A pioneering study demonstrated that interference with RNA m⁶A methylation affects clock gene transcription and by this means the length of circadian period in mice (Fustin *et al.*, 2013). More recently, artificial lighting was shown to affect RNA m⁶A methylation and corresponding gene expression in the rat lateral habenula (Li Y *et al.*, 2023), a brain region pivotal for fronto-limbic to brainstem connections and implicated in affective and substance use disorders (Ables *et al.*, 2023). Recent studies have suggested the involvement of cerebral RNA m⁶A methylation in stress response, whereas m⁶A and its regulatory gene expression levels can change in a regionally specific manner (Engel *et al.*, 2018). Several studies have found changes in RNA m⁶A methylation after fear conditioning and effects of manipulation on m⁶A methylation enzymes on fear-conditioned behaviour (Widagdo *et al.*, 2016; Walters *et al.*, 2017; Chang *et al.*, 2022). In learned helpless rats, the expression of several plasticity genes in hippocampus was downregulated along with enrichment in their m⁶A methylation (Roy *et al.*, 2022). In summary, RNA m⁶A methylation serves as a crucial regulation mechanism at the epitranscriptomic level to shape synaptic plasticity and behaviour (Malovic and Pandey, 2023) and involvement of RNA m⁶A methylation appears in multiple aversive behavioural conditions, but so far evidence for a role of m⁶A methylation in anxiety has remained correlational.

In silico-based approach that departed from the crystal structure of the METTL3/METTL14 complex with S-adenosyl-L-homocysteine (Wang P *et al.*, 2016) enabled some of us to identify the first small molecule ligands that bind to METTL3/METTL14/WTAP and function as RNA methylation activators (Selberg *et al.*, 2019). In particular, as measured using the surface plasmon resonance, the compounds increased 200-fold the

binding efficiency of the RNA methylating substrate S-adenosyl-methionine to the METTL3/METTL14/WTAP protein complex. The treatment of cells with these compounds activating the METTL3/METTL14/WTAP complex led to the increase of m⁶A-modified nucleotides in the substrate RNA (Selberg *et al.*, 2021a). The cytotoxicity screening on the number of endpoints did not show any aberrations up to the 200 μM concentration of compounds (acute oral toxicity LD₅₀ > 2 000 mg/kg). The compounds do not inhibit hERG channel and the monoamine oxidase. The compounds have good water solubility (>100 μM), and the half-lives of the best compound CHMA1004 are t_{1/2} = 354 min in human plasma and t_{1/2} = 60.5 min in rat plasma, respectively (data available on request). The computational modelling on other methyltransferases (DNA and histone methyltransferases) did not show any strong binding of the m⁶A methylation activators to these enzymes, thus they are predicted to be very specific to METTL3/METTL14/WTAP protein complex. Two of these compounds were recently found to support the survival of mouse dopamine neurons in growth factor deprivation assay and to protect them from 6-OHDA toxicity. Importantly, CHMA1004 (methyl-piperazine-2-carboxylate) protected stem cell-derived human dopamine neurons against 6-OHDA-induced cell death. Furthermore, intrastriatal administration of CHMA1004 attenuated amphetamine-induced ipsilateral rotations after 6-OHDA treatment and increased dopamine neuron fibre density in the rat model of PD (Yu *et al.*, 2023). Given the massive arborisation of dopaminergic axons (Matsuda *et al.*, 2009), dopaminergic neural projections may be particularly sensitive to the epitranscriptomic regulation of mRNA translation that occurs in the axonal and dendritic ribosomes (Biever *et al.*, 2020).

Thus, taking into account the indirect evidence reviewed above on the role of RNA m⁶A methylation in anxiety and depression, and the implication of dopaminergic neurotransmission in these disorders that often are co-morbid with addictions (Rehm *et al.*, 2016), we aimed to examine the effect of the novel METTL3/METTL14 activator compound CHMA1004 in a battery of anxiolytic and antidepressant screening tests in the rat, its effect on emission of ultrasonic vocalisations indicative of positive and negative affect in the rat, and effects of acute amphetamine treatment. The effect of subchronic administration of CHMA1004 on monoamine neurochemistry in several brain regions and on gene expression in striatum, and its acute effect on dopamine overflow, were also examined.

Materials and methods

Animals

In the behavioural experiments, twenty-seven Wistar rats (15 female and 12 male; 3 months of age at the beginning of the experiment; body weight 290 ± 4 g and 499 ± 13 g, respectively; bred locally, parent rats obtained from Charles River) were given CHMA1004 (1 mg/kg or 5 mg/kg) dissolved in saline intraperitoneally (IP; 1 ml/kg) once in a day. Control group received vehicle (saline). Each group consisted of 5 female and 4 male rats. In the microdialysis experiment, 19 male and 16 female rats with the same background were used; their allocation to treatment groups is given in the Microdialysis section. Animals were housed 4–5 per cage in standard transparent polypropylene cages under controlled light cycle (lights on 08:00–20:00) and temperature (22°–24°C), with free access to water and food pellets (v1534–000 universal maintenance diet, ssniff Spezialdiäten GmbH, Soest,

Germany). The studies were approved by the Ethical Committee for Animal Experiments of the Estonian Ministry of Rural Affairs (permission no 149).

General procedure

The procedure is illustrated in Figure 1. Thirty minutes after the first IP administration of CHMA1004 or vehicle, an open field test was conducted with recording of ultrasonic vocalisations (USVs). A series of behavioural tests were performed in the course of daily drug treatment, while all behavioural tests were started 30 min after drug administration. After 17 or 18 days of daily drug treatment, rats were sacrificed 30 min after the last injection by decapitation, the brains dissected as previously described (Liiver *et al.*, 2023) and samples immediately frozen in liquid nitrogen. All procedures, except the sucrose preference test, occurred during the lights-on phase. Animals were transported 30 min before the start of testing to the room of behavioural experiments to habituate, and tested (within the sex group) in randomised order. All animal experiments were in compliance with the ARRIVE guidelines and were carried out in accordance with EU Directive 2010/63/EU for animal experiments; the experimental protocol was approved by the Animal Experimentation Committee at the Estonian Ministry of Rural Affairs.

Open field test

Open field test was carried out in a 0.5 × 1.0 m metal box with side walls 40 cm high (Tõnissaar *et al.*, 2008). The area was divided into eight squares of equal size. Time spent moving around was recorded, and square crossings and rearing in 4 min were counted, as was the number of faecal boli left on the open field. The apparatus was cleaned with laboratory tissue dampened with water after each animal.

USVs

The microphone of an ultrasound recording device (Avisoft Ultra Sound Gate 116–200, Avisoft Bioacoustics, Berlin, Germany) was located about 30 cm from the floor of the apparatus, recording USVs with a sampling rate of 300 kHz in 16-bit format on a computer hard drive (Kõiv *et al.*, 2021). The files were recorded with Avisoft-RECORDER software and later analysed with Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) software. Spectrograms were created using the Fast Fourier Transform algorithm (1024 FFT length, 75% frame, Hamming window, and 75% time window overlap). USVs were manually marked and saved on the spectrogram by an observer blind to experimental conditions. Fifty kHz USVs with frequencies over 35 kHz, including flat, frequency-modulated and trill calls) and 22-kHz (<35 kHz) USVs were counted.

Elevated zero-maze

The elevated zero-maze test (Shepherd *et al.*, 1994) was conducted as previously described (Matto *et al.*, 1997). An elevated annular platform (outer Ø 105 cm, width 10 cm, 72 cm above the floor) was equally divided into two opposing enclosed quadrants (height of the walls 28 cm) that were connected by open quadrants (height of the edge 1 cm, 5–11 Lx in the open part). The open quadrants were also divided into three equidistant parts to quantify the locomotor activity. The rat was placed at the outset of one of the closed quadrants and was observed at a distance for 5 min. The measures taken were stretched-attend postures toward the open quadrants,

latency to enter the open quadrant with all four paws (s), entries into the open quadrants, and time spent (s) in the open quadrants. The apparatus was cleaned with moist tissue after every rat.

Social interaction test

The social interaction test introduced by Sandra File (reviewed by File and Hyde, 1978) was conducted as described previously (Tõnissaar *et al.*, 2004) with the variance that animals were not single-housed. Two unfamiliar, weight-matched rats were placed in the opposite corners of a brightly lit chamber (30 × 30 × 60 cm) with floor covered with wood shavings. The behaviour of the animals was concurrently observed for 10 min and the total time spent in active social behaviour (allogrooming, sniffing the partner, crawling under and over, following) was recorded.

Forced swimming test

The forced swimming test (Porsolt *et al.*, 1978) was carried out as described previously (Mällo *et al.*, 2009). On the first day of the experiment, the procedure lasted 15 min and the re-exposure 24 h later lasted 5 min. The measurements were based on the behavioural categories described by Armario *et al.* (1988); the first 5 min period of the first day was counted. A rat was judged to be immobile when it remained floating in the water with all limbs motionless or made minimal movements in order to maintain its head above the water and to struggle whenever it made intense movements of all the four limbs with the two front paws breaking the surface of the water or touching the walls of the tank. The time spent in swimming represents the rat making active swimming motions, more than necessary to merely maintain its head above water.

Amphetamine treatment effect

D-Amphetamine (1 mg/kg IP) was dissolved in saline and administered 15 min prior to the animal being placed in a standard group-housing cage (55 × 33 cm) for assessment of stimulant-induced locomotor response and ultrasonic vocalisation (Kõiv *et al.*, 2016). Horizontal locomotion was assessed from a 12 min video by counting line crossings with four paws of the boundaries of six equal squares on the cage floor, rearings were counted when the animal stood on hind legs with the body at least at a 45° angle. Data were analysed by 2 min bins.

Locomotion and ultrasound vocalisations in a novel home-cage-like environment

Measurement of USVs and spontaneous horizontal and vertical locomotion was conducted in a standard novel home-cage-like box (for 8 min) with a similar protocol as previously described for amphetamine-induced behavioural measurements.

Measurement of sucrose intake and preference

The sucrose preference test was carried out during the dark phase with no previous food or water deprivation (Kõiv *et al.*, 2019). The animals were placed into single cages 1 h before the sucrose preference testing and group-housed again after that. Food was available during sucrose preference test. Two bottles were used, one filled with 1% sucrose solution and the other with water. Liquid consumption was measured by weighing previously weighed sucrose solution and water bottles 1h and 12 h after the beginning of the sucrose preference test.

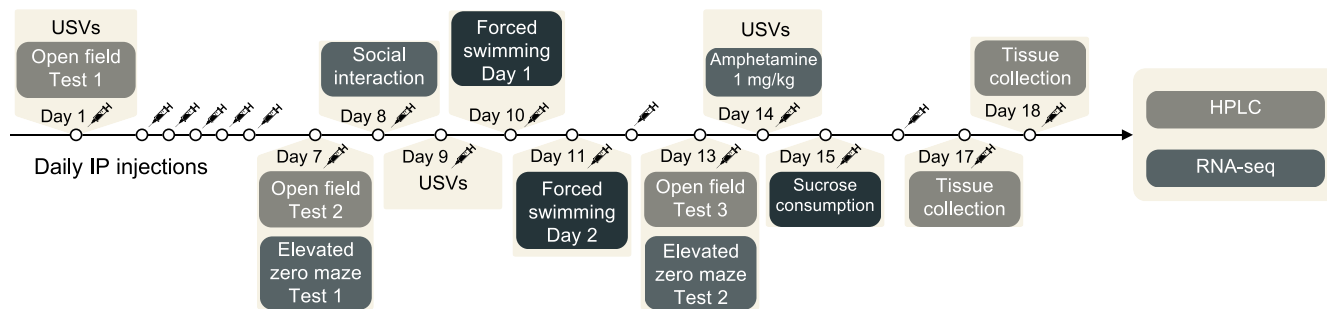


Fig. 1. Experimental design of behavioural and *ex vivo* biochemical studies of the effect of acute and subchronic treatment with CHMA1004. Note: HPLC, high-performance liquid chromatography; IP, intraperitoneal; RNA-seq, RNA sequencing; USVs, recording of ultrasonic vocalisation.

High-performance liquid chromatography (HPLC) in *ex vivo* studies

Monoamines and their metabolites were assayed by HPLC with electrochemical (amperometric) detection as described previously (Köiv et al., 2019). Rat brain tissues were homogenised with an ultrasonic homogeniser (Bandelin Sonopuls, Bandelin Electronic, Berlin, Germany) in ice-cold solution of 0.1 M perchloric acid containing 5 mM of sodium bisulphite and 0.4 mM EDTA to avoid oxidation. The homogenate was then centrifuged at 14 000 r.p.m. for 10 min at 4°C. Aliquots (10 µl) of the supernatant obtained were chromatographed on a Luna C18(2) column (150 mm × 2 mm, 5 µm). The separation was done in isocratic elution mode at column temperature of 30°C, using the mobile phase containing 0.05 M sodium citrate buffer at pH 3.7, 0.02 mM EDTA, 1 mM KCl, 1 mM sodium octanesulphonate and 7.5% acetonitrile. The two used chromatography systems consisted of an isocratic pump (HP1100, Agilent, Waldbronn, Germany or Shimadzu LC-20AD, Japan), a temperature-regulated autosampler (HP1100 or Shimadzu SIL-20AC), a temperature-regulated column compartment and an HP 1049 electrochemical detector (Agilent, Waldbronn, Germany) with a glassy carbon electrode (HP flow cell or Antec VT-03 flow cell). The measurements were done at an electrode potential of +0.7 V versus the Ag/AgCl reference electrode. The limits of detection at signal-to-noise ratio = 3 were as follows (expressed as pmol/mg tissue for each): 0.08 for dopamine (DA), 0.10 for homovanillic acid (HVA), 0.05 for 3,4-dihydroxyphenylacetic acid (DOPAC), 0.08 for serotonin (5-HT), 0.04 for 5-hydroxyindoleacetic acid (5-HIAA), 0.07 for noradrenaline (NA), 0.03 for normetanephrine (NMN), and 0.01 for 3-methoxytyramine (3-MT).

RNA extraction and mRNA sequencing

RNA was extracted from female rat striatal samples using FavorPrep™ Tissue Total RNA Mini Kit (FATRK 001-2, Favorgen, Taiwan) according to the manufacturer's protocol. Briefly, the FARB lysis buffer, containing β-mercaptoethanol, was added to the tissue sample, grinding was performed with a micropestle followed by homogenisation through a 20-G needle syringe. The lysate was cleared by using the filter column followed by addition of one volume of 70% ethanol. The sample was then transferred to the FARB Mini Column, washed and dried by centrifugation at 18,000 g for three minutes. Total RNA was eluted in 30 µl of nuclease-free water, concentration was measured by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at -80°C.

Sequencing was performed by Novogene Co., Ltd (Beijing, China). Quality control and preprocessing of the fastq files, containing raw reads from Illumina 150bp paired end sequencing, was conducted with fastp v0.23.2 using default parameters (Chen S et al., 2018). Strand-specific alignment to the rat rn7 genome was carried out using HISAT2 v2.2.1. (paired end, Strand-FR). FeatureCounts v1.6.4 (Liao et al., 2014) was used for transcript counting using *Rattus norvegicus.mRatBN7.2.108.gtf* annotation from Ensembl database as a reference (Martin et al., 2023). Differential gene expression between pairwise groups was performed with DESeq2 v2.11.40.6 (Love et al., 2014) using poscounts method for size factor estimation. Genes with *p*-value smaller than 0.05 were considered differentially expressed. SynGO portal was used for ID conversion (Koopmans et al., 2019). GO enrichment analysis was performed with DAVID 2021 (Sherman et al., 2022). Two samples were excluded from the subsequent analysis because of low alignment rate (<5%) to the reference rn7 genome, and one sample was considered as an outlier by visual inspection of PCA plot and omitted from subsequent analysis; consequently, further analysis included 4 rats per group. Volcano plot was created using R ggplot2-based tool in Galaxy (Version 0.0.3) using output from DESeq2.

Microdialysis experiments

In vivo microdialysis was carried out to assess the effect of acute treatment with CHMA1004 on extracellular levels of dopamine in striatum, essentially as previously described (O'Leary et al., 2016). The animals were anaesthetised with ketamine/medetomidine anaesthesia (60 mg/kg and 0.5 mg/kg IP, respectively) and mounted in a Stoelting stereotaxic frame. A self-made concentric Y-shaped microdialysis probe with 7 mm shaft length and 3 mm active tip was implanted into the left dorsal striatum according to the following coordinates: AP +0.7; ML +3.0; DV -7.0, according to Paxinos and Watson (1986). The dialysis membrane used was polyacrylonitrile/sodium methyl sulphonate copolymer (Filtral 12; i.d.: 0.22 mm; o.d.: 0.31 mm; AN 69, Hospal, Bologna, Italy). Two stainless steel screws and dental cement were used to fix the probe to the skull. After the surgery, anaesthesia was reversed by administration of atipamezole (1 mg/kg, SC) and meloxicam (1–2 mg/kg, SC) was administered for post-operative analgesia. The rats were placed in 21 cm × 36 cm × 18 cm individual cages in which they remained throughout the microdialysis experiment. Rats were given about 24 h for recovery and microdialysis procedure was conducted in awake freely moving animals. The microdialysis probe was connected via FEP tubing (i.d. 0.12 mm, AgnTho's AB, Lidingö, Sweden) to a syringe pump (SP101, World Precision Instruments, Inc., Sarasota,

FL, USA) and to a refrigerated micro-sampler (Univentor 820, Univentor Limited, BLB029 Bulebel Industrial Estate, Zejtun ZTN 3000, MALTA) and perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄; pH 7.20–7.22) at a constant rate of 1.5 µl/min. After connecting the animal to the microdialysis system, the perfusate was discarded during the first 60 min to allow stabilisation. Six baseline samples were collected, followed by an i.p. injection of either saline (male rats; $n = 2$), CHMA1004 1 mg/kg (male, $n = 6$; female, $n = 6$), CHMA1004 5 mg/kg (male, $n = 6$; female, $n = 6$) or amphetamine 0.5 mg/kg (male, $n = 4$; female, $n = 4$), after which another 12 samples were collected. All samples were collected at 15 min intervals. Sample vials were pre-filled with 7.5 µl of 0.02 M acetic acid to prevent oxidation of dopamine. The quantity of dopamine and serotonin in the microdialysis samples was determined by high-performance liquid chromatography with electrochemical detection. The chromatography system consisted of a Shimadzu LC-10AD pump and CBM-20A controller (Shimadzu Corporation, Kyoto, Japan), a Luna C18(2) 5 µm column (150 mm × 2 mm) kept at 30°C and Decade II digital electrochemical amperometric detector (Antec Leyden BV, the Netherlands) with electrochemical flow cell VT-03 (2 mm GC WE, ISAAC reference electrode, Antec Leyden BV, the Netherlands). The mobile phase consisted of 0.05 M sodium citrate buffered to pH 5.3, 2 mM KCl, 0.02 mM EDTA, 3.5 mM sodium octyl sulphate and 14% acetonitrile. The mobile phase was pumped through the column at a rate of 0.2 ml/min. 5-HT and DA eluted from the column were measured with a glassy carbon working electrode maintained at a potential of +0.4 V versus Ag/AgCl reference electrode. Data were acquired using a Shimadzu LC Solution system.

Data analysis

Behavioural and neurochemical data were first analysed by two-factor ANOVA (Sex and Treatment) with repeated measures (Test) if appropriate. Because the apparent dose dependency of the effect of CHMA1004 was in some tests different between sexes, this was followed by one-way ANOVA (CHMA1004) with male and female dosage group pooled or separate if the interaction term was closely missing $p < 0.05$. Owing to small group sizes and apparent difference in dose-response between male and female animals, subsequent pairwise comparisons were made by Fisher's least significant difference test. Significance threshold was set at 0.05. Statistical analysis was made using the IBM SPSS Statistics 26 software.

Results

Body weight and weight of adrenals

Body weight gain was similar in all treatment groups. After the 18-day treatment with CHMA1004, no statistically significant effect was found on adrenal weight (data not shown).

Effect of acute and repeated administration of CHMA1004 on open field behaviour

Acute treatment with CHMA1004 increased time spent in active movement on the open field (Figure 2); in female rats, this effect was statistically significant at 1 mg/kg and still observable after repeated treatment (Supplementary Fig. 1) despite being smaller as the activity became lower (Treatment × Sex $F_{2,21} = 3.49$; $p < 0.05$; Test $F_{2,42} = 33.5$; $p < 0.001$). This increase of activity was statistically significant only in female rats while at the dose of

5 mg/kg a similar tendency appeared in males. Similar group differences were reflected in rearings (Treatment $F_{2,21} = 4.27$; $p < 0.05$; Sex $F_{1,21} = 7.96$; $p < 0.05$; Treatment × Sex $F_{1,21} = 3.42$; $p = 0.052$; Test $F_{2,42} = 10.3$; $p < 0.001$), and in the number of line crossings in which case the tendency of increase in males at the dose of 5 mg/kg was the closest to statistical significance (Treatment $F_{2,21} = 3.37$; $p = 0.054$; Sex $F_{1,21} = 6.88$; $p < 0.05$; Treatment × Sex $F_{2,21} = 4.59$; $p < 0.05$; Test $F_{2,42} = 11.3$; $p < 0.001$). The number of faecal boli left on the open field at the first test, a rather sensitive anxiety measure, was larger in male rats (Figure 2), and this was reduced by CHMA1004 treatment (Sex $F_{1,21} = 21.4$; $p < 0.001$; Treatment $F_{1,21} = 3.46$; $p = 0.05$; Treatment × Sex $F_{2,42} = 4.78$; $p < 0.05$). Thus, CHMA1004 treatment elicited anxiolytic- or psychostimulant-like locomotor activation, especially in female rats, and reduced emotionality in male rats as expressed in mitigation of defecation. Acute CHMA1004 treatment had no statistically significant effect on 50-kHz USVs (Figure 2). Only one rat produced 22-kHz USVs, suggestive that neither testing nor the drug was perceived as anxiogenic. Emission of 50-kHz USVs was also low, with a tendency of more vocalisation after CHMA1004 treatment. In conclusion, acute administration of CHMA1004 was without strong negative or positive emotional valence.

Effect of repeated administration of CHMA1004 on behaviour in elevated zero-maze

Time spent in the open sections of the elevated zero-maze was longer on the second test (Test $F_{1,20} = 16.7$; $p < 0.001$), especially in females. CHMA1004 treatment had no statistically significant effect while the measure tended to be increased (Figure 3). Repeated CHMA1004 treatment increased the number of head-dips at the first zero-maze test (CHMA1004 $F_{2,20} = 5.35$; $p < 0.05$); this effect was more prominent in female rats. The number of entries into the open section was significantly higher at the second testing (Test $F_{1,20} = 18.6$; $p < 0.001$), and was significantly increased in male rats of the CHMA1004 5 mg/kg group as compared to the control at the second elevated zero-maze test (Sex × Test $F_{1,20} = 5.77$; $p < 0.05$). Stretched-attend postures were reduced at the second test (Test $F_{1,20} = 19.3$; $p < 0.001$), however the male CHMA1004 5 mg/kg treatment group had this measure reduced already at the first elevated zero-maze test (Treatment × Sex × Test $F_{2,20} = 3.09$; $p = 0.067$; CHMA1004 $F_{2,20} = 8.03$; $p < 0.01$). In sum, CHMA1004 had an anxiolytic-like effect after repeated administration.

Effect of repeated administration of CHMA1004 in social interaction test

In rats housed in groups, social interaction with an animal from another cage was very low (40–50s on average) in female rats, and was reduced in the male CHMA1004 5 mg/kg group to the level comparable to females (Sex $F_{1,20} = 27.4$; $p < 0.001$; Treatment × Sex $F_{2,20} = 3.33$; $p = 0.057$; data not shown).

Effect of repeated administration of CHMA1004 on novelty-induced USVs

Not a single rat produced any 22-kHz USVs during this test, but 50-kHz USVs were emitted by all animals and frequently by most of them. Two-way ANOVA revealed no statistically significant Treatment effect but a significant Sex effect ($F_{1,21} = 8.08$; $p < 0.01$). Male control rats had higher 50-kHz emission than

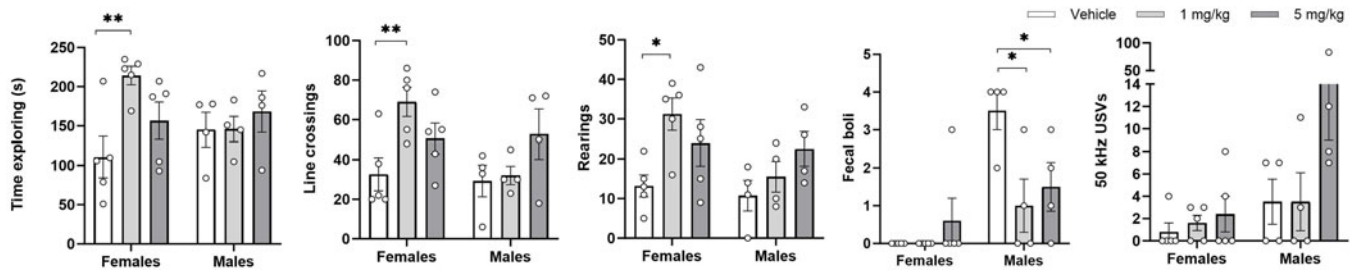


Fig. 2. Effect of acute administration of CHMA1004 on locomotion, defecation and emission of ultrasonic vocalisations in an open field test. Note: * $p < 0.05$; ** $p < 0.01$ versus vehicle. Post hoc test on rearings in male rats CHMA1004 (5 mg/kg) $p = 0.069$.

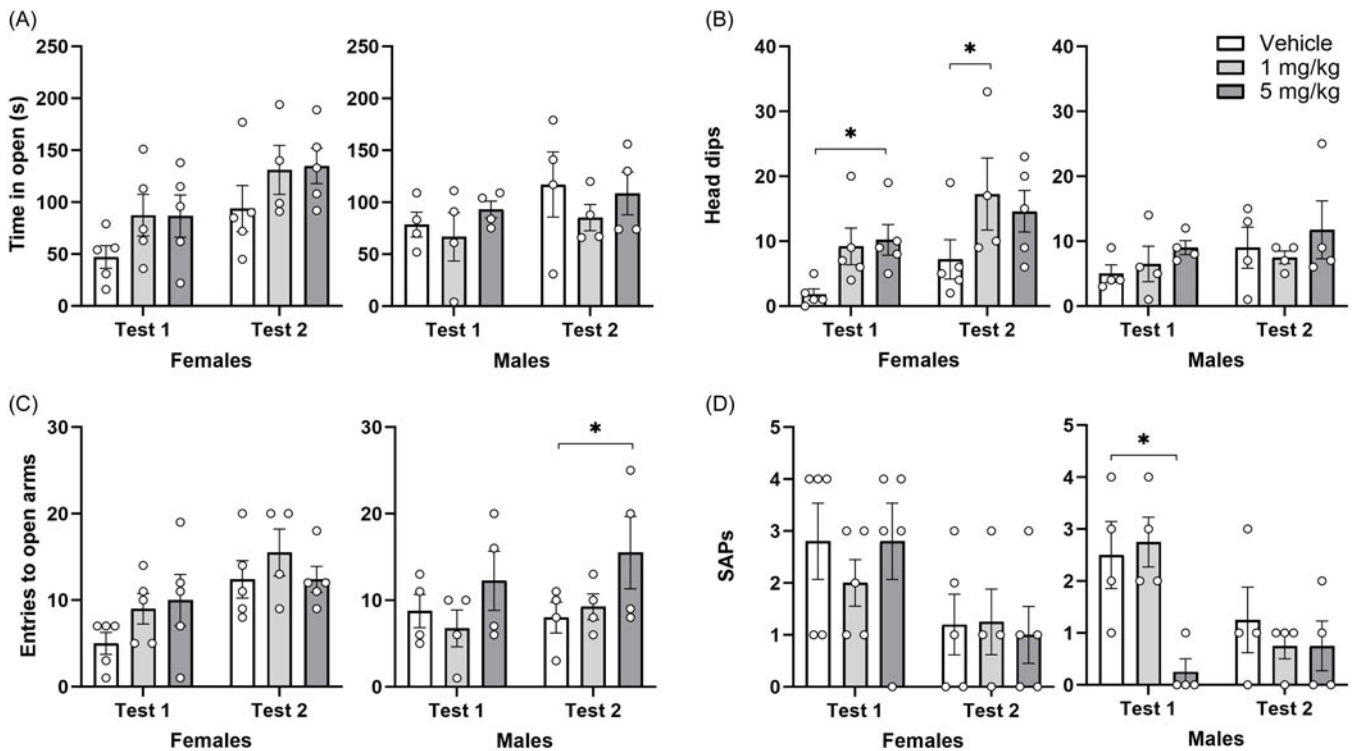


Fig. 3. Effect of repeated administration of CHMA1004 in elevated zero-maze test. Note: SAPs, number of stretched-attend postures; * $p < 0.05$ versus vehicle.

female controls, but CHMA1004 treatment tended to reduce this to the level of females (Supplementary Fig. 2).

Effect of repeated administration of CHMA1004 in the forced swimming test

In this screening test for antidepressant potential, CHMA1004 treatment had no significant effect on immobility (Figure 4), but increased swimming (Treatment F $2,21 = 3.75$; $p < 0.05$; Test F $1,21 = 40.6$; $p < 0.001$; Sex \times Test F $1,21 = 16.5$; $p < 0.001$) and reduced struggling (Test F $1,21 = 13.8$; $p < 0.01$; Sex \times Test F $1,21 = 11.9$; $p < 0.01$) in female rats on both days of forced swimming, at both dose levels.

Effect of repeated administration of CHMA1004 on effects of amphetamine

This was studied in order to assess the potential of RNA m⁶A methylation to cause sensitisation to psychostimulant action.

After acute administration of amphetamine (1 mg/kg) female rats made more rearings than males (Supplementary Fig. 3A), especially during the latter part of the test (Sex F $1,21 = 27.8$; $p < 0.001$; Test F $5,105 = 26.4$; $p < 0.001$; Sex \times Test F $1,21 = 2.88$; $p < 0.05$), for horizontal activity the differences were not statistically significant. In male rats, locomotion tended to be higher in the CHMA1004 (5 mg/kg) group, but this effect was not statistically significant (Supplementary Fig. 3AB). After treatment with amphetamine, only three male rats and no female rat emitted 22-kHz USVs, but as expected the emission of 50-kHz USVs was robust (Supplementary Fig. 3C). Female rats produced more 50-kHz USVs than males, but this was dependent on CHMA1004 treatment (Sex F $1,21 = 9.17$; $p < 0.01$; Treatment \times Sex F $2,21 = 3.52$; $p < 0.05$). Thus, in male rats subchronic CHMA1004 treatment tended to increase the emission of 50-kHz USVs, but in females this treatment reduced vocalisation, so that the large male-female difference in control groups was much smaller in CHMA1004 treatment groups.

Effect of repeated administration of CHMA1004 on sucrose intake and preference

CHMA1004 treatment did not statistically significantly affect sucrose intake or preference (data not shown), but if intake of 11 h was adjusted to body weight, an increase was found in female rats of the CHMA1004, significantly in the 1 mg/kg group, as compared to controls (Supplementary Fig. 4; Sex $F_{1,21} = 31.2$; $p < 0.001$).

Effect of repeated administration of CHMA1004 on monoamine neurochemistry

Subchronic treatment with most psychotropic medications causes adaptive changes in one or several monoamine systems, and indeed CHMA1004 affected the levels of monoamine neurotransmitters and their metabolites in several brain regions (Table 1). In striatum, CHMA1004 treatment increased the levels of NMN (Treatment $F_{2,21} = 4.30$; $p < 0.05$; Sex $F_{1,21} = 3.41$; $p < 0.05$) in females and dopamine turnover in males (Treatment \times Sex $F_{2,21} = 4.44$; $p < 0.05$). Frontal cortical NMN levels were increased by subchronic treatment with CHMA1004 in males (Treatment $F_{2,21} = 4.94$; $p < 0.05$; Treatment \times Sex $F_{2,21} = 4.73$; $p < 0.05$). Similar effect on noradrenaline levels closely missed the conventional level of significance (Treatment $F_{2,21} = 3.40$; $p = 0.0525$), and both NA and NMN tended to be higher also in females at the lower dose of CHMA1004. In locus coeruleus, CHMA1004 treatment reduced HVA levels in female rats, while the effect was opposite in males (Treatment \times Sex $F_{2,21} = 4.66$; $p < 0.05$). Ventral tegmental area was also dose-dependently affected by CHMA1004 treatment: Levels of noradrenaline and HVA were higher after 1 mg/kg (Treatment $F_{2,21} = 5.22$ and 4.21 , respectively; $p < 0.05$; post hoc tests not significant owing to small groups by sex and large male-female differences), and 5-HIAA levels would be significantly affected in the same manner if one outlier in the vehicle group were excluded (Treatment $F_{2,20} = 6.42$; $p < 0.01$). In hippocampus and in the raphe area, two-way ANOVA did not reveal any statistically significant treatment effect.

Extracellular levels of dopamine in striatum

To assess whether METTL3/14 activation could be triggering dopamine release, we conducted a separate microdialysis experiment. As expected, acute administration of saline had no effect of striatal dopamine levels, while amphetamine (0.5 mg/kg) increased dopamine release in male ($F_{51,187} = 6.88$; $p < 0.001$) and female ($F_{34,170} = 5.58$; $p < 0.001$) rats. CHMA1004 at either dose did not elicit any significant effect on extracellular dopamine levels in striatum (Figure 5).

Effect of subchronic administration of CHMA1004 on striatal gene expression

Because it is not known which epitranscriptomic changes should be expected to be most responsive to METTL3/METTL14 activation, a hypothesis-free approach, RNA sequencing was performed. Differential expression analysis resulted in statistically significant change (Wald test $p < 0.05$) of 172 and 120 genes following daily 1 mg/kg and 5 mg/kg of CHMA1004, respectively (Supplementary Table 1). Of these genes, seven were overlapping (Galns, Hps3, Misp3, Nbl1, Samd4a, Slc28a2, Spon2), with similar change in expression. Among the genes that were affected with the highest statistical significance (Figure 6), most were of specific interest, for example *Acsms5*, *Peli1*, *Pcdhb5*, *Psmb9*, *Fgr*, *Lrp10*,

Grb83, *Hes1*, *Vwa5b2*, *Mx2*, *Serpinf2*, *Nbl1* and *Mdga1*; of other differentially expressed genes, *Nxph3* would deserve particular attention (see Discussion). Pathway analyses (Supplementary Table 2) suggested the involvement of, for example, phospholipase C-activating G-protein coupled receptor signalling, positive regulation of phosphatidylinositol 3-kinase signalling metabolic pathways, cell adhesion, and mitochondrial matrix in the action of CHMA1004.

Discussion

This study revealed that acute and subchronic administration of a novel METTL3/METTL14 activator compound CHMA1004 can elicit activating and anxiolytic-like effects in the rat. Subchronic treatment with CHMA1004 affected monoaminergic neurotransmission in the brainstem and forebrain areas. These effects were region- and dose-dependent and not all identical in male and female animals, but in general were suggestive of an increase in catecholamine function. Subchronic CHMA1004 treatment also elicited many alterations in striatal gene expression that are relevant to stress response.

Acute treatment effect of CHMA1004 was assessed in an open-field test. Increased locomotor activity that was observed can be indicative of anxiolytic action, but also of a psychostimulant effect. Similarly, lower levels of locomotion do not necessarily reflect anxiety, and in the rat, ultrasonic vocalisation provides the most reliable index of strong activation of emotion circuits (Panksepp, 1998). Therefore, we simultaneously recorded USVs, as classic psychostimulants typically elicit 50-kHz USVs (Simola *et al.*, 2010; Köiv *et al.*, 2019; Wöhr, 2022). No strong psychostimulant effect was observed, suggesting that the increased locomotor activity was reflective of either reduced anxiety or a non-dopaminergic psychostimulant effect. On the other hand, rats did not produce 22-kHz USVs. Previously, administration of neuropeptide S that also displays mixed anxiolytic/psychostimulant profile in exploration-based tests (Xu *et al.*, 2004; Rizzi *et al.*, 2008) was found to elicit substantial 22-kHz vocalisation revealing predominantly negative emotional response behind behavioural activation (Köiv *et al.*, 2021). CHMA1004 did, in contrast, display an anxiolytic-like and stimulatory effect without any significant affective response in terms of USV production. That the effect of acute administration of CHMA1004 was anxiolytic, was further suggested by the reduction of defecations on open field in male rats. Previously, acute restraint stress was shown to rapidly downregulate *Mettl3* expression in the prefrontal cortex and amygdala of mice (Engel *et al.*, 2018). Altogether the facts that METTL3/METTL14 activating compound CHMA1004 can elicit an anxiolytic-like activating effect in conditions of novelty stress suggest that RNA m⁶A methylation is involved in positive stress response.

Repeated treatment with CHMA1004 did not appear to produce significant tolerance to its anxiolytic-like/activating effect. The anxiolytic effect of CHMA1004 was further substantiated by results of the elevated zero-maze test where stretched-attend postures were reduced, open quadrant entries and head-dips increased, which is not a profile of a psychostimulant effect. At the first glance, the tendency of decrease in social interaction time in male rats may appear as contradictory to an anxiolytic effect, but the test was not conducted as validated for this purpose: We could not use single housing in this experiment because this would sensitise animals to stressful stimulation. It is likely that at this locomotor activating dose CHMA1004 increased exploration of the novel physical environment on account of social behaviour.

Table 1. Monoamine levels after two and half weeks of administration of the METTL3/METTL14 activating compound CHMA1004 in male and female rats

	NA	NMN	NMN/NA	DA	DOPAC	DOPAC/DA	3-MT	HVA	5-HT	5-HIAA	5-HIAA/5-HT
Striatum											
<i>Males</i>											
Vehicle	4.80 ± 0.74	5.79 ± 0.33	1.29 ± 0.20	75.17 ± 7.47	6.34 ± 0.75	0.08 ± 0.002	4.60 ± 0.59	3.69 ± 0.34	5.84 ± 0.23	2.51 ± 0.10	0.43 ± 0.01
CHMA1004 1 mg/kg	4.79 ± 0.64	5.11 ± 0.21	1.15 ± 0.23	73.31 ± 8.26	6.66 ± 0.80	0.09 ± 0.001	4.29 ± 0.30	3.78 ± 0.41	5.78 ± 0.28	2.33 ± 0.13	0.40 ± 0.02
CHMA1004 5 mg/kg	6.04 ± 0.92	6.19 ± 0.28 [#]	1.09 ± 0.15	76.56 ± 7.12	7.78 ± 0.47	0.10 ± 0.01*	4.68 ± 0.20	4.03 ± 0.34	5.86 ± 0.54	2.53 ± 0.20	0.43 ± 0.03
<i>Females</i>											
Vehicle	5.52 ± 0.40	5.83 ± 0.33	1.07 ± 0.06	71.75 ± 5.65	7.33 ± 0.76	0.10 ± 0.01	4.02 ± 0.19	4.45 ± 0.23	5.89 ± 1.29	2.74 ± 0.18	0.55 ± 0.10
CHMA1004 1 mg/kg	5.72 ± 0.45	6.32 ± 0.38	1.12 ± 0.06	79.27 ± 7.03	6.66 ± 0.74	0.08 ± 0.01*	4.05 ± 0.36	4.65 ± 0.54	5.60 ± 0.54	2.67 ± 0.17	0.49 ± 0.03
CHMA1004 5 mg/kg	6.49 ± 0.71	7.08 ± 0.40*	1.13 ± 0.10	86.64 ± 6.87	7.82 ± 0.90	0.09 ± 0.01	4.12 ± 0.33	5.21 ± 0.42	6.61 ± 0.84	3.19 ± 0.30	0.50 ± 0.04
Frontal cortex											
<i>Males</i>											
Vehicle	6.56 ± 0.63	4.46 ± 0.31	0.68 ± 0.02	0.35 ± 0.05			0.28 ± 0.04	0.16 ± 0.03	3.07 ± 0.12	1.65 ± 0.18	0.53 ± 0.04
CHMA1004 1 mg/kg	7.94 ± 0.72	6.03 ± 0.44*	0.76 ± 0.03	0.33 ± 0.04			0.34 ± 0.10	0.20 ± 0.03	3.27 ± 0.11	1.50 ± 0.10	0.46 ± 0.03
CHMA1004 5 mg/kg	8.04 ± 0.18*	6.57 ± 0.36**	0.82 ± 0.05	0.37 ± 0.05			0.27 ± 0.04	0.20 ± 0.04	3.21 ± 0.10	1.73 ± 0.16	0.54 ± 0.06
<i>Females</i>											
Vehicle	7.48 ± 0.11	6.10 ± 0.38	0.82 ± 0.05	0.37 ± 0.03			0.33 ± 0.08	0.20 ± 0.04	3.75 ± 0.22	1.65 ± 0.10	0.45 ± 0.04
CHMA1004 1 mg/kg	8.44 ± 0.48	6.94 ± 0.45	0.84 ± 0.09	0.36 ± 0.04			0.42 ± 0.06	0.21 ± 0.02	3.31 ± 0.19	1.54 ± 0.08	0.47 ± 0.02
CHMA1004 5 mg/kg	7.50 ± 0.39	5.82 ± 0.39 [#]	0.78 ± 0.04	0.37 ± 0.04			0.22 ± 0.004	0.23 ± 0.02	3.53 ± 0.11	2.03 ± 0.40	0.57 ± 0.11
Hippocampus											
<i>Males</i>											
Vehicle	2.42 ± 0.36	0.11 ± 0.02	0.05 ± 0.01	0.21 ± 0.03	0.10 ± 0.01	0.48 ± 0.06		0.05 ± 0.01	2.17 ± 0.25	1.65 ± 0.18	0.77 ± 0.07
CHMA1004 1 mg/kg	2.28 ± 0.12	0.08 ± 0.02	0.03 ± 0.01	0.29 ± 0.09	0.10 ± 0.01	0.45 ± 0.12		0.05 ± 0.01	1.96 ± 0.13	1.43 ± 0.10	0.73 ± 0.05
CHMA1004 5 mg/kg	2.18 ± 0.11	0.09 ± 0.02	0.04 ± 0.01	0.18 ± 0.07	0.09 ± 0.02	0.59 ± 0.10		0.04 ± 0.01	2.36 ± 0.31	1.69 ± 0.19	0.78 ± 0.17
<i>Females</i>											
Vehicle	1.98 ± 0.16	0.07 ± 0.01	0.04 ± 0.01	0.27 ± 0.05	0.11 ± 0.01	0.44 ± 0.06		0.06 ± 0.01	1.92 ± 0.17	1.59 ± 0.06	0.86 ± 0.10
CHMA1004 1 mg/kg	2.09 ± 0.18	0.07 ± 0.02	0.04 ± 0.01	0.59 ± 0.19	0.18 ± 0.04	0.38 ± 0.10		0.07 ± 0.02	1.95 ± 0.14	1.69 ± 0.05	0.88 ± 0.05
CHMA1004 5 mg/kg	2.20 ± 0.08	0.07 ± 0.02	0.03 ± 0.01	0.28 ± 0.06	0.11 ± 0.02	0.44 ± 0.07		0.07 ± 0.02	1.87 ± 0.07	1.77 ± 0.11	0.95 ± 0.06
Locus coeruleus											
<i>Males</i>											
Vehicle	13.26 ± 1.45	9.00 ± 0.49	0.71 ± 0.11	0.68 ± 0.03				0.37 ± 0.05	5.65 ± 0.96	4.25 ± 0.86	0.76 ± 0.08
CHMA1004 1 mg/kg	17.87 ± 2.44	10.80 ± 0.72	0.63 ± 0.07	1.12 ± 0.35				1.34 ± 0.06*	5.68 ± 0.95	3.82 ± 0.64	0.68 ± 0.03
CHMA1004 5 mg/kg	17.85 ± 2.72	12.50 ± 3.01	0.70 ± 0.13	0.94 ± 0.29				0.95 ± 0.29	6.91 ± 2.17	4.08 ± 1.27	0.59 ± 0.03

Table 1. (Continued)

<i>Females</i>											
Vehicle	14.53 ± 1.39	11.45 ± 1.94	0.78 ± 0.11	1.20 ± 0.12				0.98 ± 0.18	9.73 ± 1.65	6.38 ± 1.07	0.66 ± 0.04
CHMA1004 1 mg/kg	14.79 ± 1.30	8.82 ± 1.11	0.61 ± 0.10	0.79 ± 0.05				0.47 ± 0.03*	5.97 ± 0.53	3.55 ± 0.61	0.58 ± 0.07
CHMA1004 5 mg/kg	14.26 ± 1.45	10.52 ± 0.63	0.77 ± 0.09	0.78 ± 0.19				0.48 ± 0.04*	7.20 ± 0.61	4.30 ± 0.46	0.60 ± 0.06
Raphe											
<i>Males</i>											
Vehicle	6.38 ± 0.31	0.18 ± 0.06	0.03 ± 0.01	1.71 ± 0.19	0.51 ± 0.06	0.30 ± 0.02		0.62 ± 0.09	6.36 ± 0.68	7.71 ± 0.59	1.27 ± 0.21
CHMA1004 1 mg/kg	5.82 ± 0.52	0.12 ± 0.03	0.02 ± 0.005	1.39 ± 0.09	0.38 ± 0.03	0.27 ± 0.01		0.53 ± 0.08	6.49 ± 0.22	6.58 ± 0.21	1.02 ± 0.05
CHMA1004 5 mg/kg	6.22 ± 0.73	0.16 ± 0.05	0.03 ± 0.01	1.43 ± 0.32	0.42 ± 0.11	0.29 ± 0.02		0.39 ± 0.10	6.01 ± 1.15	6.90 ± 0.96	1.21 ± 0.14
<i>Females</i>											
Vehicle	5.52 ± 1.07	0.20 ± 0.05	0.04 ± 0.01	1.74 ± 0.42	0.45 ± 0.08	0.28 ± 0.04		0.45 ± 0.10	6.62 ± 0.89	6.34 ± 0.81	0.96 ± 0.05
CHMA1004 1 mg/kg	6.09 ± 0.15	0.15 ± 0.03	0.03 ± 0.005	1.48 ± 0.19	0.40 ± 0.04	0.27 ± 0.02		0.47 ± 0.05	7.16 ± 0.25	7.04 ± 0.77	0.98 ± 0.08
CHMA1004 5 mg/kg	6.11 ± 0.23	0.14 ± 0.03	0.02 ± 0.01	1.25 ± 0.07	0.41 ± 0.07	0.33 ± 0.05		0.42 ± 0.04	7.32 ± 0.31	7.64 ± 0.30	1.05 ± 0.05
Ventral tegmental area											
<i>Males</i>											
Vehicle	4.50 ± 0.86	0.12 ± 0.02	0.03 ± 0.005	2.55 ± 1.22	0.71 ± 0.36	0.31 ± 0.08	0.42 ± 0.15	0.82 ± 0.27	6.66 ± 1.08	3.93 ± 0.93	0.61 ± 0.11
CHMA1004 1 mg/kg	8.70 ± 1.89 ^a	0.35 ± 0.13	0.03 ± 0.003	4.51 ± 1.48	1.06 ± 0.29	0.26 ± 0.03	1.07 ± 0.93	1.53 ± 0.32	7.14 ± 1.29	5.95 ± 0.66	0.69 ± 0.14
CHMA1004 5 mg/kg	6.26 ± 2.18	0.34 ± 0.24	0.03 ± 0.007	1.78 ± 0.50	0.85 ± 0.29	0.63 ± 0.28	0.48 ± 0.32	0.93 ± 0.42	8.09 ± 1.58	3.80 ± 0.11	0.60 ± 0.14
<i>Females</i>											
Vehicle	5.28 ± 0.41	0.06 ± 0.01	0.60 ± 0.42	3.71 ± 1.43	0.71 ± 0.27	0.19 ± 0.01	0.39 ± 0.13	0.83 ± 0.15	6.81 ± 1.28	6.10 ± 1.60	1.12 ± 0.49
CHMA1004 1 mg/kg	8.02 ± 1.54	0.21 ± 0.12	0.89 ± 0.86	6.16 ± 1.38	1.45 ± 0.36	0.23 ± 0.01	1.03 ± 0.53	1.65 ± 0.39	10.62 ± 2.26	6.21 ± 0.84	0.64 ± 0.07
CHMA1004 5 mg/kg	4.79 ± 0.61	0.08 ± 0.02	0.74 ± 0.45	3.75 ± 1.15	1.04 ± 0.26	0.38 ± 0.17	0.67 ± 0.46	0.79 ± 0.17 [#]	6.38 ± 1.36	4.08 ± 0.50	0.69 ± 0.08

Monoamine levels are expressed as pmol/mg tissue. * $p < 0.05$; ** $p < 0.01$ versus vehicle; [#] $p < 0.05$ versus CHMA1004 (1 mg/kg); ^a $p = 0.055$ versus vehicle.

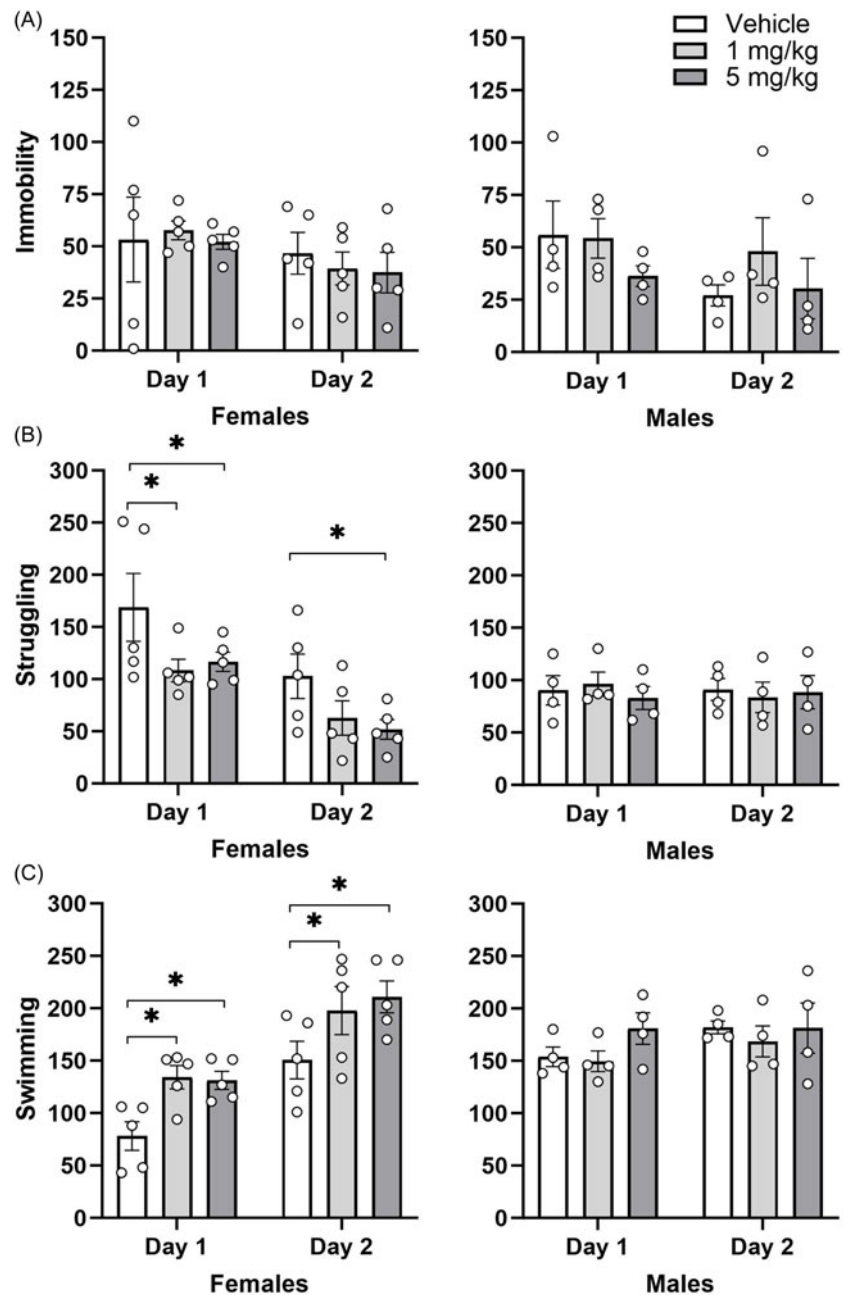


Fig. 4. Effect of repeated administration of CHMA1004 in the forced swimming test. Note: * $p < 0.05$ versus vehicle.

Similar is the effect of amphetamine in the social interaction test (File and Hyde, 1979; unpublished results from this laboratory).

The forced swimming test has been the preferred screening tool for novel drugs with antidepressant potential, while the interpretation of results with this test has been subject to multiple interpretations (e.g. Harro, 2019). Subchronic CHMA1004 treatment did not affect immobility but increased swimming and reduced struggling in female rats. The characteristic response to the first generation antidepressant drugs such as tricyclics is reduction of immobility (Porsolt *et al.*, 1978) while the selective serotonin reuptake inhibitors increase swimming (Armario *et al.*, 1988). It should be noted that in the forced swimming test used as a screening tool mostly acute drug treatment after the first swimming session is applied. In the present study, the drug had been repeatedly administered before the test and immobility was relatively low in these repeatedly handled rats. As expected,

swimming increased and struggling decreased when CHMA1004 treated animals were submitted to forced swimming the second time. Previous studies applying chronic stress or partial serotonergic denervation have suggested that a behavioural activation that is present already at the first swimming session is indicative of higher expression of impulsivity (Harro *et al.*, 2001). Thus, the present results could best be interpreted as subchronic CHMA1004 treatment presenting an impulsivity-reducing effect. This possibility should be examined further in behavioural tests specifically targeting different aspects of impulsivity (Dalley and Robbins, 2017). In another test of antidepressant prediction, the sucrose preference test, sucrose preference was not affected but consumption as adjusted to body weight was increased by CHMA1004 treatment in females. Again, the test is validated in conditions of chronic stress-induced reduction of sucrose intake, and as part of the present test, battery informs little about potential

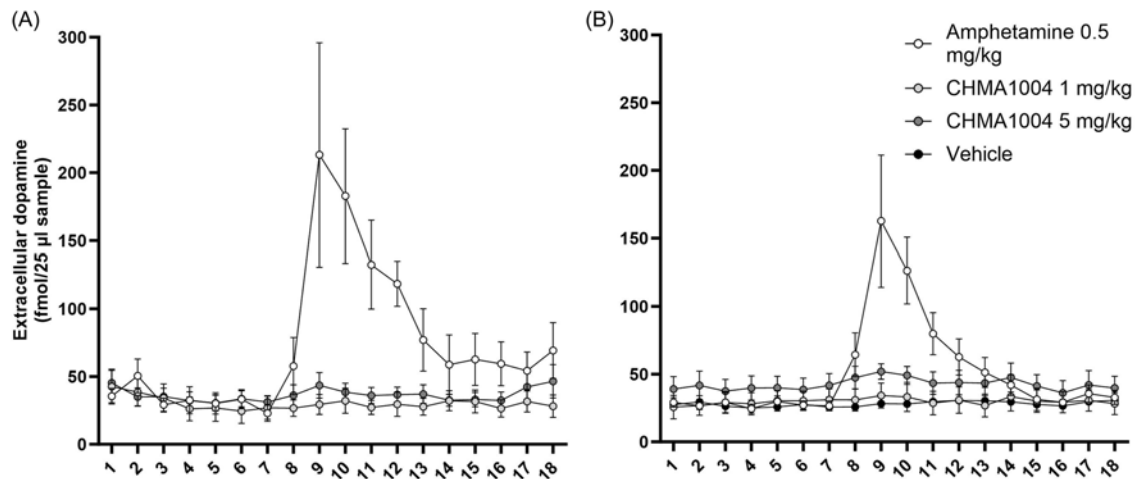


Fig. 5. Extracellular levels of dopamine after acute administration of amphetamine or CHMA1004. Note: A: female rats; B: male rats.

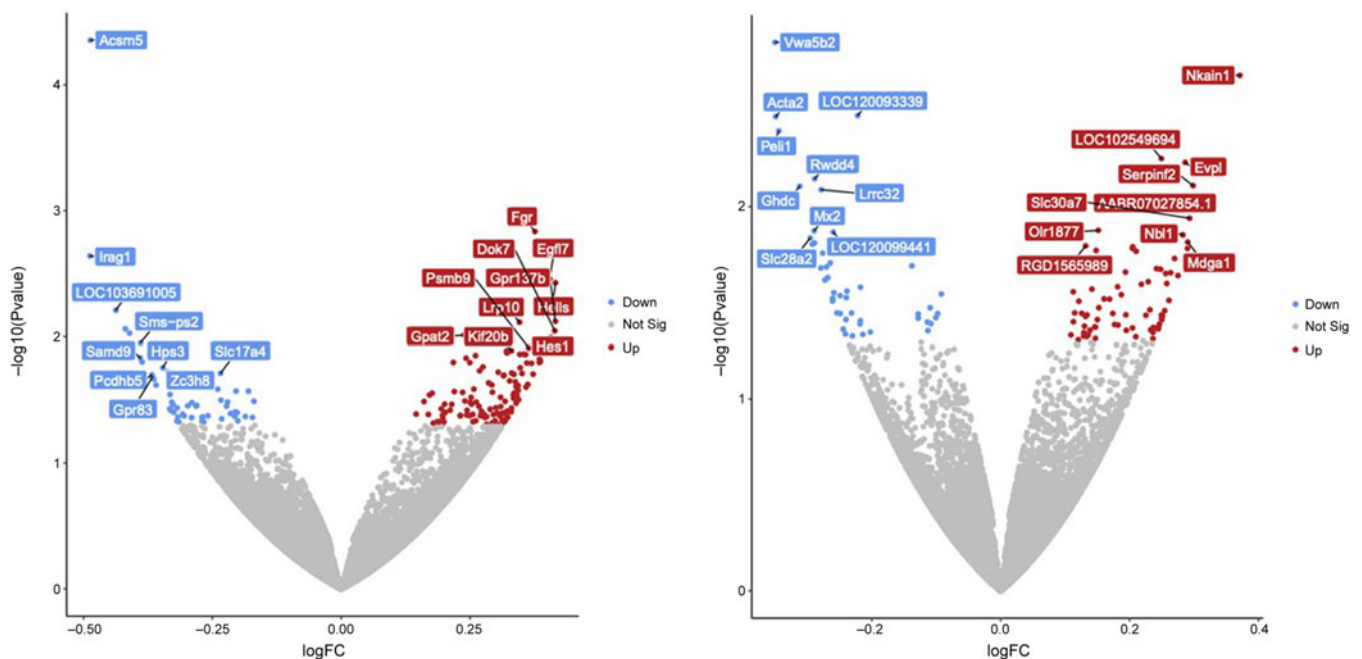


Fig. 6. Effect of repeated administration of CHMA1004 on striatal gene expression in female rats. Note: Volcano plots of the differentially expressed gene analysis with DESeq2 between control and CHMA1004 1 mg/kg (left) or 5 mg/kg (right). Significantly differentially expressed genes ($p < 0.05$) are labelled blue (downregulated genes) or red (upregulated genes). The names of the top 10 genes with the lowest p -values are shown.

antidepressant effect but suggests that subchronic CHMA1004 treatment is not stressful/anhedonic and rather may increase the appetitive value of carbohydrates. In this context it is also relevant that long-term treatment with CHMA1004 did not increase adrenal weight, again suggesting that this treatment was not strongly stressful; indeed, it may be important to notice that at the doses that were behaviourally most effective (1 mg/kg in females and 5 mg/kg in males, respectively), a tendency of reduction of adrenal weight appeared.

Fifty kHz USVs are indicative of positive affect in the rat (Burgdorf *et al.*, 2001; Brudzynski and Granon, 2023), and in adult animals, the most powerful stimulus to elicit this response is psychostimulant drugs such as amphetamine. Novel home-cage-like environment can also elicit the 50-kHz response. Subchronic

CHMA1004 treatment did not modify the effect of either novel cage or amphetamine treatment significantly, but statistical power to detect its effect may have been too low, because substantial differences between male and female control groups appeared. A general limitation in this study is the number of animals of each sex was small. This was to reduce the overall number of rats used and simultaneously provide comparison of male and female animals. It was not foreseen that dose dependency varies between males and females, and the significantly larger body weight of males may have been a contributory factor to this. Nevertheless, it can be concluded that CHMA1004 did not elicit substantial psychostimulant-like effects or produced sensitisation on its own. CHMA1004 treatment enhanced locomotor activity in response to amphetamine challenge in male rats, but their activity remained below of

females. Altogether the potential of CHMA1004 to increase amphetamine-like stimulant responses appeared low.

Recent studies have highlighted the fact that dysregulation of m⁶A mRNA methylation occurs in neurodegeneration, and dopamine neurons are dependent on proper regulation of m⁶A mRNA methylation (Chen *et al.*, 2019). Inhibition of m⁶A RNA demethylases Fto and Alkbh5, which results in increased m⁶A levels, can support the survival of dopamine neurons (Selberg *et al.*, 2021a). Furthermore, the METTL3/METTL14 activating compound CHMA1004 was also found to support survival of cultured dopamine neurons that were deprived of growth factors and to protect neurons from 6-OHDA toxicity (Yu *et al.*, 2023). Importantly, intrastriatal administration of CHMA1004 improved motor behaviour and increased tyrosine hydroxylase positive fibre density in mice after 6-OHDA lesion; it also protected human cultured dopamine neurons in that study. Hence, we measured monoamine levels *ex vivo* in striatum after long-term administration of CHMA1004, and having observed alterations in catecholamines, performed further measurements in a few other brain regions. The most prominent effect of subchronic treatment with CHMA1004 was observed in VTA at the lower dose level. Dopaminergic measures tended to be altered also in the other brainstem regions and several alterations were observed in measures of catecholaminergic neurotransmission, mostly increasing with dose. These results suggest a complex reorganisation of monoamine systems by a METTL3/METTL14 activating drug, possibly primarily in the dopamine neurons. Acute CHMA1004 treatment did however not produce any increase in striatal extracellular dopamine levels, as might have been suggested by its behaviourally stimulatory effects, which is consistent with no increase in 50-kHz USVs after either acute or subchronic CHMA1004 treatment.

Most of proposed pathogenetic mechanisms of depression, relating to neurogenesis, activity of the HPA axis, glial function, neuroinflammation and synaptic transmission have all been associated with mRNA m⁶A methylation (Li C *et al.*, 2023). One genetic association study could link a variance in the RNA m⁶A demethylase encoding gene *ALKBH5* with major depression in a large sample (Du *et al.*, 2015) and another associated with depression in an *FTO* variant (Samaan *et al.*, 2013). The biochemical pathways involved in the association of mRNA m⁶A methylation with anxiety or depression-related behavioural changes have however remained completely unknown. To extend knowledge beyond the monoamine systems and to analyse what mRNAs are regulated by METTL3/METTL14 activator CHMA1004 we also performed RNA sequencing in striatum, where CHMA1004 had been found acting on dopamine neurons *in vivo* (Yu *et al.*, 2023) and where the authors had observed an increase in *Mettl3* expression after an anxiety-reducing manipulation (unpublished). This was done in female rats. The low and high dose of CHMA1004 resulted in a different landscape of differential gene expression, suggesting that response to subchronic drug treatment involved dose-dependent adaptive changes that had also been apparent in behavioural and biochemical effects; however, expression of several genes was altered in a similar manner. Many effects of CHMA1004 on gene expression agree with what is known of m⁶A in neurodegeneration and affective disorders (see Introduction), and the capacity of the drug to protect dopamine neurons (Yu *et al.*, 2023) as well as its behavioural effects in the present study. For example, *Galns* and *Samd4a* have been implicated in neuronal viability and plasticity; *Galns* that encodes a lysosomal exohydrolase has been considered a gene related to

Parkinson's disease (Wettergren *et al.*, 2012) and *Samd4a* is a RNA binding protein that can bind to a variety of target mRNAs through stem-loop structures, also known as Smaug recognition elements (SREs). Notably, *Samd4a* can regulate the mRNA stability, degradation and translation (Wang and Zhang, 2023). *Fgr* (Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog) and *Psmb9* (proteasome subunit beta type-9), two innate immunity genes, both of which were affected by CHMA1004 treatment, each were recently associated with specific genetic forms of Parkinson's disease (Shani *et al.*, 2023). Another differentially expressed gene, *Lrp10* (low-density lipoprotein receptor-related protein 10), has similarly been linked to Parkinson's disease but also other neurodegenerative diseases, whereas its role has been attributed to astrocytic vesicle trafficking (Grochowska *et al.*, 2021), and its contribution to neurodegeneration suggested to be significant specifically in females (Guo *et al.*, 2023). CHMA1004 treatment elicited upregulation of several cognition-related genes such as *Acta2*, *Mx2*, *Spon2* and *Peli1*. *Peli1*, encoding Pellino E3 ubiquitin protein ligase 1, was recently shown to mediate methamphetamine-induced neuroinflammation whereas its downregulation was protective (Xu *et al.*, 2023); similarly, the reduction of *Peli1* function reduced the lipopolysaccharide-induced inflammatory response in microglia (Dai *et al.*, 2019). Given that neuroinflammatory mechanisms are set in the brain by stress (Black, 2002), the reduction of *Peli1* expression by CHMA1004 treatment may reflect a drug effect facilitating adaptive stress response to the experimental procedures. *Mx2*, an interferon-inducible GTP-binding protein, has been associated with the beneficial effect of physical exercise in a mouse model of Alzheimer's disease (Widjaya *et al.*, 2023), and *Spon2* that can induce neurite outgrowth with learning effects in monkeys (Matsunaga *et al.*, 2015). *Serpinf2* that encodes a serine protease inhibitor has a link to Alzheimer's disease (De Jager *et al.*, 2014), but was recently found as differentially expressed in a rat model of depression (Liao *et al.*, 2023). Depression-related is also *Mdga1*, encoding a cell adhesion molecule (Li *et al.*, 2022), as well as a few other differentially expressed genes. *Pcdhb5* or protocadherin beta-5 has been reported as a gene with altered expression in suicide victims (Thalmeier *et al.*, 2008). It belongs to the group of cadherin-like cell adhesion molecules and knowledge on its function is limited but likely to include the establishment of specific neural connections. *Nbl1*, a candidate neuroblastoma suppressor gene has also been associated with suicide (Han *et al.*, 2023).

In turn, regulation of *Vwa5b2* at the DNA methylation level has been related to lithium response in bipolar disorder (Marie-Claire *et al.*, 2020). Yet another prominent effect of CHMA1004 was downregulation of *Acsf5* that encodes a mitochondrial enzyme that catalyses the activation of fatty acids by coenzyme A. While very little is known of the brain function of *Acsf5*, it has been identified as a top attention-deficit/hyperactivity disorder (ADHD)-related gene in a bioinformatics transcriptome study (Qi *et al.*, 2019), and ADHD is a vulnerability factor for several other psychiatric disorders including anxiety disorders, depression and bipolar disorder (Chen Q *et al.*, 2018). A few other genes with substantially altered expression by CHMA1004 treatment have been linked to anxiety and stress response: expression of *Hes1*, an essential Notch signalling effector was associated with neuronal excitability in amygdala and with anxiety in the mouse (Matsuzaki *et al.*, 2019), and knockdown of *Gpr83*, a glucocorticoid-regulated receptor has been associated with anxiety in female mice and a decrease in receptor expression to dexamethasone treatment,

whereas anxiety was reduced in males (Fakira *et al.*, 2021). Indeed, a thorough study on the role of m⁶A methylation in stress response regulation reported that the response of m⁶A levels upon glucocorticoid receptor stimulation was altered in patients with major depression (Engel *et al.*, 2018). In that study, one of the most prominent alterations by knocking out the gene responsible for m⁶A methylation, *Mettl3*, was downregulation of *Nxph3*, encoding neurexophilin 3. The METTL3/METTL14 activator CHMA1004, in contrast, increased the expression of *Nxph3* in the present study. Neurexophilin 3 is a ligand of synaptic α -neurexins thus contributing to the regulation of neurotransmitter release, and it has a role in sensorimotor gating and motor coordination (Beglopoulos *et al.*, 2005).

The anxiolytic-like effects of a METTL3/METTL14 activating compound appear as inconsistent with findings that downregulation of *Fto* and increased m⁶A methylation can lead to anxiety- and depression-like behaviours in mice (Liu *et al.*, 2021; Wang XL *et al.*, 2022). In these studies, the FTO-related anxious behaviours were associated with mRNA methylation and expression, respectively, of β_2 -adrenoceptor in hippocampus or matrix metalloproteinase 9 (MMP-9) in anterior cingulate cortex. In our study, neither *Adrb2* nor MMP-9 expression was altered by CHMA1004 in striatum. While we did not examine the same brain regions, the contrasting behavioural findings suggest that METTL3/METTL14 activation by systemic administration of a small molecule compound affects RNA m⁶A methylation dissimilarly from downregulation of *Fto* by other type of interventions. Another study demonstrated that increased RNA m⁶A methylation of fatty acid amide hydrolase mRNA by reducing the function of ALKBH5 attenuated depressive-like behaviours (Huang *et al.*, 2020), which is in line with our findings and further suggests that some of the multiple mechanisms recruited in modifying RNA m⁶A methylation have a potential in the management of psychiatric disorders. Furthermore, treadmill exercise was shown to counteract the anxiety-provoking effects of chronic stress in an RNA m⁶A methylation-dependent manner (Yan *et al.*, 2023), providing support to the notion that METTL3/METTL14 activating drugs have anxiolytic and activating effects by promoting physiological mechanisms. As the next step, it is desirable to conduct transcriptomic analysis also in male rats, and RNA m⁶A sequencing to specify which transcriptomic changes correspond directly to drug effect at the epitranscriptomic levels.

Despite the study's several limitations (small number of observations and limited statistical power due to different dose-response profile in males and females; potential carry-over effects in a test battery), the overall pattern of results suggested that treatment with CHMA1004 can elicit a variety of sizeable behavioural changes. Conclusively, the first-in-class METTL3/METTL14 activator compound methyl-piperazine-2-carboxylate (CHMA1004) elicited anxiolytic-like effects after acute systemic administration, and its anxiolytic-like and activating properties were consistently observed in a battery of behavioural tests during subchronic treatment. While behaviourally activating, CHMA1004 did not exhibit strong psychostimulant-like properties. Subchronic administration of CHMA1004 increased locomotor activity and led to alterations in catecholaminergic neurotransmission and changes in striatal gene expression linked to dopamine neuron viability, neurodegeneration, depression, anxiety and stress response. Thus, pharmacological activation of RNA m⁶A methylation has potential for neuropsychiatric drug development.

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Author contribution. MKk, MKn, MS and JH designed the study; MK, KL, MŠ, IT, KL and JH collected the data; MK, KL, IT, TO and KL analysed the data, all authors revised paper critically for important intellectual content.

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Competing interests. MKn and JH are in the advisory board of Chemestmed Ltd that develops compounds that affect RNA m⁶A methylation. They are not paid for this role. Chemestmed did not provide support to the presented study except making the compound freely available. JH has received lecturer fees from Lundbeck. The authors declare no competing interests.

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