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Effects of DNA methyltransferase inhibition on pattern separation performance in mice

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ABSTRACT

Enhancement of synaptic plasticity through changes in neuronal gene expression is a prerequisite for improved cognitive performance. Moreover, several studies have shown that DNA methylation is able to affect the expression of (e.g. plasticity) genes that are important for several cognitive functions. In this study, the effect of the DNA methyltransferase (DNMT) inhibitor RG108 was assessed on object pattern separation (OPS) task in mice. In addition, its effect on the expression of target genes was monitored. Administration of RG108 before the test led to a short-lasting, dose-dependent increase in pattern separation memory that was not present anymore after 48 h. Furthermore, treatment with RG108 did not enhance long-term memory of the animals when tested after a 24 h inter-trial interval in the same task. At the transcriptomic level, acute treatment with RG108 was accompanied by increased expression of *Bdnf1*, while expression of *Bdnf4*, *Bdnf9*, *Gria1* and *Hdac2* was not altered within 1 h after treatment. Methylation analysis of 14 loci in the promoter region of *Bdnf1* revealed a counterintuitive increase in the levels of DNA methylation at three CpG sites. Taken together, these results indicate that acute administration of RG108 has a short-lasting pro-cognitive effect on object pattern separation that could be explained by increased *Bdnf1* expression. The observed increase in *Bdnf1* methylation suggests a complex interplay between *Bdnf* methylation-demethylation that promotes *Bdnf1* expression and associated cognitive performance. Considering that impaired pattern separation could constitute the underlying problem of a wide range of mental and cognitive disorders, pharmacological agents including DNA methylation inhibitors that improve pattern separation could be compelling targets for the treatment of these disorders. In that respect, future studies are needed in order to determine the effect of chronic administration of such agents.

1. Introduction

Pattern separation is the ability to make distinct representations from highly overlapping information, a process which is important for memory encoding (Clelland et al., 2009). For correct pattern separation, old information needs to be retrieved and compared to new information. If the information is similar, but not exactly the same, it needs to be stored separately (Kirwan & Stark, 2007). The concept of pattern separation was initially described from computational-neuronal models (Marr, Willshaw, & McNaughton, 1991) and only recently it has been shown to be an integral part of normal neuronal functioning (Clelland et al., 2009). In turn, the study of pattern separation gained more interest due to evidence indicating that pattern separation is one of the underlying cognitive processes that are impaired in neurodegenerative and psychiatric disorders, like anxiety (Kheirbek, Klemenhagen, Sahay, & Hen, 2012) and schizophrenia (Tamminga, Stan, & Wagner, 2010). It is suggested that impairments in pattern separation is an endophenotype of these disorders and being expressed as the inability of individuals to distinguish between similar daily cues, resulting in panic attacks and psychotic behavior (Das, Ivleva, Wagner, Stark, & Tamminga, 2014; Kheirbek et al., 2012; Mineka & Zinbarg, 2006; Tamminga et al., 2010). Taking this into account, pattern

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separation could be a promising test for future diagnosis and treatment of mental disorders.

Pattern separation is a process that takes place in the hippocampus and more specifically in the dentate gyrus (DG) and Cornu Ammonis region 3 (CA3) (Morris, Churchwell, Kesner, & Gilbert, 2012). The main source of input in the hippocampus is derived from the enthorinal cortex (EC) that mainly projects to the granule cells in the DG. From the DG the information is sent to the CA3, and from CA3 to Cornu Ammonis region 1 (CA1) (Myers & Scharfman, 2011). The process of forming distinct representations out of overlapping stimuli can only be accomplished because the DG granule cells have small place fields and can therefore disperse the input from the EC. Subsequently, the information is relayed to the CA3 region via the mossy fiber synapses (Kheirbek et al., 2012). Animal studies showed that pattern separation is based on two types of neuronal processing between the DG and CA3 (Leutgeb, 2008). The encoding of small differences at a given location takes place at the granule cells of DG. The CA3 region adds another level of pattern separation when the differences in a location are more pronounced, by activating different neuronal subpopulations (Leutgeb, Leutgeb, Moser, & Moser, 2007).

Learning and memory involving pattern separation requires changes in synaptic plasticity and associated neuronal gene expression (Feng et al., 2010), the latter of which has been shown to depend on epigenetic alterations (Feng, Fouse, & Fan, 2007). As such, the orchestrated action of DNA methylation and demethylation could define transcription of genes related to mnemonic processes. DNA methylation is controlled by DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl-group at CpG sites of DNA. Accordingly, a high degree of DNA methylation, especially at the promoter region of a gene, is often associated with reduced gene expression by preventing transcription factor binding (Watt & Molloy, 1988) or by recruitment of methyl-CpG binding domain (MBD) proteins. These proteins form a complex with histone deacetylases (HDACs), promoting histone tail deacetylation, which subsequently leads to a transformation of chromatin into a condensed, repressive state (Guoping & Hutnick, 2005). However, it remains to be elucidated whether such epigenetic mechanisms may directly affect pattern separation.

In the present study, we aimed to investigate the effect of the non-specific DNMT inhibitor RG108 on pattern separation performance and therefore provide first evidence regarding the influence of an epigenetic mechanism on pattern separation memory. Next, in order to get more insight into the effect of DNMT inhibition, we analyzed the hippocampal expression of relevant target genes for plasticity and memory function after treatment with RG108. The genes of interest were histone deacetylase 2 (*Hdac2*), brain-derived neurotrophic factor 1, 4 and 9 (*Bdnf1, 4* and *9*) and glutamate ionotropic receptor AMPA type subunit 1 (*Gria1*). We chose to determine the expression of *Hdac2* because -HDAC2 works in close concert with DNMT's, while the remaining genes were selected due to their association with memory function. Our results indicate that administration of RG108 increases the expression of *Bdnf1*, while the expression levels of the other genes remained unaltered. Finally, we opted to get a first indication whether the increase in *Bdnf1* expression is accompanied with differences in the methylation pattern, by analyzing the methylation levels of 14 CpG loci at its promoter region.

2. Materials & methods

2.1. Animals

For this study, 35 three-months-old male C57BL/6 mice were used (Charles River Laboratories International, Inc., Sulzfeld, Germany). The mice were housed individually on sawdust bedding in standard Tecniplast individually ventilated greenline cage at 21 °C ± 2 °C, with a humidity of 50% ± 10%, under reversed light/dark cycle conditions, meaning that lights were off from 7:00 to 19:00 in order to perform the behavioral task in the active period of the mice under low illumination (20 lx). Furthermore, background noise by a radio was maintained to accustom the mice to noise. Food and water were available *ad libitum*. All experimental procedures were approved by the local ethics committee of Maastricht University for animal experiments and met governmental guidelines.

2.2. Reagents

RG108 was commercially obtained from Tocris Bioscience (Bristol, UK). The compound was dissolved in sterile physiological saline (B. Braun Melsungen AG, Melsungen, Germany) to produce an injection volume of 4 ml/kg at four different concentrations: 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg and 3 mg/kg. The injection was given intraperitoneally (i.p.) thirty minutes before the first trial of the OPS task. The dose and the timing of the treatment were based on a previous study (Sales & Joca, 2016).

2.3. Object pattern separation (OPS) task

The OPS task assesses subtle differences in spatial pattern separation in rodents by utilizing the smallest distance at which the mice are able to distinguish between the original and the novel location of an object during the choice trial. The task was performed as described previously (Van Hagen, Van Goethem, Lagatta, & Prickaerts, 2015). For the test, we utilized a circular arena made of polyvinyl chloride (PVC) with a diameter and height of 40 cm. The one half of the arena that was facing the experimenter was made of transparent PVC, whereas the other half was colored white. Two different types of objects were used in order to prevent familiarization, namely two massive aluminum cubes with tapering tops (4.5 cm × 4.5 cm × 8.5 cm), or two massive metal cubes (2.5 cm × 5 cm × 7.5 cm) with two holes (diameter 1.5 cm). Both set of objects could not be moved by the mice.

The OPS task was divided in two trials in which animals were allowed to freely explore two identical objects. The objects were oriented in the same way during the whole experiment. Exploration was defined as pointing the nose to the object at a distance of no more than 1 cm and/or touching the object. Sitting on or leaning to an object was not considered to be exploratory behavior. Before the start of the experiment, mice were handled daily as described elsewhere for two weeks by the experimenter and were put in the arena to habituate them with the environment and the exprerimental proceduce (van Goethem, van Hagen, & Prickaerts, 2018). Prior to the first (T1) and second trial (T2), the natural exploratory behavior of the mice was stimulated by placing them in an empty cage for four minutes. After these four minutes, the mice were placed in the arena, facing the middle of the transparent half. During T1, they had four minutes to explore the two identical objects placed on a horizontal line in the middle of the arena. After a predetermined interval in their home cage, the animals were put back into the arena for T2 that also lasted four minutes. For T2, one of the two objects was displaced along a vertical axis in a position shown to be appropriate for investigating pattern separation mechanisms (Van Hagen et al., 2015). The left and right objects were randomly displaced to avoid place preference bias. The time that the animals spent exploring the objects during T1 and T2 was recorded manually using a computer.

Four direct measures were obtained: a1, a2, a3, and b. a1 and a2 indicate how much time the mice spent exploring each object location during T1, whereas a3 and b indicate the time spent on the stationary and the displaced object during T2, respectively. From these four measures the variables e1, e2 and d2 were calculated. e1 and e2 are the total exploration time of both objects in T1 (e1 = a1 + a2) and T2 (e2 = a3 + b), respectively. The d2 index is the ratio of the difference in exploration time between the stationary and the moved object in T2 divided by the total exploration in T2 (d2 = b − a3/b + a3), which makes this cognitive measure independent of possible changes in

exploration (Akkerman, Prickaerts, Steinbusch, & Blokland, 2012). A minimum amount of exploration, i.e. ten seconds, in both trials was used as a cutoff in order to reliable assess pattern separation (Akkerman et al., 2012; Şık, van Nieuwehuyzen, Prickaerts, & Blokland, 2003). If an animal scored below that threshold in one or both trials it was excluded from the analysis.

2.4. Drug administration

In order to determine the pro-cognitive effect of RG108 administration, animals were randomly divided in three experimental groups (vehicle, 0.1 mg/kg RG108, 0.3 mg/kg RG108) and tested with 1 h interval between the trials. Vehicle treatment was repeated in all animals after 48 h to test a possible carry-over effect. Finally, we examined whether an acute dose of RG108 had an effect on long-term memory. For that purpose, animals were allocated in five experimental groups (vehicle, 0.1 mg/kg RG108, 0.3 mg/kg RG108, 1 mg/kg RG108, 3 mg/kg RG108) and tested using a 24 h inter-trial interval. Considering that each animal was tested repeatedly, a 48 h wash-out period was implemented between testing drug conditions. Additionally, the mice were allocated in the groups in such a way to avoid repeated testing of the same condition in each mouse. The experimenter was always blind to the drug conditions that were tested.

For the gene expression analysis the same cohort of animals was injected with vehicle, 0.1 mg/kg RG108 and 0.3 mg/kg RG108 thirty minutes prior to T1 and sacrificed thirty minutes after T1. The animals were decapitated, their brains were excised and the hippocampus was dissected, immediately snap-frozen in liquid nitrogen and stored at −80 °C. As previously mentioned, the animals were sacrificed thirty minutes after T1, while for the behavioral study the animals were tested 1 h after T1. The rationale of sacrificing the mice at an earlier time point was to detect differences in gene expression that, via altered protein expression, could explain improved cognitive performance later on.

2.5. RNA isolation

RNA was isolated from the dorsal hippocampus using TRIzol® Reagent according to the manufacturer's protocol (Life Technologies, Carlsbad, California, USA). Briefly, 1 ml TRIzol® Reagent was added per 50–100 mg hippocampal tissue and the samples were homogenized with a Mini BeadBeater (Biospec products Inc, Bartlesville, Oklahoma, US) using 0.5 mm beads. After incubating the homogenized samples for five minutes at room temperature, 0.2 ml chloroform was added per 1 ml of TRIzol® Reagent and shaken for fifteen seconds, following incubation at room temperature for two minutes. Afterwards, the samples were centrifuged at 12,000g for fifteen minutes at room temperature. Finally, the aqueous phase was collected and 0.5 ml of 100% isopropanol per 1 ml TRIzol® Reagent was added. After an incubation of ten minutes at room temperature and centrifugation at 12,000g for ten minutes at 4 °C, the extracted RNA was washed with 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent. The sample was vortexed and centrifuged at 7500g for five minutes at 4 °C and the supernatant was discarded. The dry RNA pellet was re-suspended in Diethyl pyrocarbonate (DEPC) water following incubation at 55–60 °C for ten minutes. RNA concentration and quality was measured with a Nanodrop spectrophotometer ND-1000 (Isogen Life Science, De Meern, the Netherlands).

2.6. cDNA synthesis

The iScript™ cDNA Synthesis Kit was obtained from Bio-Rad Laboratories, Inc. (Hercules, California, USA) and cDNA was synthesized according to the manufacturer's protocol. In short, 4 µl 5 × iScript reaction mix was added to 1 µl iScript reverse transcriptase. RNA template and nuclease free water were added, so that the end volume added up to 20 µl and the mixture contained 750 ng RNA. Finally, the complete reaction mix was incubated for five minutes at room temperature, thirty minutes at 42 °C and five minutes at 85 °C. After the incubation, 10 µl DEPC water was added to end up with an approximate cDNA concentration of 25 ng/µl.

2.7. qPCR

The synthesized cDNA was used for TaqMan based qPCR assays. Gene expression was analyzed for *Hdac2, Bdnf1, 4, 9, Gria1* and three housekeeping genes (Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), Beta-2 microglobulin (*B2m*), TATA-binding protein (*Tbp*)). The reaction mixture for each well in the 96-wells plate contained 1 µl 20 × TaqMan assay, 10 µl 2 × Taqman Universal Master Mix II with Uracil N-Glycosylase (UNG) (Applied Biosytems, Foster City, California, USA), 8 µl DEPC water and 1 µl of the respective cDNA (2 µl for *Bdnf9*). All samples were pipetted and measured in duplicates.

All qPCR reactions were performed using the light cycler 480 (Roche, Basel, Switzerland) under the following conditions: one cycle at 50 °C for two minutes to incubate UNG, one pre-incubation cycle at 95 °C for ten minutes, 45 (55 for *Bdnf9*) amplification cycles at 95 °C for fifteen seconds, sixty seconds at 60 °C, one second at 72 °C and thirty seconds cooling at 40 °C. For all qPCR reactions, the following TaqMan Assay probe sets (ThermoFisher Scientific) were used: *Hdac2*, Mm00515108_m1; *Bdnf1*, Mm01334047_m1; *Bdnf4*, Mm01334042_m1; *Bdnf9*, Mm04230564_m1; *Gria1*, Mm00433753_m1; *Hprt*, Mm00446968_m1; *B2m*, Mm00437762_m1 and *Tbp*, Mm00446973_m1. Prior to qPCR analysis, primer efficiency was checked with standard cDNA (pooled from four samples) curve from 0.1 to 100 ng cDNA as well as with the predicted amplicon length that was checked by gel-electrophoresis. Two different software programs, Conversion_LC_480 and LinregPCR, were used to analyze the data.

2.8. DNA methylation analysis

DNA was extracted from dorsal hippocampus using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. Two amplicons within the CpG island of the promoter region of *Bdnf* Exon I were chosen for pyrosequensing analysis (Fig. 3A). PCR and sequencing primers (Supplementary Table 1) were designed with the PyroMark Assay Design 2.0. Software (Qiagen). Genomic DNA (500 ng) was bisulfite-converted using the EpiTect Fast Bisulfite Conversion kit (Qiagen) according to the manufacturer's instructions. PCR amplifications were performed using the PyroMark PCR kit (Qiagen) using the following conditions: initial denaturation for fifteen minutes at 94 °C, followed by 45 cycles for thirty seconds at 95 °C for denaturation, thirty seconds at 60 °C, thirty seconds at 72 °C for elongation and four minutes at 72 °C. The PCR products were purified using a streptavidin sepharose High Performance beads (34 µm, GE Healthcare) and the PyroMark Q96 Vacuum Workstation (Qiagen) according to the manufacturer's instructions. PCR templates were sequenced on the PyroMark Q96 ID (Qiagen) using the PyroMark Gold Q96 CDT Reagents kit (Qiagen). Pyrosequencing results were analyzed using the PyroMark CpG software (Qiagen). As control, we used in all experiments, unmethylated (0%), 50% methylated, fully methylated (100%) and no-template controls in the bisulfite-conversion. Samples that did not pass quality control test were repeated or excluded.

2.9. Statistics

For the experiments with drug administration, one-way ANOVAs followed by post-hoc Dunnett's tests were performed in order to detect significant differences between experimental groups. Additionally, one-sample Student's t-tests were performed for assessing whether the d2 index, for each experimental condition separately, differed significantly from zero.

Table 1
Mean values (± SEM) of the different OPS measures after 1 h interval.

Dose RG108	e1 (s)	e2 (s)	d2	N
Vehicle	39.93 (5.33)	54.44 (7.90)	0.19 (0.06) #	11
0.1 mg/kg	38.74 (5.31)	47.47 (4.88)	0.32 (0.05) ###	12
0.3 mg/kg	40.24 (4.58)	57.87 (4.04)	0.46 (0.05) ###	12

The table displays the mean exploration times in T1 (e1) and T2 (e2) and the discrimination performance (d2) in the different treatment conditions. The SEM is presented between brackets. One sample t-tests were performed on the d2 measures to reveal significant differences from zero. A significant difference from zero (one-sample *t*-test; indicated by hashes; #: $p < 0.05$, ###: $p < 0.001$) indicates that the animals were able to discriminate the location of the object from T1.

For the qPCR study, all samples were normalized with the value of the standard (100 ng/ml) and corrected for the geomean of the three housekeeping genes. Treatment effect was evaluated for each gene by using one-way ANOVA with a previous outlier correction. Outliers were tested with Dixon's Q test (Dixon, 1959a, 1959b). When the overall ANOVA was significant, LSD post-hoc analysis was performed.

Regarding the methylation of the CpG islands, we performed one-way ANOVA for each CpG site followed by Dunnett's post-hoc test to determine significant differences between the treatment groups. The relationship between percentage of DNA methylation and expression of *Bdnf1* gene was analyzed by calculating the Pearson correlation coefficient (r_p) next to linear regression for calculating the best fitted line and confidence intervals. Statistical significance was adjusted for multiple comparisons by Bonferroni's correction.

3. Results

3.1. Acute administration of the DNMT inhibitor RG108 improves mice performance in the OPS tested after 1 h inter-trial interval

Table 1 summarizes the results after 1 h inter-trial interval in the OPS per treatment condition. There were no differences in exploration times between treatment conditions for both T1 ($F_{2,32} = 0.025$, n.s.) and T2 ($F_{2,32} = 0.884$, n.s.). As expected, since the mice were tested after a short retention interval (Fig. 1A), the d2 values of all the conditions differ significantly from chance level (vehicle: $p = 0.015$; 0.1 mg/kg RG108: $p = 0.0001$; 0.3 mg/kg RG108: $p < 0.0001$; Table 1).

One-way ANOVA demonstrates a significant effect of drug treatment on d2 ($F_{2,32} = 6.188$; $p = 0.005$). Dunnett's post-hoc analysis revealed that a single injection with 0.3 mg/kg RG108 improves significantly memory performance compared to control ($p = 0.003$; Fig. 1D). This indicates that the d2 value increases dose-dependently, with the highest dose of 0.3 mg/kg showing a significant pro-cognitive effect in comparison to the vehicle.

3.2. Acute administration of RG108 does not affect mice performance in the OPS tested after 48 h

In order to examine a possible carry-over effect of the drug, mice were tested again in the OPS 48 h after the initial acute administration of the drug. To have similar testing conditions as before, all the animals received a vehicle injection prior to T1 and tested in T2 after 1 h inter-trial interval (Fig. 1B). Exploration levels did not differ significantly between groups for both T1 ($F_{2,32} = 0.368$, n.s.) and T2 ($F_{2,32} = 1.22$, n.s.). In addition, all groups remembered the object's location, as confirmed by one sample t-tests (vehicle: $p = 0.0001$; 0.1 mg/kg RG108: $p = 0.0015$; 0.3 mg/kg RG108: $p < 0.0001$; Table 2). Finally, one-way ANOVA showed no difference in d2 values between the groups ($F_{2,32} = 0.503$, n.s.; Fig. 1E), indicating that the effect of the treatment is absent after 48 h.

3.3. Acute administration of RG108 does not affect long-term memory of mice tested after 24 h inter-trial interval in the OPS

In order to test whether acute administration of RG108 could improve long-term memory, mice were injected with either vehicle or one of four different concentrations of RG108 (0.1 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg) and pattern separation performance was measured after an interval of 24 h (Fig. 1C). The exploration time of the different groups did not differ in both trials (T1: $F_{4,53} = 0{,}187$, n.s.; T2: $F_{4,53} = 2.203$, n.s.) and there was no difference in the discrimination index as compared to zero (Table 3). The latter indicates that the treatment did not improve OPS performance after a long 24 h inter-trial interval (Fig. 1F).

3.4. Effect of acute treatment with RG108 on gene expression of target genes

In order to investigate whether treatment with the DNMT inhibitor alters expression of target genes, mice received acute treatment with vehicle or 0.1 mg/kg RG108 or 0.3 mg/kg RG108 thirty minutes prior to T1 and sacrificed 1 h later. Expression of the target genes *Gria1*, *Hdac2*, *Bdnf1*, *Bdnf4* and *Bdnf9*, as well as, the expression of three housekeeping genes was analyzed in the dorsal hippocampus by qPCR. As expected, the housekeeping genes do not show any significant differences in mRNA expression (data not shown). Although *Gria1*, *Hdac2*, *Bdnf4* and *Bdnf9* do not show significant differences in mRNA expression between the treated groups (*Gria1*: $F_{2,29} = 0.724$; n.s., *Hdac2*: $F_{2,30} = 0.971$; n.s., *Bdnf4*: $F_{2,31} = 1.059$; n.s., *Bdnf9*: $F_{2,26} = 0.039$; n.s.), treatment with 0.1 mg/kg RG108 and 0.3 mg/kg RG108 increased the expression of *Bdnf1*, when compared to vehicle ($F_{2,27} = 3.435$; $p = 0.047$; LSD post-hoc: $p = 0.022$ and $p = 0.042$, respectively; Fig. 2).

3.5. Effect of acute treatment with RG108 on methylation pattern of Bdnf1 promoter region

Considering that treatment with both doses increased *Bdnf1* expression, we sought to determine whether this increase is related to changes in the DNA methylation pattern at the promoter of exon I in the *Bdnf* gene. Although mice were treated with a DNMT inhibitor, we measured an increase in certain CpG sites in the promoter I. Specifically, one-way ANOVA showed a significant effect of RG108 administration on the DNA methylation level at CpG2 ($F_{2,29} = 4.993$, $p = 0.014$), CpG3 ($F_{2,27} = 3.439$, $p = 0.047$) and CpG6 ($F_{2,27} = 6.127$, $p = 0.006$). Dunnett's post hoc analysis showed that treatment with 0.1 mg/kg RG108 increased methylation in all the above CpGs ($p = 0.037$, $p = 0.039$, $p = 0.003$, respectively), while treatment with 0.3 mg/kg RG108 promoted methylation only for the CpG2 ($p = 0.011$). In contrast, the methylation status of the other CpGs tested was not different in the experimental groups as revealed by one-way ANOVA (CpG4: $F_{2,31} = 3.439$, n.s.; CpG5: $F_{2,27} = 1.628$, n.s.; CpG7: $F_{2,29} = 1.458$, n.s.; CpG10: $F_{2,62} = 0.924$, n.s.; CpG11: $F_{2,62} = 0.284$, n.s.; CpG12: $F_{2,62} = 0.955$, n.s.; CpG13: $F_{2,62} = 1.141$, n.s.; CpG23: $F_{2,31} = 1.984$, n.s.; CpG24: $F_{2,31} = 1.177$, n.s.; CpG25: $F2_{,31} = 0.603$, n.s.; CpG27: $F_{2,31} = 0.153$, n.s.; Fig. 3B).

As methylation levels were increased in three CpG sites of *Bdnf1*, we performed Pearson correlation analysis to determine possible correlation with the observed increase in *Bdnf1* expression. In the vehicle-treated animals, the percentage of methylation in CpG2 and 3 showed significant negative correlation with *Bdnf1* expression (CpG2: $r_p = -0.857$, $p = 0.021$; CpG3: $r_p = -0.951$, $p = 0.003$; Fig. 4A-B), while a trend was observed for CpG6 ($r_p = -0.872$, $p = 0.071$; Fig. 4C). These results indicate that a decrease in methylation is related to increased *Bdnf1* expression as could be expected. Treatment with 0.1 mg/kg RG108 showed no significant correlation between methylation levels and *Bdnf1* expression (CpG2: $r_p = 0.123$, n.s.; CpG3: $r_p = 0.299$, n.s.; CpG6: $r_p = 0.263$, n.s.; Fig. 4D-F). After treatment with 0.3 mg/kg

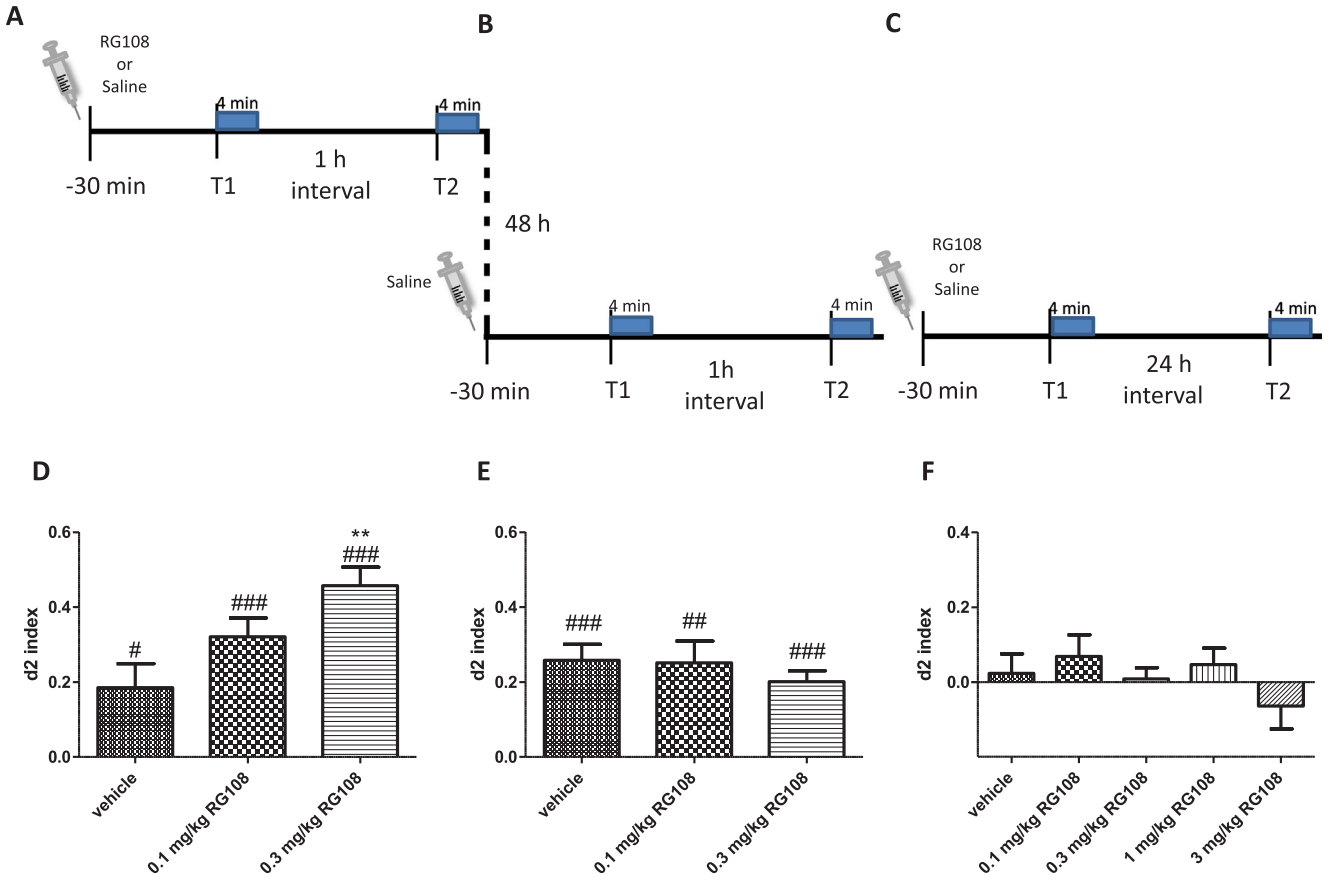

Fig. 1. The effect of RG108 treatment (i.p.) on mice performance in OPS with different inter-trial intervals. (A) In order to determine the effect of RG108 in short-term memory, animals were treated thirty minutes prior to T1 with T2 taking place after 1 h interval. (B) 48 h after the initial treatment the same cohort of animals underwent the same test in order to determine a carry-over effect of RG108. The animals were injected with saline thirty minutes prior to T1 in order to maintain the same experimental conditions from the previous test. (C) The animals were treated with RG108 thirty minutes prior to T1 and tested after 24 h inter-trial interval in T2 for examining a treatment effect on long-term memory. (D) OPS performance after RG108 treatment and a 1 h retention interval showed that all the treated groups are able to discriminate between the old and the new location of the objects as compared to zero. Additionally, treatment with the highest dose of 0.3 mg/kg thirty minutes before testing improves animals' d2 index in comparison to the vehicle. RG108 treated groups, N = 12; vehicle group, N = 11. (E) OPS performance 48 h after the previous treatment conducted again with a 1 h interval between T1 and T2. The animal's performance differs significantly from chance level, yet there is no apparent treatment effect between the groups anymore. RG108 treated groups, N = 12; vehicle group, N = 11. (F) OPS performance after RG108 treatment and 24 h inter-trial interval reveals that the treatment did not affect animals' memory at any of the doses tested. Vehicle and RG108 0.3 – 3 mg/kg groups, N = 12; RG108 0.1 mg/kg group, N = 11. Data are shown as mean ± SEM. A significant difference from zero is depicted with hashes (one sample t-tests, #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$). A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett's test, **: $p < 0.01$).

Table 2
Mean values (± SEM) of the different OPS measures conducted 48 h after initial treatment with 1 h inter-trial interval between T1 and T2.

Dose RG108	e1 (s)	e2 (s)	d2	N
Vehicle	46.07 (4.36)	42.89 (4.30)	0.26 (0.04) ###	12
0.1 mg/kg	46.89 (4.05)	46.16 (5.05)	0.25 (0.06) ##	11
0.3 mg/kg	42.54 (2.93)	53.63 (5.68)	0.20 (0.03) ###	12

The table displays the mean exploration times in T1 (e1) and T2 (e2) and the discrimination performance (d2) in the different treatment conditions. The SEM is presented between brackets. One sample t-tests were performed on the d2 measures to reveal significant differences from zero. A significant difference from zero (one-sample *t*-test; indicated by hashes; ##: $p < 0.01$, ###: $p < 0.001$) indicates that the animals were able to discriminate the location of the object from T1.

RG108, we observed a negative correlation between percentage of methylation-*Bdnf1* expression for CpG3 and 6 (CpG3: $r_p = -0.734$, n.s.; CpG6: $r_p = -0.533$, n.s.; Fig. 4H-I), while a positive correlation was detected for CpG2 ($r_p = 0.187$, p = n.s.; Fig. 4G). At any case, the correlation was not significant.

Table 3
Mean values (± SEM) of the different OPS measures after a 24 h interval.

Dose RG108	e1 (s)	e2 (s)	d2	N
Vehicle	55.07 (8.81)	34.08 (3.86)	0.02 (0.05)	12
0.1 mg/kg	50.34 (3.82)	34.81 (3.10)	0.07 (0.06)	11
0.3 mg/kg	49.85 (5.38)	37.95 (2.82)	0.01 (0.03)	12
1 mg/kg	54.05 (4.94)	49.73 (6.61)	0.05 (0.04)	12
3 mg/kg	49.81 (4.76)	42.51 (4.27)	−0.06 (0.06)	11

The table displays the mean exploration times in T1 (e1) and T2 (e2) and the discrimination performance (d2) in the different treatment conditions. The SEM is presented between brackets. One sample t-tests were performed on the d2 measures and revealed no significant differences from zero.

4. Discussion

The objective of the present study was to examine the effect of the DNMT inhibitor RG108 on pattern separation performance in mice, and to pinpoint the underlying gene expression and methylation changes. Administration of RG108 prior to T1 showed a dose-dependent improvement of pattern separation performance after 1 h retention interval. Additionally, mice treated with 0.3 mg/kg RG108 showed an

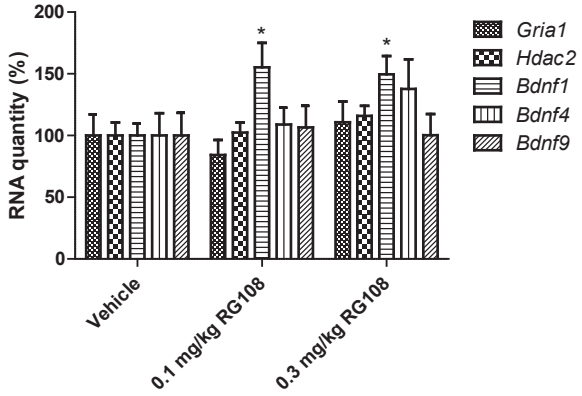

Fig. 2. RNA expression level of target genes 1 h after RG108 treatment (i.p.). Effects of RG108 0.1 mg/kg and 0.3 mg/kg compared to vehicle group on the expression levels of *Gria1*, *Hdac2*, *Bdnf1*, *Bdnf4*, and *Bdnf9* in the mouse dorsal hippocampus, showed increased expression in *Bdnf1* for both doses tested. Values are percentages of the means ± SEM compared to the vehicle group (corrected for geomean of the three housekeeping genes tested). A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by LSD test, *: $p < 0.05$). RG108 0.1 and 0.3 mg/kg groups, N = 12; vehicle group, N = 11.

improved performance in comparison to vehicle-treated animals. Although these results indicate that DNMT inhibition improves pattern separation in mice, this pro-cognitive effect of RG108 was only observed in acute conditions, as the treatment effect on pattern separation was not present after 24 and 48 h.

As shown by qPCR analysis, *Bdnf1* expression was up-regulated by approximately fifty percent after injection of RG108. A possible explanation is that the overall methylation in promoter of exon I is decreased by the compound, therefore facilitating its gene expression. The rodent *Bdnf* gene has a complex structure consisting of eight (I-VIII) 5′ non-coding exons, each driven by a specific promoter, and one 3′ coding exon (IX). Several splice variants have been described, all consisting of the 3′ coding exons and differing in the number of 5′ non-coding exons. Interestingly, multiple promoters are postulated to allow for spatio-temporal regulation of BDNF transcripts in the CNS (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). Distinct subcellular distribution of the different splice variants and, subsequently, the BDNF protein, is achieved by restricted regulation of *Bdnf* mRNA trafficking (Chiaruttini, Sonego, Baj, Simonato, & Tongiorgi, 2008). For example, BDNF1 was shown to be expressed in the soma and dendrites of neuronal cells (Chiaruttini et al., 2009; Pattabiraman et al., 2005), where it contributes to the synthesis of neurotransmitters (Loudes, Petit, Kordon, & Faivre-Bauman, 1999) and the local synthesis of BDNF (Kang, Jia, Suh, Tang, & Schuman, 1996; Tongiorgi, Righi, & Cattaneo, 1997), respectively. Considering the important role of BDNF in neuronal functioning, we could speculate that the observed increase in *Bdnf1* expression after treatment with RG108 could account for the pro-cognitive effect of the latter in short-term OPS.

The observed increase in *Bdnf1* mRNA occurred 1 h after treatment indicates participation of BDNF in early plasticity and subsequently mnemonic processes. Most of the evidence regarding the importance of BDNF for memory formation derived from electrophysiological studies that utilized the molecular correlate of memory, referred to as long-term potentiation (LTP) (Chen, Rex, Pham, Lynch, & Gall, 2010; Cunha, Brambilla, & Thomas, 2010; Dixon, 1959). LTP consists of a labile early phase (E-LTP), lasting 1 to 3 h, followed by a more stable late phase (L-LTP) characterized by protein synthesis (Reymann & Frey, 2007). Although there is a plethora of studies establishing the importance of BDNF signaling for the L-LTP and subsequently long-term memory (Cunha et al., 2010; Edelmann, Leßmann, & Brigadski, 2014; Reymann & Frey, 2007), it has been shown that BDNF is also important for the early phase of LTP. Considering the time point that we measure gene expression, our results underscore the involvement of BDNF in the early phase of memory formation.

There is a growing body of evidence indicating the importance of pre- or post-synaptic BNDF signaling for E-LTP and early memory processes (for a review see Edelmann et al., 2014). The differential findings regarding the site of BDNF action could be the result of different stimulation protocols or stimulation of different areas in the hippocampus. A study from Mohajerani et al. reported a distinct role of BDNF signaling in the different phases of LTP (Mohajerani, Sivakumaran, Zacchi, Aguilera, & Cherubini, 2007). Specifically, it was shown that during E-LTP, BDNF acts at the presynaptic cell to enhance neurotransmitter release, while at the L-LTP its action is mainly located at the postsynaptic site, where it promotes protein synthesis. Another study showed that the transcription of *Bdnf* gene occurs within thirty minutes upon stimulation of cortical cell culture neurons (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). The above observation regarding immediate transcription of *Bdnf in vitro* was subsequently confirmed in a memory paradigm in rats. In more detail, it was shown that *Bdnf* is immediately transcribed in the CA1 region of the hippocampus during contextual learning in the fear conditioning test (Hall, Thomas, & Everitt, 2000).

Although the role of BDNF in mnemonic processes is well established, there is scarcity of evidence associating BDNF with pattern separation. Nevertheless, a recent study has shown that BDNF in the DG has an eminent role in the early memory processes of pattern separation (Bekinschtein et al., 2013). Specifically, intra-DG blockage of BDNF, either before or after the acquisition phase of the pattern separation task, impaired rats' ability to separate similar representations. Importantly, biochemical analysis showed an increase in BDNF protein levels in the DG when animals were sacrificed within 1 h after learning the pattern separation task. Finally, intrahippocampal injection of human recombinant BDNF, after the acquisition phase of the pattern separation task, enhanced discrimination of similar environmental cues. These findings provide ground evidence for the involvement of BDNF in pattern separation memory (Bekinschtein et al., 2013). Also the role of BDNF during pattern separation processes was shown to be mediated by interaction of BDNF with adult-born immature cells in the DG (Bekinschtein et al., 2014). Our study corroborates the above findings regarding the role of BDNF in pattern separation and provides further evidence for the involvement of *Bdnf1* during the early memory processes of this mnemonic task.

Despite the significant increase in *Bdnf1* mRNA levels after DNMT inhibition, the expression of *Bdnf4* and *Bdnf9* was unaltered. It is particularly surprising that *Bdnf4* is not affected by the DNMT inhibitor, as the promoter of the *Bdnf4* exon is known to be susceptible to epigenetic changes (Kotera et al., 2004) and has an eminent role in the processes of learning and memory in rodents (Lubin, 2011). A possible explanation for the above observation could be related to the differential involvement of BDNF transcripts in distinct memory processes of different cognitive tasks. For example, in a study in which the contextual fear conditioning paradigm was used, it was shown that exposure to a novel context leads to upregulation of *Bdnf1* in the hippocampus within 2 h, while consolidation of associative memory was accompanied by elevation in expression of *Bdnf4* (Lubin, Roth, & Sweatt, 2008). Interestingly, a different study in which they used the object recognition memory task showed that short-term recognition memory is positively correlated with increased methylation of *Bdnf1* in the hippocampus of the rats, though BDNF protein levels in the hippocampus were decreased. Of note, an opposite effect was observed for BDNF in the perirhinal cortex (Muñoz, Aspé, Contreras, & Palacios, 2010). Therefore, the memory process and task specific requirement of different BDNF transcripts in brain structures could explain the differential results regarding expression of the BDNF splice variants after treatment. Although in our study we did not measure protein levels after drug administration, it would be interesting to check if our increases in *Bdnf1*

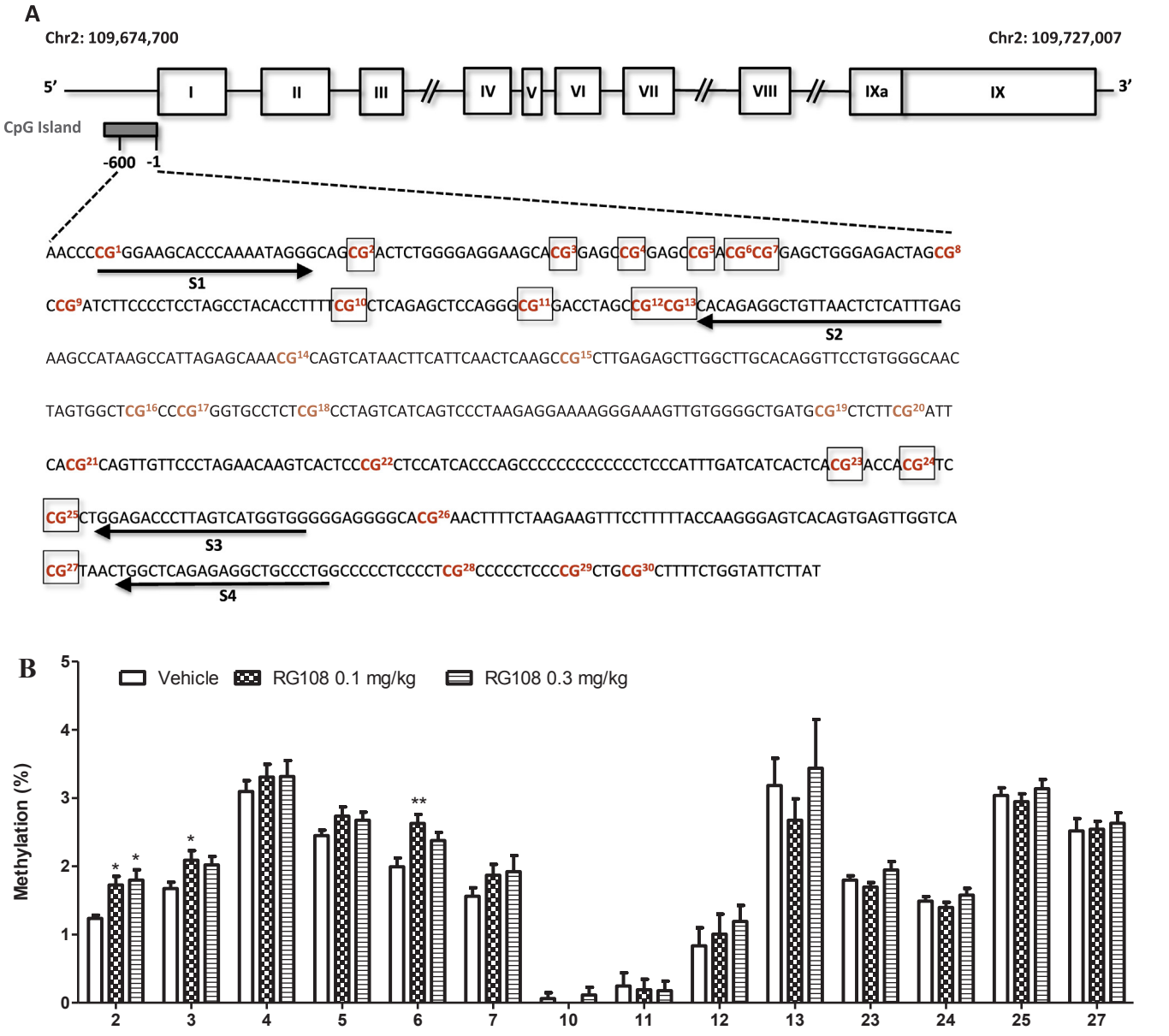

Fig. 3. Effect of the RG108 treatment (i.p.) on the methylation status at 14 CpG islands in *Bdnf* exon I. (A) The top panel presents the structure of the mouse *Bdnf* gene with sequence map of the promoter I including the 14 CpG islands that were analyzed. Boxes indicate exons, horizontal lines display introns and flanking 5′-3′ regions. The grey box shows the CpG island of exon I promoter. Numbers represent the position of the nucleotides relative to the transcription start site of exon I (−600 to −1). The lower panel represents the nucleotide sequence that was analyzed by pyrosequencing. CpGs displayed in red and in the black boxes indicate the CpGs that were studied. Pyrosequencing primers locations are indicated by black arrows (S1-4). The primers sequences are available in Supplementary Table 1. (B) Treatment with RG108 0.1 mg/kg induced a significant increase in the methylation of CpG2, 3 and 6 in the promoter region of *Bdnf1,* while treatment with RG108 0.3 mg/kg increased methylation in the CpG2. Values are percentages of the means ± SEM compared to the vehicle group. A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by Dunnett's test, *: $p < 0.05$, **: $p < 0.01$). CpG2: Vehicle and RG108 0.1 mg/kg, N = 10; RG108 0.3 mg/kg, N = 12; CpG3: all groups N = 10; CpG4-23-24-25-27: Vehicle and RG108 0.1 mg/kg, N = 11; RG108 0.3 mg/kg, N = 12; CpG7: Vehicle and RG108 0.1 mg/kg, N = 11; RG108 0.3 mg/kg, N = 10; CpG5: Vehicle, N = 9; RG108 0.1 mg/kg, N = 10; RG108 0.3 mg/kg, N = 11; CpG6: Vehicle, N = 9; RG108 0.1 mg/kg, N = 11; RG108 0.3 mg/kg, N = 10; CpG10, 11, 12, 13: Vehicle, N = 22; RG108 0.1 mg/kg, N = 21; RG108 0.3 mg/kg, N = 22.

mRNA levels could subsequently result in decreased or increased BDNF synthesis in different brain areas.

Similar to *Bdnf4,* acute administration of RG108 did not alter gene expression of *Gria1* and *Hdac2,* despite their relevance for the task. *Gria1* belongs to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) family that is known to be crucial for LTP and the strengthening of synapses that is important for learning and memory (Lisman, Yasuda, & Raghavachari, 2012). For that, rapid trafficking of GluA1- containing AMPARs at the postsynaptic membrane is required. The trafficking may involve delivery of already existing AMPARs or synthesis of new AMPARs (Ju et al., 2004; Nayak, Zastrow, Lickteig, Zahniser, & Browning, 1998; Oh, Derkach, Guire, & Soderling, 2006; Penn et al., 2017). Nevertheless, the timing of these dynamic changes is difficult to be predicted. Therefore, it is possible that 1 h after treatment represents a time-point that does not involve transcription of GluA1-AMPARs, implying it to be too early or too late in this respect.

Regarding *Hdac2*, its expression levels were determined because it works in close concert with DNMTs. Opposed to methylation, acetylation normally leads to an increase in gene-transcription, by opening the chromatin and making it more accessible to transcription factors. The strong interplay between DNMT and HDAC2 makes it interesting to

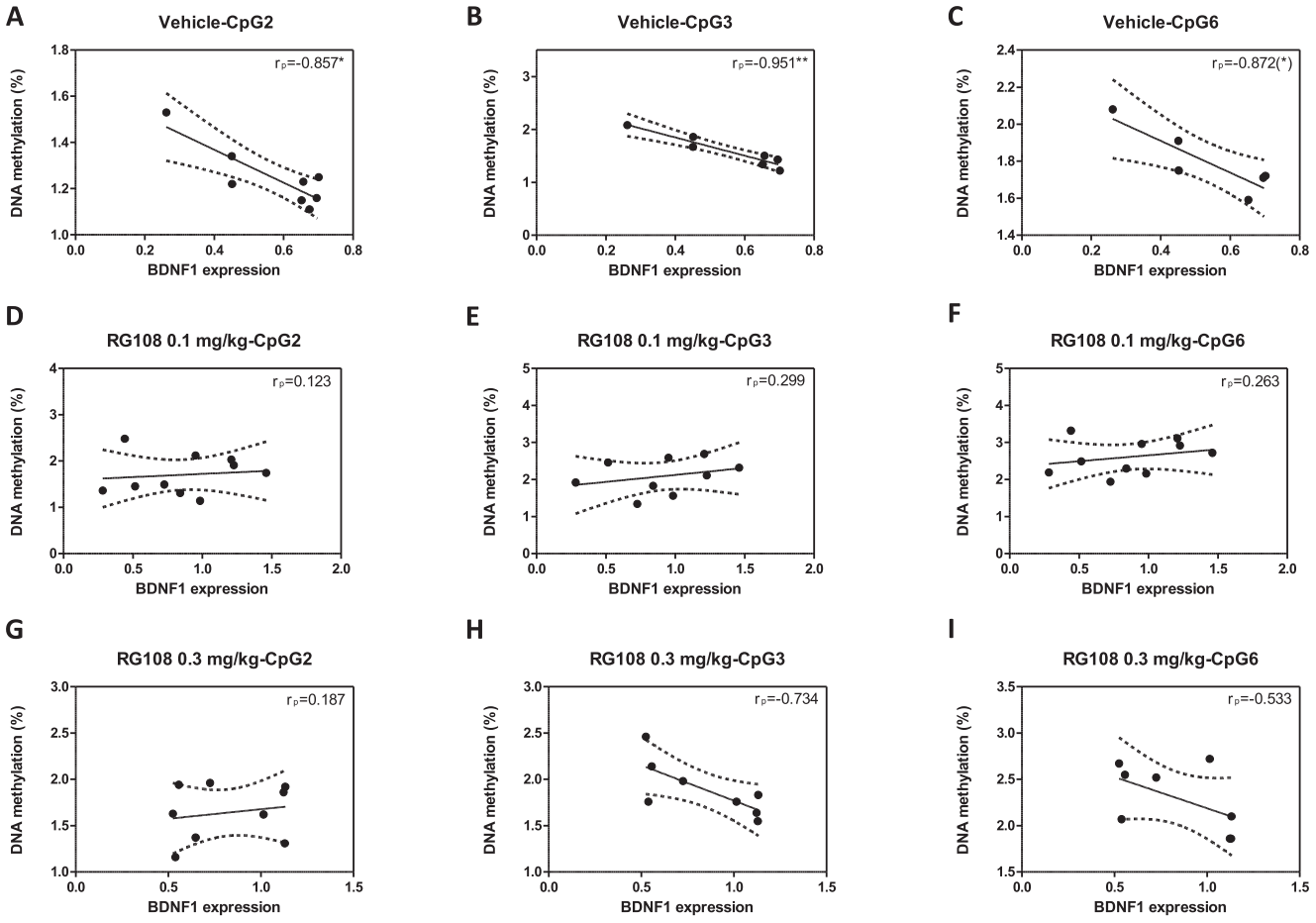

Fig. 4. Correlation between percentage of methylation and expression levels of *Bdnf1*. (A–C) In the vehicle-treated group the percentage of methylation showed a significantly negative correlation with the expression levels of *Bdnf1* for CpG2 and 3, whereas a trend for negative correlation was detected for CpG6. (D–F) In the RG108 0.1 mg/kg group, there is a positive, yet not significant, correlation between percentage of methylation and *Bdnf1* expression for the three CpG islands analyzed. (G–I) Regarding the treatment with RG108 0.3 mg/kg, there is negative correlation between methylation levels and *Bdnf1* expression for CpG3 and 6, while there is a weak positive correlation for CpG2. The scatterplots depict correlation factor (r_p), p-values and the 95% confidence intervals by dashed lines. The relationship between the percentage of DNA methylation and *Bdnf1* expression was calculated by correlation analysis. The best fitted line and confidence interval were calculated by regression analysis followed by Bonferroni's correction, (*): $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$.

assess whether DNMT inhibition also induces *Hdac2* expression changes (Feng, Fouse, & Fan, 2007; Tsankova, Renthal, Kumar, & Nestler, 2007). In contrast to *Hdac1* that is mainly expressed in astrocytes, *Hdac2* is mainly found in neurons and HDAC2 has a crucial role in normal brain development and cell survival (Hagelkruys et al., 2014). HDAC2 however is only linked to one of the two mechanism of methylation-induced gene silencing via binding in the MBDs and could therefore remain unchanged even if methylation changes (Feng et al., 2007).

In order to extend our *Bdnf1* findings, we performed DNA methylation analysis in 14 CpG sites in the promoter I of *Bdnf* and observed an increase in the methylation status of 3 of them. Specifically, for the treatment that had an intermediate effect (0.1 mg/kg) in OPS performance, there was an increase at the CpG 2, 3 and 6 in promoter I, while for the most effective treatment (0.3 mg/kg) the increased methylation was restricted in CpG2. The methylation status of the remaining CpG islands tested was unaltered between the different experimental groups. Considering that the animals were treated with a DNMT inhibitor, the observed increased methylation was not anticipated. A paradoxical effect in the action of DNMTs was also observed in a study showing that stress induced an increase in DNA methylation levels in the hippocampus and a decrease in methylation levels in the prefrontal cortex. Administration of RG108 was able to compensate for these changes, suggesting that both increase and decrease in stress-induced DNA methylation could be regulated by DNMT activity (Sales & Joca, 2016).

In contrast to the methylation that is catalyzed exclusively by DNMTs, there have been suggested several pathways that could induce demethylation (Watt & Molloy, 1988). However, the existence and influencing of enzymes that promote active demethylation remains elusive. Studies aiming to gain more insight into the demethylation mechanism in vertebrates suggested that, under specific conditions, mammalian DNMTs could act as active DNA demethylases by removing the methyl or hydroxymethyl group from 5-methylcytosine (5-mC) or 5-hydroxy-mC (5-hmC), respectively (Chen et al., 2012, 2013). Importantly, the demethylase activity of DNMTs is Ca^{2+}-dependent (Chen, Wang, & Shen, 2013). Considering that increased Ca^{2+} mediates signal transduction in neuronal populations, it could be speculated that in activated neurons DNMTs promote active demethylation. In our study, the animals were sacrificed thirty minutes after the mnemonic test raising the possibility that the action of DNMTs was shifted towards the demethylation pathway. In that case, administration of RG108 could prevent demethylation rather than methylation explaining the initially counterintuitive finding of increased *Bdnf1* methylation after treatment. Additional studies are required in order to shed light into the complex epigenetic changes occurring after DNMT inhibition as well as to determine the exact location of DNMT binding in the promoter region of *Bdnf1*.

Additionally, of outmost important is the location of the different CpG islands in relation to the transcription starting site (TSS) of the

promoter as well as their interaction with transcription activators or repressors. It has been shown that methylation at the downstream region of the TSS in the region of exon I of a gene is more related to transcriptional silencing in comparison to methylation upstream of the TSS, in the promoter region (Brenet et al., 2011; Okitsu & Hsieh, 2007). Additionally, a study of Tian et al. demonstrated that activity-dependent chromatin remodeling occurring in the promoter of *Bdnf1* follows a temporally distinct pattern (Tian et al., 2009). This phenomenon produces "waves" of transcription that cumulative determine the transcriptional outcome of the *Bdnf1* mRNA (Tian et al., 2009). The above findings also indicate that the observed increase in *Bdnf1* expression in our study possibly represents a net effect of DNA methylation/demethylation in *Bdnf1* promoter. This is also depicted on the fact that increased methylation in the CpG islands is not correlated anymore with increased expression levels of *Bdnf1*.

It could be argued that epigenetic changes in the *Bdnf1* gene occur due to handling, training and previous exposure to the learning paradigm. Rapid changes in methylation status of genes have been suggested as part of a priming mechanism based on which epigenetic modifications could take place after a certain stimulus and these changes could facilitate a faster response of the genes during the next (same) stimulation. Alternatively, it is suggested that such epigenetic changes are transient and, during the absence of the original stimulus, the methylation levels of the gene return to baseline. Along the same line, persistent methylation due to a stimulus is thought to negatively alter the ability of a neuron to respond to later stimuli, rather than contributing to it (for a review see Baker-Andresen, Ratnu, & Bredy, 2013). Considering that epigenetic changes most likely have a transient nature, we suggest that the observed changes in *Bdnf1* expression and methylation are due to treatment with RG108 and that they are not related to other experimental aspects of our study to which the animals have already habituated.

Pharmacological agents that improve pattern separation could be beneficial for the treatment of disorders that are related to impairments in pattern separation, like anxiety disorder (Kheirbek et al., 2012) and schizophrenia (Tamminga et al., 2010). In that respect, treatment with DNMT inhibitors could provide a novel approach, especially since current treatment for the above disorders lacks efficacy. Therefore, future studies could investigate the potency of DNMT inhibition in these disorders and further determine whether this effect is related to upregulation of *Bdnf* expression. Nevertheless, treatment with DNMT could have wide spread effects in the whole gene. This makes it necessary to check other brain regions than the dorsal hippocampus alone, as changes in those regions could also be the underlying cause of the observed behavior. Additionally, it is important to determine whether chronic treatment also improves pattern separation performance without introducing unwanted side effects or showing decreased efficacy (e.g. due to tolerance).

Taken together, this study gives first insights into the complex effect of DNMT inhibition on pattern separation. Acute administration of RG108 enhanced pattern separation performance measured with the OPS task and increased *Bdnf1* expression. However, this effect was only acute, and was not present anymore after 48 h. Additionally, this upregulation in *Bdnf1* expression was accompanied with increased methylation in specific CpG islands in the promoter of the gene. Further studies are needed to reveal the exact effects on DNA methylation in different brain areas as well as the effect of chronic administration of RG108. Furthermore, future studies should indicate whether pharmacological enhancement of pattern separation performance can help patients suffering from disorders like anxiety or schizophrenia.

Acknowledgments

None.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2019.02.003.

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