The effect of cocaine treatment biology essay

Science, Biology

First, the effects of acute (AC) and repeated cocaine (RC) treatment on adult mice were assessed. Our data showed that AC treatment significantly increased the locomotor activity of mice (Figure 1; two-way ANOVA repeated measures, interaction F2, 35= 7. 43, p= 0. 002; treatment effect F2, 35= 18. 9, p < 0. 0001, days effect F1, 35= 28. 81, p < 0. 0001). Furthermore, we also found that after a daily injection of cocaine for 7 days (RC), all of the mice displayed a significantly enhanced locomotion in response to the same dose of cocaine on day 7 compared with day 1 (p < 0. 001) indicating the behavioral sensitization.

1. 2. The effect of cocaine treatment on DNMT's expression in the NAc and hippocampus

Earlier studies by Miller and Sweatt (2007) have demonstrated that mRNA levels of DNMT3A and DNMT3B were upregulated in the adult rat hippocampus following contextual fear conditioning. These data suggest that DNMTs are dynamically regulated in the adult nervous system (Miller and Sweatt, 2007). Therefore, our next aim was to investigate whether or not DNMTs mRNA levels in the mouse NAc and hippocampus are altered by acute and repeated cocaine treatment. Using qPCR, we found that AC administration displayed an increase of DNMT3A mRNA levels in both time points (1. 5 h and 24 h) and DNMT3B mRNA level was increased at 24 h after AC treatment compared to saline control (Figure 2A-C). However, the RC treatment did not change significantly DNMT3A and DNMT3B mRNA levels compared to the saline control. There were also no significant changes of DNMT1 mRNA levels after AC and RC treatment. In a parallel study, the effect of acute and repeated cocaine treatment on DNMT1, DNMT3A, and

DNMT3B mRNA levels in the hippocampus at 1. 5 and 24 h after treatment was determined (Figure 3A-C). We found that the acute and repeated cocaine treatment increased DNMT3A mRNA levels 1. 5 h after treatment and DNMT3B mRNA was increased in the hippocampus only after acute cocaine treatment. There were no changes in DNMT3A and DNMT3B mRNA levels 24 h after cocaine treatment compared with the saline control. Both acute and repeated cocaine treatment did not alter the mRNA level of DNMT1 gene in the hippocampus. Thus, the time course analysis demonstrated that DNMT3A and DNMT3B expression is dynamically regulated in the adult NAc and hippocampus in response to cocaine treatment.

1. 3. Cocaine treatment alters marker genes expression in the NAc

DNMT3A and DNMT3B upregulation might cause hypermethylation of certain gene promoters associated with CpG islands and consequently downregulate the expression of these genes. Therefore, a search for genes that show a diminished expression following cocaine treatment was conducted. Several genes were tested, such as BDNF, PP1c, fosB, and adenosine A2A receptor (A2AR) that participate in cocaine-induced neuroadaptations in the NAc and in silico analysis revealed CpG islands located within promoter regions. Our qPCR results demonstrated that PP1c mRNA levels in the mouse NAc were not altered 1. 5 h after both acute and repeated cocaine treatment (Figure 4A). However, 24 h after treatment in both acute and repeated cocaine groups, the PP1c mRNA levels were significantly decreased compared with the saline control. To further confirm PP1c mRNA downregulation in the NAc,

western blot analysis was performed and we found that both acute and repeated cocaine treatment resulted in a significantly lower PP1c protein level 24 h after treatment (Figure 4B). As expected, it was found that acute and repeated cocaine treatment resulted in upregulation of fosB mRNA levels 1. 5 h after treatment (Figure 4C), but there were no significant changes 24 h after treatment. We also found that A2AR mRNA level was significantly downregulated 1. 5 h after both acute and repeated cocaine treatment (Figure 4D). However, there were no significant changes in the mRNA levels of BDNF gene after cocaine treatment. As our aim was to investigate cocaine-induced long-lasting effects, for future DNA methylation studies, PP1c and fosB (as reference) genes were selected.

1. 4. Cocaine treatment alters DNA methylation at the PP1c and fosB promoter regions

To determine the role of DNA methylation in cocaine-induced behavioral sensitization, cocaine-induced changes of DNA methylation patterns at the PP1c promoter region was evaluated. Using MeDIP assay, we found that both acute and repeated cocaine treatment resulted in PP1c promoter associated CpG island hypermethylation 24 h after treatment (Figure 5B). To verify cocaine-induced hypermethylation at the PP1c promoter region, methylation-specific quantitative real-time PCR (MSP qPCR) was performed. Data generated using MSP qPCR demonstrated that both acute and repeated cocaine treatment elicited DNA hypermethylation at the PP1c promoter region 24 h after treatment (Figures 5A and C; two-way ANOVA, interaction $F_{2, 66} = 8.34$, $p = 0.0006$, treatment $F_{2, 66} = 2.77$, $p = 0.0699$, methylation effect $F_{1, 66} = 31.24$, $p < 0.0001$). As a DNA methylation control, levels of

unmethylated DNA within the PP1c promoter after acute and repeated cocaine treatment was measured, using primer sequences designed to amplify unmethylated CpG island sites. Thus, MeDIP and MSP qPCR methods demonstrated that acute and repeated cocaine treatment resulted in hypermethylation at the PP1c promoter region 24 h after treatment. In correlation with upregulated fosB mRNA levels, our MeDIP results demonstrated hypomethylation at the fosB promoter 1. 5 h after acute and repeated cocaine treatment (Figures 6A and B). To further confirm that acute and repeated cocaine administration altered DNA methylation at the promoter regions of PP1c and fosB genes, ChIP assay was performed. Consistent with the MeDIP and MSP qPCR results, ChIP analysis demonstrated that acute and repeated cocaine treatment increased by 1. 8- to 2-fold the PP1c promoter-associated MeCP2 binding 24 h after treatment in the NAc (Figure 7A). However, in the fosB promoter, acute and repeated cocaine treatment was associated with a significant decrease in MeCP2 binding compared with the saline control at 1. 5 h after treatment (Figure 7B).

1. 5. The effect of DNMT inhibitor zebularine on cocaine-induced DNA methylation and the development of behavioral sensitization in mice

As we found that cocaine induced DNMT3A and DNMT3B expression and PP1c promoter hypermethylation, the next aim in this study was to assess the effect of DNMT inhibitor on cocaine-induced molecular changes and development of behavioral sensitization. We selected the DNMT inhibitor zebularine for testing based on reported data (Miller and Sweatt, 2007; Lubin

et al, 2008). To correlate the molecular changes with cocaine-induced locomotor sensitization, a dose of zebularine (300 ng per 0. 5 ml, i. c. v.) that did not affect basal locomotor activity and decreased DNA methylation at the PP1c promoter region at 1. 5 h after acute treatment (Figure 8A) was selected from our pilot study. For the co-treatment experiment, all mice received i. c. v. infusion of saline or zebularine followed, after 20 min, by i. p. saline or cocaine (15 mg/kg). Locomotor activity was recorded for 60 min immediately after the last i. p. injection. Repeated saline and cocaine (S+C) treatment for 7 days displayed a significantly enhanced locomotion on day 7 compared with that of day 1 (Figure 8B; two-way ANOVA with repeated measures, interaction $F_{3, 39} = 5.69$, $p = 0.0025$, treatment $F_{3, 39} = 15.98$, $p < 0.0001$, days effect $F_{1, 39} = 10.26$, $p = 0.0027$). However, mice co-treated with zebularine and cocaine (Z+C) did not show any sensitization on day 7 compared with day 1 ($p < 0.05$). There was also no behavioural sensitization after repeated zebularine and saline (Z+S) treatments ($p < 0.05$). Therefore, our results suggest that the inhibition of DNMTs with zebularine did not affect acute cocaine-induced locomotor activity, but instead delayed cocaine-induced behavioural sensitization in mice. Finally, we studied for molecular evidence to confirm that zebularine infusions (i. c. v.) before cocaine treatment altered DNA methylation level at the PP1c promoter region in the NAc. Using MeDIP assay we found that repeated cocaine treatment (S+C) induced, at the PP1c promoter region, DNA hypermethylation 24 h after treatment compared with the saline control group (S+S), and repeated zebularine and cocaine (Z+C) co-treatment avoided this effect (Figure 9A; one-way ANOVA, Bonferroni post-test, $p < 0.$

001 S+S vs. S+C, p < 0. 001 S+C vs. Z+C, n = 6). Similarly, MSP qPCR analysis demonstrated that there was a significant decrease of DNA methylation associated with the PP1c promoter region in the Z+C group compared with the S+C group (Figure 9B; two-way ANOVA, interaction F3, 45= 80. 8, p < 0. 0001, treatment F3, 45= 39. 0, p < 0. 0001, methylation effect F1, 45= 42. 8, p < 0. 0001). Interestingly, in both studies we found significant changes of DNA methylation between the zebularine (Z+S) and the saline (S+S) control groups (p < 0. 05). In correlation with the PP1c promoter-associated CpG island methylation results, we observed that zebularine attenuated cocaine-induced downregulation of PP1c mRNA level (Figure 9C; one-way ANOVA; Bonferroni post-test, p < 0. 001 S+S vs. S+C, p < 0. 05 S+C vs. Z+C, n = 6). These results indicate that the inhibition of DNMTs by zebularine reverses cocaine-induced PP1c gene hypermethylation and mRNA downregulation in the NAc.

2. The effect of S-adenosylmethionine (SAM) on cocaine-induced DNA methylation and locomotor sensitization in mice (II)

2. 1. SAM pretreatment potentiated the development and expression of cocaine-induced locomotor sensitization in mice

In this part of study, our first aim was to evaluate the effect of SAM (4520 mg/kg/day) on the locomotor sensitization to cocaine (10 mg/kg/day) in adult mice. Our data showed that repeated cocaine treatment (S+C) and repeated SAM and cocaine co-treatment (M+C) for 7 days displayed a significantly enhanced locomotion on day 7 compared with the day 1 (Figure 10A), indicating the development of the locomotor sensitization. There were also

significant differences (p < 0. 001) between S+C and M+C groups on day 7. However, we did not find difference in locomotor activity between SAM (M+S) and saline control (S+S) groups. In cocaine (7 mg/kg i. p) challenge study (on days 14 and 28) S+C and M+C groups demonstrated a robust sensitization exhibiting more locomotor activities than S+S group (Figure 10B). Importantly, M+C group had higher expression of sensitization compared to S+C group. Cocaine challenge also increased the locomotor activity in M+S group compared to S+S group, but these changes were not substantial. These data demonstrated that exogenous SAM pretreatment did not affect acute cocaine-induced locomotor response, but instead potentiated the development and the expression of cocaine-induced locomotor sensitization in mice.

2. 2. SAM-modified cocaine-induced gene expression

An Illumina microarray was used to study persistent changes in gene expression in the NAc following repeated SAM and cocaine treatment. The samples for gene expression profiling were collected 24 hours after the final treatment. Four different treatment groups - S+S, M+S, S+C and M+C (4 samples per group) were compared and differentially expressed genes were identified by a combination of statistical significance (p < 0. 05) and a fold change (FC) filter (FC > 1. 5). In total, 482 separate transcripts were expressed differently between the M+S, S+C and M+C groups, representing 1. 88 % of the total number of transcripts analyzed whereas 98. 12 % of the transcripts remained unaltered. To assess the direction of gene expression changes induced by the treatments, the M+S, S+C and M+C treatment groups were compared to the S+S group. Our data showed that in the M+S

group a total of 18 transcripts (36%) were up- and 32 transcripts (64%) were downregulated (see Supplementary Tables S1 and S2; Paper II), representing 0. 19 % (n = 50) of the total number of transcripts analyzed. In the S+C group, 93 transcripts (38. 6%) were up- and 148 transcripts (61. 4%) were downregulated (see Tables 2 and 3; Paper II), representing 0. 94% (n = 241) of the total number of transcripts analyzed. In the M+C group, 54 transcripts (42. 5%) were up- and 73 transcripts (57. 5%) downregulated (see Supplementary Tables S3 and S4; Paper II), representing 0. 49% (n = 127) of the total number of transcripts analyzed. The comparisons between M+S, S+C, and M+C groups were also performed. Our analysis showed that 28 of 32 (87. 5%) SAM-responsive transcripts were down- and 9 of 18 (50%) were upregulated in the S+C group (see Supplementary Figures S1A and S2A; Paper II). Comparisons between M+S vs. M+C groups demonstrated that 12 of 32 (37. 5%) SAM-responsive transcripts were down- and 8 of 18 (44. 4%) were upregulated in the M+C group (see Supplementary Figures S1B and S2B; Paper II). Interestingly, we found that 64 transcripts (43. 2%) of the 148 cocaine-responsive genes were down- and 50 transcripts (53. 8%) of the 93 cocaine-responsive genes were upregulated in the M+C group (see Supplementary Figures S1C and S2C; Paper II). These data suggest that SAM pretreatment reduced 56. 8 and 46. 2% (of genes down- and upregulated by cocaine, respectively) of cocaine-induced transcripts. Gene ontology (GO) analysis was performed to group significantly regulated genes into similar biological or molecular functional categories. GO analysis showed an over-representation of downregulated genes in the M+S, S+C, and M+C groups encoding proteins involved in: (i) cell cycle, differentiation and proliferation,

(ii) developmental process, and (iii) signal transduction (see Supplementary Table S5; Paper II). The upregulated genes in all those groups were mainly aggregated into the categories of (i) multicellular organismal process, (ii) cell cycle, differentiation and proliferation, (iii) signal transduction, (iv) developmental process and/or ion transport (see Supplementary Table S6; Paper II). Table S6 shows that genes, which are related to " cell-cell signaling" and " behavior" were upregulated only in the S+C group.

2. 3. SAM pretreatment altered cocaine-induced CpG island methylation and transcriptional activity in the NAc

qPCR analysis was performed to validate the subset of gene expression changes observed in the microarray analyses. Genes chosen for qPCR validation were selected based on their potential roles in cocaine-induced neuronal plasticity and on in silico analysis that revealed CpG islands located within their promoter regions. From the microarray data, three genes for validation were selected: a) solute carrier family 17 member 7 or vesicular glutamate transporter 1 (Slc17a7) and cholecystokinin (Cck) as downregulated genes; b) galanin (Gal) as upregulated gene after repeated cocaine treatment. Using the same RNA samples as in the gene expression profiling, transcription analysis of Slc17a7 and Cck revealed a significant decrease in mRNA levels following repeated M+S, S+C, or M+C treatments in the NAc. There were significant differences ($p < 0.001$) in both genes between the S+C and the M+C groups. Using mouse cerebellum as a reference brain region, we found that the marker genes mRNA were altered in the cerebellum as well, but these changes were not as extensive as in the NAc. Slc17a7 and Cck mRNA levels comparisons in both brain tissues

demonstrated that Slc17a7 mRNA was significantly different ($p < 0.001$) between the S+C group in the NAc vs. S+C group in the cerebellum and Cck mRNA level between the S+C and M+C groups in NAc vs. S+C and M+C groups in the cerebellum (Figures 11A and B). Gal mRNA data in the NAc showed that repeated M+S, S+C and M+C treatments significantly increased ($p < 0.001$) Gal expression compared to the S+S group. Furthermore, Gal mRNA was significantly different ($p < 0.001$) between the S+C and M+C groups. In the cerebellum, we found that M+S and S+C treatments significantly ($p < 0.001$) upregulated Gal expression compared to the saline control. There were also statistical differences ($p < 0.001$) between S+C and M+C groups. Gal mRNA levels comparison in both brain regions demonstrated that there were significant differences ($p < 0.001$) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum (Figure 11C). Using MeDIP assay, Slc17a7, Cck, and Gal promoter-associated CpG island methylation analysis in the NAc was performed. For the Slc17a7 promoter, MeDIP analysis revealed that both M+S and S+C treatment resulted in promoter hypermethylation compared to the S+S group (Figure 12A). We also found that repeated SAM pretreatment significantly ($p < 0.001$) decreased Slc17a7 promoter hypermethylation compared to the S+C group. With regard to Cck promoter methylation, M+S and S+C treatments resulted in promoter hypermethylation (Figure 12B). However, there was an additive increase in Cck promoter methylation levels in the M+C group when compared with S+C treatment ($p < 0.001$). Gal MeDIP analysis demonstrated that M+S and S+C treatments induced promoter-associated CpG island hypomethylation (Figure 12C). Remarkably,

we found that repeated M+C treatment essentially reversed Gal promoter hypomethylation compared to the S+C treatment (p < 0. 001). MeDIP data in the mouse cerebellum showed that repeated M+S and S+C treatments significantly (p < 0. 001) enhanced (Slc17a7, Cck) promoter-associated CpG island methylation (see Supplementary Figures S3A, B; Paper II). Moreover, Slc17a7 and Cck promoter methylation data comparison in both brain tissues demonstrated that there were significant differences (p < 0. 001) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum. Gal MeDIP data comparison in both brain regions showed that there were significant changes (p < 0. 001) only between the S+C groups in the NAc vs. S+C groups in the cerebellum (see Supplementary Figure S3C; Paper II).

2. 4. The effect of SAM treatment on methyltransferase activity and DNMT's expression in PC12 cells

To evaluate the underlying mechanism of the SAM modifying effect at the gene and genome level, we first studied the effects of a single and repeated dose (7 days) of 0. 5 mM SAM on methyltransferase (Dnmt) activity in PC12 cells. Time-course analyses showed that SAM altered methyltransferase activity in a biphasic manner: a single SAM treatment (SST) enhanced and a repeated SAM treatment (RST) decreased Dnmt activity compared with vehicle controls (Figures 13A, B). Next, to link methyltransferase activity with DNMT3A and -3B mRNA levels, we measured the SAM-altered Dnmt activity on 1st, 3rd, 5th and 7th treatment days. On the 1st day, Dnmt activity was similar to vehicle control, on the 3rd day its activity was reduced approximately by 50%, on the 5th by 65% and on the 7th day by 82%

compared to controls (Figure 13C). We also discovered that the decrease in Dnmt activity in PC12 cells correlated with downregulation of DNMT3A mRNA level (Figure 14A), but not remarkably with DNMT3B mRNA level (Figure 14B) after repeated SAM treatment. In silico analysis of DNMT3A revealed that CpG islands located within the promoter region, therefore, we assessed DNMT3A promoter methylation following repeated SAM treatment. The MeDIP results showed that repeated SAM treatment resulted in a significant increase in methylation of the DNMT3A promoter (Figure 14C), thereby decreasing DNMT3A gene transcription in PC12 cells.

2. 5. SAM treatment hypermethylated the DNMT3A promoter and downregulated mRNA level in the NAc

To bridge our PC12 cells and mice data, we assessed DNMT3A and -3B promoter methylation patterns and transcriptional activity in the NAc. Using MeDIP, we discovered that M+S and M+C treatment increased DNMT3A promoter methylation (Figure 15A), but there was no significant change in DNMT3B promoter (Figure 15B). Next, we evaluated whether aberrant promoter methylation was associated with altered DNMT3A expression. We found that DNMT3A mRNA was significantly decreased following M+S and M+C treatments and increased following S+C treatment compared with the saline-treated group (Fig. 15C). There were significant ($p < 0.001$) differences in both DNMT3A and -3B mRNA levels after the S+C and M+C treatment in NAc (Figure 15 C, D). These data indicate that repeated SAM treatment might decrease methyltransferase activity in vitro and repeated SAM treatment is associated with hypermethylation of DNMT3A promoter region both in vitro and in vivo.