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Regulation and function of MeCP2 Ser421 phosphorylation in U50488-induced conditioned place aversion in mice

Anthony S. Zannas^{1,2,3}, Jun H. Kim¹, and Anne E. West¹

¹Dept. of Neurobiology, Duke University Medical Center, Durham, NC USA 27710

²Dept. of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC USA 27710

Abstract

Rationale—Phosphorylation of the methyl-DNA binding protein MeCP2 at Ser421 (pMeCP2-S421) is induced in corticolimbic brain regions during exposure to drugs of abuse and modulates reward-driven behaviors. However, whether pMeCP2-S421 is also involved in behavioral adaptations to aversive drugs is unknown.

Objectives—Our goal was to establish the role of pMeCP2-S421 in corticolimbic brain regions of mice upon acute treatment with the kappa opioid receptor agonist U50488 and during the expression of U50488-induced conditioned place aversion (CPA).

Methods—pMeCP2-S421 levels were measured in the nucleus accumbens (NAc), prelimbic cortex, infralimbic cortex (ILC), and basolateral amygdala (BLA) of male mice after intraperitoneal administration of U50488 and upon the expression of U50488-induced CPA. Fos was measured as marker of neural activity in the same brain regions. U50488-induced CPA and Fos levels were compared between knockin (KI) mice that lack pMeCP2-S421 and their wild-type (WT) littermates.

Results—U50488 administration acutely induced pMeCP2-S421 and Fos selectively in the NAc but did not alter MeCP2 levels in any brain region. U50488-induced CPA was associated with decreased pMeCP2-S421 in the ILC and BLA and induced Fos in the BLA. MeCP2 KI mice showed CPA indistinguishable from their WT littermates, but they also showed less BLA Fos induction upon CPA.

Correspondence: Anthony S. Zannas (aszannas@gmail.com, phone: +498930622567, fax: +498930622605) or Anne E. West (west@neuro.duke.edu, phone: +19196811909, fax: +19196844431).

³Current address: Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany 80804

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

ASZ: designed and performed the behavioral and immunohistochemistry experiments, analyzed all data, wrote the manuscript; JK: performed and analyzed immunohistochemistry; AEW: designed the behavioral and immunohistochemistry experiments, analyzed all data, wrote the manuscript.

Supplementary Material

Table 1. Chamber times for C57BL/6 mice exposed to saline or 5mg/mg U50488

Table 2. Chamber times for MeCP2 wild-type (WT) and Ser421 Ala knockin (KI) mice exposed to saline

Table 3. Chamber times for MeCP2 wild-type (WT) and Ser421Ala knockin (KI) mice exposed to 5mg/kg U50488

Conclusion—These data are the first to show that pMeCP2-S421 is induced in the brain acutely after U50488 administration but not upon U50488-induced CPA. Although pMeCP2-S421 is not required for U50488-induced CPA, this phosphorylation event may contribute to molecular plasticities in brain regions that govern aversive behaviors.

Keywords

aversive learning; conditioned place aversion; epigenetics; Fos; MeCP2

Introduction

Complex organisms survive by constantly adapting to ever-changing environments, and this adaptability relies on their brain's capacity to acquire new information and learn from environmental encounters. Aversive learning, a type of motivational learning that is instructed by the negative emotional valence of an environmental stimulus, is particularly instrumental for survival, because it enables the organism to implement behavioral adaptations that will increase the likelihood of avoiding negative consequences (Ullsperger et al. 2014). Such adaptations require structural and functional changes in brain circuits that govern the learned behavior (Volman et al. 2013). While several biological processes can drive these brain changes, accumulating evidence highlights the role of epigenetic regulation, a set of mechanisms that mediate long-lasting stimulus-induced changes in gene expression (Feil and Fraga 2011; Telese et al. 2013), in the shaping of aversive learning (Dias and Ressler 2014; Mahan et al. 2012; Miller and Sweatt 2007).

Among molecules involved in epigenetic regulation, important roles in brain function have been ascribed to the methyl-CpG binding protein 2 (MeCP2). MeCP2 binds methylated CpGs in DNA and acts as scaffold that recruits chromatin regulators and transcription complexes (Fatemi and Wade 2006). Loss-of-function mutations in human *MECP2* are the cause of Rett syndrome, a developmental disorder characterized by prominent learning deficits and impaired neural plasticity (Chahrour and Zoghbi 2007; Moretti et al. 2006). MeCP2 has been further shown to function in reward circuits of the brain and to modulate certain kinds of reward-related behaviors induced by psychostimulant drugs (Deng et al. 2010; Im et al. 2010). For example, lentiviral overexpression of MeCP2 in the nucleus accumbens (NAc) inhibits amphetamine (AMPH)-induced conditioned place preference (CPP), whereas shRNA-mediated MeCP2 knockdown enhances AMPH-induced CPP (Deng et al. 2010). MeCP2 undergoes stimulus-dependent phosphorylation at several sites, and MeCP2 phosphorylation has been shown to influence its ability to associate with other proteins (Ebert et al. 2013; Gonzales et al. 2012; Zhou et al. 2006). Thus inducible phosphorylation provides a mechanism to couple MeCP2 activity to environmental stimuli. In particular, phosphorylation of MeCP2 Ser421 (pMeCP2-S421) is induced in response to AMPH in the mouse NAc and prelimbic cortex (PLC) (Deng et al. 2010). Mice lacking pMeCP2-S421 show accelerated AMPH-induced locomotor sensitization and reduced threshold for the maintenance of cocaine self-administration, suggesting that pMeCP2-S421 serves to limit the neural and behavioral plasticities induced upon repeated exposure to psychostimulants (Deng et al. 2010; Deng et al. 2014). Beyond its role in rewarding stimuli, MeCP2 phosphorylation has been also shown to occur in the brain during morphine

withdrawal (Ciccarelli et al. 2013), suggesting that pMeCP2-S421 may be implicated in aversive states. However, no studies have examined its role in behavioral adaptations upon exposure to aversive drugs.

Aversive learning is mediated by an intricate brain circuitry comprised of prefrontal cortical and limbic regions. Among these regions, important roles in aversive behaviors have been demonstrated for the NAc (Albrechet-Souza et al. 2013; Carlezon and Thomas 2009; Wheeler and Carelli 2009), the PLC (Bi et al. 2010; Jiang et al. 2014), the infralimbic cortex (ILC) (Awad et al. 2015; He et al. 2011; Xin et al. 2014), and the basolateral amygdala (BLA) (Belova et al. 2007; He et al. 2011; Shabel and Janak 2009). These regions show extensive inter-connectivity and undergo changes in activation during aversive learning (Lesting et al. 2011; Peters et al. 2009).

The present study examined the relationship between pMeCP2-S421 and aversive learning in mice, using the well-established paradigm of conditioned place aversion (CPA) induced by the kappa opioid agonist U50488 (Mucha and Herz 1985; Wee and Koob 2010). First, we characterized the acute effects of U50488 in brain regions involved in aversive behaviors by measuring pMeCP2-S421 levels in the mouse NAC, PLC, ILC, and BLA after intraperitoneal administration of U50488. In the same brain regions, we then examined if pMeCP2-S421 is regulated during aversive behaviors by measuring pMeCP2-S421 upon the expression of U50488-induced CPA. To examine whether pMeCP2 changes are coupled with changes in neuronal activity, we assessed the levels of the immediate early gene product Fos in the same brain regions and time points. Lastly, we examined whether pMeCP2-S421 is either necessary for aversive learning or shapes neural responses upon the expression of CPA by comparing U50488-induced CPA and brain Fos levels between MeCP2 Ser421Ala knockin (KI) mice, which lack pMeCP2-S421, and their wild-type (WT) littermates.

Method

Animals

We purchased adult (8- to 10-week-old) male C57BL/6J mice from Jackson Laboratories. We crossed *Mecp2*^{Ser421Ala/+} female mice to C57BL/6J males to generate *Mecp2*^{Ser421Ala/Y} (knockin, KI) and *Mecp2*^{+/Y} (wildtype, WT) male littermates as was previously described (Cohen et al. 2011). The mice were genotyped by PCR on tail DNA with primers that differentially detect the WT and KI alleles. Genotyping was performed as previously described (Cohen et al. 2011). Continually crossing females carrying the MeCP2 knockin allele to C57BL/6J males imported from Jackson Laboratories allowed us to maintain the line as congenic on a C57BL/6J background.

All experiments used male mice 2–5 months old. We gave mice free access to standard laboratory chow and water and housed them in a humidity- and temperature-controlled room on a 14/10 h light/dark cycle, with four or five males in a cage. Lights went on at 7:00 A.M., and all behavioral experiments were conducted between 10:00 A.M. and 3:00 P.M. in the light cycle. All animal procedures were performed in accordance with the Duke University animal care committee's regulations.

Acute U50488 Exposure

C57BL6 male mice were placed in the open field for 30 min prior to injection of Vehicle (saline) or 5mg/kg i.p. U50488 (Tocris). 2 hrs later mice were transcardially perfused with 4% paraformaldehyde (PFA) and 0.1 M PBS. Brains were postfixed overnight in PBS with 4% PFA and then transferred to 20% sucrose in PBS with 4% PFA.

Immunofluorescent staining of brain sections

Brains were cut to 40µm coronal sections on a freezing microtome, and brain regions (prelimbic and infralimbic regions of the medial prefrontal cortex, nucleus accumbens, and basolateral amygdala) were identified by anatomical landmarks. Sections were blocked in 10% normal goat serum with 0.3% Triton X-100 in PBS and were incubated overnight in primary antibodies, which included rabbit anti-phospho-Ser421 MeCP2 (1:15,000; (Deng et al. 2010)) and rabbit anti-Fos (1:10,000; Ab-5; Millipore). Because both antibodies were raised in rabbit, sections could not be co-stained for both pMeCP2-S421 and Fos; thus we used adjacent sections from each animal for these analyses. Sections were visualized by application of donkey anti-rabbit Cy3 or donkey anti-rabbit Cy2 (1:500; Jackson ImmunoResearch) secondary antibodies. Hoechst dye was added to visualize nuclei and the sections were mounted in Vectashield (Vector Laboratories). To minimize bleaching, sections were stored at all stages in dark conditions and were exposed for the minimum duration of time to bright field illumination.

Image Analysis

Images were captured on a Leica DMI4000 inverted fluorescence microscope using a Leica DFC365FX camera, and quantified using the Count Nuclei module in MetaMorph 7 Image Analysis software (Molecular Devices) to evaluate both cell numbers and integrated intensities of the pMeCP2-S421 or Fos immunoreactivities. We used quantitative immunostaining protocols that have been previously used successfully to measure the somewhat sparse pMeCP2-S421 and Fos induction that is seen in vivo following many stimuli (Deng et al. 2010; Moratalla et al. 1996). Within a single experiment, identical parameters (intensity of positive signal above background and size range that signals a nucleus) were used to analyze images obtained with uniform capture exposures for all conditions. Data are graphed as integrated intensity, which is a summation of all intensities within all positive nuclei. As has been previously reported, the levels of pMeCP2-S421 in vehicle treated controls differ between brain regions (Deng et al. 2010; Zhou et al. 2006). Thus, for each brain region or condition, the absolute average integrated intensity under control conditions was scaled to a value of 100 and the experimental conditions are graphed relative to that number to show fold change with stimulation or genetic modification.

Conditioned Place Aversion

CPA testing was conducted in a three-chambered apparatus equipped with infrared diodes (Med Associates, St. Albans, VT). The apparatus consists of two equally-sized outer compartments (16.8 × 12.7 × 12.7 cm) -a black-colored chamber with steel grid rod floor and a white-colored chamber with steel mesh floor- separated by a smaller gray start box (7.2 × 12.7 × 12.7 cm) with manually operated doors. Each compartment contained no

bedding and the apparatus was cleaned and sanitized after each conditioning or testing session. Both C57BL/6J and MeCP2 Ser421Ala KI mice and their WT littermates were used for CPA as described in the text. CPA testing was conducted over 8 days. On the first day, mice were acclimated to the apparatus by placing them into the center chamber and allowing them free access to the entire apparatus for 30 min. We used these data to determine the baseline more preferred chamber for each mouse, and this chamber was assigned as the “drug-paired” chamber. For each mouse, we determined the baseline (on acclimation day) preference score, which was calculated as previously described (Wilkinson and Bevins 2008) by the ratio of the time spent in the drug-paired chamber divided by the total time spent in both black-colored and white-colored chambers. Because strong baseline preferences can impede CPA learning, we excluded mice with baseline preference scores above or equal to 0.65. This resulted in exclusion of 2 mice from the C57BL/6J study and 3 mice from the MeCP2 Ser421Ala KI/WT study.

Conditioning began on day 2 and proceeded for 6 consecutive days. Vehicle-treated (control) mice received saline in alternative chambers on each day. Drug-treated mice were treated with U50488 (5mg/kg i.p.) on days 2,4, and 6 in the baseline more preferred chamber and with saline on days 3,5, and 7 in the alternate chamber. Finally on day 8, the challenge day, all mice were placed in the center chamber and allowed free access to the entire apparatus for 30 min with no drug exposures. On challenge day, total time spent in each chamber was measured and preference scores were determined as above. By comparing the time spent in the two chambers (drug/saline) and how their difference changes during conditioning, the preference score captures the animal’s true change in place preference, irrespective for example of shifts in time spent in the neutral (gray) chamber of the apparatus. 2 hours following behavioral analysis on challenge day, mice were perfused for brain immunostaining or were killed with CO₂ and brain regions were harvested for western blotting.

Western Blotting

Brain regions were dounced in 20μL 1% SDS and boiled for 3 min. They were then adjusted to 100μL in RIPA buffer (10mM Tris pH7.5, 1mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150mM NaCl), and insoluble material was spun out at 13,000 RPM for 3 min. Samples were subjected to BCA protein assay, and 30μg of total cell lysate/sample was run on 8% SDS-PAGE and transferred to nitrocellulose for Western blotting with the following primary antibodies: mouse anti-actin (1:10,000; MAB1501; Millipore), rabbit anti-phospho-Ser421 MeCP2 (1:1,000; Deng et al., 2010), mouse anti-MeCP2 (Ab5005 Abcam). Secondary antibodies were goat anti-rabbit 770 and goat anti-mouse 680 (1:1000; Biotium). Fluorescent immunoreactivity was imaged on a LI-COR Odyssey and quantified using ImageJ. MeCP2 was normalized to actin expression in the same lane. Normalized MeCP2 levels in WT mice for each single brain region were scaled to a value of 100, and MeCP2 KI levels are graphed relative to WT in that brain region.

Statistical Analyses

We performed all statistical analyses using SigmaPlot (version 13.0) or Excel. The data are depicted as means and standard error of the means (SEMs). Immunofluorescence intensity

and protein expression on western blots was compared between genotypes or treatments by a Student's unpaired t test. All of the western data and most of the immunofluorescence data showed a normal distribution (Kolmogorov-Smirnov test $p>0.05$). However, some baseline Fos expression datasets were skewed toward zero, which is to be expected for immediate-early gene expression, as it is completely absent in many brain regions prior to environmental stimulation. We thus additionally tested significance of all immunofluorescence datasets using the non-parametric Mann-Whitney U test. For all of these comparisons, both statistical tests yielded the same result. For all behavioral experiments, we performed two-way repeated measures analysis of variance (ANOVA) examining time (acclimation/challenge) as the within-subject factor and either treatment (Vehicle/U50488) or genotype (WT/KI) as the between-subject factor of interest. Significant effects were followed with posthoc pairwise tests using Bonferroni correction for multiple comparisons. In all cases, a two-tailed $p<0.05$ was considered statistically significant.

Results

U50488 induces pMeCP2-S421 and Fos selectively in the NAc

To determine whether exposure to aversive drugs can acutely induce pMeCP2-S421 in the medial prefrontal cortex (mPFC) and limbic brain regions, we exposed male mice to Vehicle (saline) or the kappa opioid receptor agonist U50488 (5mg/kg i.p.). 2 hrs after drug treatment, mice were perfused, and we visualized pMeCP2-S421 by immunostaining on coronal brain sections through the NAc, PLC, ILC, and the BLA (Fig. 1a). We chose this time after stimulation because previous studies show that it is in the middle of the time course of pMeCP2-S421 induction after other similar drug exposures (Deng et al. 2010; Hutchinson et al. 2012a; Hutchinson et al. 2012b; Zhou et al. 2006). Mice that received U50488 showed a significant increase of pMeCP2-S421 in the NAc compared with Vehicle-treated mice ($p=0.025$; $n = 7$ Vehicle, 7 U50488; Fig. 1b,c). By contrast, there were no significant changes in pMeCP2-S421 following U50488 exposure when we quantified immunostaining in PLC ($p=0.97$), ILC ($p=0.63$), or BLA ($p=0.26$) (Fig. 1b,c).

MeCP2 is a nuclear protein that regulates transcription, and Ser421 phosphorylation of MeCP2 is induced by calcium-activated kinases that are known to regulate neuronal activity-dependent genes (Lyons and West 2011; Tao et al. 2009; Zhou et al. 2006). To determine whether the induction of pMeCP2-S421 by exposure to U50488 correlates with the induction of activity-dependent gene transcription, we assessed the expression of the immediate-early gene product Fos in the same brain regions we evaluated for pMeCP2-S421. Similar to our observations for pMeCP2-S421, we found that U50488 significantly induced Fos expression in the NAc compared with Vehicle-treated mice ($p=0.027$; $n = 5$ Vehicle, 5 U50488; Fig. 1d,e). U50488 did not alter Fos expression in the PLC ($p=0.054$), ILC ($p=0.73$), or BLA ($p=0.30$) (Fig. 1d,e). These data show that acute exposure to U50488 induces pMeCP2-S421 and gene transcription in neurons of the NAc, an effect that may contribute to the ability of this drug to induce aversive learning.

The expression of aversive place conditioning is associated with decreased pMeCP2-S421 in the ILC and BLA

Repeated exposure to aversive drugs is sufficient to induce lasting changes in behavior that can be measured by place conditioning (Prus et al. 2009). To determine whether the expression of place conditioning is contemporaneous with regulation of pMeCP2-S421, we assessed pMeCP2-S421 levels in mPFC and limbic brain regions 2hr after the expression of CPA induced by repeated exposure to U50488, a kappa opioid agonist able to induce CPA (Mucha and Herz 1985; Wee and Koob 2010). Male C57BL/6J mice were conditioned in a three-chambered apparatus, with control mice receiving only saline as Vehicle and the experimental group receiving saline or 5mg/kg U50488 (i.p.) on alternating days in opposite chambers following the protocol in Fig. 2a. Two-way repeated measures ANOVA yielded significant effects for treatment ($F_{1,11}=5.561$, $p=0.038$) and time ($F_{1,11}=11.735$, $p=0.006$), and Bonferroni-corrected posthoc comparisons revealed that only U50488-treated mice showed reduction in preference scores during conditioning ($p=0.004$; Fig. 2b). Thus pairing with 5mg/kg (i.p.) U50488 was sufficient to induce place aversion in male C57BL/6J mice.

In addition to its regulation by phosphorylation, the expression of MeCP2 is sensitive to modulation by select stimuli. For example, chronic exposure to cocaine, fluoxetine, or nicotine is sufficient to induce the expression of MeCP2 in physiologically relevant brain regions (Cassel et al. 2006; Im et al. 2010; Pastor et al. 2011), though chronic heroin intake is not associated with changes in MeCP2 expression (Theberge et al. 2012). To determine whether U50488-induced CPA changes expression of total MeCP2 in mPFC and limbic brain regions, we compared the MeCP2 expression in these brain regions between mice that had been exposed to saline alone and mice exposed alternately to saline and 5mg/kg U50488 in the CPA paradigm. This was performed using western blotting, a method that is widely used to capture the shifts in the levels of total MeCP2, which is expressed extensively in corticolimbic brain regions (Cohen et al. 2011). These data showed no significant difference in MeCP2 expression in mPFC, NAc, or BLA between saline control and U50488 treated mice (mPFC $p=0.87$; NAc $p=0.82$, BLA $p=0.59$; $n=4-6$ per group; Fig. 2c,d).

To determine whether pMeCP2-S421 is induced during the expression of CPA, following the assessment of place preference on the final testing day, mice were perfused and coronal brain sections were immunostained for pMeCP2-S421 in the PLC, ILC, NAc, and BLA. We then compared the levels of pMeCP2-S421 in corticolimbic brain regions between mice that had been exposed to U50488 during the training period with those that had received only saline. Unlike the induction of pMeCP2-S421 we observed upon exposure to U50488, the expression of U50488-induced CPA was accompanied by significantly decreased pMeCP2-S421 immunofluorescence in the ILC and BLA (ILC $p=0.013$; BLA $p=0.034$; $n = 7$ Vehicle, 7 U50488; Fig. 2e,f). We found no significant differences in pMeCP2-S421 levels between control and CPA mice in the NAc ($p=0.10$) or PLC ($p=0.45$) (Fig. 2e,f).

To determine whether CPA is associated with stimulus-dependent changes in the expression of activity-dependent genes in corticolimbic brain regions, we measured expression of Fos on the same sections used for pMeCP2-S421 immunostaining. Following the expression of CPA, Fos expression was significantly elevated in the BLA of mice that had been exposed to U50488 relative to control mice that had received only saline ($p=0.035$; $n = 7$ Vehicle, 7

U50488; Fig. 2g,h). No differences in Fos levels were observed between U50488-treated and control mice in the PLC ($p=0.14$), ILC ($p=0.40$), or NAc ($p=0.60$) (Fig. 2g,h).

Absence of pMeCP2-S421 does not influence the behavioral expression of CPA but does impair CPA-associated gene induction

To determine whether pMeCP2-S421 is required for CPA, we assessed U50488-induced CPA in a strain of MeCP2 knockin (KI) mice in which Serine 421 of MeCP2 has been replaced by the nonphosphorylatable amino acid alanine (Fig. 3a) (Cohen et al. 2011). Consistent with previous reports on this strain (Cohen et al. 2011; Deng et al. 2014), we found that, compared with their wildtype (WT) littermates, the mice lacking phosphorylation of MeCP2 at Ser421 showed no difference in MeCP2 expression in mPFC, NAc, or BLA (relative expression MeCP2 mPFC WT= 1 ± 0.25 , KI= 0.90 ± 0.22 , $p=0.77$; NAc WT= 1 ± 0.36 , KI= 0.81 ± 0.14 , $p=0.66$; BLA WT= 1 ± 0.31 , KI= 1.4 ± 0.28 , $p=0.37$, $n=6$ WT, 6KI). Place conditioning was then compared between KI mice and their wild-type (WT) littermates. Both WT and KI mice were first assessed for baseline preference during acclimation and then received either only saline or, on alternating days, 5mg/kg U50488 (i.p.) or saline as vehicle in opposite chambers following the experimental protocol in Fig. 2a. Following conditioning, two-way repeated measures ANOVA showed that neither WT nor KI mice exposed only to saline showed a significant change in their preference for the chamber assigned as “drug-paired” at acclimation ($F_{1,11}=0.018$, $p=0.9$; Supplementary Material Table 2). By contrast, two-way repeated measures ANOVA after conditioning of both genotype groups by U50488 exposure showed significant effects for time ($F_{1,27}=12.491$, $p=0.001$) but not genotype ($F_{1,27}=0.005$, $p=0.95$), indicating that pMeCP2-S421 is not required for the behavioral expression of CPA (Fig. 3b; Supplementary Material Table 3).

These findings are consistent with previous studies in MeCP2 Ser421Ala KI mice showing that pMeCP2-S421 is not required for the expression of locomotor sensitization induced by drugs of abuse (Deng et al. 2014). Nevertheless, these mice also show subthreshold induction of locomotor sensitization, and this effect is associated with impaired induction of Fos expression in the NAc (Deng et al. 2014), suggesting that pMeCP2-S421 can shape neuronal activity within the circuits that underlie drug-induced behaviors. To examine whether pMeCP2-S421 similarly regulates neuronal activity during the expression of U50488-induced CPA, we compared Fos levels between KI and WT mice in the BLA, the brain region that showed Fos induction during CPA expression (Fig. 2). Indeed, following CPA we found significantly less Fos in the BLA of KI mice compared with their WT littermates ($p=0.014$, $n=9$ WT, 8 KI; Fig. 3c,d). By contrast, we saw no differences in Fos levels between genotypes in the PLC ($p=0.49$) or ILC ($p=0.29$), two brain regions that do not show CPA-associated inductions of Fos (Fig. 2). These data suggest that the MeCP2 Ser421Ala KI mice show an altered plasticity of select brain regions that participate in the expression of CPA.

Discussion

The present study examined the regulation and function of MeCP2 Ser421 phosphorylation in corticolimbic brain regions in a paradigm of aversive learning in mice. We first showed

that acute exposure to the aversive kappa opioid agonist U50488 induces pMeCP2-S421 selectively in the NAc, and this change is accompanied by increased neuronal activity as suggested by the induced expression of Fos in the same brain region. By contrast, the expression of U50488-induced CPA was associated with decreased pMeCP2-S421 in the ILC and BLA and induced Fos in the mouse BLA, indicating that the expression of the aversive drug-induced CPA is associated with corticolimbic pMeCP2-S421 and neuronal activity changes that are distinct from the changes observed upon acute exposure to the drug. Lastly, we found that although mice lacking pMeCP2-S421 exhibit intact behavioral expression of CPA, they showed decreased CPA-associated Fos expression in the BLA. Taken together, our findings support a model whereby U50488-induced pMeCP2-S421 contributes to molecular plasticities in brain regions that underlie aversive behaviors.

Our study is the first to show the induction of pMeCP2-S421 in the NAc after acute exposure to an aversive drug, though pMeCP2-S421 induction has been previously observed in this brain region during morphine withdrawal (Ciccarelli et al. 2013), which is also an aversive state. Given that we have previously observed pMeCP2-S421 induction in the NAc upon exposure to drugs with positive motivation valence (Deng et al. 2010), these data suggest that pMeCP2-S421 regulation reflects a shared response to emotionally salient environmental signals, irrespective of the positive or negative valence of the stimulus. This is also in accordance with previous studies showing the involvement of NAc in both reward and aversion (Carlezon and Thomas 2009; Muschamp et al. 2011; Wheeler and Carelli 2009). Interestingly, in addition to its regulation by phosphorylation, in other paradigms MeCP2 is also subject to experience-dependent changes in its expression via the microRNA miR-212 (Im et al. 2010). In line with our findings of the non-specific induction of pMeCP2-S421 in opposite motivational states, changes in MeCP2 expression may similarly be shared among stimuli of opposite valence. This is supported by a previous study using two different doses of nicotine to induce either CPA or CPP, where both paradigms induced comparable increases in the expression of MeCP2 in the NAc but differential regulation of other molecular markers, such as the cAMP-response element-binding protein (Pastor et al. 2011). Taken together, these findings suggest that MeCP2 may modulate motivational learning by responding in the NAc irrespective of the valence of the stimulus, but this shared initial response could be coupled with upstream or downstream molecular events that are specific to the motivational valence of the stimulus.

Both rewarding and aversive stimuli are salient to mice, and they share the ability to induce the associative learning that underlies the behavioral expression of conditioned place aversion or preference. However, what is most striking about both the current study of CPA, as well as previous studies of MeCP2 Ser421 in other behavioral tasks, such as spatial learning (Cohen et al. 2011; Li et al. 2011) or social defeat stress (Hutchinson et al. 2012b), is that they uniformly show pMeCP2-S421 is not required for the forms of learning tested. Instead, past studies have shown that pMeCP2-S421 plays a modulatory role by limiting the development and/or the extent of synaptic and behavioral plasticities. For example, MeCP2 Ser421Ala KI mice more rapidly develop behavioral sensitization to repeated AMPH, and this correlates with the precocious appearance in these mice of intrinsic excitability changes in striatal neurons that are thought to underlie the expression of this behavioral response (Deng et al. 2014). Accordingly, although we show that pMeCP2-S421 is not required for

the expression of CPA, the reduced Fos induction in the BLA of MeCP2 KI mice suggests that pMeCP2-S421 may modulate the molecular plasticity of brain circuits involved in aversive learning.

How might pMeCP2-S421 influence mesolimbic brain circuits during motivational learning? MeCP2 binds both methylated DNA and other transcriptional regulatory proteins, and via these interactions it is thought to play a global modulatory role in the regulation of gene transcription (Lyst and Bird 2015). However, whether there are specific gene targets of MeCP2 that explain its requirement for proper neuronal function is not fully understood, and which functions of MeCP2 are affected by its phosphorylation at Ser421 remains unknown. Intriguingly, pMeCP2-S421 may show divergent effects on gene transcription across different brain regions, as suggested by our observation that pMeCP2-S421 and Fos increase concomitantly in the NAc but change in opposite directions in the BLA. Such opposite changes could reflect the indirect effects of MeCP2 changes propagating across distinct neuronal subpopulations of the BLA. While the present study did not distinguish the regulation of pMeCP2-S421 within specific neuronal subpopulations or within subregions with distinct functional roles -such as the central nucleus, an amygdalar subregion that can undergo molecular changes distinct and even opposite to the basolateral amygdala during aversive learning (Frenois et al. 2005; Lucas et al. 2012; Lucas et al. 2008)- our findings suggest that pMeCP2-S421 may contribute, through either direct or indirect mechanisms, to the regulation of stimulus-inducible transcriptional programs in brain circuits that govern aversive behaviors. Future studies of neuronal excitability in corticolimbic brain regions in the MeCP2 Ser421Ala KI mice may provide further insights into the circuit plasticities and the molecular mechanisms underlying different modes of motivational learning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Administration of U50488 induces MeCP2 Ser421 phosphorylation (pMeCP2-S421) and Fos in the nucleus accumbens (NAc). **a**, Atlas figures from Franklin and Paxinos (2007) showing the anatomical position of the fields we quantified for NAc, Prelimbic Cortex (PLC), Infralimbic Cortex (ILC), and basolateral amygdala (BLA) throughout this study. **b**, Representative brain sections immunolabeled for pMeCP2-S421 from mice that were injected with Vehicle or U50488. The anterior commissure is at the upper left corner of each NAc section, and medial is to the right. PLC is above the white dotted line, ILC is below the

white dotted line, and medial is to the right. BLA is bounded by the dotted lines, and medial is to the right. **c**, Quantification of the integrated intensity of pMeCP2-S421 immunofluorescence in the brain regions noted. Intensity was scaled in order to set Vehicle levels for each brain region equal to 100 so that relative percent change with U50488 could be displayed. $n = 7$ Vehicle, 7 U50488; $*p < 0.05$ Vehicle vs. U50488. Scale bars, NAc (60 μ m), ILC/PLC (100 μ m), BLA (100 μ m). **d**, Representative brain sections immunolabeled for Fos from mice that were injected with Vehicle or U50488. **e**, Quantification of the integrated intensity of Fos immunofluorescence in the NAc, PLC, ILC, and BLA. Intensity was scaled in order to set Vehicle levels for each brain region equal to 100 so that relative percent change with U50488 could be displayed. $n = 5$ Vehicle, 5 U50488; $*p < 0.05$ Vehicle vs. U50488. Scale bars: NAc (60 μ m), ILC/PLC (100 μ m), BLA (100 μ m). In all graphs, bars indicate averages and error bars show S.E.M.

Fig 2.

U50488 induces conditioned place aversion (CPA) that is associated with decreased pMeCP2-S421 in the infralimbic cortex (ILC) and basolateral amygdala (BLA) and with Fos induction in the BLA. **a**, Procedure for place conditioning. P, designated drug-paired chamber; NP, non-paired (vehicle) chamber. **b**, Preference for the designated drug-paired chamber at acclimation and at challenge after pairing with Vehicle or upon CPA induced by U50488. Two-way repeated measures ANOVA yielded significant effects for both treatment ($F_{1,11}=5.561$, $p=0.038$) and time ($F_{1,11}=11.735$, $p=0.006$). $n = 6$ Vehicle, 7 U50488;

$p < 0.01$, n.s., not significant, posthoc Bonferroni-corrected p values. **c**, Western blot of MeCP2 protein in medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and BLA of mice exposed to Vehicle or U50488 in the CPA paradigm shown in **a**. Actin is shown as a loading control. **d**, Quantification of the western blots shown in **c**. MeCP2 expression is normalized to the expression of actin in each lane. $n = 4-6$ per condition. **e**, Representative brain sections immunolabeled for pMeCP2-S421 in mice that were injected with Vehicle or underwent U50488-induced CPA. **f**, Quantification of the integrated intensity of pMeCP2-S421 immunofluorescence in the NAc, Prelimbic Cortex (PLC), ILC, and BLA. Intensity was scaled in order to set Vehicle levels for each brain region equal to 100% so that relative percent change with U50488 could be displayed. $n = 7$ Vehicle, 7 U50488; $*p < 0.05$ Vehicle vs. U50488. **g**, Representative brain sections immunolabeled for Fos in mice that were injected with Vehicle or underwent CPA induced by U50488. **h**, Quantification of the integrated intensity of Fos immunofluorescence in the Nucleus Accumbens (NAc), Prelimbic Cortex (PLC), Infralimbic Cortex (ILC), and BLA after CPA. Intensity was scaled in order to set Vehicle levels for each brain region equal to 100 so that relative percent change with U50488 could be displayed. $n = 7$ Vehicle, 7 U50488. Scale bars in **e** and **g**, NAc (60 μ m), ILC/PLC (100 μ m), BLA (100 μ m). In all graphs, bars indicate averages and error bar show S.E.M.

Fig 3.

Loss of pMeCP2-S421 does not affect the expression of U50488-induced conditioned place aversion (CPA), but impairs CPA-associated Fos expression. **a**, Western blot of MeCP2 and pMeCP2-S421 in medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and basolateral amygdala (BLA) of drug-naïve MeCP2 Ser421 knockin (KI) mice and their wildtype (WT) littermates. Actin is shown as a loading control. **b**, Preference for the designated drug-paired chamber at acclimation and at challenge after U50488-induced conditioning of MeCP2 Ser421Ala KI mice and their WT littermates. Two-way repeated

measures ANOVA showed significant effects for time ($F_{1,27}=12.491$, $p=0.001$) but not genotype ($F_{1,27}=0.005$, $p=0.95$). U50488 $n=13$ WT, 16 KI. $\#p<0.01$, $*p<0.05$, posthoc Bonferroni-corrected p values. **c**, Representative brain sections immunolabeled for Fos in MeCP2 wildtype WT and MeCP2 Ser421Ala knockin KI mice that were injected with U50488 in CPA. **d**, Quantification of the integrated intensity of Fos immunofluorescence in the BLA, where Fos is induced after CPA, as well as the Prelimbic Cortex (PLC) and Infralimbic Cortex (ILC), two brain regions that do not show CPA-induced Fos. Intensity was scaled in order to set WT levels for each brain region equal to 100 so that relative percent difference in the MeCP2 Ser421Ala KI mice could be displayed. $n = 9$ WT, 8 KI. $*p < 0.05$ WT vs. KI. Scale bars in **c**, 100 μ m, **e**, 60 μ m. In all graphs, bars indicate average and error bar show S.E.M.