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Chromatin Alterations in Response to Forced Swimming Underlie Increased Prodynorphin Transcription

Brian Reed^a, Nancy Fang^{a,1}, Brandon Blackwell-Mayer^a, Shasha Chen^a, Vadim Yuferov^a, Yan Zhou^a, and Mary Jeanne Kreek^a

^a The Laboratory of the Biology of Addictive Diseases, The Rockefeller University, New York, NY, USA 10065

Abstract

Antagonism of the kappa opioid receptor (KOR) has been reported to have anti-depressant-like properties. The dynorphin/KOR system is a crucial neurochemical substrate underlying the pathologies of addictive diseases, affective disorders and other disease states. However, the molecular underpinnings and neuroanatomical localization of the dysregulation of this system have not yet been fully elucidated. Utilizing the Porsolt Forced Swim Test (FST), an acute stressor commonly used as in rodent models measuring antidepressant efficacy, male Sprague-Dawley rats were subject to forced swimming for 15 minutes, treated 1 hour with vehicle or nor-BNI (5 or 10 mg/kg), and then 1 day later subject to FST for five minutes. In accordance with previous findings, nor-BNI dose dependently increased climbing time and reduced immobility. In comparison to control animals not exposed to FST, we observed a significant elevation in prodynorphin (pDyn) mRNA levels following FST using real-time optical PCR in the caudate putamen but not in the nucleus accumbens, hypothalamus, amygdala, frontal cortex, or hippocampus. Nor-BNI treatment did not affect pDyn mRNA levels in comparison to animals that received vehicle. The corresponding brain regions from the opposite hemisphere were analyzed for underlying chromatin modifications of the prodynorphin gene promoter region using chromatin immunoprecipitation with antibodies against specifically methylated histones H3K27Me2, H3K27Me3, H3K4Me2, and H3K4Me3, as well as CREB-1 and MeCP2. Significant alterations in proteins bound to DNA in the Cre-3, Cre-4, and Sp1 regions of the prodynorphin promoter were found in the caudate putamen of the FST saline-treated animals compared to control animals, with no changes observed in the hippocampus. Epigenetic changes resulting in elevated dynorphin levels specifically in the caudate putamen may in part underlie the enduring effects of stress.

Keywords

Stress; Dynorphin; Histones; CREB; MeCP2; Caudate-Putamen

Affective disorders and addiction to drugs of abuse have a high propensity for comorbidity, with groups that are vulnerable to one often showing vulnerability to the other. For instance, people who have experienced significant traumas are at greater risk for the development of

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Corresponding Author: Brian Reed The Laboratory of the Biology of Addictive Diseases The Rockefeller University Box 171 1230 York Avenue New York, NY USA 10065 Ph. +1.212.327.8248 Fx. +1.212.327.8574 reedb@rockefeller.edu.

¹Present address - University of Pittsburgh School of Medicine, Pittsburgh, PA, USA 15261

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both atypical depression(Levitan et al., 1998) and addiction(Lo and Cheng, 2007). The propensity for comorbidity of depression and addiction are suggestive of overlapping neurochemical and neuroanatomical substrates. These substrates have been investigated intensively in animal models of both depression and addiction (Nierenberg et al., 2008, Qi et al., 2011).

The kappa-opioid system has recently been under intensive investigation as a potential new therapeutic target (Kreek et al., 2002, Carlezon et al., 2009). Stress-induced behaviors in animal models of depression and addiction have been demonstrated to be blocked by kappa-opioid receptor antagonists. Nor-BNI has been demonstrated to be effective in attenuating immobility by rodents in the Porsolt Forced Swim Test (Pliakas et al., 2001, Mague et al., 2003, McLaughlin et al., 2003, Land et al., 2008, Chartoff et al., 2012), a robust model which has been shown to have predictive value for antidepressant efficacy(Porsolt et al., 1977). In the case of addiction, nor-BNI and JDtic, both long-duration kappa opioid antagonists, have been shown to attenuate stress-induced cocaine seeking in self-administration paradigms(Beardsley et al., 2005, Redila and Chavkin, 2008). Together, these results support the possibility of the kappa opioid receptor as a potential therapeutic target for both depression and addictions, as well as in cases where these disorders are comorbid.

Given the efficacy of kappa opioid receptor antagonists in animal behavioral models of depression and addiction, furthering our understanding of the mechanism underlying the stress-involvement in these disorders requires investigation of the dynorphin-kappa opioid receptor system. Although investigations into stress-induced changes in the dynorphin-kappa-opioid receptor system have been conducted, they have generally had a narrow neuroanatomical focus(Shirayama et al., 2004, Chartoff et al., 2009); the dynorphin-kappa opioid receptor systems are localized in several brain regions, including the hypothalamus, amygdala, and frontal cortex, which play important roles in the regulation of stress-responsivity, as well as in mesolimbic brain regions which are crucial to reward-motivated behavior. In our current studies, we have endeavored to determine which brain regions might be responsible for the observed effects of kappa-opioid antagonists on stress-induced behaviors, with semiquantitative measurements of the levels of prodynorphin mRNA in specific brain regions.

In the study of the neurochemical underpinnings of addictions, depression, and other diseases, the measurement of mRNA levels of genes which encode for proteins suspected to be involved in dysregulation underlying altered neuropsychological states has been of incredible importance to advancing our mechanistic understanding. Epigenetic changes in chromatin structure and DNA methylation in the promoter region of the genes of interest are thought to largely be responsible for alterations in mRNA expression levels, via regulation of transcription start site accessibility and RNA polymerase initiation. These epigenetic changes, which can include specific histone modifications, binding of transcription factors and other DNA binding proteins, and complexes of a number of different proteins, drive, at least in part, adaptations in protein levels which control cell signaling(Kumar et al., 2005, Tsankova et al., 2007, Wilkinson et al., 2009, Maze et al., 2010). These complex pathways of transcriptional control are not yet fully understood, but may eventually provide a different level of therapeutic targeting for dysfunctional neurochemical signaling.

Experimental Procedures

Animals

A total of 24 male Sprague Dawley rats (Charles River) weighing approximately 285-330 g were used. Rats were housed individually in plastic cages with wood shavings and additional paper bedding, and were maintained on a 12 hour light (9:00-21:00)/12 hour dark

cycle with free access to food and water except during testing. Experiments were conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH).

Test Compounds

Norbinaltorphimine (nor-BNI) was donated by the National Institute of Health- National Institutes of Drug Addiction Division of Drug Supply and Analytical Services. Nor-BNI was dissolved in sterile saline and administered systemically via the intraperitoneal route at the indicated doses of 5 and 10 mg/kg.

Forced Swim Test (FST)

Eighteen rats were exposed to the Porsolt FST in this study (Porsolt et al., 1977). The rats were exposed initially to forced swim stress in a 15 minute swim session on day one in a 28.4 L Nalgene cylindrical tank (Fisher Scientific, Pittsburgh, PA) filled to 75% of total volume with water (25°C). The rats were then placed in clean cages under warm lights for 15 minutes and then returned to their home cages. There were three treatment groups—6 rats per group received either 5 mg/kg or 10 mg/kg nor-BNI or saline administered intraperitoneally, targeted at one hour after the first swim exposure. The rats were subjected 24 hours later to 5 minutes under identical swim conditions. The 5 minute duration for the actual testing period for comparison of scoring has been found sufficient to demonstrate the effects of the initial forced swim stress on swim behaviors, as well as the attenuation of such effects by effective antidepressant therapeutics (Porsolt et al., 1977). FST sessions were videotaped and scored. Six rats used as controls were not subjected to FST and were placed in new cages for 15 minutes on day 1, and 5 minutes on day 2, during the time period that study animals were placed in the water cylinders.

FST Scoring

Persons unaware of which animals were subject to each treatment condition scored the videotapes of the FST session. In the behavioral testing, rats were rated in 5-second intervals throughout the 5 minute retest session for the dominant behavior exhibited. These fell into three categories: immobility, swimming, and climbing (modified from Detke (Detke et al., 1995)). Immobility was categorized as the rat making only movements to keep its head above the water, swimming if the rat was making active movements to move around or swim below the water's surface, and climbing if the paws were actively thrashing above the water at the cylinder wall in attempt to climb out. Data analysis for the determination of effects of treatment on the behavioral parameters was carried out using one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc analysis to determine statistical significance ($p < 0.05$).

Brain Region Dissections

Rats were anesthetized using brief exposure (<20 seconds) to CO₂ and sacrificed 30 minutes after their FST retest session. Whole brains were removed from decapitated rats and immediately frozen at -80°C. The caudate putamen, nucleus accumbens, hypothalamus, frontal cortex, hippocampus, and amygdala were isolated by gross dissection, separating the left and right sides of the brain into individual containers, and frozen at -80°C until further processing. One side of the brain was used to measure relative mRNA expression of the prodynorphin gene. The other hemisphere was used for chromatin immunoprecipitation (ChIP) assays.

mRNA Expression Analysis

Dissected brain regions from animals receiving nor-BNI (5 or 10 mg/kg) or vehicle and subject to FST, and naïve control were processed for prodynorphin mRNA quantitation.

RNA isolation was performed using TRIzol® reagent kit (Invitrogen, Carlsbad, CA). Total RNA (14 µl, range of 1-3 ng/µl) was used to synthesize cDNA with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Primers specific for the prodynorphin gene and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene (Applied Biosystems, Foster City, CA) were used. GAPDH is ubiquitously expressed and was measured here as a “house-keeping” gene for normalization (Schlussman et al., 2010). Prodynorphin and GAPDH expression levels were determined with ABI Prism 7900HT Sequence Detection System (Applied Biosystems) in a total volume of 12 µl in the presence of TaqMan® Universal PCR Master Mix (Applied Biosystems), with 2 µl of 1:10 diluted cDNA. The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. A dissociation stage was included to ensure the purity of the PCR products. All samples were run in quadruplicate and reported values were normalized to GAPDH. Ct values, at which the fluorescent values associated with PCR amplification crossed a threshold defined by the user, were detected with SDS 2.1 software (Applied Biosystems). Normalized values were obtained by calculating $\Delta Ct = Ct_{GAPDH} - Ct_{pdyn}$, with the fold difference determined as $2^{-(\Delta Ct)}$. The mean and SEM of these values for the different treatment groups were analyzed using a one-way ANOVA followed by Newman-Keuls post-hoc analysis to determine statistical significance ($p < 0.05$).

Chromatin Immunoprecipitation Assay

Brain tissue from the caudate putamen of animals exposed to FST and receiving vehicle (n=6) and of control animals that were not exposed to force swim test (n=6) were processed into chromatin by use of published protocols (Tsankova et al., 2004) with some modifications. Each half-brain region was thawed, minced into ~1 mm-sized pieces, placed in 0.5 mL PBS and immediately crosslinked with formaldehyde (1%). The samples were incubated at 37°C for 10 min. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M. The tissue was washed four times with cold PBS containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

The samples were centrifuged ($100 \times g$) and re-suspended in homogenization buffer (10 mM Tris, pH 8.1, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail). Next, the samples were homogenized with a Brinkman Polytron PT 3000 homogenizer (Kinematica, Bohemia, NY) twice for ten seconds ($18,000 \times g$), and the homogenate centrifuged ($10,000 \times g$, 4°C, 5 min). The supernatant was decanted and the pellet was re-suspended in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitor cocktail). The sample was placed on ice for 15 min to ensure solubilization of the cells and re-homogenized under the same conditions. Next, the extracted chromatin was sheared using via sonication (Model 150 V/T, BioLogics Inc., Manassas, VA). Each sample was sonicated fifteen times on ice, 15 sec each, at 75% of maximum power, with at least 1 min between each sonication step.

The chromatin lysate was centrifuged ($1700 \times g$, 4°C, 10 min) and the supernatant was transferred to a new microcentrifuge tube. Equal amounts of chromatin lysate (50 µl) were diluted with dilution buffer (20 mM Tris, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, protease inhibitor cocktail) to a final volume of 500 µl. Five µl were saved as “input” for later normalization.

The chromatin solution was pre-cleared with protein-A Dynabeads (Invitrogen) coated with salmon sperm DNA and bovine serum albumin (BSA) for 30 min, with rotation at 4°C. The supernatant was collected and immunoprecipitated overnight at 4°C in separate aliquots with 2 µg of CREB-1 antibody (Santa-Cruz, Santa Cruz, CA), 1 µg of MeCP2 antibody (AbCam, Cambridge, MA), 1 µg H3K27Me2 antibody (AbCam), 1 µg H3K27Me3 antibody (AbCam), 1 µg H3K4Me2 antibody (Fisher Scientific), or 2 µg H3K4Me3 antibody (Fisher Scientific). As a control, one sample per brain region was incubated without antibody.

(nonspecific binding). After immunoprecipitation, the DNA-histone complex was collected with 20 μ g protein-A Dynabeads (Invitrogen) coated with salmon sperm DNA and BSA for 1.5 hrs at 4°C. The beads were washed, in succession (to minimize nonspecifically bound protein and oligonucleotides), with low salt buffer, high salt buffer, and LiCl containing buffer, and then washed twice with 10 mM Tris, pH 8.1/ 1 mM EDTA buffer. The samples were eluted with 125 μ l elution buffer (1% SDS, 0.1 M NaHCO₃), twice in succession, and incubated at 25°C for 15 min with shaking. Crosslinks were reversed via incubation of the samples at 65°C for 4 hr with 60 μ l of 1 M NaCl. “Input” chromatin lysate was diluted with elution buffer to a 1:10 dilution for a final volume of 50 μ l and also dissociated under high salt conditions. Proteins were digested using proteinase K treatment at 45°C for 2 hr. The DNA was then extracted with phenol/chloroform/ chloroform, precipitated with 100% ethanol, and resuspended in 50 μ l PCR-grade water. “Input” DNA samples were purified with QIAQuick PCR purification kits (Qiagen, Valencia, CA) due to poor recovery with phenol/chloroform/chloroform extraction.

Quantification of DNA by real-time PCR

Levels of immunoprecipitated DNA associated with antibody targets of interest by quantitative real-time PCR). Custom primers were designed to amplify regions in the prodynorphin gene promoter containing known Cre-sites and an Sp1 site (Douglass et al., 1994). The primers 5'-GATACGGTGAAACAAACAAGCGGCAA-3' and 5'-GGGCATCATCCTCTTCCT-3' amplified a region containing CRE-1 and CRE-2 sites. The primers 5'-GGAAGAGGATGATGCCCA-3' and 5'-CATACTTCCCTGCAGCTGTCC-3' amplified a region containing the CRE-3 site. The primers 5'-GAGCAAGCCGACATATCCAGT-3' and 5'-GGGTTTAGAACTGGCCACA-3' amplified a region containing the Sp1 site. The primers 5'-AGAACTGCCATAGGGGGATT-3' and 5'-ATCTTACCTGCGTGCTGCTT-3' amplified a region containing the CRE-4 site and the transcription start site. Amplification reactions were run in quadruplicate in presence of SYBR-Green (Applied Biosystems). The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 30 sec, 54 °C for 15 sec and 72°C for 30 sec. A dissociation stage was included to ensure the purity of the PCR products. Ct values from each sample were determined using SDS 2.1 (Applied Biosystems). Normalized values were obtained by calculating $\Delta Ct = Ct_{GAPDH} - Ct_{pdyn}$, with the fold difference determined as $2^{-(\Delta Ct)}$. Mean and SEM values were calculated for each fold difference between treatment and control groups. These values analyzed with two-tailed T-tests to determine statistical significance ($p < 0.05$).

Results

Forced Swim Test

Rats were exposed to the classical Porsolt Forced Swim Test (FST) (Porsolt et al., 1977). One hour following the initial exposure to 15 minutes of forced swim stress, animals were injected with saline or one of two doses of nor-BNI (5 or 10 mg/kg) prior to testing 24 hours later in a five minute session. Following blind scoring for immobility, swimming, and climbing, we found a main effect of treatment on immobility measures ($F_{(2,15)} = 4.42$, $p < 0.05$) (Figure 1A), but not on climbing or swimming (Figure 1B and 1C). Post-hoc analyses revealed that 10 mg/kg nor-BNI significantly reduced immobility in comparison with saline controls ($p < 0.05$). For the climbing measurements, one way ANOVA with planned comparisons between groups receiving nor-BNI and the group receiving saline revealed a significant increase in climbing in response to treatment with nor-BNI ($F_{(1,15)} = 5.43$, $p < 0.05$) (Figure 1B).

Prodynorphin mRNA Gene Expression

We measured the relative levels of prodynorphin mRNA, via real time optical PCR normalized to GAPDH, in select brain regions known to express prodynorphin dissected from animals treated with vehicle or 10 mg/kg nor-BNI and subject to FST, as well as control animals not subject to FST. There was a main effect of treatment on mRNA expression of prodynorphin in the caudate-putamen in animals subjected to the FST ($F_{(2,15)} = 4.45$, $p < 0.05$). Post-hoc analyses revealed that both FST group, treated with saline or nor-BNI, had elevated prodynorphin mRNA in this brain region compared to control animals ($p < 0.05$) (Figure 2). There was no significant difference of prodynorphin mRNA levels between animals receiving saline and nor-BNI (10 mg/kg). Also, there was no change in prodynorphin mRNA levels observed in other brain regions, including the nucleus accumbens, hypothalamus, frontal cortex, hippocampus, and amygdala (Figure 2).

Chromatin Immunoprecipitation

We performed ChIP assays using antibodies against select histone methylation states, histone H3-K4Me₂ and Me₃, which are generally associated with increases in transcriptional activity, and histone H3-K27Me₂ and Me₃, which are associated with decreases in transcriptional activity, as well as antibodies against phospho-CREB, which is a well-described transcription factor driving prodynorphin gene expression (Pliakas et al., 2001, Briand and Blendy, 2010) and MeCP2, which primarily binds to methylated CpG DNA (Guy et al., 2011) (Table 1). The association of proteins bound to DNA in the Cre-3, Cre-4, and Sp1 regions of the prodynorphin promoter were found to be altered in the caudate putamen of the FST saline-treated animals ($n=6$) compared to control animals ($n=6$) (Table 2, Figure 3). Within the Cre-3 primer region of the prodynorphin gene promoter, there was a significant increase in association of phospho-CREB and a decrease in the association of MeCP2, H3K27Me₂ and H3K4Me₃ proteins in animals exposed to force swim test compared to control animals. Within the Cre-4 region, there was a significant increase of phospho-CREB and decrease of H3K4Me₃ in animals exposed to force swim test compared to control animals. Finally, there was also a significant decrease of H3K4Me₃ proteins in the Sp1 region of the prodynorphin promoter region. The direction of change in association of these proteins with the prodynorphin gene promoter is generally consistent with the increase in dynorphin gene expression in the caudate putamen, with the exception of the reduction seen in H3K4Me₃. Of note, for the Cre-1/2 region, no statistically significant changes were observed. The rt-PCR signal was generally lower for this amplicon (i.e. C_t , the cycle during which the signal crossed threshold, was higher), both for input DNA and for immunoprecipitated DNA, potentially reflecting enhanced susceptibility to cleavage in the sonication step of the assay. Consequently, the relative variability was considerably greater than for the other promoter regions.

There was no significant difference in the association of any of the tested chromatin proteins and prodynorphin gene promoter regions between FST and control animals in the hippocampus (Table 3).

It should be noted that significance was assigned using a p-value of 0.05, determined using the student T-test, without correction for multiple comparisons. The most often used method in statistical analyses for controlling for multiple comparisons, the Bonferroni correction, is very conservative. The level of expected false positive significant differences for a given brain region, with 24 independent measurements, at a level of $p < 0.05$, is 1.2, based on the Poisson distribution. In the striatum, we found 7 differences at this level of confidence, highly suggestive that many of these differences are in fact real, and not resulting from random variance. The presence of considerably greater number of results with p-values less than 0.05 than expected based on the Poisson distribution is compelling argument to not

reject these differences as insignificant. We have included both the point-wise and experiment-wise (corrected for multiple comparisons using the Bonferroni correction) p-values in tables 2 and 3.

Discussion

We conducted these studies in rodent models with the goal of elucidating the response of prodynorphin, the precursor for dynorphin peptides which interact with the kappa opioid receptor system, to an acute stressor, forced swimming, to better understand the mechanisms which may underlie the effects of kappa opioid receptor antagonists in the Porsolt Forced Swim test. The kappa opioid receptor system has an elevated expression in human brain compared to rodents (Peckys and Lendwehrmeyer, 1999, Mathieu-Kia et al., 2001), and thus the kappa opioid receptors may play an even more pronounced role in stress-related disorders in humans. The current studies may be helpful in guiding future studies of the role of the kappa-opioid receptor system in humans.

We observed an increase in the levels of prodynorphin mRNA only in the caudate putamen, 30 minutes following the completion of the classical Porsolt FST. The attenuation of time spent immobile during the five minute test following systemic administration of nor-BNI, as previously reported by other investigators (Pliakas et al., 2001, Mague et al., 2003, McLaughlin et al., 2003, Carr et al., 2010) and reproduced in the current studies, indicates that the endogenous kappa-opioid receptor/dynorphinergic system serves to mediate, at least in part, the behavioral effects of swim stress. Although pharmacological studies have clearly demonstrated a role for the dynorphin/kappa-opioid receptor system in stress-induced behaviors, mechanistic, pharmacodynamic and anatomical questions in relation to this role persist. The goal of the current study was to investigate the responsiveness of prodynorphin gene expression to forced swim stress in specific brain regions in which kappa opioid receptor activity plays a role, coupled with investigation of chromatin structural changes in the promoter region of the prodynorphin gene which may regulate any observed alterations in mRNA levels.

Within the scope of the current investigations, we initially focused on determining the brain regions in which changes in dynorphinergic signaling are induced in response to swim stress by investigating mRNA levels of prodynorphin in several brain regions at a single time point. Each of the brain regions studied contains a functioning kappa-opioid receptor system, including prodynorphin expression. Any or all of these regions could be involved in mediating the nor-BNI sensitive behavior. We observed changes in prodynorphin gene expression only in the caudate putamen, with no change observed in the hypothalamus, frontal cortex, amygdala, nucleus accumbens, or hippocampus. To verify the role of the caudate putamen, in general, and the stress-induced dynorphin mRNA increase, in particular, in the behavioral effects observed in the forced swim test, it will be necessary in future experiments to administer nor-BNI locally into the caudate putamen following cannulation at the same time point studied. Additionally, the time-courses of the changes in the dynorphin gene expression changes (eg. dynamics of increase and potentially a subsequent return to baseline) are of particular interest and will be the subject of future investigations. Further, we observe the changes following acute stress; it will be important to determine whether corresponding changes are observed in response to chronic stress.

The mechanisms which underlie the selective increase of prodynorphin in particular brain regions have yet to be delineated. At the level of transcriptional regulation, control is exerted by chromatin structure, which determines accessibility at gene promoter regions and transcription start sites for the binding of enhancer /repressor complexes and the RNA polymerase regulatory factors. A number of factors are involved in the control of chromatin

structure, both globally and locally, including histone modifications and DNA methylation. There are several layers of interacting regulatory factors controlling histone modification and DNA methylation, as well as other aspects of chromatin structure.

We investigated the chromatin alterations underlying the increase in prodynorphin mRNA levels in the caudate putamen. Within the promoter region of the rat prodynorphin gene, there are 4 putative CRE sites, as well as a Sp1 site, which potentially mediate transcription factor binding (See Figure 3). We targeted these regions in our studies of changes in chromatin complexes using chromatin immunoprecipitation/PCR. We targeted two sites of histone H3 methylation, K4 and K27, as methylation at these sites is thought to correlate with increased and decreased transcription, respectively. We also targeted phospho-CREB, which has previously been shown to drive prodynorphin transcription (Pliakas et al., 2001, Briand and Blendy, 2010). While studies in cell culture have suggested that CREB binding to the CRE-3 site of the rat prodynorphin promoter represses dynorphin transcription, and phosphorylation of CREB leads to derepression (Collins-Hicok et al., 1994), our findings *in vivo* suggest that CREB binding to the CRE-3 site may correlate with activation. We observed significant increases of CREB-p binding to the CRE-3 and CRE-4 sites, with no increase in binding to the region containing the CRE-1 and CRE-2 sites, suggesting the CRE-3 and CRE-4 regions to be the important mediators of CREB-induced prodynorphin transcription in response to stress.

Investigation of the histone modifications utilized antibodies specific to the di- and trimethylation states of Lysines 4 and 27 of histone H3. The group of Nestler has previously demonstrated considerable alterations in the levels of the transcription repressive histone methylation modifications, histone H3K9-Me2/3, and histone H3K27-Me2/3 in the nucleus accumbens in response to social stress (Wilkinson et al., 2009). Global alterations in the levels of both repressive histone methylation modifications, histone H3K9-Me1/2/3 and histone H3K27-Me2/3, as well as the transcription promoting histone methylation modification, histone H3K4-Me2/3, in the hippocampus in response to restraint stress have been reported by the group of McEwen (Hunter et al., 2009). We observed decreases in the levels of association of methylated lysine 4 modifications of histone H3 with the promoter region of the prodynorphin gene in the caudate putamen, in contrast to expectations given the observed increases in prodynorphin mRNA levels. It is possible that other regions of the promoter region would exhibit increases in methylated histone H3 lysine 4, or that histone H3 lysine 4 methylation is not involved in chromatin reorganization underlying increased prodynorphin gene transcription in the caudate putamen in response to stress.

The decrease in association of the methyl DNA binding protein MeCP2 (Guy et al., 2011) to the promoter region of prodynorphin in tandem with the other chromatin alterations observed suggests the possibility that alterations in DNA methylation may be concomitant with altered prodynorphin transcription in the caudate putamen. The observed decrease in MeCP2 binding potentially indicates a decrease in the methylation of one or more CpG sites, which is consistent with increases in transcription, as methylation of a given CpG site within a gene promoter has a tendency to result in decreases in active transcription at the particular gene. The notion that forced swim stress results in decreased methylation within the prodynorphin gene promoter is as yet speculative, and will require future studies, likely using bisulfite sequencing to determine the extent of methylation for the CpG sites within the promoter region of the prodynorphin promoter, in a similar fashion to that which we previously reported in human post-mortem brain tissue (Yuforov et al., 2011). As the current studies utilized one half of the brain for mRNA analyses, and the other half for the corresponding ChIP studies, DNA was not available to DNA methylation studies with the current cohort of animals under study.

In all cases of observed chromatin alterations in the promoter region of prodynorphin, it is important to note that no corresponding changes were observed in the hippocampus, a brain region in which alterations in prodynorphin gene transcription were also not observed. The concomitant study of a brain region in which gene transcriptional changes were *not* observed is an important control for evaluating the relevance of chromatin changes in response to stimuli regulating changes in gene transcription. The simplest interpretation of our results from the chromatin immunoprecipitations in the caudate putamen and the hippocampus is that the changes in the detected associations in the caudate putamen contribute to increased transcription of prodynorphin, and the absence of such changes in the hippocampus similarly results in stable transcriptional activity of the prodynorphin gene in neuronal cells within the hippocampus.

The findings of no alteration in prodynorphin gene expression in the nucleus accumbens and hippocampus differ from findings previously published by other groups (Shirayama et al., 2004, Chartoff et al., 2009). A recent study also studied mRNA transcript levels of prodynorphin following FST using quantitative PCR (Chartoff et al., 2009), reporting an increase in prodynorphin expression in the nucleus accumbens shell, with no difference in the core. As previously mentioned, we did not observe any changes in the levels of prodynorphin mRNA in the nucleus accumbens following the forced swim test. This difference from the previously published study is possibly due to the fact that the core and shell of the nucleus accumbens were analyzed together in the same sample, and any changes in the mRNA levels in the core were masked by the presence of mRNA levels of prodynorphin in the shell which did not change. The levels of mRNA of prodynorphin in the nucleus accumbens of rats are sufficiently high to allow for subdivision, and we will investigate whether we find a similar change in dynorphin in the shell in the future; the small size of the shell subdivision may limit the scope of chromatin immunoprecipitation assays. In the chromatin immunoprecipitation assays in the caudate putamen as reported here, we were able to utilize 6 separate antibodies for chromatin immunoprecipitation from the same sample. The number of separate antibodies which may be used in parallel are directly related to the size of the region, and to maximize the number which might be used to yield epigenetic information, we initially focused our studies on the entire nucleus accumbens region. It would be of interest to investigate the changes in dynorphin mRNA in the shell versus core, as well as potentially divergent underlying epigenetic changes in the two regions. We hypothesize that the epigenetic changes in the nucleus accumbens shell would parallel those we observe in the caudate putamen.

The increase of prodynorphin mRNA in response to forced swim stress in the caudate putamen is similar to that observed in response to drugs of abuse, particularly cocaine which is known to activate the stress-response system and may be viewed as a type of chemical stressor. Cocaine results in rapid and reproducible increases in prodynorphin mRNA in the caudate putamen, with lower or no increases typically observed in the nucleus accumbens (Hurd et al., 1992, Spangler et al., 1996, Yuferov et al., 2001, Zhou et al., 2002, Fagergren et al., 2003, Schlussman et al., 2003, Schlussman et al., 2005). The mechanism whereby cocaine results in increased dynorphin mRNA in the caudate putamen is not precisely known, but may arise from a complex interplay of cocaine effects on factors regulating dynorphin expression, including dopamine, serotonin, other neuropeptides, and glucocorticoids (Angulo and McEwen, 1994, Zhang et al., 2004, Horner et al., 2005). Similarly to the findings presented here in the case of forced swimming and the findings following cocaine administration, voluntary running has been shown to result in increased dynorphin mRNA in the caudate putamen of rats (Werme et al., 2000). Given the effects of running on the stress-responsive HPA axis, reflected by increased corticosterone levels (Brown et al., 2007, Hajisoltani et al., 2010), the relative contribution of stress and motor activity to the increases in dynorphin mRNA are difficult to discern. It will be of

interest to compare, in future studies, chromatin alterations underlying cocaine-induced, as well as running-induced, increases in dynorphin expression in contrast to the swimming-induced alterations reported here.

In the case of previous reported changes in prodynorphin levels in the hippocampus (Shirayama et al., 2004), there were significant differences in the experimental methods in comparison with our study which likely explain the discrepancy. This previous study measured a different analytical endpoint, prodynorphin peptide-like immunoreactivity using immunohistochemistry in regions of the hippocampus, and also measured a different time point, four hours following the 5 min swim test portion of the FST. These investigators observed an increase in the levels of prodynorphin peptides in certain regions of the hippocampus. There have been many documented instances of discrepancies in the levels of mRNA gene expression and encoded protein/peptide levels, as there are multiple layers of regulation of levels and activity of both mRNA transcripts and proteins. Also, the measurements in the previous study were performed on slices, whereas our measurements were made on homogenized dissected brain tissue containing essentially the entire hippocampus. Hence, differences in subregions of the hippocampus which are observable in a subset of brain slices may not be of sufficient magnitude to allow for the observation of differences in the entire brain region, as measured in homogenates. It should be noted that different regulatory mechanisms of prodynorphin expression in the hippocampus compared to the striatum have been reported in the case of cocaine exposure (D'Addario et al., 2007).

Conclusions

The demonstration that prodynorphin mRNA levels selectively increase in the caudate putamen following exposure to the forced swim stress, suggests that kappa-ergic activity in the striatum may mediate the stress-induced immobility in the forced swim test that is attenuated upon treatment with the kappa-antagonist nor-BNI. Our studies suggest that the selective increase in prodynorphin expression in this region is due to corresponding selective alterations in chromatin structure in the promoter region of prodynorphin. The precise molecular mechanism, including the stress-induced signals, which cause these tissue-specific changes await further investigation, as does the dynamic profile of these changes, in terms of onset of effect and recovery to baseline, if indeed full recovery occurs. Additional questions regarding the dynamics of chromatin alterations include whether any or all of these alteration closely follow the dynamics of changes in prodynorphin mRNA levels. It should be recognized that systemic injection of nor-BNI, as performed for the currently reported studies, will result in antagonism of kappa-opioid receptors globally, and while the increase in caudate putamen of prodynorphin mRNA is correlative evidence suggesting the importance of the kappa-opioid system in this brain region, other brain regions may also play an important role in the observed behaviors, in spite of this not being reflected at the level of mRNA expression. The epigenetic changes suggest that the CRE-3 region of the dynorphin promoter play a key role as a scaffold for chromatin associated proteins involved in the observed increases in dynorphin transcription. The correlative decrease in MeCP2 at this site suggests the possibility of altered DNA methylation in this region, which will be investigated in future studies aiming to further delineate the chromatin alterations directing tissue-specific changes in dynorphin expression in response to stress.

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Highlights

‘Chromatin Alterations in Response to Forced Swim Stress Underlie Increased Prodynorphin Transcription’

Forced swim stress induces region specific rise in dynorphin mRNA in caudate putamen

MeCP2 binding to CRE site in dynorphin gene promoter is correspondingly reduced
phosphoCREB binding to select dynorphin gene promoter CRE sites is increased

Select histone modifications demonstrate reduced binding to dynorphin gene promoter

Figure 1.

Immobility, swimming, and climbing during 5 minute test session of the FST protocol. The rats were videotaped and blindly scored in 5 sec intervals for immobility, swimming, or climbing. A maximum score of 60 could be achieved if the rat displayed one behavior during the entirety of the retest session. Rats received either vehicle (blank bars), 5 mg/kg nor-BNI (wide stripe bars), or 10 mg/kg nor-BNI (thin stripe bars) i.p. 24 hr before the five min retest session on day 2. For each group, n=6. **A** There was a main effect of treatment on immobility; post-hoc tests revealed that rats receiving 10 mg/kg nor-BNI exhibited significantly lower immobility in comparison with saline treated rats (* - $p < 0.05$). While the group receiving 5 mg/kg nor-BNI exhibited a trend of lowered immobility, this effect did not reach significant ($p = 0.07$). **B** No main effect of treatment was observed in climbing scores among the three groups. One way ANOVA with planned comparison between groups receiving nor-BNI versus the group receiving saline demonstrated significantly higher climbing scores in the nor-BNI treated groups ($F_{(1,15)} = 5.43$, * - $p < 0.05$). **C** There were no significant differences in scores for swimming between any treatment groups exposed to FST.

Figure 2.

A significant increase (* - $p < 0.05$) in relative prodynorphin gene expression between rats receiving vehicle, as well as rats receiving 10 mg/kg nor-BNI, exposed to force swim test, in comparison to control rats not subject to FST, in the caudate putamen. There was no significant difference in the relative expression of prodynorphin between animals receiving vehicle exposed to force swim test and animals receiving 10 mg/kg exposed to force swim test. There were no significant differences observed in the nucleus accumbens, hypothalamus, frontal cortex, hippocampus, or amygdala. CPu – caudate putamen, NAc – nucleus accumbens, Hypo – hypothalamus, FCx – frontal cortex, Hip – hippocampus, Amyg – amygdala.

Figure 3. The rat prodynorphin gene promoter region, with transcription factor binding sites, and localization of targeted regions for determination of associated proteins in ChIP assays. The four targeted regions encompass the CRE-1 and CRE-2 sites, the CRE-3 site, and the Sp-1 site upstream of the coding region, as well as the CRE-4 site within exon 1 of the coding region.

Table 1

Description of targets of antibodies used in chromatin immunoprecipitation experiments.

ChIP Antibody Targets				
Antigen	Family	Role	Expected Effect on Transcription	References
cAMP-response element-binding protein-1 (CREB-1)	Transcription factor	Binds to cAMP-responsive element	Increase	Briand and Blendy, 2010
Methyl CpG Binding Protein 2 (MeCP2)	DNA binding protein	Binds to single methyl CpG site	Decrease	Bogdanovic and Veestra, 2009
Histone H3 dimethyl Lys27 (H3K27Me2)	Histone	Histone modification	Decrease	Kouzarides, 2007
Histone H3 trimethyl Lys27 (H3K27Me3)	Histone	Histone modification	Decrease	
Histone H3 dimethyl Lys4 (H3K4Me2)	Histone	Histone modification	Increase	
Histone H3 trimethyl Lys4 (H3K4Me3)	Histone	Histone modification	Increase	

Normalized values of specific prodynorphin promoter region DNA fragments bound to chromatin-associated proteins in FST and control rats within the caudate putamen.

Table 2

CAUDATE PUTAMEN	Primer 1 (CRE-1 & -2)			Primer 2 (CRE-3)			Primer 3 (SP1)			Primer 4 (CRE-4)		
	FST	Control	p-value	$\bar{p}$	FST	control	p-value	$\bar{p}$	FST	control	p-value	$\bar{p}$
CREB-1	2.075	5.089	0.376	1	4.160	0.164	0.047	1	0.937	3.235	0.171	1
MeCP2	0.358	0.718	0.197	1	0.387	1.166	0.025	0.600	0.073	0.221	0.137	1
H3K27Me2	9.183	267.1	0.218	1	22.62	105.8	0.043	1	8.021	37.64	0.103	1
H3K27Me3	22.31	272.0	0.241	1	37.29	98.90	0.094	1	10.24	27.70	0.091	1
H3K4Me2	0.225	2.064	0.127	1	0.800	2.759	0.125	1	0.160	0.330	0.162	1
H3K4Me3	5.167	101.1	0.136	1	9.258	77.02	0.042	1	2.504	18.68	0.018	0.432
									5.765	26.38	0.017	0.408

Values calculated using $2^{-\Delta\Delta C_t}$ where ΔC_t is the difference between the sample count and processed total DNA count

p-value – point-wise p-value, determined using student T-test.

Grey - Values not significantly different

Bold - Indicates values significantly higher, using point-wise p-value criterion, in rats subject to FST, positively correlated with prodynorphin mRNA levels ($p < 0.05$)

Bold italic - Indicates values significantly lower, using point-wise p-value criterion, in rats subject to FST, negatively correlated with prodynorphin mRNA levels ($p < 0.05$)

$\bar{p}$ p- experiment-wise p-value, calculated using the Bonferroni correction, for 24 repeated measurements.

Table 3

Normalized values of specific prodynorphin promoter region DNA fragments bound to chromatin-associated proteins in FST and control rats within the hippocampus.

HIPPOCAMPUS	Primer 1 (CRE-1 & 2)				Primer 2 (CRE-3)				Primer 3 (SP1)				Primer 4 (CRE-4)			
	FST	control	p-value	p- $\dot{\tau}$	FST	control	p-value	p- $\dot{\tau}$	FST	control	p-value	p- $\dot{\tau}$	FST	control	p-value	p- $\dot{\tau}$
CREB-1	0.050	0.073	0.455	1	0.128	0.100	0.694	1	0.033	0.048	0.578	1	0.078	0.080	0.928	1
MeCP2	0.011	0.014	0.312	1	0.017	0.018	0.819	1	0.003	0.002	0.535	1	0.006	0.008	0.885	1
H3K27Me2	2.583	0.926	0.327	1	2.340	1.277	0.463	1	1.110	0.560	0.328	1	1.093	0.754	0.556	1
H3K27Me3	0.248	0.699	0.438	1	0.361	0.438	0.737	1	0.205	0.178	0.855	1	0.370	0.479	0.669	1
H3K4Me2	0.020	0.029	0.322	1	0.036	0.029	0.756	1	0.004	0.007	0.498	1	0.037	0.032	0.830	1
H3K4Me3	0.049	0.066	0.521	1	0.126	0.100	0.732	1	0.057	0.034	0.473	1	0.245	0.198	0.727	1

Values calculated using $2^{-\Delta Ct}$ where ΔCt is the difference between the sample count and processed total DNA count

p-value – point-wise p-value, determined using student T-test.

[†]p-experiment-wise p-value, calculated using the Bonferroni correction, for 24 repeated measurements.