Stress-induced epigenetic regulation of κ -opioid receptor gene involves transcription factor c-Myc

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Exposure to stress is associated with adverse emotional and behavioral responses. Whereas the κ -opioid receptor (KOR) system is known to mediate some of the effects, it is unclear whether and how stress affects epigenetic regulation of this gene. Because the KOR gene can use two promoters (Pr1 and Pr2) and two polyadenylation signals (PA1 and PA2), it is also interesting whether and how these distinct regulatory mechanisms are differentially modulated by stress. The current study examined the effects of stress on these different regulatory mechanisms of the KOR gene. Results showed that stress selectively increased the expression of KOR mRNA isoforms controlled by Pr1 and terminated at PA1 in specific brain areas including the medial-prefrontal cortex, hippocampus, brainstem, and sensorimotor cortex, but not in the amygdala or hypothalamus. These effects correlated with altered epigenetic state of KOR Pr1 chromatin, as well as elevation and increased recruitment of the principal transcription factor c-Myc, which could activate Pr1. Stress-induced modulation of Pr1 was further validated using glutamate-sensitive murine hippocampal cell line, HT22. The results revealed a common molecular mechanism underlying the effect of stress on selected chromatin regions of this gene at the cellular level and in the context of whole animal and identified a critical role for c-Myc in stress-triggered epigenetic regulation of the KOR gene locus. This study sheds light on the mechanisms of stress-induced epigenetic regulation that targets specific chromatin segments and suggests certain KOR transcripts and its principal transcription factor c-Myc as potential targets for brain-area-specific intervention.

The response to stress is modulated by environmental, genetic, and epigenetic factors (1). Stress increases the risk for depression, anxiety, and drug-seeking behavior (2, 3). Among the systems implicated in the modulation of stress response is the dynorphin/ κ -opioid receptor (KOR) system. Mice subjected to behavioral stress demonstrate increase in dynorphin secretion and KOR activation in stress-related brain areas (4, 5). Administering KOR antagonists, or deleting the dynorphin/KOR gene, can block stress-induced anxiogenic, prodepressive, and proaddictive effects (6–10). Pharmacotherapy aimed to selectively block KOR has been suggested as a useful strategy in the treatment of anxiety, depression, and drug abuse (4, 11, 12).

Neurotransmitter/peptide systems and signal transduction can be responsible for dynorphin/KOR-dependent behaviors (11, 13), but plasticity at the prodynorphin/KOR gene expression itself may contribute to the enduring effects of stress. Exposure to stress and recovery affects dynorphin and KOR mRNA levels in hypothalamus, hippocampus, striatum, and amygdala (14–18), and differential KOR mRNA levels were detected in stress-sensitive rodent strains [Wistar-Kyoto rats (19), BALB/cJ, and DBA/2J mice (20)]. However, these results are complicated by inconsistent findings. These inconsistencies might be explained by the complexity of the KOR gene (21, 22). The mouse KOR gene generates three 5' isoforms, A, B, and C through alternative splicing and promoter use [promoter 1 (Pr1) vs. promoter 2 (Pr2)] (21), and two alternative polyadenylation (PA) sites, PA1 and PA2. By combining 5' variation and alternative PA use, at least six RNA isoforms can be generated from the KOR gene, each with distinct stability, translation, and transport efficiency, providing a rich reservoir for regulating receptor production

and/or distribution (23). The development of neuropathic pain following nerve injury correlates with differential expression of KOR mRNA isoforms (24), and rodent strains with elevated stress susceptibility (1) have differential expression of KOR mRNA isoforms in the cortex, hypothalamus, and locus coeruleus (19, 20). It is possible that stress differentially modulates KOR gene regulation in stress-related brain areas in a specific manner. However, the underlying mechanism is unclear.

Epigenetic regulation is one potential mechanism mediating stress effects on gene expression, which can be gene specific (i.e., glial cell-line–derived neurotrophic factor gene) (1) and/or brain area specific (i.e., hippocampus) (25). Epigenetic regulation of the KOR gene in culture systems includes inputs like retinoic acid, nitric oxide and nerve growth factor (26–29). However, whether and how the diverse promoter regions and PA sites of the KOR gene are subjected to differential epigenetic regulation in animals is unclear. This study first determines the effects of behavioral stress on KOR gene regulation at the level of specific transcript expression, polyadenylation site use, and epigenetic modulation, in brain areas implicated in stress response, followed by the assessment of specific epigenetic markers and binding of relevant transcription factors and the specific chromatin-modifying enzyme histone deacetylase 1 (HDAC1).

On the basis of a potential contribution of oxidative stress to the etiology of stress and anxiety (30, 31), and finding that vitamin A depletion, also implicated in oxidative stress (32), up-regulates KOR mRNA expression in brains (33), the study also assesses stress effects on an immortalized murine HT22 hippocampal cell line that is sensitive to high glutamate concentrations (34). Results demonstrate epigenetic effects selectively on Pr1 and a functional role for transcription factor c-Myc in mediating such effects.

Results

Complete statistical summaries of behavior, gene expression by RT-PCR, chromatin immunoprecipitation (ChIP), and Western blotting data are provided in the *SI Appendix*, Tables S1–S4, respectively.

Repeated Exposure to the Forced Swim Paradigm Results in Behavioral Stress. We adopted the paradigm of repeated exposure to forced swimming to induce stress (5, 9, 35). This procedure produced a robust stress response, demonstrated by a reduction in the percentage of time spent in the open arms of the elevated plus maze (EPM) (Fig. 1A) and in the percentage of entries into the open arms of the EPM (Fig. 1B). Additional indications of a stress response are the reduction in distance traveled in central/total area of the open field (Fig. 1C) and number of entries into (Fig. 1D) and time spent in the central area of the open field (Fig. 1E).

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Fig. 1. Effects of repeated exposure to the forced swim procedure on behavioral stress indicators. Mean and SE of (A) percentage of time spent in the open arms of the EPM, (B) percentage of entries into the open arms of the EPM, (C) distance traveled in central/total area of the open field, (D) number of entries into the central area of the open field and (E) time spent in the central area of the open field in mice unexposed (no stress, NS) or exposed to stress (forced swim stress, FSS). Significantly different from NS condition ($*P < 0.05$). Data represent three individual experiments. No experiment or interaction of experiment $\times$ condition effect was found ($n = 25-27$).

Behavioral Stress Mediates KOR mRNA Regulation in an Isoform-Selective and Brain-Area-Specific Manner. Fig. 2*A* depicts detection of KOR isoforms A, B, and C and PA1 and PA2; Fig. 2*B* depicts investigated brain areas. No effect on any of the KOR isoforms was detected in the amygdala or hypothalamus (Fig. 2*C* and *D*). On the contrary, increased expression of isoform B was detected in the hippocampus, brainstem, and sensorimotor cortex, and increased expression of isoform A was detected in the medial prefrontal cortex (mPFC). In these four brain areas, increase in mRNA containing PA1, but not PA2, was also detected (Fig. 2*E-H*). Changes in the expression of isoforms A and B, but not C, suggest that this type of stress selectively induced epigenetic changes on Pr1 but not Pr2, in the affected areas, and that stress affects transcripts containing PA1 but not PA2.

Behavioral Stress-Induced Epigenetic Modification of KOR mRNA Regulation in the Brainstem. We then examined specific epigenetic markers and the recruitment of specific transcription factors to Pr1 and Pr2 (36). Primers were designed to encompass specific motifs of the promoter regions (Fig. 3*A*). Limited by sample size, and guided by previous findings showing that Pr2 is active only in certain brain areas, particularly the brainstem (21), this brain area was first examined for the abundance of AcH4 and H3K4me2 marks, as well as the recruitment of HDAC1 and c-Myc to the KOR gene.

Fig. 3 shows that HDAC1 recruitment was reduced, acetylation of histone 4 was increased, and recruitment of c-Myc was increased on Pr1 segments containing an enhancer (E) and GC box. A trend toward reduced dimethylation of histone 3 at lysine 4 was also found (Fig. 3*B* and *C*), consistent with the selective up-regulation of KOR mRNA controlled by Pr1. In line with the lack of effect on isoform C, which is controlled by Pr2, no change in AcH4 or H3Kme status was detected on Pr2. Interestingly, within the Pr2 segment containing an alternative splicing site that would generate isoform B, HDAC1 recruitment was significantly reduced (Fig. 3*D-F*).

Brain-Area-Specific Epigenetic Regulation of KOR. The epigenetic markers were further assessed in hippocampus and hypothalamus regions, because KOR expression was most significantly affected by stress in the first, but not in the second region. The

amygdala and mPFC were not analyzed due to sample size limitation.

Fig. 4*A* shows an increase in c-Myc and a trend toward reduction in HDAC1 binding on the E box of Pr1 in the hippocampus. Similar to the result seen in the brainstem, a reduction in recruitment of HDAC1 to the alternative splicing site that would generate isoform B was also detected in the hippocampus (Fig. 4*C*). As predicted, the effect in the hypothalamus was negligible (Fig. 4*D*). Interestingly, there was also a reduction in HDAC1 recruitment to the alternative splicing of the hypothalamus (Fig. 4*F*), although the expression of isoforms A and B did not change significantly in this brain area (Fig. 2*C*).

Behavioral Stress-Induced Regulation of KOR and c-Myc Protein Level in the Brainstem, Hippocampus, and Hypothalamus. The expression level of relevant transcription factors and KOR itself were assessed. As can be seen, in responsive brain areas, the increase in c-Myc recruitment was accompanied by an increase in c-Myc protein level (Fig. 5*A-C*). Importantly, KOR protein level was also significantly elevated in the brainstem and hippocampus, but not in the hypothalamus (Fig. 5*A-C*).

Glutamate-Induced Oxidative Stress Affects KOR Gene Regulatory Mechanisms in HT22 Cells. Oxidative stress might contribute, at least partially, to the etiology of stress and anxiety (30, 31). In HT22, a subclone of H4 immortalized hippocampal cell line sensitive to glutamate (34), only isoform A is stably expressed. Following 5 mM glutamate treatment for 18 h [eliminating 65% of viable cells, Fig. 6*A*, unpaired *T* test, $t(17) = 8.393$, $P < 0.0001$], isoform A, as well as KOR and c-Myc proteins, were significantly elevated (Fig. 6*B-E*). Moreover, epigenetic changes similar to those seen in the hippocampus (increased c-Myc recruitment, reduced HDAC1 binding, increased histone 4 acetylation) were detected on Pr1. Reduced HDAC1 binding to the alternative splicing region was also detected (Fig. 6*F-I*). To determine the functional role for c-Myc in mediating stress-induced epigenetic changes on the KOR gene, RNA interference-mediated silencing was performed. The results demonstrate that glutamate-induced elevation of isoform A is abolished under a c-Myc silencing condition (silencing, 70%; Fig. 6*J* and *K*), supporting a causal relationship between c-Myc level and KOR gene's Pr1 activity.

Discussion

Stress is a complex experience for which the adverse effects are well documented, but many of the underlying mechanisms are unclear and controversial. The current study explored the effects of behavioral stress on epigenetic regulation of the KOR gene and identified a specific chromatin target on the KOR locus, Pr1, within a specific brain circuitry. The results also uncovered the functional role for a specific transcription factor, c-Myc, targeting Pr1 in the process.

The current study, demonstrating experience-related plasticity of the KOR gene, is in line with previous reports showing changes in mRNA expression of KOR (14-16) and dynorphin (17, 18) following the induction of behavioral stress or in stress-sensitive rodent lines (19, 20). However, different from most previous studies, the current research used a detailed assessment of the different KOR isoforms and polyadenylation sites in an attempt to take into account the complexity of this gene's regulatory elements conserved in human and mice (21, 22). Because all of the known KOR mRNA isoforms produce the same polypeptide, Western blot analysis alone apparently cannot discriminate the potentially different effects exerted on the various KOR mRNA species. However, by exploiting the well-documented sequence variation among these KOR mRNA isoforms, we are able to demonstrate that swim stress selectively affects transcripts controlled by Pr1, but not Pr2. Using this strategy, this current study provides evidence for selective targeting of swim stress on specific regulatory machinery that may augment the production of KOR protein. These findings provide molecular explanation for previous findings showing brain-

Fig. 2. Effects on the expression level of KOR mRNA isoforms. Map describing KOR mRNA isoforms, with forward primers indicated by arrows (A). Mouse brain areas (B). Quantitative analyses of isoforms A, B, C and PA1 and PA2 in the hypothalamus (C), amygdala (D), hippocampus (E), sensorimotor cortex (F), brainstem (G), and medial prefrontal cortex (H) in mice unexposed (NS) or exposed to repeated stress (FSS). Specific signal was normalized to actin signal and resulting data were normalized to isoform A-NS group, set arbitrarily as the value of 1. Significantly different from NS condition (* $P < 0.05$). Data represent a minimum of two independent experiments, each in duplicates. No experiment or interaction of experiment $\times$ brain area or isoform effect was found ($n = 4-7$, amygdala and medial prefrontal cortex, $n = 5-7$, hypothalamus, hippocampus, sensorimotor cortex, and brainstem).

area-specific differential expression of KOR isoforms A and B (20) in mice strains with distinct stress sensitivity (1), as well as preferential expression of Pr1-driven transcripts in the dorsal root ganglia of mice developing neuropathic pain (24). The rich reservoir offered by the different RNA isoforms could be relevant to various needs for receptor production and/or distribution under different physiological states. The complexity of the KOR gene regulatory elements might have contributed to the controversy previously found in the field [i.e., stress-induced KOR mRNA reduction (14) vs. increase (15) in the striatum, no effect (16) vs. increased (15) KOR mRNA in the nucleus accumbens]. To this end, it is important to note that the different isoforms of KOR mRNA exhibit distinct stability, translation efficiency, RNA transport efficiency, and CNS expression patterns (23, 37).

The current research, demonstrating the effects of stress on KOR epigenetic processes is in line with the indicated ability of external stimuli, including stressors such as forced swim exposure, to affect chromatin remodeling, DNA methylation, and histone modification of stress-associated genes and/or within stress-sensitive brain area (25). The change in c-Myc is most significant, which is consistent with studies of cell cultures (26-28). The increased acetylation of histone 4, reduced lysine dimethylation of histone 3, and reduced binding of HDAC1 are also

consistent with changes on Pr1 chromatin in studying cultured cells where this promoter is found to be active (36).

All brain areas explored in the current study have been chosen on the basis of their potential connection with stress and anxiety (38). However, stress effects on KOR regulation were found to be restricted to the hippocampus, brainstem, mPFC, and sensorimotor cortex, sparing the amygdala and hypothalamus. One explanation might involve the temporal and functional differences between these areas. The amygdala is crucially involved in the acquisition of fear conditioning and expression of fear response, whereas the hippocampus and mPFC are involved in processes of learning and remembering the significance of a threat as well as in inhibitory control of stress response and in extinction, respectively (38). The brainstem contains the locus coeruleus (LC), a regulatory center of arousal, attention, and adaptive behavioral responses (39) and the dorsal raphe nucleus, found to regulate stress, affective state, and analgesic response (6). The paradigm used in this study involved a repeated form of stress induction, which might be different from acute paradigms or fear-inducing paradigms, and as such, might not involve the amygdala and its projection to the hypothalamic-pituitary-adrenal axis. In line with this suggestion, both acute stress and fear paradigms have been found to affect KOR activation and transcription within the basolateral amygdala (BLA) (8, 14), whereas acute stress was found to affect KOR transcription in the paraventricular nucleus of the

hypothalamus (16). In addition, it is important to take into account the heterogeneity of the described brain areas. For instance, whereas the current research did not distinguish the central nucleus of the amygdala (CeA) from the BLA, different neurochemistry or neurotransmission within these areas might have obscured the pattern of results. Related to this complication, differential activation of KOR in the BLA vs. the CeA has been

previously reported (7). Future studies, assessing different nuclei of the amygdala and using fear vs. stress models and acute vs. repeated and/or chronic stress induction might be able to test this possibility. Additionally, because forced swim exposure has been found to affect physiological processes including energy homeostasis and to yield motoric burden (40), its effects on KOR epigenetic programs as observed here might also result from certain

Fig. 5. Effects of repeated forced swim exposure on KOR and c-Myc protein levels in the brainstem, hippocampus, and hypothalamus. Western blot analyses of KOR and c-Myc protein levels in the brainstem (A), hippocampus (B), and hypothalamus (C) of mice unexposed (NS) or exposed to stress (FSS). Significantly different from NS condition ($*P < 0.05$). Samples were run in duplicates ($n = 6-8$).

physiological changes in these animals. Future studies should also address these complications.

This study does not elucidate whether up-regulation of KOR mediates the demonstrated KOR-dependent increase in stress-related behaviors (7–10) or whether it represents a compensatory mechanism, activated in response to stress. One possibility might involve the ability of presynaptic KOR to inhibit the release of endogenous excitatory amino acids (including glutamate) and attenuate excitatory transmission in the hippocampus (41), LC (42), and mPFC (43) following the induction of stress (42, 44, 45). Presynaptic inhibition of noradrenaline release by KOR has also been postulated (46) and may mediate stress-induced increase in noradrenaline release in the mPFC and hippocampus (47, 48). Hence, within this specific circuit, increased stress-induced mediation of KOR regulation may provide a mechanism activated to counterbalance over excitation to restore homeostatic condition.

In conclusion, the current research assesses the effect of stress on epigenetic alteration on KOR locus in an *in vivo* model. In a relevant cell model, the study also partially addresses the mechanistic basis. Through these studies, a common mediator for epigenetic regulation of the KOR gene by behavioral and

oxidative stress has been uncovered. The study also shed light on specific brain circuits, mRNA isoforms, and transcription factors as targets for future investigation of the effects of stress on KOR gene plasticity and the potential involvement of KOR in the pathophysiology of stress and anxiety.

Materials and Methods

For subjects and behavioral treatments, see *SI Appendix*.

Cell Culture and Viability Assay. HT22 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS, 1% penicillin, 1% streptomycin. Cell viability assay was conducted following 18 h of 5-mM glutamate treatment and viability was assessed by measuring cell ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), using the TOX-1 kit (Sigma-Aldrich). Absorbance was measured at 544 nm and percent survival was calculated.

Materials. Antibodies were from Santa Cruz Biotechnology, [anti-c-Myc, sc-40; anti-HDAC1, sc-6298; and anti-β-actin, sc-47778], Millipore [anti-acetyl-Histone H4, 06-866, anti-dimethyl-Histone H3 (Lys4), 07-030], and Abcam (anti-KOR, ab10566). The enzymes were purchased from New England Biolabs (BclI, BglII, EcoRI, and BanII). L-Glutamic acid was from Sigma-Aldrich.

Fig. 6. Effects of glutamate-induced oxidative stress on the level of KOR mRNA, protein, and epigenetic modifications in hippocampal HT22 cells. (A) Data (means of triplicate) are expressed as percentage of survival relative to control. (B and C) KOR isoform A mRNA. (D and E) Western blots of KOR and c-Myc protein levels. (F–I) ChIP analysis of the levels of ACh4, HDAC1, and c-Myc binding to Pr1-fragment 1 (F and I), Pr1-fragment 2 (G and I), and splicing (H and I). (J and K) RT-PCR analyses of KOR isoform A mRNA and c-Myc. Significantly different from control condition ($*P < 0.05$).

Reverse Transcriptase PCR (RT-PCR). Total RNA extracted with TRIzol (Invitrogen) was reverse transcribed by using the Omniscript RT kit (205113; Qiagen) and PCR amplified (SI Appendix, Table S5). Ratios of optical density of the target genes normalized to β -actin were determined for quasi-quantified analysis using ImageJ software. Specificity of PCR product was validated using enzyme digestion (KOR isoforms A and B, BglII, EcoRI; KOR isoform C, BglII, BclI; c-myc, BanII).

Chromatin Immunoprecipitation (ChIP). Tissues from four to eight animals were pooled together to yield a total of 240–300 mg sample and used for chromatin immunoprecipitation (ChIP) assay as described previously (28). DNA was analyzed for Pr1 and Pr2 regions, the E-box vs. the GC-box (GGGCGG sequence) regions of Pr1 and the AP2 vs. splicing regions of intron 1 (see SI Appendix, Table S5 for primers).

Western Blotting. Cells/tissues from three to four animals were pulled together. Blots were incubated overnight at 4 °C with the primary indicated antibodies (1:1,000), incubated for 1 h with the corresponding HRP-conjugated second-

ary antibodies at room temperature and developed with a Super Signal West Pico kit (Thermo Fisher Scientific).

RNA Interference-Mediated Silencing of c-Myc. Transfection was performed by using Hiperfect (Qiagen) according to the manufacturer's instructions with c-Myc-specific siRNA plasmids (target 1, CCCAAGGTAGTGATCTCTCAA; target 2, CTGGTGCATAAACTGACCTAA) and scrambled siRNA control plasmid (Qiagen). Thirty hours following transfection, cells were treated with 5 mM glutamate for an additional 18 h.

Data Analysis. Analyses of the data were performed using an appropriate analysis of variance. Significant effects were followed up with Bonferroni's post hoc tests. In all cases, *P* values were two tailed, and the comparisons were considered statistically significant when *P* < 0.05. Data are presented as the mean $\pm$ SEM.

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