HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2017 August 15.

Published in final edited form as:

Brain Res. 2016 August 15; 1645: 71–74. doi:10.1016/j.brainres.2015.12.039.

Reflections on: "A General Role for Adaptations in G-Proteins and the Cyclic AMP System in Mediating the Chronic Actions of Morphine and Cocaine on Neuronal Function"

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Abstract

In 1991 we demonstrated that chronic morphine exposure increased levels of adenylyl cyclase and protein kinase A (PKA) in several regions of the rat central nervous system as inferred from measures of enzyme activity in crude extracts (Terwilliger et al., 1991). These findings led us to hypothesize that a concerted upregulation of the cAMP pathway is a general mechanism of opiate tolerance and dependence. Moreover, in the same study we showed similar induction of adenylyl cyclase and PKA activity in nucleus accumbens (NAc) in response to chronic administration of cocaine, but not of several non-abused psychoactive drugs. Morphine and cocaine also induced equivalent changes in inhibitory G protein subunits in this brain region. We thus extended our hypothesis to suggest that, particularly within brain reward regions such as NAc, cAMP pathway upregulation represents a common mechanism of reward tolerance and dependence shared by several classes of drugs of abuse. Research since that time, by many laboratories, has provided substantial support for these hypotheses. Specifically, opiates in several CNS regions including NAc, and cocaine more selectively in NAc, induce expression of certain adenylyl cyclase isoforms and PKA subunits via the transcription factor, CREB, and these transcriptional adaptations serve a homeostatic function to oppose drug action. In certain brain regions, such as locus coeruleus, these adaptations mediate aspects of physical opiate dependence and withdrawal, whereas in NAc they mediate reward tolerance and dependence that drives increased drug self-administration. This work has had important implications for understanding the molecular basis of addiction.

Keywords

G alpha subunits; adenylyl cyclase; protein kinase A; CREB; opiates; stimulants; drugs of abuse; addiction

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Original Article Abstract

"A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function"

Previous studies have shown that chronic morphine increases levels of the G-protein subunits $G_{i\alpha}$ and $G_{o\alpha}$, adenylate cyclase, cyclic AMP-dependent protein kinase, and certain phosphoproteins in the rat locus coeruleus, but not in several other brain regions studied, and that chronic morphine decreases levels of $G_{i\alpha}$ and increases levels of adenylate cyclase in dorsal root ganglion/spinal cord (DRG-SC) co-cultures. These findings led us to survey the effects of chronic morphine on the G-protein/cyclic AMP system in a large number of brain regions to determine how widespread such regulation might be. We found that while most regions showed no regulation in response to chronic morphine, nucleus accumbens (NAc) and amygdala did show increases in adenylate cyclase and cyclic AMP-dependent protein kinase activity, and thalamus showed an increase in cyclic AMP-dependent protein kinase activity only. An increase in cyclic AMP-dependent protein kinase activity was also observed in DRG-SC co-cultures. Morphine regulation of G-proteins was variable, with decreased levels of $G_{i\alpha}$ seen in the NAc, increased levels of $G_{i\alpha}$ and $G_{o\alpha}$ amygdala, and no change in thalamus or the other brain regions studied. Interestingly, chronic treatment of rats with cocaine, but not with several non-abused drugs, produced similar changes compared to morphine in G-proteins, adenylate cyclase, and cyclic AMP-dependent protein kinase in the NAc, but not in the other brain regions studied. These results indicate that regulation of the G-protein/cyclic AMP system represents a mechanism by which a number of opiate-sensitive neurons adapt to chronic morphine and thereby develop aspects of opiate tolerance and/or dependence. The findings that chronic morphine and cocaine produce similar adaptations in the NAc, a brain region important for the reinforcing actions of many types of abused substances, suggest further that common mechanisms may underlie psychological aspects of drug addiction mediated by this brain region. © 1991.

Introduction: Drug Abuse Research Circa 1990

This manuscript was published four years into my first term as a new assistant professor in the Department of Psychiatry at Yale University. At that time, virtually nothing was known about how drugs of abuse such as opiates and stimulants change the brain to produce the range of behavioral abnormalities that define a state of addiction. In the opiate field, there was nearly exclusive focus on the opioid receptor itself, which had not yet been cloned. The notion that molecules beyond the receptor might be involved in adaptations to chronic opiate exposure was novel—unheard of really, as was pointed out to me in the reviews of my first NIH grant appication in 1987 which was not funded: "There is no evidence to suggest that postreceptor signaling is important for opiate addiction." The grant was ultimately funded on the second try in 1988. Its goal was to study whether chronic opiate exposure altered proteins downstream of opioid receptors in a range of brain regions implicated in mediating the consequences of chronic opiate exposure including physical dependence, analgesic tolerance, and changes in reward and motivation associated with addiction.

By the mid-1980s it was known that opioid receptors signal through inhibitory G proteins comprised of Gαi/Gαo and Gβγ subunits, which inhibit adenylyl cyclase and protein kinase A (PKA). Very little was known about the substrates of PKA that would presumably mediate some of the functional consequences of opioid receptor signaling. We approached this question using an unbiased approach, which would be called proteomics today: we prepared extracts from several discrete brain regions of rats, exposed chronically to morphine or sham treatment, under conditions that inhibited protein kinases and protein phosphatases. We then added purified PKA + ^{32}P-labeled ATP to phosphorylate proteins in the extracts and resolved the phosphorylated proteins by 2D gel electrophoresis and autoradiography. Our initial study focused on locus ceruleus (LC), the major noradrenergic nucleus in brain, which had been implicated at the time in mediating physical opiate dependence and withdrawal (Koob et al., 1992). We identified altered phosphorylation of several known substrates of PKA (Guitart and Nestler, 1989, 1990; Guitart et al. 1990), suggesting that PKA activity itself might be induced by morphine, something we confirmed directly (Nestler and Tallman, 1988). This also prompted us to investigate whether adenylyl cyclase is regulated in LC by chronic morphine and indeed found this to be the case (Duman et al., 1988). Finally, we found that this induction of adenylyl cyclase and PKA was associated with increased levels of Gαi and Gαo in LC in response to chronic morphine administration (Nestler et al., 1989). Our findings with adenylyl cyclase and PKA were reminiscent of studies a decade earlier by Sharma et al. (1975), who showed that repeated exposure of cultured neuroblastoma x glioma cells to morphine led to increased levels of cAMP upon removal of the morphine. Our hypothesis was that the concerted upregulation of the cAMP pathway, namely, induction of adenylyl cyclase and PKA, in LC represents a homeostatic adaptation to chronic opiate exposure which serves to oppose opiate action (tolerance) and drive physical withdrawal symptoms upon removal of the opiate (dependence), thus representing the first known molecular mechanism of opiate tolerance and dependence established in vivo. It would take us through most of the 1990s and beyond to provide direct causal evidence for this hypothesis; more about that below.

Our findings in LC, and a related report concerning adenylyl cyclase by Stanley Crain and colleagues in dorsal root ganglion (DRG)-spinal cord (SC) co-cultures (Makman et al., 1988), prompted the study highlighted in this issue (Terwilliger et al., 1991), in which we surveyed whether changes in G proteins and the cAMP pathway are seen in other CNS regions in response to chronic morphine. The study relied on very crude methods: measures of G protein subunits by Western blotting and of adenylyl cyclase and PKA catalytic activity in crude extracts of discrete brain regions dissected from morphine- or sham-treated rats. It was not possible to perform Western blots on adenylyl cyclase and PKA subunits at that time due to the lack of suitable antibodies. We found no evidence for regulation of these proteins in most brain regions studied with a few notable exceptions: induction of adenylyl cyclase and PKA activity in nucleus accumbens (NAc) and amygdala, and induction of PKA only in thalamus; we also demonstrated induction of PKA in DRG-SC co-cultures. Changes in G protein subunits were more variable. These findings prompted us to update our hypothesis stated above: that upregulation of the cAMP pathway represents a general mechanism of opiate tolerance and dependence exhibited by many types of central neurons and mediates diverse behavioral consequences of chronic opiate exposure depending on the functioning of

the affected neurons and their circuitry. Thus, whereas cAMP pathway upregulation in LC contributes to physical opiate dependence and withdrawal, the same adapations in NAc—a key brain reward region—might mediate reward tolerance and dependence, and so on. This general hypothesis was supported by related work in the myenteric plexus of the gut (Wang and Gintzler, 1995).

Moreover, because the NAc had at that time come to be seen as a shared neural substrate for the rewarding actions of all drugs of abuse (Di Chiara and Imperator, 1988; Koob and Bloom, 1988), we tested whether cocaine induced similar molecular changes in this brain region. Our findings of equivalent adaptations in G protein subunits, adenylyl cyclase, and PKA in NAc of rats treated chronically with cocaine, but not with several classes of non-abused drugs, led us to hypothesize further that upregulation of the cAMP pathway represents a mechanism of reward tolerance and dependence not only for opiates but for stimulants as well (Terwilliger et al., 1991). This was one of the first demonstrations of a common molecular adaptation to opiate and stimulant drugs of abuse and contributed to the growing awareness of shared mechanisms underlying addiction syndromes (Koob et al., 1998; Nestler, 2005).

Improving the Quality of Causal Evidence

Proof that upregulation of the cAMP pathway contributes to opiate tolerance and dependence in LC, and to reward tolerance/dependence for opiates and stimulants in NAc, came slowly due to technical limitations of providing causal evidence for in vivo adaptations in brain (Nestler and Aghajanian, 1997). We showed relatively quickly, with George Aghajanian, using pharmacological activators and inhibitors of PKA, that the upregulated cAMP pathway contributes causally to the hyperexcitability of LC noradrenergic neurons seen during opiate withdrawal (Kogan et al., 1992), and over the next several years established that these adaptations in LC indeed contribute to physical opiate withdrawal utilizing local intra-LC delivery of PKA activators and inhibitors (Rasmussen et al., 1990; Punch et al., 1997). Years later we confirmed these findings using mice lacking certain isoforms (types I and VIII) of adenylyl cyclase (Cao et al., 2010). This role for an upregulated cAMP pathway in LC in mediating opiate dependence was replicated by other laboratories (Maldonado et al., 1995; Aston-Jones et al., 1997).

Moreover, based on the hypothesis that cAMP pathway upregulation was mediated in part via alterations in gene expression, we focused on CREB, which at that time was discovered as a major cAMP-regulated transcription factor (Montminy et al., 1990; Frank and Greenberg, 1994; Martin and Kandel, 1996; Goodman and Mandel, 1998). We first demonstrated that chronic morphine exposure increased CREB phosphorylation in LC (Guitart et al., 1992). Over the ensuing years, we provided direct evidence that CREB activation mediates the cascade of events underlying opiate dependence: CREB mediates induction of adenylyl cyclase and PKA subunits, hyperexcitability of LC neurons, and physical withdrawal behaviors. This was established first by use of antisense oligonucleotides delivered directly into LC to knockdown CREB expression and later by use of viral-mediated gene transfer to overexpress wildtype CREB or dominant negative CREB mutants in LC neurons (Lane-Ladd et al., 1997; Han et al., 2006; Cao et al., 2010).

Similar approaches were used to directly demonstrate a causal role of cAMP pathway-CREB upregulation in NAc in mediating reward tolerance and dependence in NAc. Transgenic reporter mice confirmed the induction of CREB in this brain region in response to chronic opiate or stimulant drugs of abuse (Shaw-Lutchman et al., 2002, 2003). Moreover, via local infusion of PKA activators or inhibitors into NAc, or by use of viral-mediated gene transfer to overexpress CREB or mutant CREB in this region, we established that cAMP pathway-CREB activation in NAc blunts the rewarding effects of opiates and of cocaine (Miserendino et al., 1995; Carlezon et al., 1998; Barrot et al., 2002). This work was extended, with Ron Duman, by use of inducible transgenic mice in which CREB or mutant CREB was overexpressed selectively in NAc and dorsal striatum, which yielded equivalent results (Sakai et al., 2002; McClung and Nestler, 2003; DiNieri et al. 2009). Importantly, such blunting of drug reward drives increased drug self-adminisration (Larson et al., 2011). Finally, with Yan Dong and Rob Malenka, we showed that CREB induction in NAc increases the excitability of NAc medium spiny neurons and that this action opposes the neurophysiological effects of cocaine on these cells (Dong et al., 2006), again confirming the role of CREB activation in NAc as a homeostatic adaptation that mediates drug tolerance and dependence. Considerable progress has also been made in identifying a range of target genes through which CREB produces these effects, including candidate gene approaches (e.g., dynorphin; Carlezon et al., 1998) and unbiased genome-wide approaches which have implicated dozens of additional potential targets (McClung and Nestler, 2003; Renthal et al., 2009), which now need to be investigated in greater detail.

Broader Implications

This history illustrates a few interesting lessons. First, it emphasizes the importance of unbiased experiments—discovery science—in revealing previously unknown mechanisms of a biological phenomenon. Our early proteomic analyses provided the initial evidence of cAMP pathway upregulation in opiate action. Second, by following initial observations, our group and others have been able to significantly expand our knowledge of the cAMP pathway in drug addiction by extending earlier work to other CNS regions and to downstream effectors of the pathway, mostly notably CREB and its target genes. Third, this work had ramifications far beyond addiction, since the discovery that CREB in NAc blunts the rewarding effects of drugs of abuse led us to consider and later establish that CREB is also induced in NAc medium spiny neurons in response to chronic stress where it serves to increase stress susceptibility and promote depression-like behavioral abnormalities (Pliakis et al., 2001; Barrot et al., 2002; Newton et al., 2002; Covington et al., 2011). This in turn provided the original foundation for the ongoing evaluation of κ opioid antagonists (the target of dynorphin) in the treatment of depression (Bruchas et al., 2010; Knoll and Carlezon, 2010). Finally, the paper upon which all of this work was based was published in *Brain Research*—it had been rejected from a couple of higher ranked journals! This underscores the dangers of focusing so much attention on top-ranked journals as well as the importance of journals such as *Brain Research* which enable the publication of a broader range of research findings.

Acknowledgments

Preparation of this review was supported by grants from the National Institute on Drug Abuse.

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