Final Research Report*

Role of Brain Stress System in Opiate Dependence and Withdrawal: Neuronal-, Cellular- and Molecular Mechanisms

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(Results related to this grant are highlighted in **bold**)

Stress and stress-associated high levels of glucocorticoid hormones are well-known risk factors in the development of drug addiction and vulnerability to relapse (Koob & Kreek, 2007; Koob, 2008). Human studies indicated that addicts often use drugs to relive their stress and anxiety. Moreover, psychological stress is known to increase vulnerability to addiction. This „self treatment" may initially decrease stress-evoked adverse effects, however, chronic exposure to addictive compounds leads to tolerance and paradoxically, drug withdrawal or cessation of the treatment results in serious stress reaction and may lead to development of anxiety disorders that is often followed by relapse. Even after long periods of abstinence, adverse stressful life events or exposure to chronic stress may precipitate relapse.
In the framework of this grant, we have revealed some important details of the cellular and molecular basis of the complex interaction between brain stress system and the reward system.

Corticotropin-releasing hormone (CRH)- the neuropeptide and neurotransmitter of the neuroendocrine- and brain stress system

The hypothalamic paraventricular nucleus (PVN) contains hypophyseotropic parvocelllar neurosecretory neurons provide corticotropin-releasing hormone (CRH) to the pituitary portal circulation to initiate the neuroendocrine stress response by stimulating ACTH release from the pituitary and consequently glucocorticoid secretion from the adrenal cortex (Vale *et al.*, 1981; Swanson *et al.*, 1983; Sawchenko & Swanson, 1985).
We have confirmed activation of the hypothalamo-pituitary-adrenocortical axis during morphine withdrawal by revealing increased levels of CRH mRNA in the hypothalamus of rats that have been chronically exposed to morphine and injected with naloxone to precipitate withdrawal (Pinter-Kubler *et al.*, 2013). During these experiments we have revealed that the activation of CRH transcription in the hypothalamus is rather quick: starting 15 min after withdrawal and lasting for about 2 hours. Furthermore, as reported previously, elevated concentrations of ACTH and corticosterone were also noted in all of our recent studies as well (Nunez *et al.*, 2010; Martin *et al.*, 2012; Pinter-Kubler *et al.*, 2013). We have also noted significant activation of prolactin secretion during naloxone precipitated morphine withdrawal that has not reported before {Pinter-Kubler, 2013 #8615).

Regulation of CRH expression in the hypothalamic neurosecretory neurons is rather complex and involves different cis-acting elements and trans-acting factors. We have previously shown that phosphorylation of cAMP-response element binding protein (CREB) plays a role in stress-induced activation of CRH expression in the hypothalamic paraventricular nucleus (Kovacs & Sawchenko, 1996). Although pCREB has been widely

used as a marker of the activation of CREB-mediated gene transcription, recent evidence suggested requirement of additional co-activators that control the kinetics of CREB target gene regulation (Liu *et al*., 2008). Recent research highlighted *the transducers of regulated CREB* activity (TORCs) as such cofactors which facilitate CREB-mediated gene transcription (Conkright *et al*., 2003). TORCs are maintained in an inactive state in the cytoplasm as a result of phosphorylation. Different stimuli lead to TORC dephosphorylation and subsequent nuclear accumulation, whereby it can freely associate with CREB.
In the experiments under this grant we have shown phosphorylation of CREB in the PVN in morphine-dependent rats during drug withdrawal which may contribute to the rapid induction of CRH transcription. Furthermore, it has been revealed that activation of CRH transcription in the neurosecretory cells of the PVH was accompanied by decrease of phospho-TORC-1 levels in the very same neuron population. It should also be noted that withdrawal-induced dephosphorylation of TORC-1 was dependent on the noradrenergic inputs originating from the brainstem similar to that seen in case of CREB phosphorylation and hypothalamic CRH transcription and HPA axis activation (Martin *et al.*, 2012).

CREB phosphorylation contributes to transcriptional activation of many other target genes which have cAMP-response element (CRE) (Montminy *et al.*, 1990). Foremost among these genes is c-fos. c-fos is induced by extracellular stimuli and encodes c-Fos protein, which is a part of AP-1 transcription factor. AP-1 is involved in regulation of various neuropeptides implicated in stress- and metabolic regulation as well as in drug addiction via long-term changes in neuronal phenotype (Hoffman & Lyo, 2002; Kovacs, 2008). Although acute morphine administration and naloxone precipitated withdrawal in morphine dependent rats results in a transient elevation of c-fos expressionin various hypothalamic and extrahypothalamic sites, these short-lasting alterations may not be responsible for those dramatic changes that are seen in the behavior and physiology of addicted animals (Laorden *et al*., 2002). In contrast to other members of the Fos family, ΔFosB is modestly induced in the brain after acute drug administration, but because of its unusual long half-life persists for weeks, even months, after the cessation of drug use. As a result, it gradually accumulates with chronic drug exposure, suggesting that ΔFosB could represent a mechanism by which drugs of abuse produce lasting changes in gene expression pattern long after the drug is withdrawn (Nestler & Aghajanian, 1997; Nestler, 2004) . The expression of ΔFosB in the brain reward system after chronic drug exposure has been widely studied (Nye & Nestler, 1996; Nestler, 2001). It has been shown that repeated administration of cocaine, amphetamine, cannabinoids or morphine increases ΔFosB levels in the nucleus accumbens (NAc) and dorsal striatum (Nestler, 2001; 2004). Given that ΔFosB is a transcription factor, it may causes behavioral plasticity through alterations in the expression of other genes (Nestler & Aghajanian, 1997).
For the first time, we have provided evidence that chronic morphine exposure results in FosB/ ΔFosB accumulation in the stress-related brain circuitry (Nunez *et al.*, 2010). Our data thus indicate that neuroadaptation to addictive substances such as morphine, observed as accumulation of ΔFosB is not limited to the reward system but manifest in other brain regions, such as the brain stress system, which have been proposed to be directly related to addiction.
Various immunochemical (Western blot and immunocytochemistry) techniques were applied to identify accumulation of ΔFosB in the hypothalamus, bed nucleus of stria terminalis (BNST), central amygdala (CeA) and nucleus of the tractus solitarius (NTS) following chronic morphine administration and withdrawal. Using combinations of

immuncytochemical identification of ΔFosB and neuropeptide markers we have revealed accumulation of ΔFosB in CRH synthesizing parvocellular neurosecretory neurons of the PVN of morphine dependent rats. Furthermore, naloxone-precipitated morphine withdrawal also resulted in an increase of double labeled profiles in the PVN. Most notably, we have detected ΔFosB immunoreactivity in the tyrozine hydroxylase (TH) containing A1 neuron population in the brainstem that provides the well-characterized noradrenergic input the stress-related CRH containing hypothalamic neurons (Nunez *et al.*, 2010).

Within the brain, expression of corticotropin-releasing hormone, CRH is not limited to the hypophyseotropic neurons of the hypothalamic paraventricular nucleus. Additional neuron populations such as cell groups in the bed nucleus of stria terminalis and central amygdala, as well as neurons in the Barrington's nucleus, scattered cells in the cortex and in the olfactory bulb are also able to synthesize CRH (Swanson *et al.*, 1983) for recent review, see (Kovacs, 2013). These neurons provide extrahypothalamic CRH that have been implicated as neurotransmitter and/or neuromodulator upon binding to CRH1R and to lesser extent to CRH2R receptors (Lu *et al.*, 2000; Kuperman & Chen, 2008). Among these CRH containing areas, cells in the "extended amygdala" (BNST and CeA) are of pivotal importance in mediating behavioral and emotional responses to various stress challenges including those that are related to drug addiction (Kiefer & Wiedemann, 2004).
In the framework of this international collaboration grant, we confirmed previous findings that morphine addiction results in long term neuronal plasticity in the extended amygdala (nucleus accumbens shell: sNAc, BNST and CeA), via expression of ΔFosB. Our research groups were the first, however, to reveal that CRH cells in the CeA and BNST became activated and express ΔFosB in morphine dependent animals and during morphine withdrawal. Furthermore, using unbiased quantitative analysis of CRH immunostained material, we have provided evidence that the number of CRH positive profiles is increased in the BNST (but not in the CeA) during morphine withdrawal. ΔFosB accumulation in the extended amygdala occurred in parallel with ΔFosB appearance in the TH neurons in the origin of ascending catecholaminergic pathways that innervate PVH and BNST/CeA and might be implicated in the negative emotional state during drug withdrawal (Nunez *et al.*, 2010).

It is very important to note that CRH gene expression is differentially regulated in the PVN and in the extended amygdala (Watts, 2005). CRH neurons in the hypophyseotropic cells of hypothalamus are under negative feedback effect of glucocorticoids via their direct or indirect action through TypeII glucocorticoid receptors (GR). By contrast, CRH transcription in the CeA and BNST is increased upon exposure to stress-levels of corticosteroid hormones (Watts & Sanchez-Watts, 1995; Kovacs, 2013). Although the cellular/molecular mechanism of facilitatory glucocorticoid action on CRH expression in the extended amygdala remained unresolved, the phenomenon is highly implicated in the negative emotional, physiological and behavioral state that seen during withdrawal in response to highly increased glucocorticoid discharge (McNally & Akil, 2002).
To reveal the contribution of hypothalamic vs. amygdala CRH in mediation of hormonal-, behavioral- and physiological symptoms of drug withdrawal we have designed experiments for site-specific silencing of the CRH gene. These included local injections of lentiviral constructs to the hypothalamic PVH or to amygdala of morphine dependent rats before withdrawal. Following our negative experience with constructs purchased from a company, at the end of 2012, we have started

collaboration with Dr. A. Chen's laboratory (Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel) who reported successful knock down of amygdala CRH expression using their own lentiviral constructs (Regev *et al.*, 2012). To this end, lentiviral constructs were designed that produce small hairpin (sh)RNA to CRH. The results of these experiments are under analysis in our laborytory.

In addition to CRH, some other members of CRF-related neuropeptide family such as urocortins (UCN1, UCN2 and UCN3) have also implicated in drug addiction (Koob, 2010). CRH and urocortins have differential affinity towards CRF receptors: CRH binds to CRHR1, UCN2 and UCN3 display high affinity to CRH2R, while urocortin1 binds to both receptors with equal affinity. It is important to note that CRH1R and CRH2Rs play a differential role in anxiogenesis activation of type 1 receptors promote anxiety, while type2 receptors have opposite effect (van Gaalen *et al.*, 2002; Merali *et al.*, 2003; Muller *et al.*, 2003). However, pharmacological blockade of both CRH receptors during morphine withdrawal results in decrease of behavioral- affective and physiological but not hormonal withdrawal symptoms (Almela *et al.*, 2012).
To assess the possible role of urocortins in the development of morphine dependence and during naloxone precipitated drug withdrawal, we detected by quantitative real time PCR the mRNA levels of UCN2 and UCN3 in the hypothalamus and found a significant increase of UCN2 but not UCN3 during withdrawal (Pinter-Kubler *et al.*, 2013).

Involvement of metabolic and reward-related neuropeptides in morphine dependence and withdrawal

Morphine dependence and naloxone-precipitated morphine withdrawal results in specific changes of **other neuropeptide** genes within the hypothalamus and extrahypothalamic brain sites that are involved in the stress- metabolic and behavioral regulation.
For instance, orexins have recently been hypothesized to modulate the extended amygdala and to contribute to the negative emotional state associated with dependence (Georgescu *et al.*, 2003).
We have revealed that naloxone-precipitated morphine withdrawal activates orexinergic neurons in the lateral hypothalamus and increases the expression of orexinA mRNA levels. These orexinergic neurons project to the extended amygdala, PVN and NTS areas that are critically involved in the reward and stress regulation. However, orexinA activates neurons only in the CeA but not BNST or PVH neurons during morphine withdrawal. Orexin1-receptor antagonist (SB334867) attenuates somatic but not hormonal withdrawal symptoms in morphine dependent rats. These results highlight differential involvement of the brain orexinergic system in development of morphine dependence and withdrawal symptoms (Laorden *et al.*, 2012).

One of the most significant somatic withdrawal symptoms in rats is the dramatic weight loss that appears during naloxone-precipitated withdrawal. Morphine-dependent rats loss up to 20-25% of their body weight shortly after naloxone injection. Morphine dependence has also been shown to have significant effect on metabolism. However, the neurobiological background of these changes was not resolved.
During the studies of this international grant we have investigated the metabolic status of morphine-dependent rats and changes of metabolic parameters during

naloxone precipitated drug withdrawal. Using a pair-feeding paradigm we have proved that morphine dependent rats in the first period of dependence loose less weight than their pair-fed controls suggesting reduced energy expenditure and/or increased metabolic efficiency. Interestingly, changes of the body weight of morphine dependent or pair fed animals was not accompanied by any significant changes of key hypothalamic neuropeptides involved in stress-and metabolic regulation such as NPY, POMC and UCNs. We have found however, increase of CRH, UCN2 and NPY mRNA levels and a very significant decrease of POMC expression in the hypothalamus of morphine dependent rats during naloxone-precipitated morphine withdrawal. These results suggest differential effects of mu-opioid dependent mechanisms in regulation of hypothalamic expression of stress- and metabolic-related neuropeptide genes (Ferenczi *et al.*, 2010; Pinter-Kubler *et al.*, 2013).

In summary, the principal findings of the studies performed in the framework of the present OTKA NN grant are the following:

1. **Morphine dependence and naloxone-precipitated morphine withdrawal results in activation of the neuroendocrine and brain stress systems in the rat brain.**
2. **Corticotropin-releasing hormone expressed in the hypophyseotropic cells of the hypothalamus as well as in the extended amygdala plays a differential role in the development of hormonal, physiological, emotional and behavioral changes in addicted animals.**
3. **cAMP-response element binding protein (CREB) and its co-activators (such as pTORC) play a critical role in activation of corticotropin-releasing hormone gene expression both in the hypothalamus and in the extended amygdala.**
4. **Ascending noradrenergic and orexinergic pathways innervating hypothalamic and extrahypothalamic sites differentially regulate the development of hormonal, somatic and psychological symptoms of morphine withdrawal.**
5. **Metabolic changes associated by drug dependence and withdrawal result in differential changes of metabolic and stress-related neuropeptides in the hypothalamus.**

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