Published in final edited form as:
Neuropharmacology. 2009 ; 56(Suppl 1): 32–43. doi:10.1016/j.neuropharm.2008.07.042.

Bidirectional Translational Research: Progress in Understanding Addictive Diseases

MJ Kreek, **SD Schlussman**, **B Reed**, **Y Zhang**, **DA Nielsen**, **O Levran**, **Y Zhou**, and **ER Butelman**
The Rockefeller University, 1230 York Avenue, New York, NY, 10065 USA

Abstract

The focus of this review is primarily on recent developments in bidirectional translational research on the addictions, within the Laboratory of the Biology of Addictive Diseases at The Rockefeller University. This review is subdivided into major interacting aspects, including: a) Investigation of neurobiological and molecular adaptations (e.g., in genes for the opioid receptors or endogenous neuropeptides) in response to cocaine or opiates, administered under laboratory conditions modeling chronic patterns of human self-exposure (e.g., chronic escalating “binge”). b) The impact of such drug exposure on the hypothalamic-pituitary-adrenal (HPA) axis and interacting neuropeptidergic systems (e.g., opioid, orexin and vasopressin). c) Molecular genetic association studies using candidate gene and whole genome approaches, to define particular systems involved in vulnerability to develop specific addictions, and response to pharmacotherapy. d) Neuroendocrine challenge studies in normal volunteers and current addictive disease patients along with former addicts in treatment, to investigate differential pharmacodynamics and responsiveness of molecular targets, in particular those also investigated in the experimental and molecular genetic approaches described above.

Keywords

Cocaine; heroin; methadone; opioid; dynorphin; beta-endorphin; addiction; mu-opioid; kappa-opioid; molecular genetics; HPA axis

As the Laboratory of the Biology of Addictive Diseases at The Rockefeller University, and as an NIH-NIDA P60 Research Center, we are honored and delighted to join in the celebration of the 35th Anniversary of NIDA. Our report herein will focus on some accomplishments of our scientists over the last five years, since we were privileged also to write a scientific tribute to the 30th Anniversary of NIDA (Kreek et al., 2004b). Selected topics only will be covered herein, primarily ones of interest both from the standpoint of basic science findings, with potential implications not only for the addictive diseases, but also for the molecular neurobiological mechanisms underlying many other human disorders, including Parkinsonism, Alzheimer's and Huntington's disease, as well as many which further elucidate normal physiology. In addition, our studies, of course, have significant implications for specific addictive diseases.

Corresponding author: Mary Jeanne Kreek, M.D., Patrick E. and Beatrice M. Haggerty Professor, Head of the Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, New York, NY 10065, Tel: 212-327-8490, Fax: 212-327-8574, kreek@rockefeller.edu.

I. In vivo effects of endogenous and synthetic opioids: Translational application of non-human primate studies, and related human studies

Neuroendocrine markers can provide, in a non-invasive manner, direct information on the pharmacodynamics of endogenous and exogenous ligands, on the responsiveness of particular receptor systems, and on the function of particular neurophysiological systems). Many of these data can be obtained from human addiction patients or normal controls. However, studies in non-human primates may be especially valuable in producing data with more extensive designs (e.g., sigmoidal dose-effect curves and quantitative antagonism experiments) or pharmacological probes that are not available for clinical use (see also Weerts et al., 2007).

As part of the Laboratory's primary focus, we have studied the effects of opioid neuropeptides on a neuroendocrine biomarker (prolactin release) and compared it with selective exogenous ligands. Prolactin release is sensitive to both kappa-and mu-agonists, and is therefore a potentially useful biomarker for the pharmacodynamics of ligands active *in vivo* at these two systems relevant to the pathophysiology of addiction.

We and others have previously found that this neuroendocrine biomarker can provide quantitative information on the relative potency and apparent efficacy of kappa-and mu-ligands (e.g., Butelman et al., 2002). Selective delta-agonists appear not to be active in this regard (Butelman et al., 2002). Furthermore, some of the opioid receptors involved in this response in primates are thought to be located in hypothalamic areas functionally outside the blood-brain barrier. Overall, this therefore provides the relatively unique opportunity to quantitatively study *in vivo* the effects of neuropeptides that may have limited ability to cross the blood-brain barrier.

A. Dynorphin A(1-17)

The endogenous full-length neuropeptide dynorphin A(1-17) (one of the main endogenous kappa-opioid ligands) was studied for its ability to stimulate prolactin release in gonadally intact female non-human primates (Butelman et al., 2004). Dynorphin A(1-17), given i.v. over a wide dose range, produced robust prolactin release, and exhibited a sigmoidal dose-effect curve (predicted by receptor theory, but rarely observed *in vivo*, due to experimental constraints). Intriguingly, in non-human primates, dynorphin A(1-17) was almost equipotent and equieffective to a synthetic high efficacy kappa-agonist U69,593, similar to its profile *in vitro*. This is therefore one of the unique situations in which an *in vivo*, non-invasive assay can be used to robustly study the pharmacodynamics of a natural sequence neuropeptide, compared with those of a synthetic agonist. Quantitative antagonism studies (apparent pK_B analysis) were carried out with naltrexone. This was consistent with the conclusion that kappa-receptors mediate the effect of dynorphin A(1-17) in this assay. Furthermore, the peripherally selective antagonist quaternary naltrexone (methylnaltrexone) blocked this effect of kappa-agonists, suggesting that opioid receptors outside the blood-brain barrier were involved. Such studies in primates using the full length dynorphin A(1-17) neuropeptide can be a valuable comparison to studies with the dynorphin A(1-13) fragment, which has been used in human experimental studies (Kreek et al., 1999; Bart et al., 2003).

B. Nalmefene

Nalmefene is a mu-selective antagonist available as a parenteral pharmacological tool, for the blockade of opioid effects in humans. We evaluated whether nalmefene alone would have effect on prolactin levels in normal healthy human volunteers (n=33) (Bart et al., 2005). We found that systemic nalmefene doses (3 or 10 mg, i.v.) caused a significant increase in prolactin levels, compared to placebo. This suggested that nalmefene had a degree of agonist tone at either mu- or kappa-receptors. Studies in collaboration with the Laboratory of Dr. Jean Bidlack

(University of Rochester) using the GTPγS *in vitro* assay determined that nalmefene is in fact a partial agonist at cloned human kappa-receptors, whereas it is solely antagonist at mu-receptors. Nalmefene's partial agonist effects at kappa-receptors may render it a particularly valuable tool for addiction pharmacotherapy, in that agonism at kappa-receptors is known to lower dopaminergic tone, which is known to be chronically affected by several addictive diseases.

C. Salvinorin A

As an illustration of the collaboration of the Center with related projects in the laboratory, we completed the first study on the neuroendocrine effects of the kappa-agonist hallucinogen salvinorin A (Butelman et al., 2007) (derived from the ethnomedical plant *Salvia divinorum*, now widely available in the United States) (see Roth et al., 2002). Salvinorin A is a diterpene, and represents a structurally unique new template for pharmacological probes of opioid receptors. Based on knowledge of kappa-agonist effects on the above biomarker, we studied the effects of a broad range of salvinorin A i.v. doses in non-human primates. Salvinorin A indeed caused robust dose-dependent prolactin release, with a very fast onset. Its potency and effectiveness were comparable to those of U69,593. Salvinorin A was sensitive to antagonism by nalmefene, at doses similar to those known to block kappa receptor effects in primates. Furthermore, gonadally intact female subjects were much more sensitive to the prolactin-releasing effects of salvinorin A than males. Overall, the present studies provide some of the first quantitative and translationally viable *in vivo* pharmacodynamic data for this novel hallucinogen, and confirm its kappa-agonist profile in a primate species.

D. Beta-endorphin

As part of the systematic comparison, the effects of beta-endorphin were studied on this biomarker in non-human primates. Beta-endorphin is a major endogenous high efficacy agonist at mu-receptors, which also has affinity at delta-receptors, *in vitro*. Based on available background information on this system, beta-endorphin was directly compared to fentanyl (a high efficacy, centrally-penetrating mu-agonist) and to loperamide (a mu-agonist which does not easily cross the blood-brain barrier). Both these compounds are in clinical use, and this further aids translational interpretation of these studies.

Fentanyl and loperamide both caused robust, dose-dependent prolactin release (fentanyl being approximately 10-fold more potent than loperamide). Surprisingly, beta-endorphin (0.01-0.32 mg/kg i.v.) produced relatively small effects on prolactin release, with 2 of 4 subjects showing almost no effect. Prior studies from other laboratories had detected effects of beta-endorphin on prolactin release (Foley et al., 1979; Catlin et al., 1980), but these are the first data that suggest a low potency and *in vivo* effectiveness of beta-endorphin in this regard. For this reason, we followed up these studies in two ways. Firstly, it is known that a particular single nucleotide polymorphism (SNP) in the non-human primate mu-receptor gene, C77G (Miller et al., 2004) has a reduced potency of beta-endorphin as its phenotype (intriguingly, this non-human primate SNP appears to be a functional ortholog of the human A118G SNP, discussed in the clinical section). However, none of these subjects had the C77G SNP. A further consideration was the potential rapid biotransformation of beta-endorphin into less active fragments. In collaboration with the Laboratory of Dr. Brian Chait (The Rockefeller University), we determined that full-length beta-endorphin was detected in these subjects until at least 5 min after injection, using a newly improved MALDI-MS method (Butelman et al., 2008). Likewise, we confirmed that the effects of loperamide could be completely blocked by the peripherally selective antagonist quaternary naltrexone, suggesting that a potential inability of beta-endorphin to cross the blood-brain barrier *per se* would not negate the possibility to be active in this biomarker assay. These studies therefore raise the possibility that the ability of beta-endorphin to mount an acute high efficacy action at mu-receptors *in vivo* may be limited.

II. Extracellular biotransformation of beta-endorphin in rat striatum and cerebrospinal fluid

In order to further understand whether beta-endorphin's agonist profile in the central nervous system (CNS) is highly related to the generation of particular biotransformation fragments, we investigated beta-endorphin biotransformation in the striatum of rats *in vivo*. These studies used a microinfusion/microdialysis technique coupled to MALDI-MS (developed in collaboration with the Laboratory of Dr. Brian Chait) (Reed et al., 2003). We observed rapid cleavage resulting in beta-endorphin 1-18, as well as several fragments resulting from further N-terminal degradation. *In vitro* studies with incubation of full-length beta-endorphin, with and without protease inhibitors, in the incubation fluid of isolated striatal slices indicate beta-endorphin is initially cleaved predominantly at the Phe18-Lys19 position, as well as at the Leu17-Phe18 position. Investigations of cerebrospinal fluid revealed similar enzymatic cleavage of beta-endorphin. The observed pattern of cleavage sites (Phe18-Lys19 and Leu17-Phe18) is consistent with published *in vitro* studies of purified insulin-degrading enzyme cleavage of beta-endorphin (Safavi et al., 1996). The binding affinities of full-length beta-endorphin, as well as previously identified beta-endorphin fragments alpha- and gamma-endorphin (beta-endorphin 1-16 and 1-17, respectively), and the novel fragment identified in the current studies, beta-endorphin 1-18, at heterologously-expressed mu-opioid, delta-opioid, and kappa-opioid receptors, respectively, were determined. The affinity of the truncation fragments is reduced at each of the receptors compared to the affinity of full length beta-endorphin (Reed et al., 2008).

III. Steady-dose and escalating-dose “binge” administration of cocaine alter expression of behavioral stereotypy and striatal preprodynorphin mRNA levels in rats

A common feature of human drug dependence is the development, over time, of tolerance to the reinforcing effects, which frequently results in the self-administration of increasing amounts of drugs (American Psychiatric Association (1994) *Diagnostic and Statistical Manual of Mental Disorders (4thedition) Washington D.C.)*. In order to more closely model this pattern of chronic drug abuse observed in humans, we modified our “binge” pattern of cocaine administration to include an escalation in the dose of cocaine administered during the course of a 14-day study (Schlussman et al., 2005) and compared the effects of chronic “binge” pattern administration of steady-dose versus escalating dose “binge” cocaine on the expression of behavioral stereotypy and the levels of preprodynorphin (ppDyn) mRNA in the caudate-putamen.

Male Fischer rats were assigned to one of three groups. Animals in the steady-dose or the escalating-dose “binge” pattern cocaine administration groups received three daily injections of cocaine in a “binge” pattern. Animals in the steady-dose “binge” group received a cocaine dose of 15 mg/kg/injection (for a total daily dose of 45 mg/kg). Animals in the escalating-dose “binge” group received 15 mg/kg/injection on days 1-3, 20 mg/kg/injection on days 4-6, 25 mg/kg/injection on days 7-9 and 30 mg/kg/injection for the remainder of the study, for a maximum daily dose of 90 mg/kg. Control animals received saline administered in the same pattern. Expression of behavioral stereotypy was scored, in the home cage, using a modification of the rating scale described by Daunais and McGinty (1995) and used previously by our group (e.g., Schlussman et al., 1998; Schlussman et al., 2003a).

To determine ppDyn mRNA levels in the caudate-putamen, RNA was isolated (Chomczynski et al., 1987) and levels of mRNA for ppDyn and total RNA (18S RNA) were quantified by

solution hybridization RNase protection (see e.g., Branch et al., 1992; Schlussman et al., 2003b).

Animals expressed a “focused stereotypy” consisting of high frequency, low amplitude head movements. Expression of this behavior was time- and dose-dependent. As cocaine dose increased, so did the expression of this behavior. The expression of “focused stereotypy” was significantly higher in the escalating- than in the steady-dose animals. The expression of this behavior showed sensitization, that is, in both cocaine groups, this behavior increased over time.

Cocaine caused a significant increase in ppDyn mRNA levels in the caudate-putamen. Both steady-dose and escalating-dose cocaine administration elevated ppDyn mRNA in this region, however there was no difference in ppDyn mRNA levels between the cocaine groups. This is in agreement with an earlier study in which we showed that the level of ppDyn mRNA did not differ among groups of rats that had received 10 or 30 mg/kg of cocaine in a “binge” pattern for 14 days (Spangler et al., 1993). They are also in agreement with our report of similar levels of ppDyn mRNA in the caudate-putamen of rats which self-administered either low (0.5 mg/infusion) or high (2.0 mg/infusion) doses of cocaine for 14 days under extended-access conditions (Mantsch et al., 2004). Taken together, these data suggest that the dynorphin response to cocaine in the caudate-putamen is not dose-dependent. Alternatively, we may be observing a ceiling effect in which even lower doses of cocaine, below 10 mg/kg, may produce lower levels of ppDyn response. It should be noted that other groups report a dose-dependency in the ppDyn mRNA response to cocaine (e.g., Daunais and McGinty, 1995; Fagergren et al., 2003). The difference between these studies may be related to the method of measuring mRNA (quantitative RNase protection – solution hybridization versus *in situ* hybridization). The present studies demonstrate that escalation of cocaine dose, over time, results in a significantly different behavioral pattern and highlights the importance of translational research moving from the clinic to the bench (Schlussman et al., 2005).

IV. Cocaine self-administration in mice is related to the phosphorylation state of DARPP-32

The reinforcing effect of cocaine is associated with increases in dopamine in the striatum. The dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32) mediates intracellular events following activation of dopamine receptors. DARPP-32 is phosphorylated at multiple sites by different protein kinases but little is known about the functional role of these different sites. Cocaine self-administration and striatal levels of dopamine after acute “binge” cocaine administration were measured in separate lines of mice with alanine mutations introduced into DARPP-32 at either Thr34 (protein kinase A site, Thr34A), Thr75 (cyclin dependent kinase 5 site, Thr75A), Ser97 (casein kinase 2 site, Ser97A) or Ser130 (casein kinase 1 site, Ser130A) (Zhang et al., 2006). Acquisition of stable cocaine self-administration required more time in Thr34A-/- mice. Both Thr34A- and Ser130A-DARPP-32 mutant mice self-administered more cocaine than their respective wildtype controls. Also, acute cocaine-induced increases of dopamine in dorsal striatum were attenuated in the Thr34A- and Ser130A-DARPP-32 phosphomutant mice compared to wildtype mice. Also, levels of P-Thr34- and P-Ser130-DARPP-32 were reduced after cocaine self-administration in wildtype mice. Thus, phosphorylation states of Thr34- and Ser130-DARPP-32 play important roles in modulating the reinforcing effects of cocaine.

V. Effect of chronic “binge cocaine” on basal levels and cocaine-induced increases of dopamine in the caudate-putamen and nucleus accumbens of C57BL/6J and 129/J mice

In vivo microdialysis was used to measure the effect of chronic “binge” administration of cocaine on basal and cocaine-induced dopamine release in the caudate-putamen and nucleus accumbens of C57BL/6J and 129/J mice (Zhang et al., 2003). Mice were implanted with a guide cannula in the caudate-putamen or nucleus accumbens. After recovery, mice in one group received “binge” pattern cocaine administration for 13 days while another group received saline in the same pattern. On the day before microdialysis, dialysis probes were lowered into the caudate-putamen and nucleus accumbens. The next morning, after baseline dopamine collection, all animals received “binge” cocaine administration. Chronic “binge” cocaine administration led to a lowering of both basal dopamine and the actual levels of cocaine-induced increases of dopamine in the two brain regions. However, the incremental rise over baseline levels of dopamine in response to chronic cocaine administration further increases in the nucleus accumbens of both mouse strains.

VI. The plant-derived hallucinogen, salvinorin A, like dynorphin A(1-17), decreases basal dopamine levels in the caudate-putamen and produces conditioned place aversion in mice by agonist actions on kappa-opioid receptors

As mentioned above, salvinorin A is a naturally-occurring hallucinogen and a potent and selective kappa-agonist *in vitro* (Roth et al., 2002). Kappa-agonists decrease dopamine levels in the caudate-putamen and cause conditioned place aversion in experimental animals. We have shown that both endogenous and synthetic kappa-agonists decrease cocaine-induced dopamine release in mice (Zhang et al., 2004a, b). Therefore, we studied the effect of salvinorin A on basal dopamine levels in the caudate-putamen, and whether salvinorin A induces a conditioned place preference or aversion with associated changes in locomotor activity (Zhang, et al., 2005). In the first experiment, effects of salvinorin A on basal dopamine levels in the caudate-putamen were measured with *in vivo* microdialysis. The second experiment examined whether salvinorin A led to conditioned place preference (or aversion) with associated changes in locomotor activity in a conditioned place preference paradigm. Salvinorin A decreased dopamine levels in a dose- dependent manner. The larger doses of salvinorin A studied (1.0 and 3.2 mg/kg, i.p.) decreased dopamine levels compared to vehicle, and this effect was completely blocked by pre-injection with 10 mg/kg of the kappa-antagonist nor-BNI. The same doses of salvinorin A caused conditioned place aversion and decreased locomotor activity. These findings suggest that salvinorin A decreased dopamine levels through kappa-receptors in the caudate-putamen. The inhibitory effect of salvinorin A on striatal dopamine may contribute to its induction of conditioned place aversion and associated decreases in locomotor activity. These findings are consistent with the *in vitro* characterization of salvinorin A as a kappa-receptor agonist, and attest to its prominent regulation of dopaminergic systems also affected by drugs of abuse.

VII. Steady-state methadone by osmotic pumps decreases cocaine-seeking behavior in animal models

Methadone (a selective, mu-agonist, long-acting in humans) is widely used in treatment of short-acting opiate (primarily heroin) addiction and for management of chronic pain. Methadone has a short half-life in rodents, about 60 min in mice, 90 min in rats (Burstein et

al., 1980), and, in contrast, a long half-life of 24 to 28 hours in humans for the racemic mixture as is used in most human pharmacotherapy and in most, but not all, rodent studies (Kreek, 1973b). In several animal studies (Kreek et al., 1983; Zhou et al., 1996), therefore, methadone was delivered through osmotic pumps to mimic steady-state methadone maintenance in humans (Kreek, 1973a,b).

There is a growing body of evidence that methadone maintenance can decrease cocaine use in heroin-cocaine codependent persons. In an earlier study, Borg and colleagues in our laboratory (Borg et al., 1995) found that 69% of all patients in a methadone maintenance treatment program for severe opiate addiction who entered treatment with cocaine and heroin codependency stopped regular cocaine abuse when maintained on a long-term basis on an average methadone dose of 67 mg/day. (This earlier study was done at a time that the purity of heroin was lower than it is in recent years, and thus higher doses of methadone were not needed, and also when the actual amount of cocaine used was less for a variety of different reasons, primarily economic.) A recent study by Peles and colleagues (2006b) also reported that around 70% of patients who entered the methadone maintenance treatment program with cocaine- heroin codependency stopped all use of cocaine after one year of methadone maintenance treatment, at which time mean methadone stabilized doses were 176 mg/day.

Higher doses of methadone may be required in patients that display severe cocaine abuse, because cocaine exposure can increase mu-opioid receptors in specific brain regions in humans (Zubieta et al., 1996; Gorelick et al., 2005). In our early and recent animal studies, we have found that there is a significant increase in mu-opioid receptor density after chronic (14 days) “binge” pattern cocaine administration, which is found in both the mesocorticolimbic and nigrostriatal dopaminergic systems, including the nucleus accumbens, amygdala, anterior cingulate, and caudate-putamen (Unterwald et al., 1992). Of interest, this increase persists into chronic (14 days) withdrawal from chronic escalating-dose “binge” cocaine administration (Bailey et al., 2005). After acute cocaine exposure (1 or 5 days), an increase in mu-opioid receptor mRNA levels has been found in the same specific brain regions (Azaryan et al., 1996; Yuferov et al., 1999). Using positron emission tomography (PET) technology, up-regulation of mu-opioid receptor binding has been observed in cocaine-dependent individuals, and it has been associated with cocaine craving (Zubieta et al., 1996; Gorelick et al., 2005). We have shown that there is a relative endorphin deficiency in cocaine addicts and also in chronically cocaine-abusing, methadone-maintained former heroin addicts, just as we reported previously that there is a persistent relative endorphin deficiency in medication-free, illicit-heroin-free, former heroin addicts (Schluger et al., 2001).

In collaboration with Leri and colleagues (University of Guelph, Canada), we investigated the effect of high-dose steady-state methadone on cocaine conditioned place preference (CPP) and cocaine intravenous self-administration, rodent models for cocaine wanting/liking and taking behaviors. We found that (1) rats implanted with osmotic pumps delivering steady-state methadone prior to cocaine conditioning did not express cocaine CPP; (2) rats with steady-state methadone after cocaine conditioning displayed neither spontaneous nor cocaine-precipitated CPP; and (3) steady-state methadone did not alter the intravenous self-administration (continuous schedule of reinforcement) of various doses of cocaine. Further, mu-opioid receptor mRNA levels in the nucleus accumbens core were significantly elevated in rats after exposure to cocaine conditioning. However, the upregulation of mu-opioid receptor mRNA levels was reduced by steady-state methadone in a dose-dependent manner. Our results suggest that high-dose steady-state methadone does not alter the direct reinforcing effect of cocaine but blocks spontaneous and cocaine-precipitated cocaine seeking, possibly by preventing mu-opioid receptor alterations in the nucleus accumbens core induced by cocaine conditioning (Leri et al., 2006). Our results in animal studies parallel and support the clinic findings in former heroin and cocaine codependent individuals maintained on high-dose

methadone who consume less cocaine. Notably, gas liquid chromatography revealed that steady-state methadone at 30 mg/kg/day in rats yielded plasma methadone levels (within the range of 450-490 ng/ml) found to be effective in reducing cocaine abuse in opiate-dependent individuals (Peles et al., 2006a,b).

VIII. Role of dopamine D1, D2, and D3 receptors in modulation of gene expression of mu-opioid receptor and proopiomelanocortin (POMC)

In humans, elevations of mu-opioid receptor binding potential have been reported to be associated with cocaine craving during early abstinence (which our group interprets due, in part, to the relative endorphin deficiency based on other findings in clinical research) (Zubieta et al., 1996; Schluger et al., 2001; Gorelick et al., 2005). Dopamine D1 or D3 receptor homozygous knockout (D1-/- or D3-/-) mice offer the opportunity to examine the roles of these specific receptors in regulating mu-opioid receptor gene expression in response to cocaine. In another recent study, in collaboration with Xu and colleagues at the University of Chicago, we found an increase in basal mu-opioid receptor mRNA levels in the frontal cortex of either D1 or D3 receptor deficient mice. Acute “binge” cocaine unexpectedly returned the frontal cortex mu-opioid receptor mRNA levels in D1 or D3 receptor deficient mice to that in wild type controls. Although the interactions are complicated, our findings suggest that: both dopamine D1 and D3 receptors may be involved in the mu-opioid receptor gene regulation (Zhou et al., 2007).

Recently, we also investigated potential roles that dopamine D1 or D2 receptors could play in regulation of POMC gene (encoding beta-endorphin) expression in the hypothalamus in response to acute “binge” cocaine. We found that the dopamine D2 receptor blockade by a selective antagonist sulpiride increased POMC mRNA levels in the hypothalamus, indicating that dopamine D2 receptor exerts a tonic inhibitory effect on hypothalamic POMC gene expression. The POMC mRNA increases induced by the dopamine D2 receptor blockade were attenuated by acute “binge” cocaine. Neither the dopamine D2 receptor blockade nor acute “binge” cocaine altered POMC mRNA levels in the amygdala. In contrast to the dopamine D2 receptor, the dopamine D1 receptor blockade by a selective antagonist SCH23390, acute “binge” cocaine or their combination had no effect on hypothalamic POMC mRNA levels. These results suggest a specific role for dopamine D2 receptor in acute cocaine's effects on hypothalamic POMC gene expression (Zhou et al., 2004).

IX. Involvement of mu-opioid receptors, orexin and preprodynorphin gene expression in the lateral hypothalamus in drug reward processes, animal model of opioid dependence or drug withdrawal (anhedonia)

Studies from our laboratory and others have examined the effects of chronic opioid agonists, or antagonists with opioid withdrawal, on mu-opioid receptor mRNA levels in different brain regions. A decrease, an increase or no changes have been reported, and these apparently contradictory results may depend on differences in the dose and route of the opioid agonist or antagonist administered, exposure time and the brain regions examined. In many previous studies from other laboratories, morphine or opioid agonists were chronically administered by pellets. This differs from heroin addicts who use an intermittent pattern of self-administration, in which they could experience both rewarding effects and chronic stress induced by repeated heroin injection and withdrawal (Kreek et al., 1998). Therefore, an administration paradigm of chronic intermittent escalating-dose morphine or heroin was developed in our laboratory, in order to mimic the multiple and escalating doses that human heroin abusers seek daily to achieve rewarding effects, and also exposes them to withdrawal in between-dose intervals.

As reviewed (Kreek et al., 1998; Koob et al., 2007), endogenous mu-opioid system, especially POMC-derived beta-endorphin, exert inhibitory effects on the hypothalamic-pituitary-adrenal (HPA) axis in both humans and rodents. Furthermore, the lateral portion of the hypothalamus is an important brain region for reward and other motivated behaviors. However, the question of whether morphine withdrawal influences mu-opioid receptor gene expression in the lateral hypothalamus has not yet been studied. We conducted this particular study in rats to determine the effects of acute single-dose morphine administration, 10-day chronic intermittent escalating-dose morphine administration or its 12-hour spontaneous withdrawal, on mu-opioid receptor mRNA levels (Zhou et al., 2006). We specifically selected several brain regions considered to play an important role in the reinforcing or motivational effects of drugs of abuse, such as the nucleus accumbens core, amygdala, lateral hypothalamus and caudate-putamen (Kreek et al., 1998). We found that either acute or chronic morphine did not modify mu-opioid receptor mRNA levels in the brain regions listed above. In contrast, acute spontaneous morphine withdrawal led to an increase in mu-opioid receptor mRNA levels in the nucleus accumbens core, lateral hypothalamus and caudate-putamen, but not in the amygdala. Our data clearly showed that morphine withdrawal increases mu-opioid receptor mRNA levels in a region-specific manner. Our finding of increased mu-opioid receptor gene expression by morphine withdrawal suggests that endogenous opioid agonists and exogenous opiates also have an inhibitory effect on mu-opioid receptor gene expression (Zhou et al., 2006).

Our laboratory has recently investigated the modulation of orexin gene expression in animal models of drug addiction. Most of the lateral hypothalamus orexin neurons express the preprodynorphin (ppDyn) gene, and around half of lateral hypothalamus orexin neurons also express mu-opioid receptors (Chou et al., 2001; Georgescu et al., 2003). It has been established that the hypothalamic orexins are involved in the regulation of sleep, arousal, feeding, and stress. There is a growing body of evidence suggesting a novel role for the orexins in regulation of drug seeking-related behaviors. Therefore, we examined levels of the ppDyn and orexin mRNAs in the lateral hypothalamus. During the aversive state of acute withdrawal from chronic escalating-dose morphine, we found increased orexin mRNA levels in rat lateral hypothalamus, supporting our hypothesis that enhanced lateral hypothalamus orexin neuronal activity, resulting from its increased gene expression, contributes to negative affective states in opiate withdrawal (Zhou et al., 2006).

To investigate cocaine-induced reward processes (as measured by cocaine place conditioning), our current study further showed that orexin mRNA level was decreased only in the lateral hypothalamus (but not perifornical area-dorsomedial hypothalamus region) after expression of cocaine place preference. In contrast, acute withdrawal from chronic (14 days) escalating-dose cocaine administration resulted in increases in both lateral hypothalamus orexin and ppDyn mRNA levels. These data further support the hypothesis that the lateral hypothalamus orexin/ppDyn system may be a critical component of neural circuitry underlying the negative affective consequences (anhedonia) of drug withdrawal (Zhou et al., 2008a).

In a related study, kappa-opioid receptor binding was investigated after chronic (14 days) escalating-dose cocaine administration and chronic (14 days) withdrawal therefrom. We found a downregulation of kappa-opioid receptors in the basolateral amygdala and septum of the rat withdrawn for 14 days from chronic escalating dose binge cocaine administration, suggesting a persistent lowering of KOP-r binding after a long time of discontinuation of the drug (Bailey et al., 2007).

X. Involvement of arginine vasopressin (AVP) and V1b receptor in drug withdrawal and heroin seeking precipitated by stress and by heroin

Vasopressinergic neuronal activity in the amygdala and hypothalamus may represent an important element in the neurobiology of stress-related behaviors. Our laboratory has begun to explore the role of AVP in drug addiction, by examining AVP gene expression in the rat amygdala and hypothalamus after chronic drug exposure or during early and late spontaneous withdrawal. We first examined AVP gene expression in the rat amygdala after chronic (14 days) “binge” cocaine administration, and its early (1 day) withdrawal. Similar to an increase in CRF mRNA in the amygdala in early cocaine withdrawal (Zhou et al., 2003), we found that there was an activated amygdalar AVP gene expression by early cocaine withdrawal. This increase was completely blocked by opioid receptor antagonist naloxone pretreatment, suggesting an opioid receptor-mediated mechanism (Zhou et al., 2005). Our findings further indicated a potential role for AVP in the aversive consequences of early drug withdrawal, and led us to examine opiate withdrawal and heroin seeking.

Indeed, we found that amygdalar AVP mRNA levels were also increased during early heroin withdrawal from chronic (10 days) intermittent escalating-dose heroin. Of note, this heroin withdrawal-induced AVP mRNA increase was replicated in a separate study after early spontaneous morphine withdrawal (Zhou et al., 2008b). In collaboration with Leri and colleagues, we found that foot shock stress increased AVP mRNA level in the amygdala of rats withdrawn from heroin self-administration, but not in heroin-naïve rats, suggesting that AVP and its receptors may be involved in stress-induced reinstatement of heroin-seeking behavior (an animal model for studying relapse to drug abuse in humans). We immediately went on to investigate whether the blockade of central AVP receptors (V1a or V1b receptor) would alter heroin seeking during tests of reinstatement of self-administration induced by foot shock stress and by heroin priming, and HPA hormonal responses to foot shock. A selective V1b receptor antagonist (SSR149415) (but not V1a receptor antagonist dP(Tyr(Me)^2,Arg-NH_2^{9})AVP, unpublished observation) dose-dependently attenuated foot shock-induced reinstatement, blocked heroin-induced reinstatement, and blunted the HPA activation by foot shock. In conclusion, these data in rats suggest that the stress-responsive AVP/V1b receptor system, including the amygdala, may be critical components of the neural circuitry underlying the aversive emotional consequences of drug withdrawal, and the effect of negative emotional states on drug-seeking behavior. To date, this V1b antagonist is the only systemically available compound identified to reduce stress- and drug-induced reinstatement of heroin seeking. It may be worthwhile to explore the usefulness of systemically-effective V1b receptor antagonists for the management of withdrawal and for the prevention of drug relapse (Zhou et al., 2008b).

XI. Human molecular genetic research on vulnerability to develop specific addictions and pharmacogenetic aspects of treatment

A major focus of the research conducted in the Laboratory of the Biology of Addictive Diseases during the past five years has been the identification of new gene variants that contribute to vulnerability to develop specific addictions, as well as establishing the function of these and of previously known variants. The vulnerability to develop addiction has a complex genetic basis in which multiple variants may work in various combinations decreasing or increasing addiction vulnerability. The effect of one gene may be masked by variants in other genes with which it interacts (epistasis). Variants may also interact with the environment to influence phenotype. Several genes have been associated with the vulnerability to develop an addictive disease (for reviews see: Kreek et al., 2005a, b; Kreek et al., 2007).

A. The mu-opioid receptor gene (*OPRM1*)

The mu-opioid receptor is centrally involved in the development of the addictive diseases. The stress-responsive HPA axis is under tonic inhibitory control of the mu-opioid receptors. Studies with mu-opioid receptor agonists and antagonists have shown that acute agonist "on/off" binding of the mu-opioid receptor represses the stress axis. One common variant, A118G (rs1799971), in the *OPRM1* gene, has been shown to be a functional variant (Bond et al., 1998). The A118G variant causes an asparagine (Asn) to aspartic acid (Asp) substitution at residue 40 in the extra-cellular amino-terminus; this removes one of five potential N-glycosylation sites of the receptor. The Asp40 variant binds beta-endorphin with greater affinity, and activates K+ channels to a greater extent than the prototype receptor (Bond et al., 1998). It has also been reported that the A118G allele expresses approximately one-half the *OPRM1* mRNA and about one-tenth the mu-opioid receptor protein (Beyer et al., 2004; Zhang, Wang, et al., 2005; Kroslak et al., 2007). Therefore, subjects with the A118G allele would probably have greater response of the variant receptor but also have reduced receptor expression.

We have assessed the functionality of the *OPRM1* A118G variant on cell surface expression of the mu-opioid receptor, agonist-binding affinity, and receptor signaling via inhibition of adenylyl cyclase activity. To do this, we expressed the prototype (118A/Asn40) and the variant (118G/Asp40) mu-opioid receptors in two cell lines, human-derived HEK293 cells and Syrian hamster-derived AV-12 cells (Kroslak et al., 2007). This study also focused on the important second-messenger system for this receptor, reduction in adenylyl cyclase activity. When stably expressed in both HEK293 and AV-12 cells, the 118G receptor was expressed with lower receptor levels than the 118A receptor. In addition, stable expression of the variant and prototype receptors was characterized by differences in forskolin-induced cAMP accumulation as well as differences in agonist-induced accumulation of cAMP (EC50) for morphine, methadone, and the mu-opioid receptor agonist DAMGO, but not for the endogenous neuropeptide beta-endorphin. In contrast, when transiently-expressed in HEK293 cells, the 118G receptor showed only a minor difference in cell surface receptor levels compared to the 118A receptor, with no differences in EC50 values for DAMGO or beta-endorphin. Treatment with an antibiotic, tunicamycin, that inhibits the synthesis of N-linked glycoproteins abolished the expression of both receptor variants onto the plasma membrane, indicating that glycosylation may play a substantial role in mu-opioid receptor function. Further site-directed mutagenesis studies, using each of the glycosylation sites of the mu-opioid receptor, will be necessary to document the importance of N-linked glycosylation sites for receptor function and expression.

In a study designed to assess the role of the A118G variant in modulating the HPA axis (Bart et al., 2006), we found a significant increase in basal levels of plasma cortisol in healthy subjects with at least one *OPRM1* 118G allele. Fifty-nine healthy adults with no history of substance abuse or dependence (including nicotine), were genotyped for the A118G variant. Subjects were admitted to a stress-minimized inpatient unit and plasma adrenocorticotropic hormone (ACTH) and cortisol levels were determined in blood samples drawn at multiple time points. Groups did not differ in levels of ACTH. Basal levels of ACTH and cortisol did not differ between genders. Subjects with one or two copies 118G allele had significantly higher cortisol levels than subjects with two copies of the 118A allele. Further work is needed to address whether the differences in basal and stress-induced cortisol response in subjects with an *OPRM1* 118G allele are due to an alteration in receptor expression or function, and if these differences contribute to the development of psychiatric disorders characterized by altered response of the HPA axis.

Animal and human studies have shown that both naltrexone and nalmefene, mu-opioid receptor antagonists, are efficacious for the treatment of alcoholism, reducing the amount of alcohol

consumed and the number of days spent in treatment (for review see O'Leary et al., 2001; O'Malley et al., 2003 and also O'Malley et al., 2002). Therefore, the *OPRM1* gene is a candidate for genetic association studies of alcoholism. Association studies of the A118G polymorphism in alcoholism specifically are conflicting, partly due to ethnic heterogeneity and admixtures.

In a recent study (Bart et al., 2005), we found a significant association between genotypes with the 118G allele and alcohol dependence in central Sweden. We focused on a population from central Sweden in order to reduce the potential effect of population admixture. A total of 389 alcohol-dependent subjects and 170 healthy volunteers were genotyped for the A118G and the C17T variants but no subjects with the C17T polymorphism were identified. Genotype and allele distribution did not vary between genders. Analysis by genotype (autosomal dominant mode of inheritance) revealed significantly greater odds ratio (OR) for alcohol dependence in subjects with a 118G allele (OR = 1.92). The attributable risk due to genotypes with a 118G allele was 11.1%. In this study, we controlled for the type of alcoholism and found no difference in allele distribution between type 1 and type 2 alcoholics, that differ in age of onset and the severity of symptoms, usually including aggressive behaviors.

From our bench and clinical studies, we had predicted that alcohol-dependent subjects with one or two copies of the 118G allele would respond better to naltrexone pharmacotherapy than individuals homozygous for the 118A allele. This prediction has been borne out in two studies, a non-prospective study (Oslin et al., 2003) and a large prospective study (Anton et al., 2008), which have shown that alcohol-dependent subjects with one or two copies of the 118G allele were more responsive to naltrexone pharmacotherapy.

B. The prodynorphin gene (*PDYN*)

Dynorphin peptides are endogenous opioid neuropeptides with multiple physiological functions in the brain. Dynorphins are the endogenous ligand of the kappa-opioid receptor. Binding of dynorphin, particularly dynorphin A, to kappa-opioid receptors reduces dopaminergic tone under basal conditions or after taking drugs of abuse (Kreek et al, 2004a). Twelve hundred nucleotides upstream of the human dynorphin gene (*PDYN*) is a 68-base pair tandem repeat polymorphism which usually is reported to be present in 1, 2, 3, or 4 copies. Zimprich and colleagues have reported increase in dynorphin gene expression when 3 or 4 copies are present as compared with 1 or 2 (Zimprich et al., 2000). The repeat polymorphism contains a near canonical AP-1 transcription factor binding site and it was shown that three or four repeat copies result in greater transcriptional activation.

In the following study (Williams et al., 2007), we wanted to test the hypothesis that genotypes with three or four repeats may be associated with vulnerability to develop cocaine or cocaine/ alcohol dependence. We studied a cohort of 302 subjects that were ascertained and characterized extensively by DSM-IV and Addiction Severity Index (ASI) criteria; 127 healthy subjects; 82 subjects with only cocaine dependence; and 93 subjects with cocaine/ alcohol codependency. The promoter region of *PDYN* was PCR amplified from genomic DNA and analyzed via gel electrophoresis. The data was stratified by the three major ethnic groups: African American, Caucasian, and Hispanic. Genotypes were grouped into three categories: short (repeat copies:1,1; 1,2; 2,2), short/long (repeat copies: 1,3; 2,3; 1,4; 2,4) and long (repeat copies: 3,3; 3,4; 4,4). A significant difference was found in grouped genotype distribution among ethnicities in controls. A point-wise significant difference in genotype frequency between the groups was found across ethnicities, between the cocaine/alcohol-codependent group and the controls. This difference was not significant after correction for multiple testing. The long genotype category was found at higher frequency in the cocaine/alcohol codependency group compared with controls, in African Americans. Hence, three or four copies of the 68-base pair tandem repeat gave an increased risk of developing cocaine/alcohol codependency in African Americans.

C. The melanocortin receptor type 2 gene (*MC2R*) (ACTH receptor)

To follow our hypothesis that altered stress responsivity plays a role in substance abuse, we have studied the melanocortin receptor type 2. This receptor is involved in the regulation of adrenal cortisol secretion, which is a component of stress-response. Other laboratories have reported that a variant of *MC2R*, -179A>G, results in a reduction in adrenal biosynthetic activity (Reich et al., 2005). In our study (Proudnikov et al., 2008) we resequenced the promoter region and exons 1 and 2 of the *MC2R* gene in 272 subjects including Caucasians, Hispanics and African Americans, and identified five novel variants. These variants were then genotyped in a total of 632 subjects using TaqMan assays. We found significant differences in genotype frequency among ethnic groups and also an association of the -184A allele and a haplotype (AACT, -184G>A, -179A>G, 833A>C and 1005C>T, respectively) with a protective effect from heroin addiction in Hispanics. In addition, a haplotype (GACT) also consisting of these four variants was significantly associated with heroin addiction.

D. Genome-wide association study of opiate addiction

A genome-wide association study was conducted using microarray technology to identify genes that may be associated with vulnerability to develop heroin addiction (Nielsen et al., 2008). DNA from former severe heroin addicts (meeting Federal criteria for methadone maintenance) and control subjects, all Caucasian, were analyzed using 10K Affymetrix GeneChips (Nielsen et al., 2008). We found that, when using separate analyses for autosomal and X chromosomal variants, the most significant association of allele frequency with heroin addiction was with the autosomal variants rs965972, located in the Unigene cluster Hs.147755, a cluster of three ESTs cloned from testis and kidney, found in a region predicted to have high regulatory potential (Taylor et al., 2006) and is a T to C transition, creating a potential CREB transcription factor binding site. The next most significant variant was rs1986513 located in a region of high conservation at 4q28.1. This variant is an A to T transversion and creates potential binding sites for the TFIID, and GATA-1, 2, and 3 transcription factors (Nielsen et al., 2008).

The three variants with the strongest association to heroin addiction by genotype frequency were rs1714984, located in an intron of the transcription factor myocardin gene *MYCOD*, rs965972 (discussed above), and rs1867898, which is found in a region of high regulatory potential (Nielsen et al., 2008). One genotype pattern (AG-TT-GG) consisting of these later three variants (rs1714984, rs965972, and rs1867898) was found to be significantly associated with developing heroin addiction (OR = 6.25) and explained 27% of the population attributable risk for heroin addiction in this cohort, and another (GG-CT-GG) with protection from developing heroin addiction (OR = 0.13), and lacking this genotype pattern explained 83% of the population attributable risk for developing heroin addiction in Caucasians. Evidence was found for involvement in heroin addiction of the genes coding for: the mu-opioid receptor; the metabotropic receptors mGluR6 and mGluR8; nuclear receptor NR4A2; and cryptochrome 1 (photolyase-like). This whole genome association approach has confirmed the role of *OPRM1* in heroin addiction and has identified several new genes that potentially are associated with this disease (Nielsen et al., 2008).

E The P-glycoprotein gene (*ABCB1*)

As mentioned above, methadone is a mu-agonist used for treating opiate dependence. The range of effective methadone doses is broad, and this inter-individual variability in dose-effectiveness may be caused by genetic factors. Methadone is a substrate of P-glycoprotein 170 (*ABCB1, MDR1*). Our hypothesis is that *ABCB1* variants may play a role in methadone absorption and distribution. In the following study (Levran et al., 2008), we assessed the association between *ABCB1* SNPs and methadone dose requirements in 98 methadone-maintained patients from Israel. We found significant difference in genotype frequencies

between the “higher” (>150 mg/day) and “lower” (≤150 mg/day) methadone dose groups for SNP 1236C>T (rs1128503). Furthermore, individuals bearing the 3-locus genotype pattern TT-TT-TT (rs1045642, rs2032582 and rs1128503) have an OR=5.26 of requiring higher doses, while individuals heterozygous for these three SNPs have an OR=2.86 of stabilizing at lower doses. In conclusion, specific *ABCB1* variants may have clinical relevance in this population.

F. High-throughput molecular haplotype analysis

Recently, we designed a new method of molecular haplotyping (allelic assignment) of single nucleotide polymorphisms by use of a variation of the fluorescent PCR technique, TaqMan (Proudnikov et al., 2004, 2006). This method is based on the PCR amplification of the allele of interest using allele-specific primers designed for the terminal variants of the haplotype. Monitoring of the amplification occurs in real-time using TaqMan probes specific for the internal variant of the haplotype. Using this technique, we successfully determined haplotypes with polymorphisms separated by three to up to several hundred nucleotides. We applied this technique for haplotyping of the polymorphisms in the kappa opioid receptor (*OPRK1*) (Proudnikov et al., 2004) and the serotonin receptor 1B (*HTR1B*) genes (Proudnikov et al., 2006).

G. The BiolAD-DB System

To store the large amount of clinical and genetic data generated in our studies on the addictive diseases, we have created a sophisticated bioinformatics system (Nielsen et al., 2007). This system, known as the BiolAD-DB system is a research bioinformatics system for inputting, validating, organizing, archiving, analyzing, and processing of complex clinical and genetic data. The database schema employs design principles for handling complex clinical information, such as response items in genetic questionnaires using a conventional database approach combined with the Entity Attribute Value (EAV) representation (Nadkarni et al., 1999). Data access and validation is provided by the BiolAD-DB client application, which features a data validation engine tightly coupled to a graphical user interface. A MySQL version of the BiolAD-DB system, including schema, client installation instructions, and links to associated websites, is freely available at http://www.rockefeller.edu/biolad-db/.

XII. Other Recent Clinical Research Studies

We have conducted numerous other studies, especially both in clinical coupled with laboratory-based research, and more applied clinical research in the field. These have included studies in which we have further a) explored the stress-responsive hypothalamic-pituitary-adrenal (HPA) axis and elucidated the dual targeting of selective mu-opioid receptor antagonists, b) conducted further studies of adolescent former heroin-addicted methadone treatment patients in opioid agonist pharmacotherapy, c) performed special studies on dose-dependent pharmacology and cardiovascular effects of full opioid agonist treatment with methadone, and d) identified heretofore unrecognized silent hepatitis B virus carrier state in former long-term heroin addicts, successfully managed in methadone maintenance treatment.

A. Hypothalamic-pituitary-adrenal (HPA) Axis

We have conducted rigorous studies of the HPA axis, with emphasis on glucocorticoid negative feedback of the HPA axis in methadone-maintained former heroin addicts, both without and with ongoing cocaine dependence (Aouizerate et al., 2006). We also have conducted studies in collaboration with Sinha and colleagues at Yale of the impact of individualized narratives on either stress- or drug-related topics, on cocaine craving and on objective measurements of the HPA response, and then followed up subjects after release from a protected clinical research inpatient setting (Sinha et al., 2006). Time to relapse and magnitude of relapse to cocaine was found to relate to the HPA axis response to the stress- and drug-induced narratives. In other

basic clinical research studies, we have found that nalmefene is both a mu-opioid antagonist, but also a kappa-opioid receptor partial agonist, which probably pertains also for naltrexone (Bart et al., 2005). We have further put our stress-responsive work in perspective, with both laboratory-based topics discussed in a recent review article in collaboration with the group of Koob and colleagues (2007). We have also presented perspectives on the integration of our findings of the functional A118G polymorphism of the mu-opioid receptor, which alters stress responsivity, with various clinical findings related to the addictive diseases (Kreek et al., 2007; Kreek, 2008).

B. Studies of Adolescent Former Opiate Addicts in Methadone-Maintenance Treatment

We have conducted studies in adolescent opiate addicts in one of the very few opioid agonist replacement programs focused on adolescent patients with opiate addiction with entry criteria, as rigorously defined by the Federal guidelines for any entry into methadone-maintenance treatment. In one of these studies, we identified that patients who did not step-wise reduce their frequency and amount of heroin use in the first few months of treatment were at greater risk of leaving treatment, and that use of cocaine at admission, and especially continuing use of cocaine during the first six weeks of treatment, were indicators of higher heroin use, in those who did remain in treatment and who reached one year retention. We further found that in the patients who stayed in treatment for one year or more, there was a highly significant reduction or elimination of heroin use, and a reduction in cocaine use and addiction (Kellogg et al., 2006). In a second study in the same population, we identified the high prevalence of co-morbidity with depression, and therefore further looked into the impact of pharmacotherapy for depression, in addition to conventional behavioral therapy, in reducing relapse to any drug of abuse during opioid agonist treatment. After controlling for demographic and clinical characteristics, we found that pharmacological antidepressant treatment was, in fact, not significantly associated with a reduction in relapse to heroin, cocaine, or other drugs of abuse in this very young population (Galarneau et al., 2006).

C. Further Special Studies in Methadone Maintenance Patients

We have been able to conduct a rigorous study relating doses of methadone in patients receiving maintenance treatment with serum levels of methadone, including much higher doses than had been previously studied (>120mg/day). In these studies, we found that overall there was a strong correlation between methadone dose and serum levels ($R+0.53$, $p<0.0005$). Further, we found in the subset of subjects with absolutely no drug abuse, including no continuing heroin or cocaine use, the correlation was even stronger than those with any continuing drug abuse of any type (Adelson et al., 2007). In another study, we evaluated the QTc intervals as measured in electrocardiograms (EKG) of former heroin addicts in methadone maintenance treatment. This was a study of 138 patients completing all parts of the study and who all had been in methadone maintenance treatment for a minimum of 3 months (100 days) and up to 10.7 years. These subjects were receiving methadone doses ranging from 40 up to 290 mg/day. All patients had an electrocardiogram performed at the same time that bloods were drawn for determination of plasma levels of methadone, at 24 hours after the last methadone dose. Of importance, we found that methadone dose and serum levels did not correlate significantly overall with the QTc intervals. The mean QTc interval in the entire study group was 419.3 ± 32.8 ms and the mean methadone dose in this population was 171 ± 50 mg/day. However, three patients were found to have unequivocally prolonged QTc intervals (above 500 ms). These subjects were all on very high doses of methadone. Of interest, two of the three persons identified with prolonged QTc intervals died after two years of follow-up, but the cause of death was not attributed to cardiac origin or related to the QTc interval prolongation. Another 19 subjects were identified with prolonged QTc intervals by current usual definition, although usually with no apparent clinical implications (between 450 and 499 ms). None of these patients with QTc intervals greater than 450 ms had any cardiac problems. It is of considerable interest that all 22 subjects

with QTc intervals above 450 ms, including the three with QTc intervals greater than 500 ms, were receiving daily doses of methadone greater than 120mg/day, the upper limit of methadone doses in all previous prospective and special cross-sectional studies of cardiac function, in methadone-maintained subjects (Peles et al., 2006a).

D. Studies of Hepatitis A, B and C in Former Opiate Addicts in Methadone Maintenance Treatment

We have made a novel finding, the unexpected presence of hepatitis B DNA, that is, "silent hepatitis B," in clinically well patients (Bart et al., 2008). In a cross-sectional study of 103 adults in long-term methadone-maintenance treatment in a single clinic, we looked for evidence of past or current infection with hepatitis A, B and C in specially obtained blood specimens. We found that more than 40% of subjects had markers for all three viruses. Hepatitis C virus was the most prevalent (84%), followed by hepatitis B (65%), and hepatitis A (46%). Of note, no subject had hepatitis B surface antigen. However when we specifically looked for hepatitis B virus DNA, using a highly sensitive technique, much more sensitive than commercial tests, we found 26% of all subjects had the silent hepatitis B virus present. These subjects all had hepatitis B core antibody, but as their only hepatitis B virus marker. This finding of silent hepatitis B virus infection with identifiable virus in long-term methadone-maintained patients must be interpreted as a potential health risk, both for the individual in any setting of any future immune suppression, and, less possibly, as a source of contamination for blood supplies (Bart et al., 2008). In another report, we have reviewed current issues for hepatitis C infection treatment in patients in long-term opiate-agonist treatment with methadone maintenance (Novick et al., 2008). These papers are of potential considerable importance for individual and public health issues.

XIII. Summary: Early History of Laboratory and Challenges for the Future

In our celebration of the 30th Anniversary of NIDA report of five years ago, we detailed some the early history of our Laboratory, as well as the more recent history. It is important to re-emphasize that our own work, in particular, the work of Mary Jeanne Kreek, M.D., began in late 1963, when Professor Vincent P. Dole made the decision to transform his laboratory at the then named Rockefeller Institute for Medical Research, from one focusing on hypertension and lipid metabolism, to a laboratory focused on heroin addiction and its treatment. At that time, Dr. Kreek was recruited to join his group in early 1964. The late Dr. Marie Nyswander, a psychiatrist who had been working in the field, was also recruited to work at Rockefeller at that time. During the first six months of 1964, two major things happened. First, this newly coalesced research team of three, in retrospect, introduced a frame shift in the paradigm of thinking about addiction. Specifically, they articulated the hypothesis that heroin addiction (and later extended to all addictive diseases) is a disease of the brain, with resultant behavioral manifestations of "drug hunger" and drug self-administration, despite negative consequences to self and to others. Further, they hypothesized that heroin addiction is not simply a criminal behavior or due alone to antisocial personality or other personality disorder (Dole et al., 1966). This hypothesis led directly to the mandate to attempt to develop an effective pharmacotherapy for heroin addiction, to combine with abstinence-based behavioral treatment, which, up to that time, had been successful in less than 20% of unselected heroin addicts. During that first six months, this research trio, using the Rockefeller University Hospital, selected the orally-effective synthetic compound methadone; they postulated (although there was no proof yet, since specific opiate receptors had not been identified) that this compound acted at opiate receptors and, further, inferred from the limited research in pain management, that this compound was much longer-acting than morphine or heroin in humans.

In this early work, conducted with very long-term heroin addicts (mean of 14 years of heroin addiction) and with a past history of many attempts and failures at detoxification and drug-free treatment, it was shown that, if methadone were initiated in treatment doses of 20-40mg/day and slowly increased to full treatment doses of 80-120mg/day, no euphoria or "high" was felt, and withdrawal symptoms were prevented. Further, they were able to show in two series of four-week-long double-blind Latin square design studies that the superimposition of the short-acting narcotic, such as heroin itself, or morphine or hydromorphone (Dilaudid™), against a background of oral methadone treatment, could not be perceived. Thus, these studies defined two mechanisms of action of methadone: first, they showed unequivocally that this compound provided pharmacodynamic long action (24 hours or more) and that, during such treatment, and no euphoria or "high," and no opiate withdrawal signs and symptoms were perceived. Further, they showed that the expressed thoughts and goals of each patient changed from those seeking and using narcotics, to trying to re-establish social contacts and to become rehabilitated or habilitated to finish some level of education and to train for some type of occupation. They documented that the mechanism of action of methadone was through the development of opioid tolerance and cross-tolerance to other opiates. They were able to show that the classical behavioral phenomenon of extinction would occur, and could be of great help in management of heroin addiction. It was soon found that over 70% of heroin addicts coming into methadone maintenance treatment would attempt to use heroin one or more times. However, when such use occurred, and the person got no euphoria (no "high"), the power of classical extinction also played a role in helping reduce, and then eliminate, heroin use. Further studies showed, contrasted with the "on-off" effects of a drug of abuse, which bench work from our laboratory and others has shown altered levels of gene expression, receptor-mediated events, physiology and behavior, that during steady-state treatment, there is no disruption of any molecular function, physiology or behavior, rather normalization of all functions disrupted during cycles of addiction occurs. Such normalization, obviously, is essential, and should continue to be our goal in seeking for new potential therapeutic agents.

Our laboratory received its first NIH grant in 1975, at the time when Mary Jeanne Kreek became the head of the very atypical independent Laboratory of the Biology of Addictive Diseases, which was without precedent at that time at The Rockefeller University. In 1978, an NIH-NIDA Senior Research Scientist Career Award was competed for and awarded to Dr. Kreek. Without this award, which continued until early 2008, there is no question that her work, and the work of the entire Laboratory of the Biology of Addictive Diseases, would have had to stop its research.

Our ongoing P60 Center grant has allowed us to codify our collaborations among different institutions in the New York City area, the nation, and worldwide, while focusing our work on molecular neurobiology and basic clinical research. The Center provides a platform with several cores which can be utilized by our own laboratory, and that of our Center collaborators, as well as other NIDA-funded collaborators at different institutions.

As we continue with our ultimate goal of developing new, more effective techniques for primary prevention, early intervention, as well as long-term chronic treatment of addictive diseases, and especially the attempts to develop such treatments for stimulant drugs of abuse, such as cocaine, for which we have no treatments, we remain concerned about a major continuing problem. As stated five years ago, an overwhelming challenge, and one not within our immediate domain of expertise, is how to unearth the roots of and then reverse the stigma against those suffering from addictive diseases, those who offer treatments for addictive diseases, and though increasingly less frequently, against those doing research related to the addictions. It is still of deep concern that some scientists who perform addictive diseases research often choose not to mention the fact that their work is focused on addiction, or could have direct relevance for addiction. Indeed, most, if not all, of the work in our laboratory and

Center, like that of innumerable other scientists, has general applicability to many neurological, neurodegenerative and specific genetic diseases. Our work, like the work of many others, is not parochial. However, to deny the very important potential impact on the development of better methods for treatment, prevention and early intervention of addictions, is to deny the fact that these diseases are needful of our research and later treatment, only leads to further stigmatization. Therefore, to quote directly a closing statement of the report we were honored to write five years ago, “As basic laboratory-based scientists and clinical scientists, we must educate the scientific, clinical, and general public of our scientific findings related to the addictive diseases. Ultimately, we can only hope that insight and knowledge will win out over stigma.”

Acknowledgments

For assistance in the writing of the manuscript, we wish to thank Dr. Ann Ho, Dr. Roberto Picetti, and for assistance in preparation, Mr. Kitt Lavoie and Ms. Susan Russo. The present studies were funded by NIH NIDA P60 DA05130 and DA00049; NIH-NIMH R01MH-79880 (MJK); specific studies were in collaboration with NIH-NIDA DA11113 and 017369 (ERB); R03 DA22266 (DAN). Clinical studies were supported by the UL1RR024143 grant from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on NCRR is available at http://www.ncrr.nih.gov/. Information on Re-engineering the Clinical Research Enterprise can be obtained from http://nihroadmap.nih.gov/clinicalresearch/overview-translational.asp.

References

Adelson M, Peles E, Bodner G, Kreek MJ. Correlation between high methadone doses and methadone serum levels in methadone maintenance treatment (MMT) patients. J Addict Dis 2007;26:15–26. [PubMed: 17439864]

Anton RF, Oroszi G, O'Malley S, Couper D, Swift R, Pettinati H, Goldman D. An evaluation of mu-opioid receptor (OPRM1) as a predictor of naltrexone response in the treatment of alcohol dependence: results from the Combined Pharmacotherapies and Behavioral Interventions for Alcohol Dependence (COMBINE) study. Arch Gen Psychiatry 2008;65:135–144. [PubMed: 18250251]

Aouizerate B, Ho A, Schluger JH, Perret G, Borg L, Le Moal M, Piazza PV, Kreek MJ. Glucocorticoid negative feedback in methadone maintained former heroin addicts with ongoing cocaine dependence: Dose-response to dexamethasone suppression. Addict Biol 2006;11:84–96. [PubMed: 16759341]

Azaryan AV, Coughlin LJ, Buzas B, Clock BJ, Cox BM. Effect of chronic cocaine treatment on mu- and delta-opioid receptor mRNA levels in dopaminergically innervated brain regions. J Neurochem 1996;66:443–448. [PubMed: 8592112]

Bailey A, Gianotti R, Ho A, Kreek MJ. Persistent upregulation of μ-opioid but not adenosine receptors in brains of long-term withdrawn escalating-dose “binge” cocaine treated rats. Synapse 2005;57:160–166. [PubMed: 15945065]

Bailey A, Gianotti R, Ho A, Kreek MJ. Downregulation of κ-opioid receptors in basolateral amygdala and septum of rats withdrawn for 14 days from an escalating-dose “binge” cocaine administration paradigm. Synapse 2007;61:820–826. [PubMed: 17621646]

Bart G, Borg L, Schluger JH, Green M, Ho A, Kreek MJ. Suppressed prolactin response to dynorphin A1-13 in methadone-maintained versus control subjects. J Pharmacol Exp Ther 2003;306:581–587. [PubMed: 12730354]

Bart G, Kreek MJ, Ott J, LaForge KS, Proudnikov D, Pollak L, Heilig M. Increased attributable risk related to a functional μ-opioid receptor gene polymorphism in association with alcohol dependence in central Sweden. Neuropsychopharmacology 2005;30:417–422. [PubMed: 15525999]

Bart G, LaForge KS, Borg L, Lilly C, Ho A, Kreek MJ. Altered levels of basal cortisol in healthy subjects with a 118G allele in exon 1 of the mu opioid receptor gene. Neuropsychopharmacology 2006;31:2313–2317. [PubMed: 16794569]

Bart G, Piccolo P, Zhang L, Jacobson I, Schaefer R, Kreek MJ. Markers for hepatitis A, B, and C in methadone maintained patients: an unexpectedly high coinfection with silent hepatitis B. Addiction 2008;103:681–686. [PubMed: 18339114]

Bart G, Schluger JH, Borg L, Ho A, Bidlack JM, Kreek MJ. Nalmefene induced elevation in serum prolactin in normal human volunteers: partial kappa opioid agonist activity? Neuropsychopharmacology 2005;30:2254–2262. [PubMed: 15988468]

Beyer A, Koch T, Schröder H, Schulz S, Höllt V. Effect of the A118G polymorphism on binding affinity, potency and agonist-mediated endocytosis, desensitization, and resensitization of the human mu-opioid receptor. J Neurochem 2004;89:553–560. [PubMed: 15086512]

Bond C, LaForge KS, Tian M, Melia D, Zhang S, Borg L, Gong J, Schluger J, Strong JA, Leal SM, Tischfield JA, Kreek MJ, Yu L. Single-nucleotide polymorphism in the human mu opioid receptor gene alters β-endorphin binding and activity: possible implications for opiate addiction. Proc Natl Acad Sci USA 1998;95:9608–9613. [PubMed: 9689128]

Borg L, Ho A, Peters JE, Kreek MJ. Availability of reliable serum methadone determination for management of symptomatic patients. J Addict Dis 1995;14:83–91. [PubMed: 8555281]

Branch AD, Unterwald EM, Lee SE, Kreek MJ. Quantitation of preproenkephalin mRNA levels in brain regions from male Fischer rats following chronic cocaine treatment using a recently developed solution hybridization assay. Brain Res Mol Brain Res 1992;14:231–238. [PubMed: 1279343]

Burstein Y, Grady RW, Kreek MJ, Rausen AR, Peterson CM. Thrombocytosis in the offspring of female mice receiving dl-methadone. Proc Soc Exp Biol Med 1980;164:275–279. [PubMed: 7403097]

Butelman ER, Ball JW, Kreek MJ. Comparison of the discriminative and neuroendocrine effects of centrally-penetrating κ-opioid agonists in rhesus monkeys. Psychopharmacology 2002;164:115–120. [PubMed: 12373425]

Butelman ER, Ball JW, Kreek MJ. Peripheral selectivity and apparent efficacy of dynorphins: Comparison to non-peptidic κ-opioid agonists in rhesus monkeys. Psychoneuroendocrinology 2004;29:292–304.

Butelman ER, Mandau M, Tidgewell K, Prisinzano TE, Kreek MJ. Effects of salvinorin A, a κ-opioid hallucinogen, on a neuroendocrine biomarker assay in non-human primates with high κ-receptor homology to humans. J Pharmacol Exp Ther 2007;320:300–306. [PubMed: 17060493]

Butelman ER, Reed B, Chait BT, Mandau M, Yuferov V, Kreek MJ. Limited effects of β-endorphin compared to loperamide or fentanyl in a neuroendocrine biomarker assay in non-human primates. Psychoneuroendocrinology 2008;33:292–304. [PubMed: 18171605]

Caltin DH, Poland RE, Gorelick DA, Gerner RH, Hui KK, Rubin RT, Li CH. Intravenous infusion of beta-endorphin increases serum prolactin, but not growth hormone or cortisol, in depressed subjects and withdrawing methadone addicts. J Clin Endocrinol Metab 1980;50:1021–1025. [PubMed: 7372783]

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159. [PubMed: 2440339]

Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, Willie JT, Beuchmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, Scammell TE. Orexin (hypocretin) neurons contain dynorphin. J Neurosci 2001;21:RC168. [PubMed: 11567079]

Daunais JB, McGinty JF. Cocaine binges differentially alter striatal preprodynorphin and zif/268 mRNAs. Brain Res Mol Brain Res 1995;29:201–210. [PubMed: 7609608]

Dole VP, Nyswander ME, Kreek MJ. Narcotic blockade. Arch Intern Med 1966;118:304–309. [PubMed: 4162686]

Fagergren P, Smith HR, Daunais JB, Nader MA, Porrino LJ, Hurd YL. Temporal upregulation of prodynorphin mRNA in the primate striatum after cocaine self-administration. Eur J Neurosci 2003;17:2212–2218. [PubMed: 12786988]

Foley KM, Kourides IA, Inturrisi CE, Kaiko RF, Zaroulis CG, Posner JB, Houde RW, Li CH. β-Endorphin: analgesic and hormonal effects in humans. Proc Natl Acad Sci USA 1979;76:5377–5381. [PubMed: 291954]

Galarneau DW, Teres JJ, Leon AC, Melia D, Lilly C, Kellogg S, Khuri E, Kreek MJ. Antidepressant treatment patterns in a novel methadone maintenance clinic targeting young adults:an observation study. J Addict Dis 2006;25:47–52. [PubMed: 16785219]

Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, Yanagisawa M, Nester EJ, Dileone RJ. Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J Neurosci 2003;23:2106–3111.

Gorelick DA, Kim YK, Bencherif B, Boyd SJ, Nelson R, Copersino M, Endres CJ, Dannals RF, Frost JJ. Imaging brain mu-opioid receptors in abstinent cocaine users: time course and relation to cocaine craving. Biol Psychiatry 2005;57:1573–1582. [PubMed: 15953495]

Kellogg SH, Lin A, Melia D, Ho A, Khuri E, Bell K, Ducat E, Oosterhuis E, Kreek MJ. Adolescent and young adult heroin patients: Drug use and success in methadone maintenance treatment. J Addict Dis 2006;25:15–25. [PubMed: 16956865]

Koob GF, Kreek MJ. Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am J Psychiatry 2007;164:1149–1159. [PubMed: 17671276]

Kreek MJ. Medical safety and side effects of methadone in tolerant individuals. JAMA 1973a;223:665–667. [PubMed: 4739193]

Kreek MJ. Plasma and urine levels of methadone. NY State J Med 1973b;73:2773–2777.

Kreek MJ. Role of a functional human gene polymorphism in stress responsivity and addictions. Clin Pharmacol Ther 2008;83:615–618. [PubMed: 18323858]

Kreek MJ, Bart G, Lilly C, LaForge KS, Nielsen DA. Pharmacogenetics and human molecular genetics of opiate and cocaine addictions and their treatments. Pharmacol Rev 2005a;57:1–26. [PubMed: 15734726]

Kreek MJ, Dodes L, Kane S, Knobler J, Martin R. Long-term methadone maintenance therapy: Effects on liver function. Ann Intern Med 1972;77:598–602. [PubMed: 4629927]

Kreek MJ, Koob GF. Drug dependence: stress and dysregulation of brain reward pathways. Drug Alcohol Depend 1998;51:23–47. [PubMed: 9716928]

Kreek MJ, LaForge KS. Stress responsivity, addiction, and a functional variant of the human mu-opioid receptor gene. Mol Interv 2007;7:74–78. [PubMed: 17468387]

Kreek MJ, Nielsen DA, LaForge KS. Genes associated with addiction: alcoholism, opiate, and cocaine addiction. Neuromolecular Med 2004a;5:85–108. [PubMed: 15001815]

Kreek MJ, Schluger J, Borg L, Gunduz M, Ho A. Dynorphin A1-13 causes elevation of serum levels of prolactin through an opioid receptor mechanism in humans: gender differences and implications for modulation of dopaminergic tone in the treatment of addictions. J Pharmacol Exp Ther 1999;288:260–269. [PubMed: 9862779]

Kreek MJ, Schlussman SD, Bart G, LaForge KS, Butelman ER. Evolving perspectives on neurobiological research on the addictions: celebration of the 30th anniversary of NIDA. Neuropharmacology 2004b; 47:324–344. [PubMed: 15464148]

Kreek MJ, Wardlaw SL, Hartman N. Circadian rhythms and levels of beta-endorphin, ACTH, and cortisol during chronic methadone maintenance treatment in humans. Life Sci 1983;33:409–413. [PubMed: 6319894]

Kroslak T, LaForge KS, Gianotti RJ, Ho A, Nielsen DA, Kreek MJ. The single nucleotide polymorphism A118G alters functional properties of the human mu opioid receptor. J Neurochem 2007;103:77–87. [PubMed: 17877633]

Leri F, Zhou Y, Goddard B, Cummins E, Kreek MJ. Effects of high dose methadone maintenance on cocaine place conditioning, cocaine self-administration, and mu-opioid receptor mRNA expression in the rat brain. Neuropsychopharmacology 2006;31:1462–1474. [PubMed: 16237390]

Levran O, O'Hara K, Peles E, Li D, Barral S, Ray B, Borg L, Ott J, Adelson M, Kreek MJ. *ABCB1 (MDR1)* Genetic variants are associated with methadone doses required for effective treatment of heroin dependence. Hum Mol Genet 2008;17:2219–2227. [PubMed: 18424454]

Mantsch JR, Yuferov V, Mathieu-Kia AM, Ho A, Kreek MJ. Effects of extended access to high versus low cocaine doses on self-administration, cocaine-induced reinstatement and brain mRNA levels in rats. Psychopharmacology (Berl) 2004;175:26–36. [PubMed: 15042275]

Miller GM, Bendor J, Tiefenbacher S, Yang H, Novak MA, Madras BK. A mu-opioid receptor single nucleotide polymorphism in rhesus monkey: association with stress response and aggression. Mol Psychiatry 2004;9:99–108. [PubMed: 14699447]

Nadkarni PM, Marenco L, Chen R, Skoufos E, Shepherd G, Miller P. Organization of heterogeneous scientific data using the EAV/CR representation. J Am Med Inform Assoc 1999;6:478–493. [PubMed: 10579606]

Nielsen DA, Ji F, Yuferov V, Ho A, Chen A, Levran O, Ott J, Kreek MJ. changed in text to delete b Genotype patterns that contribute to increased risk for or protection from developing heroin addiction. Mol Psychiatry 2008;13:417–428. [PubMed: 18195715]

Nielsen DA, Leidner M, Haynes C, Krauthammer M, Kreek MJ. The BiolAD-DB system: an informatics system for clinical and genetic data. Mol Diagn Ther 2007;11:15–19. [PubMed: 17286447]

Novick DM, Kreek MJ. Critical issues in the treatment of hepatitis C virus infection in methadone maintenance patients. Addiction 2008;103:905–918. [PubMed: 18422827]

O'Leary G, Borrow J, Weiss RD. Opioid antagonists in the treatment of alcohol dependence. Curr Psychiatry Rep 2001;3:484–488. [PubMed: 11707162]

O'Malley SS, Froehlich JC. Advances in the use of naltrexone: an integration of preclinical and clinical findings. Recent Dev Alcohol 2003;16:217–245. [PubMed: 12638640]

O'Malley SS, Krishnan-Sarin S, Farren C, Sinha R, Kreek MJ. Naltrexone decreases craving and alcohol self-administration in alcohol-dependent subjects and activates the hypothalamo-pituitary-adrenocortical axis. Psychopharmacology (Berl) 2002;160:19–29. [PubMed: 11862370]

Oslin DW, Berrettini W, Kranzler HR, Pettinati H, Gelernter J, Volpicelli JR, O'Brien CP. A functional polymorphism of the mu-opioid receptor gene is associated with naltrexone response in alcohol-dependent patients. Neuropsychopharmacology 2003;28:1546–1552. [PubMed: 12813472]

Peles E, Bodner G, Kreek MJ, Rados V, Adelson M. Corrected-QT intervals as related to methadone dose and serum level in methadone maintenance treatment (MMT) patients – a cross-sectional study. Addiction 2006a;102:289–300. [PubMed: 17222284]

Peles E, Kreek MJ, Kellogg S, Adelson M. High methadone dose significantly reduces cocaine use in methadone maintenance treatment (MMT) patients. J Addict Dis 2006b;25:43–50. [PubMed: 16597572]

Proudnikov D, LaForge KS, Kreek MJ. High-throughput molecular haplotype analysis (allelic assignment) of single-nucleotide polymorphisms by fluorescent polymerase chain reaction. Anal Biochem 2004;335:165–167. [PubMed: 15519584]

Proudnikov D, LaForge KS, Hofflich H, Levenstein M, Gordon D, Barral S, Ott J, Kreek MJ. Association analysis of polymorphisms in serotonin 1B receptor gene and heroin addiction: a comparison of molecular and statistically estimated haplotypes. Pharmacogenet Genomics 2006;16:25–36. [PubMed: 16344719]

Proudnikov D, Hamon S, Ott J, Kreek MJ. Association of polymorphisms in the melanocortin receptor type 2 (MC2R, ACTH receptor) gene with heroin addiction. Neuroscience Lett 2008;435:234–239.

Reed B, Bidlack JM, Chait BT, Kreek MJ. Extracellular biotransformation of β-endorphin in rat striatum and cerebrospinal fluid. J Neuroendocrinol 2008;20:606–616. [PubMed: 18363801]

Reed B, Zhang Y, Chait BT, Kreek MJ. Dynorphin A(1-17) biotransformation in striatum of freely moving rats using microdialysis and matrix-assisted laser desorption/ionization mass spectrometry. J Neurochem 2003;86:815–823. [PubMed: 12887680]

Reisch N, Slawik M, Zwermann O, Beuschlein F, Reincke M. Genetic influence of an ACTH receptor promoter polymorphism on adrenal androgen secretion. Eur J Endocrinol 2005;153:711–715. [PubMed: 16260430]

Roth BL, Baner K, Westkaemper R, Siebert D, Rice KC, Steinberg S, Ernsberger P, Rothman RB. Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist. Proc Natl Acad Sci USA 2002;99:11934–11939. [PubMed: 12192085]

Safavi A, Miller BC, Cottam L, Hersh LB. Identification of gamma-endorphin-generating enzyme as insulin-degrading enzyme. Biochemistry 1996;35:14318–14325. [PubMed: 8916918]

Schluger JH, Borg L, Ho A, Kreek MJ. Altered HPA axis responsivity to metyrapone testing in methadone maintained former heroin addicts with ongoing cocaine addiction. Neuropsychopharmacology 2001;24:568–575. [PubMed: 11282257]

Schlussman SD, Ho A, Zhou Y, Curtis AE, Kreek MJ. Effects of “binge” pattern cocaine on stereotypy and locomotor activity in C57BL/6J and 129/J mice. Pharmacol Biochem Behav 1998;60:593–599. [PubMed: 9632245]

Schlussman SD, Zhang Y, Kane S, Stewart CL, Ho A, Kreek MJ. Locomotion, stereotypy, and dopamine D1 receptors after chronic “binge” cocaine in C57BL/6J and 129/J mice. Pharmacol Biochem Behav 2003a;75:123–131. [PubMed: 12759120]

Schlussman SD, Zhang Y, Yuferov V, LaForge KS, Ho A, Kreek MJ. Acute 'binge' cocaine administration elevates dynorphin mRNA in the caudate putamen of C57BL/6J but not 129/J mice. Brain Res 2003b;974:249–253. [PubMed: 12742644]

Schlussman SD, Zhou Y, Bailey A, Ho A, Kreek MJ. Steady-dose and escalating-dose "binge" administration of cocaine alter expression of behavioral stereotypy and striatal preprodynorphin mRNA levels in rats. Brain Res Bull 2005;67:169–175. [PubMed: 16144651]

Sinha R, Garcia M, Paliwal P, Kreek MJ, Rounsaville BJ. Stress-induced cocaine craving and hypothalamic-pituitary-adrenal responses are predictive of cocaine relapse outcomes. Arch Gen Psychiatry 2006;63:324–331. [PubMed: 16520439]

Spangler R, Unterwald EM, Kreek MJ. 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. Brain Res Mol Brain Res 1993;19:323–327. [PubMed: 7694032]

Taylor J, Tyekucheva S, King DC, Hardison RC, Miller W, Chiaromonte F. ESPERR: Learning strong and weak signals in genomic sequence alignments to identify functional elements. Genome Res 2006;16:1596–1604. [PubMed: 17053093]

Unterwald EM, Horne-King J, Kreek MJ. Chronic cocaine alters brain mu opioid receptors. Brain Res 1992;584:314–318. [PubMed: 1325249]

Yuferov V, Zhou Y, Spangler R, Maggos CE, Ho A, Kreek MJ. Acute "binge" cocaine increases mu-opioid receptor mRNA levels in areas of the rat mesolimbic mesocortical dopamine system. Brain Res Bull 1999;48:109–112. [PubMed: 10210176]

Weerts EM, Fantegrossi WE, Goodwin AK. The value of nonhuman primates in drug abuse research. Exp Clin Psychopharmacol 2007;15:309–327. [PubMed: 17696678]

Williams TJ, LaForge KS, Gordon D, Bart G, Kellogg S, Ott J, Kreek MJ. Prodynorphin gene promoter repeat associated with cocaine/alcohol codependence. Addict Biol 2007;12:496–502. [PubMed: 17559549]

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Effect of the endogenous κ-opioid agonist dynorphin A(1-17) on cocaine-evoked increases in striatal dopamine levels and cocaine-induced place preference in C57BL/6J mice. Psychopharmacology (Berl) 2004a;172:422–429. [PubMed: 14712335]

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Effect of the κ-opioid agonist R-84760 on cocaine-induced increases in striatal dopamine levels and cocaine-induced place preference in C57BL/6J mice. Psychopharmacology (Berl) 2004b;173:146–152. [PubMed: 14712342]

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at κ-opioid receptors. Psychopharmacology (Berl) 2005;179:551–558. [PubMed: 15682306]

Zhang Y, Schlussman SD, Ho A, Kreek MJ. Effect of chronic "binge cocaine" on basal levels and cocaine-induced increases of dopamine in the caudate putamen and nucleus accumbens of C57BL/6J and 129/J mice. Synapse 2003;50:191–199. [PubMed: 14515336]

Zhang Y, Svenningsson P, Picetti R, Schlussman SD, Nairn AC, Ho A, Greengard P, Kreek MJ. Cocaine self-administration in mice is inversely related to phosphorylation at Thr34 (protein kinase A site) and Ser130 (kinase CK1 site) of DARPP-32. J Neurosci 2006;26:2645–2651. [PubMed: 16525043]

Zhang Y, Wang D, Johnson AD, Papp AC, Sadee W. Allelic expression imbalance of human mu opioid receptor (OPRM1) caused by variant A118G. J Biol Chem 2005;280:32618–32624. [PubMed: 16046395]

Zhou Y, Adomako-Mensah J, Yuferov V, Ho A, Zhang JH, Xu M, Kreek MJ. Effects of acute "binge" cocaine on mRNA levels of μ opioid receptor and neuropeptides in dopamine D1 or D3 receptor knockout mice. Synapse 2007;61:50–59. [PubMed: 17068774]

Zhou Y, Bendor J, Hofmann L, Randesi M, Ho A, Kreek MJ. Mu opioid receptor and orexin/hypocretin mRNA levels in the lateral hypothalamus and striatum are enhanced by morphine withdrawal. J Endocrinol 2006;191:137–145. [PubMed: 17065397]

Zhou Y, Bendor J, Yuferov V, Schlussman SD, Ho A, Kreek MJ. Amygdalar vasopressin mRNA increases in acute cocaine withdrawal: evidence for opioid receptor modulation. Neuroscience 2005;134:391–397.

Zhou Y, Cui CL, Schlussman SD, Choi J, Ho A, Han JS, Kreek MJ. Effect of cocaine place conditioning, chronic escalating-dose “binge” pattern cocaine administration and acute withdrawal on orexin/hypocretin and preprodynorphin gene expressions in lateral hypothalamus of Fischer and Sprague-Dawley rats. Neuroscience 2008a;153:1225–1234. [PubMed: 18436386]

Zhou Y, Leri F, Cummins E, Hoeschele M, Kreek MJ. Involvement of arginine vasopressin and V1b receptor in heroin withdrawal and heroin seeking precipitated by stress and by heroin. Neuropsychopharmacology 2008b;33:226–236. [PubMed: 17443128]

Zhou Y, Spangler R, Ho A, Kreek MJ. Increased CRH mRNA levels in the rat amygdala during acute withdrawal from chronic “binge” cocaine. Mol Brain Res 2003;114:73–79. [PubMed: 12782395]

Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ. Steady-state methadone in rats does not change mRNA levels of corticotropin-releasing factor, its pituitary receptor or pro-opiomelanocortin. Eur J Pharmacol 1996;315:31–35. [PubMed: 8960861]

Zhou Y, Spangler R, Yuferov V, Schlussman SD, Ho A, Kreek MJ. Effects of selective D1- or D2-like dopamine receptor antagonists with acute “binge” pattern cocaine on corticotropin-releasing hormone and proopiomelanocortin mRNA levels in the hypothalamus. Mol Brain Res 2004;130:61–67. [PubMed: 15519677]

Zimprich A, Kraus J, Wöltje M, Mayer P, Rauch E, Höllt V. An allelic variation in the human prodynorphin gene promoter alters stimulus-induced expression. J Neurochem 2000;74:472–477. [PubMed: 10646497]

Zubieta JK, Gorelick DA, Stauffer R, Ravert HT, Dannals RF, Frost JJ. Increased mu opioid receptor binding detected by PET in cocaine-dependent men is associated with cocaine craving. Nat Med 1996;2:1225–1229. [PubMed: 8898749]