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THE MOLECULAR BASIS OF ASSOCIATIVE TOLERANCE TO OPIATES

By

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Abstract

The Pavlovian conditioning analysis of drug tolerance emphasizes that cues present at the time of drug administration become associated with drug-induced disturbances. These disturbances elicit unconditional responses that compensate for the pharmacological perturbation. The drug-compensatory responses eventually come to be elicited by drug-paired cues. These conditional compensatory responses (CCRs) mediate tolerance by counteracting the drug effect when the drug is administered in the presence of cues previously paired with the drug.

Although there are many studies evaluating the molecular mechanisms underlying opiate drug tolerance, there are few experiments examining the role of Pavlovian conditioning in modulating the intracellular mechanisms that are hypothesized to mediate opiate tolerance. The results reported in Chapter 2 suggest that external predrug cues signalling morphine, and not simply chronic administration of morphine, are crucial in inducing morphine tolerance, striatal c-Fos, and AP-1 DNA binding.

Besides external cues, internal cues also are important in mediating opiate tolerance and striatal c-Fos. The yoked-control experiments of Chapter 3 demonstrate that interoceptive cues inherent in self-administration are important for striatal c-Fos expression. Self-administering heroin (SA-H) animals exhibit less ataxia and more striatal c-Fos expression than their yoked partners (Y-H).

The role of c-Fos is to activate other genes. The CCK2 receptor gene may be modulated by c-Fos and has been implicated in associative opiate tolerance. The in situ hybridization experiments reported in Chapter 4 were designed to investigate, in several

brain areas, the RNA encoding the CCK2 receptor in SA-H, Y-H, and yoked-saline (Y-S) rats. Although no differences were found between the groups in the brain areas examined, the cDNA array experiments in Chapter 5 showed that the CCK2 receptor is up-regulated in the nucleus accumbens (NA_{cc}) of the SA-H rat compared to the Y-H rat. Furthermore, several other genes were also differentially expressed between SA-H and Y-H groups. The results of these experiments have helped narrow down those genes that may be involved in the associative opiate tolerance. Further investigation with more sophisticated gene screening techniques (e.g., gene chips) may elucidate the molecular mechanisms underlying the learned aspects of opiate tolerance.

Para meus queridos pais

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Chapter 5

Preface

This thesis includes introductory text that is based, in part, on a published manuscript (Siegel, Baptista, Kim, McDonald, and Weise-Kelly, 2000), experiments based on a published manuscript (Baptista, Siegel, MacQueen and Young, 1998), and a manuscript submitted for publication (Baptista, Weise-Kelly, Siegel, MacQueen and Young, submitted). Due to the multiple authors on these manuscripts, my contribution to each is explained here.

Chapter 1 is based, in part, on material presented in Siegel, Baptista, Kim, McDonald, and Weise-Kelly, 2000. I was a co-author of this review paper and some of the relevant material is presented in this thesis. Chapters 2 and 3 include experiments that are relevant to this thesis.

List of Abbreviations

AOP	anti-opioid peptide
AP-1	activator protein 1
CCK	cholecystokinin
CCK2	cholecystokinin receptor 2
CCR	conditional compensatory response
CR	conditional response
CS	conditional stimuli
CREB	cAMP response element binding
NA_{cc}	nucleus accumbens
PM	paired morphine
PS	paired saline
SAC	self-administration cues
SA-H	self-administering heroin
UCR	unconditional response
UCS	unconditional stimulus
UPM	unpaired morphine
UPS	unpaired saline
VTA	ventral tegmental area
Y-H	yoked-heroin
Y-S	yoked-saline

Chapter 1: Introduction

Early chroniclers of drug effects noted that responsivity to drugs often decreased as a function of experience with the drug. For example, in 1612 Jean Mousin, physician to the King of France, wondered why individuals sometimes became progressively more sober while they were continuing to drink alcoholic beverages. Although the term tolerance was not used until some years later, it appears that Mousin observed the phenomenon now termed acute tolerance – decreased responsiveness to a drug within the course of a single administration (Kalant, 1998).

Acute Tolerance and Withdrawal

Acute tolerance has been investigated extensively with respect to ethanol (e.g., LeBlanc, Kalant, & Gibbins, 1975), as well as other drugs, such as opiates. For example, over the course of a single, long administration of morphine, accomplished by gradual infusion via an implanted morphine pellet, the analgesic effect of the drug decreases (e.g., Tilson, Rech, & Stolman, 1973; Wei & Way, 1975).

The existence of acute tolerance is evidence that pharmacological stimulation initiates adaptive responses that compensate for the primary drug effect (Haefely, 1986; Ramsay & Woods, 1997; Siegel & Allan, 1998). The observed effect of a drug is, therefore, the net result of primary, drug-induced changes and these secondary, compensatory responses. Further evidence for drug-compensatory responses may be seen when the drug effect is abruptly terminated (e.g., by cessation of the delivery of ethanol vapor to the environment, or by removal of a morphine pellet). The compensatory response, now having little to compensate for, may now be seen. Thus,

upon termination of an ethanol effect (and the anticonvulsant effect of the drug), a decrease in seizure threshold is noted (e.g., McQuarrie & Fingl, 1958). Similarly, upon termination of a morphine infusion (and the analgesic effect of the drug), an increased sensitivity to painful stimuli is noted (e.g., Tilson et al., 1973; Wei & Way, 1975). Such compensatory responses, seen following termination of drug administration, are termed acute withdrawal symptoms.

Chronic Tolerance and Withdrawal

Typically, drugs are not administered via constant long infusions. Rather, administration is by means of a brief injection, and the effects are measured following the termination of the injection. It has been known for many years that when such measurements are made following each of a series of drug administrations, the drug effect frequently is noted to become progressively smaller over the course of these administrations. This decreasing effect, seen following each successive administration of a drug, is termed chronic tolerance (for historical reviews of chronic tolerance to ethanol and opiates, see Kalant, 1998, and DuMez, 1919, respectively). The term tolerance, as it is generally used, refers to such chronic tolerance.

Chronic tolerance, like acute tolerance, is mediated by compensatory responding. That is, at some time following a series of drug administrations, if the drug no longer is administered, pharmacological aftereffects may be seen. These withdrawal symptoms, seen after chronic administration, may be termed chronic withdrawal symptoms, but generally are termed simply withdrawal symptoms.

Acute tolerance results from drug-compensatory processes reflexively elicited by a drug (e.g., Haefely, 1986). Chronic tolerance (hereafter termed tolerance) results, at least in part, from drug-compensatory processes elicited not only by the drug effect, but also by cues that, in the past, have been associated with the drug effect. That is, learning contributes to tolerance.

Tolerance and Learning

As early as the 1960s, some investigators proposed that a complete analysis of tolerance requires an appreciation of associative principles. For example, in 1965, Cohen, Keats, Krivoy, and Ungar suggested that "the development of tolerance can be considered a form of learning" (p. 383), because actinomycin D, an inhibitor of protein synthesis, retarded the development of tolerance (much as it retards the acquisition of other learned responses). Results of subsequent research demonstrated that many metabolic inhibitors impede the development of morphine tolerance, as do several other manipulations that retard learning (e.g., electroconvulsive shock or frontal cortical stimulation). Moreover, several pituitary peptides that antagonize metabolic inhibitors, and facilitate learning, also facilitate the acquisition of tolerance (see Siegel, 1983, for a historical summary of research concerning the relationship between learning and tolerance).

In addition, some researchers proposed that learning contributed to tolerance because tolerance often was very well retained. That is, if an organism has acquired tolerance to a drug, this tolerance may be manifest even after a prolonged, drug-free period. For example, tolerance to the analgesic effect of morphine in rats persists over a drug-free

period of months – indeed, perhaps even a year (Cochin & Kornetsky, 1964; Kornetsky & Bain, 1968). Since learned responses typically display very substantial retention (e.g., Kimble, 1961, p. 281), some investigators suggested that tolerance is "a reaction analogous to memory" (Cochin, 1970, p.19).

The contribution of learning to tolerance, and the importance of drug-associated environmental cues to tolerance, is incorporated in an analysis of tolerance that emphasizes Pavlovian conditioning principles.

Pavlovian Conditioning and Tolerance

Pavlov (1927, pp. 35ff) suggested that the administration of a drug could be viewed as a conditioning trial; the drug effect serves as the unconditional stimulus (US), and the immediately antecedent environmental cues served as the conditional stimulus (CS). Some years ago, Siegel (1975) suggested that "conditioned drug responses are commonly opposite in direction to the unconditioned effects of the drug" (Siegel, 1975, p. 499), and these "compensatory" conditional responses (CRs) attenuated the drug effect and mediated tolerance. The pharmacological CR, then, was conceived as being opposite in direction to the pharmacological unconditional response (UR) – at least in instances in which tolerance occurred – a position contrary to Pavlov's view that the CR was similar to the UR.

The conditioning analysis of drug administration subsequently has undergone several important modifications, primarily as a result of critical analyses of pharmacological conditioning by several authors (B. R. Dworkin, 1993; Eikelboom & Stewart, 1982; Poulos & Cappell, 1991; Ramsay & Woods, 1997; Wikler, 1973). It is now apparent that

the initial application of the Pavlovian conditioning paradigm to drug administration was somewhat superficial. The UR to a pharmacological stimulus, in common with reflex responses to other stimuli, consists of responses generated by the central nervous system (CNS). The drug effect that initiates these CNS-mediated responses is the US (not the UR). For many effects of drugs, the UR consists of responses that compensate for drug-induced perturbations. These unconditionally-elicited compensatory responses are responsible for acute tolerance (Ramsay & Woods, 1997). After some pairings of the pre-drug CS and pharmacological US, drug-compensatory responses can be elicited by pre-drug cues. These conditional compensatory responses (CCRs) mediate the development of tolerance by counteracting the drug effect. As noted by B. R. Dworkin (1993), the analysis now closely follows Pavlov's (1927) conceptualization of conditioning – "Conditioned drug responses, when adequately isolated, dissected, and understood, exemplify in an uncomplicated way the phenomenon first described by Pavlov: The conditioned reflex resembles the unconditioned reflex, and as it develops, it augments the effect of the unconditioned reflex" (B. R. Dworkin, 1993, p. 38).

Typically, CCRs are observed by presenting the usual pre-drug cues in the absence of the drug. Perhaps the first demonstration of a CCR was provided by Subkov and Zilov over 60 years ago. They injected dogs with epinephrine (adrenaline) on a number of occasions (one injection every few days), and noted that the tachycardiac effect of the drug decreased over the course of repeated injections (i.e., tolerance developed). On a final test session, they placed the dog in the injection stand and administered an inert substance (Ringer's solution). On this test, a decrease in heart rate was observed: "It

follows that the mere reproduction of the experimental conditions in which the animal is accustomed to receive adrenaline is alone sufficient to set in motion the mechanism, by means of which the animal counteracts the high vascular pressure produced by adrenaline" (Subkov & Zilov, 1937, p. 295).

Subsequently, CCRs have been demonstrated with many drugs (see Siegel, 1991, 1999a), including commonly abused drugs, such as opiates (e.g., Grisel, Wiertelak, Watkins, & Maier, 1994; Krank, Hinson, & Siegel, 1981; Raffa & Porreca, 1986), ethanol (e.g., Larson & Siegel, 1998; Siegel, 1987), and caffeine (Andrews, Blumenthal & Flaten, 1998; Rozin, Reff, Mark, & Schull, 1984).

The original phenomenon implicating CCRs in tolerance has been termed the "situational-specificity of tolerance" (Siegel, 1978, p. 345). After tolerance is established by repeatedly administering the drug in a particular environment, tolerance often is more pronounced in that drug-paired environment than in an alternative environment.

Situational-Specificity of Tolerance

Situational-specificity of tolerance has been demonstrated in experiments that have cues explicitly paired with a drug effect, or that have used opportunistic designs that rely on the subjects' extra-experimental conditioning histories.

Experimental designs. There are several experimental designs that have been used to demonstrate situational-specificity of tolerance (see Siegel, 1983). For example, the paired/unpaired design was used both by Siegel, Hinson, and Krank (1978) to demonstrate the situational-specificity of tolerance, and by Baptista, Siegel, MacQueen, and Young (1998) to evaluate the neurochemical basis of the phenomenon. In these

experiments, rats were assigned to paired or unpaired conditions. For paired rats, pretest morphine injections were signalled by an audiovisual cue. Unpaired rats received their pretest drug injections and cue presentations in an unpaired manner. Following the last pretest injection, analgesia was assessed in the presence of the audiovisual cue. Despite the fact that paired and unpaired rats received the same number of morphine injections, at the same doses, at the same intervals, paired rats were more tolerant to morphine-induced analgesia than were unpaired rats.

Opportunistic designs. An example of an opportunistic design is that used by McCusker and Brown (1990). In their experiment, one group of (human) subjects was given alcohol in a familiar context (beer in a simulated bar, the beer-bar group), and another group was administered the same dose of alcohol in an unusual form and context (alcohol mixed in carbonated water and consumed in an office setting, the alcohol-office group). Subjects in the beer-bar group were less impaired on cognitive and motor tasks than were the subjects in the alcohol-office group. More recently, Remington, Roberts, and Glautier (1997) reported that the same amount of alcohol induced less impairment when college students consumed the alcohol in an alcohol-associated beverage (beer) rather than in a novel liquid (a blue, peppermint-flavored beverage).

Situational-specificity of tolerance to the lethal effects of drugs. The most dramatic demonstrations of the situational-specificity of tolerance concern tolerance to the lethal effects of drugs. Following a series of drug administrations involving escalating doses, each in the context of the same cues, tolerance develops to the potentially lethal effect of that drug as long as it is administered in the usual context. Altering the context of drug

administration increases the lethality of several drugs, including heroin (Siegel, Hinson, Krank, & McCully, 1982), pentobarbital (Vila, 1989), and alcohol (Melchior, 1990; Melchior & Tabakoff, 1982, but see Neumann & Ellis, 1986; Tsibulsky & Amit, 1993). There are clinical reports suggesting that an alteration in pre-drug cues may be responsible for some instances of opiate overdoses experienced by drug addicts (Siegel, 1984), and by patients that receive drugs for pain relief (Siegel & Ellsworth, 1986; Siegel & Kim, in press).

Generality of the situational-specificity of tolerance. Situational-specificity has been demonstrated with respect to tolerance to many effects of a variety of drugs: opiates (reviewed by Siegel, 1991), naloxone (Goodison & Siegel, 1995b), ethanol (e.g., Lê , Poulos, & Cappell, 1979; Seeley, Hawkins, Ramsay, Wilkinson, & Woods, 1996), nicotine (e.g., Cepeda-Benito, Reynosa, & McDaniel, 1998; Epstein, Caggiula, & Stiller, 1989), pentobarbital (e.g., Cappell, Roach, & Poulos, 1981), phencyclidine (Smith, 1991), immunoenhancing drugs (Dyck, Driedger, Nemeth, Osachuk, & Greenberg, 1987), cholecystokinin (Goodison & Siegel, 1995a), carisoprodol (Flaten, Simonsen, Waterloo, & Olsen, 1997), haloperidol (Poulos & Hinson, 1982) and several benzodiazepines (Greeley & Cappell, 1985; King, Bouton, & Musty, 1987; Siegel, 1986b). It has been reported in many species, from snails (Kavaliers & Hirst, 1986) to humans (e.g., Dafters & Anderson, 1982). Situational-specificity typically also is seen with respect to cross-tolerance. Thus, rats tolerant to Drug A in a particular context also display cross-tolerance to Drug B if Drug B is administered in that context, but not if

Drug B is administered in an alternative context (e.g., El-Ghundi, Kalant, Lê, & Khanna, 1989; Goodison & Siegel, 1995b; but see Carter & Tiffany, 1996).

The fact that tolerance displays situational-specificity is consistent with the conditioning analysis of tolerance. That is, drug-associated cues elicit CCRs that attenuate the drug effect, thus tolerance is greater when assessed in the presence of drug-associated cues than when it is assessed elsewhere.

Parallels between Pavlovian Conditioning and Tolerance

If conditioning processes contribute to tolerance, it would be expected that nonpharmacological manipulations of putative CSs (cues present at the time of drug administration), known to affect the course of Pavlovian conditioning, should similarly affect the course of CCR acquisition and thus tolerance. The results of many such manipulations have been assessed. Because these data are extensively reviewed elsewhere (e.g., Goudie, 1990; Ramsay & Woods, 1997; Siegel, 1989, 1991), they are summarized only briefly here.

Extinction of tolerance. The magnitude of established CRs is decreased by extinction, i.e., repeated presentations of the CS without the US, or unpaired presentations of both the CS and US. Similarly, tolerance to both the lethal (Siegel, Hinson & Krank, 1979) and analgesic (e.g., Siegel, Sherman & Mitchell, 1980) effects of morphine is attenuated by repeated presentation of the pre-drug cues. Once tolerance to the behaviorally-sedating effect of morphine is established by repeated presentation of the drug in the presence of distinctive cues, this tolerance is attenuated by subsequent unpaired presentation of these cues and the drug (Faneslow & German, 1982). Extinction

of morphine tolerance is seen with a variety of routes of administration, e.g., subcutaneously (Siegel et al., 1980) and directly into the ventricles of the brain (MacRae & Siegel, 1987). Furthermore, tolerance to a variety of effects of ethanol, amphetamine, midazolam (a short-acting benzodiazepine), and the synthetic polynucleotide, Poly I:C, also can be extinguished (see reviews by Siegel, 1989, 1991).

In addition to the parallels between conditioning and tolerance summarized above, the two phenomena are similar in other respects. Like other conditional responses, drug tolerance displays inhibitory learning (Faneslow & German, 1982; Hinson & Siegel, 1986; Siegel, Hinson, & Krank, 1981), stimulus generalization (e.g., Caggiula et al., 1991), and a flattening of the generalization gradient as a result of extending the interval between acquisition and assessment (Feinberg & Riccio, 1990). Tolerance also displays sensory preconditioning (Dafters, Hetherington, & McCartney, 1983), and a variety of compound conditioning effects, such as overshadowing (e.g., Dafters & Bach, 1985; Walter & Riccio, 1983) and blocking (Dafters et al., 1983).

In summary, there are many demonstrations that, following a series of drug administrations, drug-paired stimuli elicit CCRs. Furthermore, tolerance often is situationally-specific; that is, tolerance is more pronounced when assessed in the presence of drug-paired cues than when assessed in the presence of alternative cues. In addition, there are many parallels between tolerance and other conditional responses: Non-pharmacological manipulations that attenuate conditioning (e.g., latent inhibition and extinction), as well as pharmacological manipulations that facilitate conditioning (e.g., glucose and anticholinergic drugs), similarly modulate the acquisition of tolerance.

Researchers are also starting to discover the conditional neurochemical and molecular-biological events that are elicited by drug-paired cues and mediate the associative contribution to tolerance. In the following Chapters, experiments are conducted in order to determine the role of gene expression in opiate tolerance mediated by Pavlovian conditioning.

Chapter 2: Introduction

Although there is substantial evidence that conditioning contributes to tolerance, there has been little research concerning the physiological events that mediate this contribution. Some researchers have noted conditional metabolic or drug-dispositional changes that may function as CCRs (Melchior & Tabakoff, 1985; Roffman & Lal, 1974). More recent research has concerned conditional pharmacodynamic alterations – what happens in the brain in response to pre-drug cues?

Conditional Neurochemical Alterations

Conditional release of taurine and ethanol tolerance. The amygdala has been implicated in both learning and drug effects (see Quertemont, de Neuville, & De Witte, 1998a). Quertemont et al. (1998a) used microdialysis to evaluate neurochemical changes in the amygdala during repeated ethanol administrations, and during presentation of a distinctive olfactory cue that had been paired with ethanol. They reported that ethanol elicited an increase in extracellular taurine. Taurine is a neuromodulator believed to attenuate ionic and osmotic changes that occur after ethanol administration. Furthermore, taurine decreases the aversive effects of ethanol, as measured by aversion to the side of a two-choice chamber that contains an ethanol-paired odor (Quertemont, Goffaux, Vlaminck, Wolf, & De Witte, 1998b). In rats with a history of ethanol administration in the presence of a vinegar odor CS, administration of saline in the presence of the olfactory CS elicited an increase in taurine microdialysate content. Quertemont et al.

(1998a) suggested that such conditional release of taurine is a CCR in rats presented with a CS for ethanol, and this CCR contributes to tolerance.

Conditional anti-opioid peptide activity. Some evaluations of neurochemical alterations that mediate opiate tolerance have focused on anti-opioid peptides (AOPs). There is evidence that AOPs are released by the central nervous system in response to opiate stimulation, and that they contribute to tolerance by attenuating the effect of the drug (Rothman, 1992). Although several putative AOPs have been proposed, one that has received considerable attention is cholecystokinin (CCK). This will be discussed more fully in chapter four.

Conditional Intracellular Alterations

In addition to studying learned modifications of neurotransmitter activity, researchers interested in the biological bases of associative contributions to tolerance have begun to study the learned modifications in intracellular events that mediate tolerance. The structural changes in the central nervous system that are responsible for learning and drug effects require gene activation. It is plausible that the situational specificity of tolerance seen in a paired/unpaired design could also be mediated by situationally-specific gene transcription.

In the paired/unpaired design, rats are assigned to paired or unpaired conditions. Paired rats receive pretest morphine injections that are signalled by an audiovisual cue. Unpaired rats receive their pretest morphine injections and cue presentations in an

unpaired manner. Despite the fact that paired and unpaired rats receive the same number of morphine injections, at the same doses, and at the same intervals, paired rats are more tolerant to morphine-induced analgesia than unpaired rats in the presence of the audiovisual cue (Siegel et al., 1978; Baptista et al., 1998). Early immediate genes could mediate this phenomenon.

Early immediate genes such as cAMP response element binding (CREB) have been implicated in both morphine tolerance development (Maldonado et al., 1996) and learning (Bourtchuladze et al., 1994; Impey et al., 1996; Kaang, Kandel, & Grant, 1993; Yin, Del Vecchio, Zhou, & Tully, 1995). It has been proposed that CREB activates another early immediate gene, c-fos. This gene encodes a transcription factor, c-Fos, and it has been implicated in memory tasks (Mileusinc, Anokhin, & Rose, 1996), fear conditioning (Milanovic et al., 1998; Radulovic, Kammermeier, & Spiess, 1998), and long-term potentiation (Abraham, Dragunow, & Tate, 1991; Kaczmarek, 1992; Lanahan, Lyford, Stevenson, Worley, & Barnes, 1997).

The c-Fos protein combines with other proteins to form an activator protein 1 complex-AP-1 (Angel et al., 1988; Bohmann et al., 1987). This AP-1 complex binds to and activates genes. There is considerable evidence that c-Fos (especially striatal c-Fos) and the AP-1 complex mediates the action of many common drugs of abuse (Graybiel, Mortalla, & Robertson, 1990; Hope, Kosofsky, Hyman, & Nestler, 1992; Liu, Nickolenko, & Sharp, 1994). Chronic morphine administration is known to cause an up-regulation in the cAMP intracellular pathway, that culminates in c-Fos expression (Collier, 1980; Nye & Nestler, 1996).

Nye and Nestler (1996) reported that chronic morphine induces striatal c-Fos expression and AP-1 DNA binding, but did not evaluate the role of learning in this effect. Using the paired/unpaired design, we demonstrate that striatal c-Fos expression and AP-1 DNA binding only occurs when there is a reliable cue available to the organism signalling the arrival of the drug effect.

Chapter 2

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Pre-drug cues modulate morphine tolerance, striatal c-Fos, and AP-1 DNA binding

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Key Words: c-Fos; AP-1; Morphine tolerance; Striatum; Pavlovian conditioning

Running Title: Striatal c-Fos and morphine tolerance

Abstract

To evaluate the molecular mechanisms that mediate the effect of learning on morphine tolerance in rats, we examined striatal c-Fos, and c-Jun protein expression, and AP-1 DNA binding. Morphine paired with a conditioned stimulus (CS) led to analgesic tolerance in the presence of the CS. Rats receiving morphine unpaired with the CS displayed significantly less tolerance than paired morphine animals. Striatal c-Fos protein levels and AP-1 DNA binding activity were increased in rats receiving paired morphine compared with rats that did not receive morphine but not in rats receiving morphine without the CS. No differences were found in c-Jun levels. These results suggest that Pavlovian conditioning may account, in part, for the molecular mechanisms associated with morphine tolerance.

Key Words: c-Fos, c-Jun, AP-1, Striatum, Morphine tolerance, Pavlovian conditioning.

Introduction

Drug tolerance refers to the decreasing response to a drug over the course of successive administrations. There is considerable evidence indicating that learning contributes to tolerance.[1,2,3] For example, CREB knockout mice and animals receiving injections of MK-801 exhibit both retarded tolerance development and learning deficits.[4,5,6,7] Furthermore, manipulations of drug-paired contextual cues affects the development of tolerance.[8,9] Siegel et al.[10] reported that rats that received morphine paired with a conditioned stimulus (CS) were tolerant to the analgesic effect of the drug when tested in the presence of the CS, whereas those with a history of morphine unpaired with a CS were not (a phenomenon termed "situational specificity of tolerance"). Although many studies have investigated the situational specificity of tolerance to the analgesic effect of morphine, little is known about the molecular mechanisms that contribute to such specificity.

The transcription factor, c-Fos, is sensitive both to non-pharmacological conditioning manipulations and to drug tolerance. In a conditioned emotional response paradigm, c-Fos expression is increased in limbic regions of the rat following exposure to a CS that signals footshock.[11] Furthermore, c-Fos in the paraventricular nucleus of the hypothalamus (PVN) and the locus coeruleus (LC) is sensitive to the situational specificity of ethanol tolerance.[12] It has also been reported that environmental cues regulate c-Fos in the suprachiasmatic nucleus,[13] suggesting that c-Fos may play a tissue specific role in mediating Pavlovian conditioning.

Acute and chronic morphine administration has also consistently been shown to induce c-Fos, Fos-related antigens, and increase AP-1 binding in rat striatum.[14,15] The possible role of c-Fos in the learned component of morphine tolerance was not examined in these earlier studies. In the present study, one group of animals received morphine paired with a CS while another group received morphine in the absence of the CS. If c-Fos and the AP-1 complex regulates the learned aspects of morphine tolerance, it should be induced to a greater extent in rats receiving morphine paired with a CS than in rats receiving the CS and morphine in an unpaired manner.

Materials and Methods

Conditioning and Morphine Tolerance Development: Conditioning was conducted using a modified version of the protocol of Siegel et al.[10] Rats (N=32) were housed continuously in ventilated and non-illuminated chambers. They were injected subcutaneously (s.c.) with morphine or saline once a day for 10 days during the tolerance development phase of the experiment. Paired morphine (PM) group rats were injected with morphine (5 mg/kg on day 1, 10 mg/kg on day 2, and 20 mg/kg for the remaining 8 days) in the presence of the CS. Before a PM animal was injected, the box was opened exposing the rat to the overhead light and the ventilation fan turned off. The overhead light and the absence of the ventilation noise consisted of the CS. Fifteen minutes after exposing an animal to the CS, the rat was injected and remained exposed to the CS for another 30 min. Unpaired morphine (UPM) group rats also were injected with the same doses of morphine as PM rats once a day for 10 days, but the drug was always injected in

the absence of the CS (animals were quickly injected in dim red light and the ventilation fans remained on). UPM rats, however, received equal exposure to the CS as PM rats, but the CS was presented randomly with respect to the injection. Paired saline (PS) and Unpaired saline (UPS) groups experienced the same protocol but did not receive morphine during tolerance development; rather they were injected with physiological saline. Twenty-four hours after the last injection, all rats were presented with the CS (15 min) and tested for analgesia 30 min post-injection. During the 3 daily test sessions, which followed tolerance development, rats received an injection of 20 mg/kg of morphine s.c. and were placed on a hot-plate (54°C) until a paw-lick was observed or 30 seconds elapsed. Paw-lick latency was scored by an observer unaware of the rat's group assignment.

Western Blotting: Additional rats (N=36) were injected s.c. with morphine or saline once a day for 10 days. They were assigned to groups PS, PM, UPS, and UPM, as described previously. Twenty-four hours after the last tolerance development session, all animals were rapidly decapitated in the presence of the CS and their brains were removed. Bilateral 1 mm coronal slices of the striatum were excised from each rat and immediately placed on dry ice. Samples were homogenized in 20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg /ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. Samples were incubated for 30 min at 4°C and centrifuged at 15,000 X g for 25 min. Samples were boiled and subjected to a 10% SDS-polyacrylamide gel

electrophoresis at 100 V for 1 1/2 hours. Western blotting was conducted under blind conditions. A typical gel (10 lanes total) contained 4 independent samples representing the four different conditions. The remaining six lanes contained a molecular marker and a linear range of control samples that were used for all the gels. The gels were transferred electrophoretically onto PVDF membrane, blocked with 5% skim milk and incubated with either antisera which recognize 4-17 residues of human c-Fos (1:2000; Calbiochem), or antisera which recognize c-Jun, JunB, and JunD (1:2000; SantaCruz) overnight. The blots were then incubated in secondary antibody (SantaCruz) for 1 hour and bands were detected using ECL (Amersham). Immunoblotting was quantified by densitometry and a blot that did not show control sample linearity was removed from the analysis.

Electromobility Gel Shift Assays (EMSA): Fifteen rats were sacrificed in the presence of the CS after being subjected to the administration regime previously described. The brains were removed and 1 mm thick bilateral coronal slices were excised in order to isolate striatal nuclei. Samples were homogenized in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethysulfonyl fluoride. Supernatants were discarded and the pellets re-suspended in lysis buffer containing 20 mM HEPES, 1.5 mM $MgCl_2$, 0.4 mM NaCl, 25% glycerol, 0.4 mM EDTA, 0.5 mM DTT, and 1 mM of phenylmethylsulfonyl fluoride. Samples were then centrifuged at 14,000 X g for 20 min at 4°C and the supernatants used for the binding assay. For AP-1 binding assays, 10 μg of protein was added to each lane. The double

stranded AP-1 consensus sequence (5'CGCTTGATGACTCAGCCGGAA3'; SantaCruz) was 5' end-labelled with γ^{32}P(ATP) by T4 kinase. For the supershift lane, c-Fos anti-serum was incubated with the protein for 1 hour prior to the addition of the probe. The specificity of the AP-1 oligo was previously determined in our laboratory.[16] The samples were electrophoresed at 100 V for 1 1/2 hours in a 6% acrylamide gel. Gel shift assays were conducted under blind conditions. The gel was dried and exposed to x-ray film. Densitometry was performed on the AP-1 bands.

Results

Conditioning and Morphine Tolerance Development: Low paw-lick latency values indicate minimal morphine-induced analgesia. As shown in Fig. 1, only the PM group displayed tolerance (latency score of 18 seconds) on the first test session. Rats in the UPM group showed a similar paw-lick latency score as rats in the saline groups (injected with morphine for the first time on this test session). These findings indicate that UPM, PS, and UPS groups displayed no evidence of tolerance to the analgesic effect of morphine on the first test session. There was a significant interaction between the conditions across the three test sessions, [$F(6,21)=5.30$, $p<.01$] and a significant difference comparing the PM group to the control groups ($p<.01$).

Fig 1 here

On the second and third test sessions, the UPM group began to show tolerance to the analgesic effect of morphine (Fig. 1). There was no significant paw-lick mean

difference between PM and UPM groups on both the second and third test session. The PS and UPS groups still showed latency values of 30 seconds. Although UPM rats were not given the opportunity to learn the association between the CS and the primary drug effect, they nonetheless acquired tolerance faster than the saline control rats over the three test sessions.

Western blots: Figure 2A shows the percent change from PS of c-Fos and c-Jun expression, and AP-1 binding in the striatum for PM and UPM conditions. Figure 2B shows representative Western blots (c-Fos and c-Jun) and an EMSA for PS, PM, and UPM groups. As shown in Fig. 2A, c-Fos levels were increased in the PM group compared to the PS condition. A Sign-test confirmed this difference to be significant ($Z=2.65$, $p<.05$). In contrast, there was a significant decrease in c-Fos levels in the UPM condition compared to the PS control ($Z=1.77$, $p<.05$). There were no significant differences between the UPS group, PS group, and UPM group on the first test session (data not shown) nor were there any differences found in striatal c-Jun levels.

Fig 2 here

Electromobility Gel Shift Assay: UPS c-Fos and c-Jun levels were not significantly different from PS (data not shown), the gel shift assay included PS, PM, and UPM rats. As shown in Fig. 2, AP-1 DNA binding in the striatum was significantly increased in the

PM group compared to control ($Z = 2.23$, $p < .05$). However, UPM rats showed no significant difference in AP-1 DNA binding compared to control.

Discussion

PM animals exhibit tolerance to the analgesic effect of morphine, show elevated c-Fos protein expression, and increased AP-1 binding activity in the striatum as compared to PS animals. In contrast, UPM rats do not exhibit tolerance when first tested in the presence of the CS, show decreases in striatal c-Fos protein expression but no change in AP-1 binding activity. This indicates that the induction of c-Fos cannot be attributed solely to the repeated exposure of the animal to morphine. In accordance with acute studies,[15] morphine did not induce the expression of c-Jun levels in the striatum.

It has been suggested that morphine acts on μ receptors in the substantia nigra which are located around dopamine neurons in the ventral tegmental area.[17] These dopamine neurons project to the striatum and the nucleus accumbens. Morphine inhibits GABA release onto the nigral dopamine neurons and thus, disinhibits the cells.[18] The resulting increase in dopamine transmission induces immediate early genes in the striatum. An intriguing possibility is that the conditioning manipulations may have interacted with this brain circuit and prevented the induction of c-Fos in the UPM group. Furthermore, preprodynorphin, which is expressed in striatal neurons and which has an AP-1 site in its promoter region, may be a down-stream target affected by the conditioning trials. Both opiates and cocaine affect striatal preprodynorphin expression[19,20,21] but it remains to be determined whether an AP-1 complex mediates

these changes. Vasoactive intestinal peptide, cholecystokinin, neuropeptide Y, neurotensin, and nerve growth factor are all genes that contain AP-1 sites in their promoter regions. These genes and others may also be involved in the learned aspects of morphine tolerance.[22,23,24]

Although c-Fos levels and AP-1 binding were correlated with the behavioural expression of tolerance on the first test session, they cannot account for the facilitated acquisition of tolerance seen in the UPM group following the three test sessions. This suggests that chronic exposure to morphine alone can facilitate the development of tolerance. It appears that this non-associative component to morphine tolerance may be mediated by something other than c-Fos and the AP-1 complex.

Conclusion

Learning contributes to the expression of morphine tolerance. Such tolerance, mediated by Pavlovian conditioning, is accompanied by changes in c-Fos expression and AP-1 binding in the striatum.

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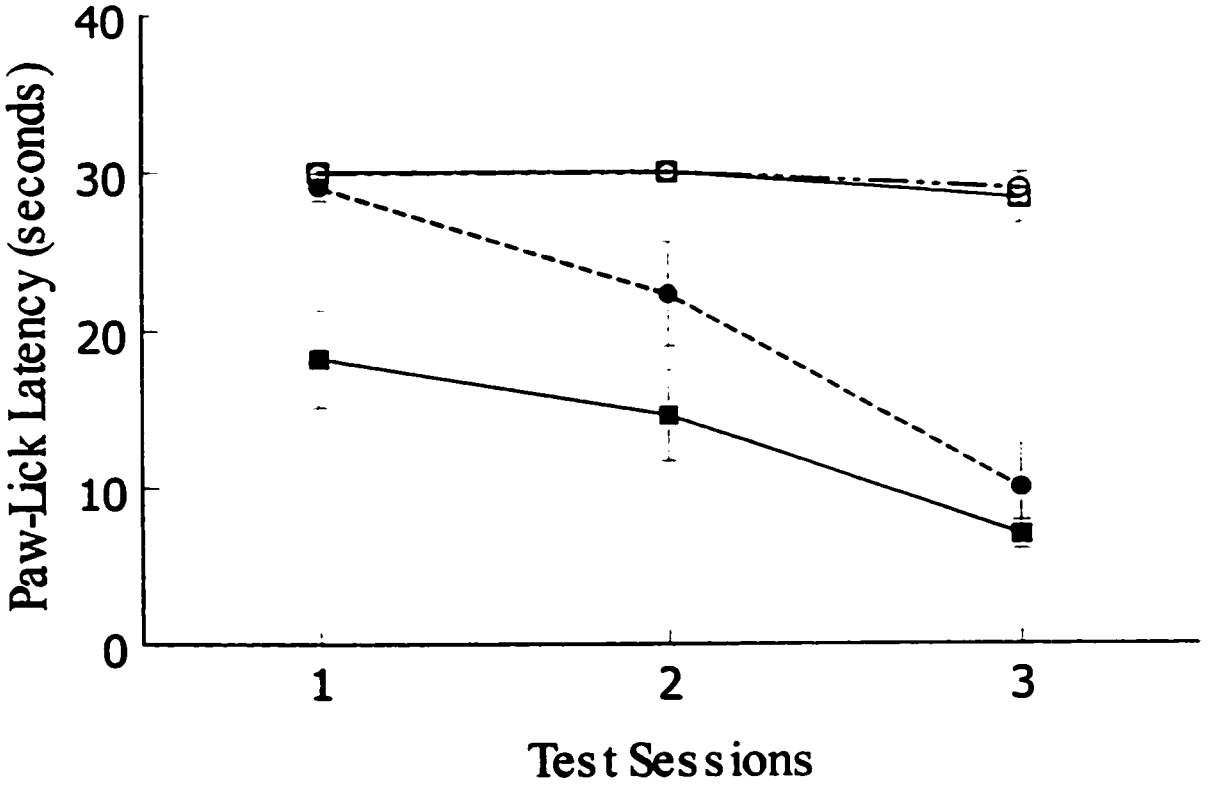
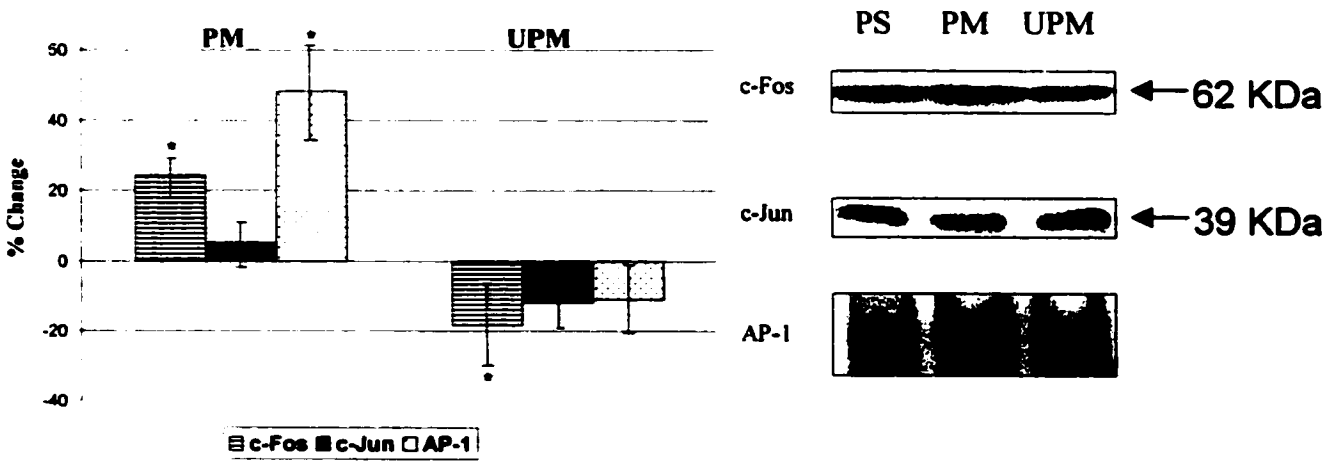
Figure Captions

FIG. 1. Morphine analgesia assessment for PS (□), PM (■), UPS (○), and UPM (●) rats across three test sessions. Low paw-lick latency values (seconds) indicate minimal morphine-induced analgesia. On the first test session, PM rats (n=7) displayed tolerance to the analgesic effect of morphine. However, PS (n=8), UPS (n=10), and UPM (n=7) rats displayed no evidence of tolerance to the analgesic effect of morphine. On the second and third test sessions, the difference between PM and UPM rats was not significant. However, PS and UPS rats still displayed no evidence of tolerance to the analgesic effect of morphine.

FIG. 2. c-Fos, and c-Jun expression and AP-1 binding in the striatum of PS (n=8 for c-Fos, n=9 for c-Jun, and n=5 for AP-1 binding), PM (n=7 for c-Fos, n=9 for c-Jun, and n=5 for AP-1 binding) and UPM (n=8 for c-Fos, n=9 for c-Jun, and n=5 for AP-1 binding). (A) Percent change from PS of c-Fos, and c-Jun levels, and AP-1 binding in the striatum for PM and UPM groups, *$p<0.05$. (B) Representative Western blots (c-Fos and c-Jun) and a representative EMSA (AP-1 binding) for PS, PM, and UPM groups.


40
30
20
10
0
Paw-Lick Latency (seconds)
1
2
3
Test Sessions


PM
UPM
50
40
20
0
-20
-40
% Change
c-Fos c-Jun AP-1
PS PM UPM
c-Fos
62 KDa
c-Jun
39 KDa
AP-1

Chapter 3: Introduction

Results summarized in Chapter 2 indicated that striatal c-Fos levels and AP-1 DNA binding were elevated in the paired morphine group, and not in the unpaired morphine group. These paired morphine rats had a reliable external cue that signalled the drug effect. Although the paired/unpaired design is useful in determining the role of environmental cues in drug tolerance, there is evidence that a variety of other stimuli may become associated with a drug and control the display of tolerance. For example, distinctive flavors (McNally & Westbrook, 1998), ambient temperatures (Kavaliers & Hirst, 1986), or magnetic fields (Kavaliers & Ossenkopp, 1985) may, after being paired with morphine administration, influence the display of morphine tolerance. One type of cue that has been studied in our laboratory is the cue incidental to self-administration.

Typically, humans self-administer the drugs that they use. Such self-administration is a characteristic of both illicit (e.g., cocaine and heroin) and licit (e.g., nicotine and ethanol) drug use. Although some psychopharmacology researchers investigate effects of drugs that are self-administered (especially the rewarding effects), most researchers administer the drug to subjects. Thus, much of what we know about the effects of drugs, such as the development of drug tolerance, is based on results of studies in which the experimenter – not the subject – administered the drug. However, there are findings indicating that the self-administration contingency modulates the acquisition and/or expression of tolerance; organisms that self-administer a drug generally are more tolerant than organisms that passively receive the drug. In the first demonstration that the self-administration contingency modulates tolerance, Mello & Mendelson (1970) allowed

alcoholic men to ingest alcohol in each of two conditions: When they wished (spontaneous condition), or only during experimenter-determined intervals (programmed condition). Tolerance was greater in the same individuals following the spontaneous condition than following the programmed condition. More recently, Ehrman, Ternes, O'Brien, and McLellan (1992) evaluated the effects of 4 mg hydromorphone in detoxified opiate abusers under two conditions: When they intravenously self-administered the drug, and when the drug was infused by the experimenter. Ehrman et al. (1992) reported that several effects of hydromorphone were greater when the drug was passively-received than when it was self-administered, and concluded that "tolerance was observed when the subjects injected the opiate, but not when the same dose was received by unsignaled intravenous infusion" (p. 218).

An especially elegant procedure for evaluating the role of self-administration in drug effects is the yoked-control design. With this design, each time a subject assigned to a self-administration (SA) group makes a particular response (e.g., presses a lever in an operant chamber), the same amount of drug is administered to that subject and to another, yoked (Y) subject. Thus, both SA and Y subjects receive the same dose of the drug, equally often, and at the same intervals. Several investigators have reported that, after some drug experience, the effects of the drug are greater in Y than in SA animals (i.e., tolerance is less pronounced in Y animals). Thus, SA rats are more tolerant than are Y rats to the effect of intravenous nicotine on adrenal hormones (Donny, Caggiula, Knopf, & Brown, 19995), to the effect of intravenous cocaine on mortality (S.I. Dworkin, Mirkis

& Smith, 1995; S.M. Dworkin, Volkmer & S.I. Dworkin, 1988), and to the effect of oral ethanol on ataxia (Weise-Kelly & Siegel, 2001).

Recently, the effect of the self-administration contingency on the ataxic effect of heroin was evaluated (Weise-Kelly & Siegel, 2001). The authors found that heroin has a different behavioural effect in self-administering (SA-H) rats than in Yoked (Y-H) rats. SA-H rats did not initially demonstrate ataxia during the self-administration sessions. In fact, SA-H rats developed heroin-induced enhancement in maintaining their balance on a tilting plane over sessions. This enhanced ability to maintain balance on a tilted plane has been termed *hypertaxia* (Weise-Kelly & Siegel, 2001). In contrast, Y-H rats were initially ataxic but developed tolerance after repeated trials. In the following experiment, we used the yoked-control design to examine whether striatal c-Fos levels in animals that have self-administered heroin were different compared to their yoked partners.

Although the nucleus accumbens has been implicated in heroin self-administration (Vaccarino et al., 1985; Zito et al., 1985), there is evidence that c-Fos expression is not chronically regulated in this brain area (Nye & Nestler, 1996). We decided to investigate the striatum again to see if interoceptive cues, like environmental cues, also modulate c-Fos levels. Furthermore, heroin was chosen as the drug reinforcer instead of morphine for several reasons. Firstly, heroin is routinely used in opioid self-administration experiments. Secondly, the dopamine mesolimbic pathway, the same brain circuit involved in morphine tolerance, is believed to be responsible for maintaining self-administration. Finally, it has already been demonstrated by Weise-Kelly and Siegel (2001) that heroin has a different ataxic effect on SA-H rats as compared to Y-H rats.

After the final session, animals from the Weise-Kelly & Siegel (2001) experiment were sacrificed. The striatums were dissected, homogenized, and the protein extracted. Western blotting was conducted on the samples and analyzed by densitometry. It was found that the self-administering animals exhibited higher striatal c-Fos levels than their yoked partners. A discussion of the possible molecular mechanism underlying this effect follows.

Chapter 3

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Heroin Self-Administering and Yoked Rats Exhibit Differential Striatal c-Fos Expression.

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Running Title: Striatal c-Fos and Heroin Self-administration

Abstract

The yoked-control design was used to evaluate the effects of the heroin self-administration contingency on striatal c-Fos and c-Jun expression. Previously, it has been shown that self-administering rats were more tolerant to the ataxic effects of heroin as compared to their yoked partners, even though both self-administering and yoked rats received the same dose of the drug, equally often, and at the same intervals. In the present study, we show that these same self-administering rats exhibit higher striatal c-Fos levels than their yoked partners. There were no changes in striatal c-Jun expression between the groups. This suggests that there may be some interoceptive cue inherent in self-administration that contributes to the behavioral and neurochemical differences observed between self-administering rats and rats that passively receive the drug.

Introduction

Results of many experiments indicate that drug tolerance is modulated by drug-associated cues present at the time of tolerance testing. The contribution of such cues has been incorporated in a Pavlovian conditioning analysis of tolerance. Using the usual conditioning terminology, cues accompanying the primary drug effect function as conditional stimuli (CSs). The direct effect of the drug constitutes the unconditional stimulus (UCS). Prior to any learning, this pharmacological stimulation unconditionally elicits responses (UCRs) that compensate for the drug-induced disturbances. After some pairings of the pre-drug CS and pharmacological UCS, drug-compensatory responses are elicited as conditional responses (CRs). Such CRs that mimic the compensatory response unconditionally elicited by a drug have been termed compensatory conditional responses (CCRs) (Dworkin 1993; Kim et al. 1999). These CCRs attenuate the effect of the drug and contribute to tolerance.

The original phenomenon implicating CCRs in tolerance has been termed the "situational-specificity of tolerance" (Siegel 1978). After tolerance is established by repeatedly administering the drug in a particular environment, tolerance often is more pronounced in that drug-paired environment than in an alternative environment. For example, Siegel et al. (1978) demonstrated the situational-specificity of tolerance using the paired/unpaired design. In these experiments, rats were assigned to same-tested or different-tested conditions. For same-tested rats, pretest morphine injections were signalled by an audiovisual cue. Different-tested rats received their pretest drug injections and cue presentations in an unpaired manner. Following the last pretest

injection, analgesia was assessed in the presence of the audiovisual cue. Despite the fact that same- and different- tested rats received the same number of morphine injections, at the same doses, and at the same intervals, same-tested rats were more tolerant to morphine-induced analgesia than different-tested rats. More recently, Baptista et al. (1998) replicated these Siegel et al. (1978) findings, and further evaluated striatal c-Fos levels and AP-1 binding in paired in unpaired rats. They found that rats not only showed behavioral evidence of situational-specificity of tolerance (i.e., paired morphine rats were more tolerant to the analgesic effect of morphine than unpaired morphine rats), but also demonstrated situational-specificity of c-Fos expression and AP-1 binding (i.e., paired morphine rats displayed higher striatal c-Fos levels and AP-1 binding than unpaired morphine rats). These findings have also been confirmed in cortical and other limbic areas using immunocytochemistry (Schroder et al. 2000).

Evaluations of situational-specificity of tolerance typically have manipulated exteroceptive cues (e.g., audio-visual, environmental stimuli). There is evidence, however, that a variety of stimuli may become associated with a drug and control the display of tolerance. In addition to drug-paired exteroceptive stimuli, there also are interoceptive stimuli that are paired with a drug effect and thus may elicit CCRs that mediate tolerance. For example, cues incidental to self-administration modulate the expression of tolerance. If drug delivery is contingent on a response, interoceptive response-initiating (or response-produced) cues are paired with the drug effect. Such internal, self-administration cues (SACs), like internal intra-administration cues, become associated with the drug effect and mediate tolerance. Behavioral evidence that SACs

modulate tolerance is provided by the results of experiments indicating that tolerance is more pronounced in organisms that self-administer a drug than in organisms that passively receive the drug (Dworkin et al. 1995; Ehrman et al. 1992; Johanson and Schuster 1981; Mello and Mendelson 1970).

An especially elegant procedure for evaluating the role of self-administration in drug effects is the yoked-control design. With this design, each time a subject assigned to a self-administration (SA) group makes a designated response, the same amount of drug is administered to that subject and to another, yoked (Y) subject. Thus, both SA and Y subjects receive the same dose of the drug, equally often, and at the same intervals. Using this procedure, Weise-Kelly and Siegel (2001) recently reported that heroin-induced ataxia was more pronounced in rats that intravenously self-administered the drug than in rats yoked to these self-administrators. The purpose of this experiment was to evaluate differences in c-Fos expression between SA heroin and Y heroin rats.

Materials and Methods

Heroin Self-Administration

Long-Evans rats from the Weise-Kelly and Siegel (2001) study were used in the present study. These animals had been surgically implanted with chronic catheters in the right jugular vein under ketamine and xylazine anaesthesia. After 1 week of recovery, animals were randomly placed in one of three conditions: Heroin self-administration (SA-H), yoked-heroin (Y-H), or yoked-saline (Y-S). A lever press by an SA-H rat resulted in a 3-sec infusion of a .1 mg/ml heroin solution (i.e., .0105 mg of heroin in .105

ml of solution) at a rate of .035 ml/sec, for itself and the Y-H animal. The Y-S rat received an infusion of .105 ml of saline. Levers were non-functional in the Y-H and Y-S chambers. Ataxia was assessed in all animals with a tilting plane following each self-administration trial.

Western Blotting

Immediately after the last ataxia measure, all animals (N=27) were rapidly decapitated and their brains were removed. One millimetre thick bilateral coronal slices of the striatum were excised from each rat and immediately placed on dry ice. Samples were homogenized in 20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg /ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. Samples were incubated for 30 min at 4°C and centrifuged at 15,000 X g for 25 min. Samples were boiled and subjected to a 10% SDS-polyacrylamide gel electrophoresis at 100 V for 1 1/2 hours. Western blotting was conducted under blind conditions. The gels were transferred electrophoretically onto PVDF membrane, blocked with 5% skim milk and incubated with either antisera which recognizes 4-17 residues of human c-Fos (1:2000; Calbiochem), or antisera which recognizes c-Jun, JunB, and JunD (1:2000; SantaCruz) overnight. The blots were then incubated in secondary antibody (SantaCruz) for 1 hour and bands were detected using ECL (Amersham). Immunoblotting was quantified by densitometry.

Results

Western Blots

Figure 1 shows representative Western blots (c-Fos and c-Jun) for Y-S, SA-H, and Y-H groups and the percent change from Y-S of c-Fos and c-Jun expression in the striatum for SA-H and Y-H groups. As shown in Figure 1, striatal c-Fos levels were lower in the Y-H condition as compared to the SA-H group. A Wilcoxon Signed Ranks test confirmed this difference to be significant ($T=6$, $p<.05$). Striatal c-Fos levels were not significantly different between the SA-H group and Y-S group. However, Y-H rats had lower striatal c-Fos levels than Y-S rats, $T=6$, $p<.05$. There was no significant difference in striatal c-Jun levels between SA-H and Y-H groups, SA-H and S-Y groups, and H-Y and S-Y groups ($T=6$, n.s., $T=4$, n.s., and $T=3$, n.s., respectively).

Fig 1 here

Discussion

Weise-Kelly and Siegel (2001) found that SA-H rats did not display ataxia over the course of the self-administration sessions. In fact, these animals exhibited an enhanced ability to maintain their balance on a tilting plane over sessions. In contrast, both Y-H and Y-S rats were ataxic during the initial sessions but developed tolerance after repeated administrations. Striatal c-Fos expression was also different between the groups. Y-H rats exhibited lower striatal c-Fos levels than SA-H rats.

Studies indicate that the striatum is an important brain area mediating the effects of heroin. Striatal dynorphin A, dynorphin B, [Met5]-enkephalin and substance P are increased following a drug expecting state in animals that self-administered heroin (Cappendijik et al. 1999), while animals that have just self-administered heroin show decreases in striatal beta-endorphin (Sweep et al. 1988). Given that Baptista et al. (1998) found that passively received chronic morphine induces striatal c-Fos expression in rats that receive a reliable external pre-drug signal, and that both morphine and heroin act on μ opiate receptors, we predicted that SA-H rats would show higher striatal c-Fos levels than Y-S rats. In fact, Pontieri et al. (1997) found that c-fos mRNA expression in the striatum of heroin drug sensitized rats was increased after a heroin challenge. This suggests that chronic heroin administration leads to elevated levels of the c-Fos protein. Although the SA-H group exhibited higher striatal c-Fos levels than the Y-H group, SA-H rats' striatal c-Fos levels were not any higher than control. It is plausible that chronic self-administration of heroin does not produce the same behavioral and neurochemical changes that are seen in a sensitization (e.g., Pontieri et al. 1997) or a classical conditioning experiment (e.g., Baptista et al. 1998).

The induction of c-Fos in the striatum requires the concurrent activation of both D_1 and NMDA receptors (Sharp et al. 1995). The former are located postsynaptically on GABAergic neurons in the striatum (Di Chiara and North 1992) while NMDA receptors are located on presynaptic terminals or dopaminergic cell bodies (Martinez-Fong et al. 1992; Wang 1991) that originate from the substantia nigra and terminate in the striatum. It has been hypothesized that the opiate binds to μ opiate receptors on GABAergic

neurons in the substantia nigra (Di Chiara and North 1992). This in turn releases the GABAergic inhibitory control over the dopaminergic cells in the substantia nigra and allows for an efflux of dopamine into the striatum. The induction of c-Fos will occur in GABAergic neurons of the striatum if dopamine binds to D_1 receptors while NMDA receptors are activated. It is plausible that since the Y-H rat is not provided with an interoceptive cue signalling heroin, there is no concurrent activation of both D_1 and NMDA receptors. NMDA receptors have been implicated in memory and may act as coincident detectors (Swartzwelder et al. 1989). A reliable interoceptive cue may produce a release of cortical glutamate that will bind to NMDA receptors in anticipation of the drug effect. This may explain why rats in the SA-H group have an enhanced ability to maintain their balance compared to their yoked partners.

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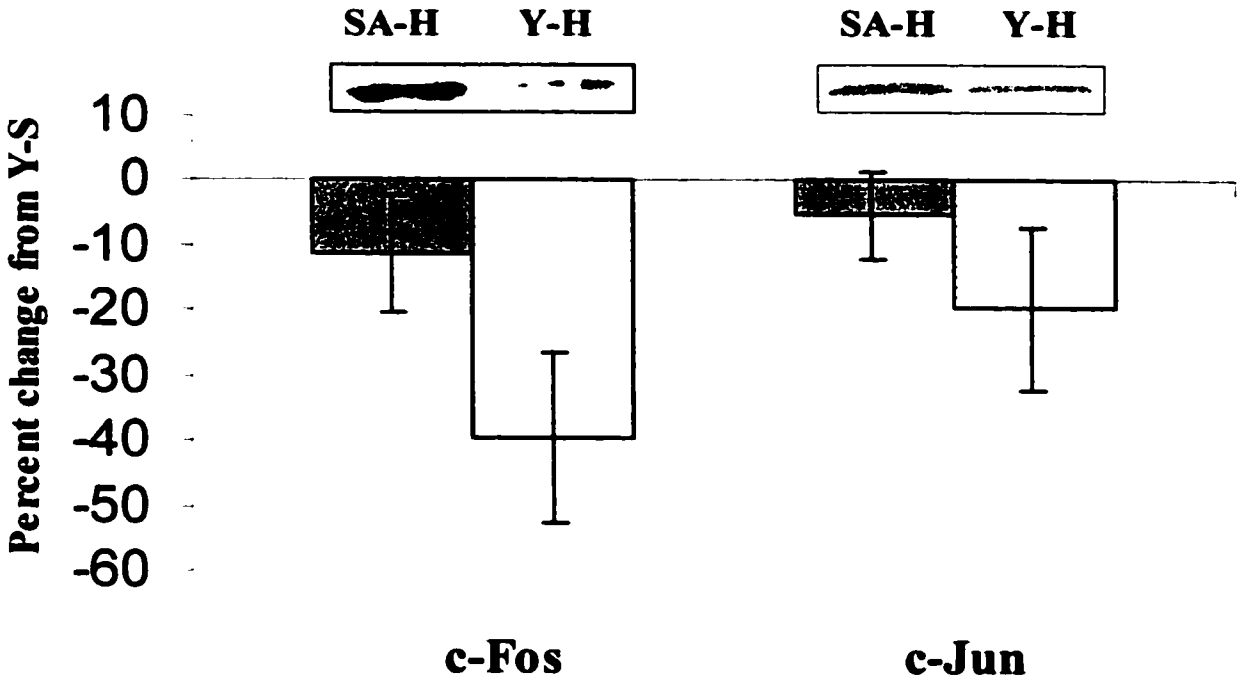
Figure Captions

FIG. 1. Percent change from Y-S of c-Fos, and c-Jun levels in the striatum for SA-H and Y-H groups. Representative Western blots (c-Fos and c-Jun) for SA-H and Y-H groups are shown above the bar graphs.


SA-H
Y-H
SA-H
Y-H
Percent change from Y-S
10
0
-10
-20
-30
-40
-50
-60
c-Fos
c-Jun

Chapter 4

CCK2 Receptor mRNA Analysis in Brains of Heroin Self-Administering, Yoked-Heroin, and Yoked Saline Rats.

Chapter 4: Introduction

In chapter 3 we demonstrated that self-administration cues signalling heroin modulate c-Fos levels in the striatum. The role of any immediate early gene is to initiate the activation of other genes. It has been proposed that c-Fos could be activating several genes, such as vasoactive intestinal peptide, neuropeptide Y, neurotensin, nerve growth factor, and cholecystokinin (CCK), by binding to their AP-1 sites in their promoter regions (Sharp, Liu, Nickolenko, & Bontempi, 1995).

The CCK gene is a likely down-stream target of c-Fos that encodes an anti-opioid peptide. It has been implicated in opiate tolerance (Han, 1995; Mitchell, Lowe, & Fields, 1998) and the conditional compensatory responding that mediates associative opiate tolerance (Kim et al., 1999; Siegel et al., 1999). Much of the work done on opiate tolerance and the CCK system has focused on manipulating the CCK2 receptor (previously named the CCK_B receptor, Wiesenfeld-Hallin et al., 1999). It is plausible that c-Fos could indirectly regulate the CCK2 gene by increasing the availability of CCK. Elevated levels of endogenous CCK and administrations of exogenous CCK, act as an effective anti-opioid peptide. For example, if CCK is administered exogenously, it blocks morphine-induced analgesia in a dose-dependent manner (Han, 1995; Mitchell et al., 1998). Conversely, blocking CCK receptors potentiates morphine analgesia in rats (e.g., Zhou, Sun, Zhang, & Han, 1992) and humans (e.g., McCleane, 1998). Moreover, morphine administration accelerates the release of CCK from the central nervous system in a dose-dependent manner (Zhou et al., 1992). Treatment with CCK receptor

antagonists has been shown to prevent the development of morphine tolerance (e.g., Kellstein & Mayer, 1991) and to attenuate the expression of established morphine tolerance (e.g., Hoffmann & Weisenfeld-Hallin, 1994; Kim & Siegel, in press).

Recently, it also has been shown that an intrathecal administration of PD135,158, a CCK2 receptor antagonist, effectively blocked the expression of a hyperalgesic conditional compensatory response elicited by a cue that signaled morphine (Kim & Siegel, in press). These alterations in CCK activity may provide a mechanism for the conditional compensatory responding that mediates the behavioral expression of tolerance.

Given the involvement of the CCK2 receptor in both morphine tolerance and the CCRs that mediate associative tolerance to opiates, we decided to investigate the role of CCK2 receptors in mediating the ataxic effects seen in the Weise-Kelly and Siegel (2001) yoked-heroin control experiments. In chapter 3, we demonstrated that interoceptive cues signalling heroin modulate c-Fos levels in the striatum. This transcription factor could be activating the CCK gene since it contains an AP-1 binding site. The increased CCK activity could indirectly regulate the CCK2 receptor.

In the present experiment, we used in situ hybridization to systematically investigate the distribution of CCK2 receptor mRNA in brains of SA-H, Y-H, and Y-S rats. Those brain areas that have been traditionally involved in heroin self-administration (nucleus accumbens, striatum and cortex) and those brain areas shown to stain highly for CCK2 mRNA (olfactory tubercule and hippocampus) were analysed.

Recently, the hippocampus has been implicated in heroin addiction. Self-reports of feeling "high" have been correlated with increased regional blood flow in this brain area (Sell et al., 2000). The authors suggest that this area is important in learning the relationship between pre-drug cues and internal states associated with the effects of heroin.

Methods

Subjects and Surgical Preparation

Long-Evans rats (N=18) were surgically implanted with chronic catheters in the right jugular vein under ketamine and xylazine anaesthesia. The tip of the catheter was made of polythylene tubing and inserted approximately 1 cm from the heart. The catheter was anchored to the vein and passed subcutaneously to the back of the rat. The catheter exited the back of the rat through a lead made of plastic and nylon mesh. Heparin and ampicillin in physiological saline were flushed through the catheter and sealed with a cap made of silastic tubing. The patency of the catheters was regularly checked with heparinized saline.

Ataxia Assessment

Ataxia was measured with a tilting plane following each self-administration session. The tilting plane consists of an alley that is hinged at one end. The rat was placed in the alley, and the free end was gradually elevated. The angle of inclination at which the rat started to slip was noted. Greater levels of ataxia result in slippage at smaller angles of inclination. Heroin-induced ataxia was compared with baseline levels, and an

Prior to every session, a pre-administration slip angle was obtained by placing the animal on a tilting plane. After every 45 min session (once a day), a post-administration slip angle was recorded. A measure of impairment was computed by taking the difference, in degrees, between the post-administration slip angle and the subjects' pre-administration slip angle for that session. All rats began the experiment on a continuous reinforcement (CRF) schedule, receiving one drug infusion when an SA-H rat pressed a lever. Starting on the fourth session, each SA-H rat had the opportunity, depending on its response pattern during the previous session, to move to a fixed ratio-3 schedule (FR:3). If this response pattern continued, the SA-H rat could then move to a fixed ratio-6 schedule (FR:6). In order to move from one schedule to the next, an SA-H rat had to self-administer a heroin infusion during the first 5 min of the previous session. If an SA-H rat failed to self-administer a drug infusion during one entire session, it was moved to the previous schedule on the next session. The experiment was complete for each triad when the SA-H animal had successfully bar pressed for 8 sessions.

Tissue Preparation

Immediately after the last ataxia measure, animals were perfused with phosphate buffer and the brains were placed in ice cold 2-Methylbutane. Twenty micron coronal sections were taken at the level of the cerebral cortex, nucleus accumbens, striatum, olfactory tubercule, and the hippocampus (CA1, CA3, and dentate gyrus). Each slide contained one coronal section from a SA-H, Y-H, and Y-S animal. Five slides were

taken for each triad for a given brain area (20 microns apart), in order to account for interslide staining variability.

Oligonucleotide Labelling with DIG-dUTP and Yield Determination

The CCK2 receptor oligonucleotide (5' GTC ATC TCC AGT CGG GAA CGC GGA AGT TGC ACA CAG CAG C 3') was dissolved in sterile H_2O and added to a 1 mM solution of DIG-dUTP (4 µl of 5X reaction buffer, 4 µl of $CoCl_2$, 1 µl of DIG-11-dUTP, 1 µl of dATP, and 1 µl of Terminal Transerase to make a final reaction volume of 20 µl). The reaction components were mixed, centrifuged, and incubated at 37°C for 15 min. After the incubation period, the reaction was stopped with 2 µl of a glycogen-EDTA mixture (200 µl of 0.2M EDTA (pH 8.0) with 1 µl of 20mg/ml glycogen solution). The labelled oligonucleotide was precipitated overnight at -80°C by adding 2.5 µl of 4M LiCl and 75 µl of prechilled 100% ethanol. The following day, the solution was centrifuged (13000g) for 15 min at 4°C, the supernatant discarded and the pellet washed with ice-cold 70% ethanol. The solution was again centrifuged (13000g) for 5 min at 4°C, the supernatant discarded, the pellet dried, and re-dissolved in DEPC treated H_2O.

The yield of the labelled probe was determined by comparing a serial dilution of a control oligonucleotide with a serial dilution of the CCK2 receptor probe. The oligonucleotides were blotted on a nylon membrane and the spot intensities were compared to the control. The CCK2 receptor probe concentration was estimated at 2.5 pmol/µl.

In Situ Hybridization

The sections were allowed to dry for 1 hour, fixed with 4% paraformaldehyde for 30 minutes, washed with DEPC treated phosphate buffered saline and treated with DEPC treated 100mM glycine and 0.3% Triton X-100. The sections were then permeabilized for 25 minutes at 37°C with TE buffer containing 1 μg/ml RNase-free Proteinase K and acetylated with 0.1M Triethanolamine buffer (pH 8.0) containing 0.25% (v/v) glacial acetic acid. Prehybridization buffer was then poured over every section and allowed to incubate for 2 hours. After prehybridization, the sections were washed with ethanol and incubated with the CCK2 receptor probe overnight. The following day, the slides were prepared for immunological detection. When colour development was optimal, the reaction was stopped by incubating the slides in TE buffer for 10 min. All slides were cover slipped and densitometry was performed on the sections.

Analysis

The results of each Triad were composed of an analysis of five slides. For each section on the slide, an average density count was taken of ten samples where labelled cells were detected (signal) and an average density count was computed in the same area for ten samples where there were no labelled cells (background). The difference between signal and background comprised the percent change from background (100).

Results

Heroin Administered

Weight differences among members of a triad could have contributed to differences in doses delivered. A heavier animal would receive a lower dose of heroin than a lighter animal. An attempt was made to match the animals in each triad by body weight. Figure 1 shows that the mean doses of heroin (+ 1 SEM) delivered to subjects in the SA-H and Y-H groups during tolerance development were similar in the two groups. A repeated-measures ANOVA analyzing the mean heroin dose of SA-H and Y-H groups over sessions reveal no statistical significance ($F<1$). Therefore, any differences between the groups cannot be attributed to the doses of heroin administered.

Insert Figure 1 about here

Ataxia

The mean impairment scores (+ 1 SEM) for the SA-H, Y-H, and Y-S groups during tolerance development are shown in Figure 2. A group X session repeated measures ANOVA of these data indicated that there was a group effect [$F(2,7)=12.64$, $p<.001$]. A Tukey HSD post hoc analysis indicated that over the eight sessions, the Y-H group ($p<.001$) and Y-S group ($p<.01$) demonstrated impairment scores that were significantly different than those of the SA-H group. However, the Y-H group ($p>.05$) and the Y-S group ($p>.05$) were not significantly different from each other.

As shown in Figure 2, SA-H rats displayed no ataxia in the first session. However, over the sessions, SA-H rats improved their ability to maintain their balance on the tilting plane. This hypertaxia remained over the eight sessions. In contrast, the Y-H rats showed ataxia in the initial sessions and were consistently more impaired than their SA-H counterparts over the trials. Y-S rats also displayed some hypertaxia but not as much as the SA-H group.

Insert Figure 2 about here

In Situ Hybridization

The signal for CCK2 receptor mRNA could not be detected in the striatum. In all other brain areas examined, there were no significant differences in CCK2 receptor mRNA between the groups (Figures 3-8).

Insert Figures 3-8 about here

Discussion

It was hypothesised that there would be differences in CCK2 receptor mRNA levels between the groups in different brain areas. There is much evidence that CCK2 receptors are important in opiate tolerance (Hoffmann & Weisenfeld-Hallin, 1994; Kellstein &

Mayer, 1991) and compensatory conditional responses (Kim et al., 1999; Siegel et al., 1999). However, no differences were found in any of the brain regions analysed. Although the rats in the SA-H group was more tolerant to the ataxic effects of heroin than the rats in the Y-H group, we could not provide evidence that these behavioural differences were due to alterations in CCK2 receptor gene transcription. There are several possible reasons why differences in CCK2 receptor mRNA were not detected.

First, it is plausible that the heroin dose used in this experiment was not sufficient to induce intracellular changes. This dose, however, elicited even larger differences in ataxia between the SA-H and Y-H groups than the Weise-Kelly & Siegel (2000) study. This could be due to the fact that the mean heroin dose administered over the eight sessions in this experiment (0.16mg/kg) was slightly larger than the mean heroin dose administered in the Weise-Kelly & Siegel (2000) study (0.10mg/kg). Therefore, it appears that dosage is not a factor that contributed to the failure to detect CCK2 receptor RNA differences between the groups.

Another possibility is that differences in CCK2 receptor mRNA transcription may have occurred during tolerance development and but were no longer evident after the last session. It is plausible that the behavioural differences might still be evident days after gene expression occurred. If this is the case, then one would expect to see changes in both CCK2 receptor mRNA levels and the product of this gene expression, CCK2 receptors, during tolerance development but only see the latter altered after the last behavioural test. A time course study of both mRNA levels and protein levels would clarify this issue.

Furthermore, failure to detect CCK2 receptor mRNA alterations could be due to the fact that the brain areas examined are not the regions where the CCK2 gene is induced during self-administration. In future studies, other brain areas such as the cerebellum could be examined. However, there is controversy over this brain area since some researchers are unable to detect a CCK2 receptor mRNA signal in the cerebellum (Pelaprat et al., 1987) while others report a positive signal (Honda, Wada , Battey, & Wank, 1993; Jagerschmidt, Popovici, O'Donohue, & Roques, 1994).

In chapter 5, we decided to concentrate more closely on the NA_{cc} since this brain region is implicated in heroin self-administration (Vaccarino, Bloom, & Koob, 1985; Zito, Vickers, & Roberts, 1985). Using technology that was recently made available, we decided to gene screen the NA_{cc} of SA-H and Y-H rats in order to narrow down those genes that are important in mediating the effect of the self-administration contingency.

Figure Captions

Figure1. Mean dose of heroin administered (± 1 SEM) (mg/kg) to SA-H and Y-H rats over 8 sessions.

Figure2. Mean impairment scores for (± 1 SEM) SA-H, Y-H, and Y-S rats over 8 sessions.

Figure3. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the NA_{cc} in SA-H, Y-H, and Y-S rats.

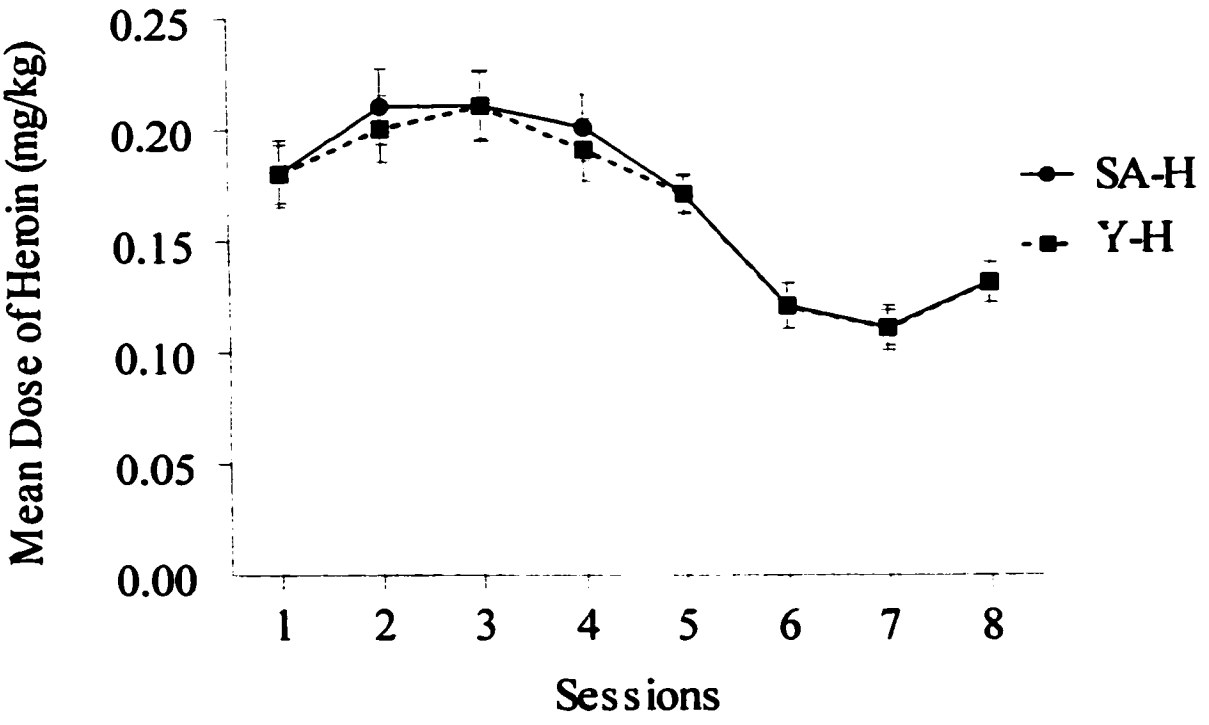
Figure4. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the frontal cortex in SA-H, Y-H, and Y-S rats.

Figure5. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the CA1 in SA-H, Y-H, and Y-S rats.

Figure6. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the CA3 in SA-H, Y-H, and Y-S rats.

Figure7. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the dentate gyrus in SA-H, Y-H, and Y-S rats.

Figure8. Mean CCK2 receptor RNA density (± 1 SEM) (change from background) in the olfactory tubercule in SA-H, Y-H, and Y-S rats.


Mean Dose of Heroin (mg/kg)
0.25
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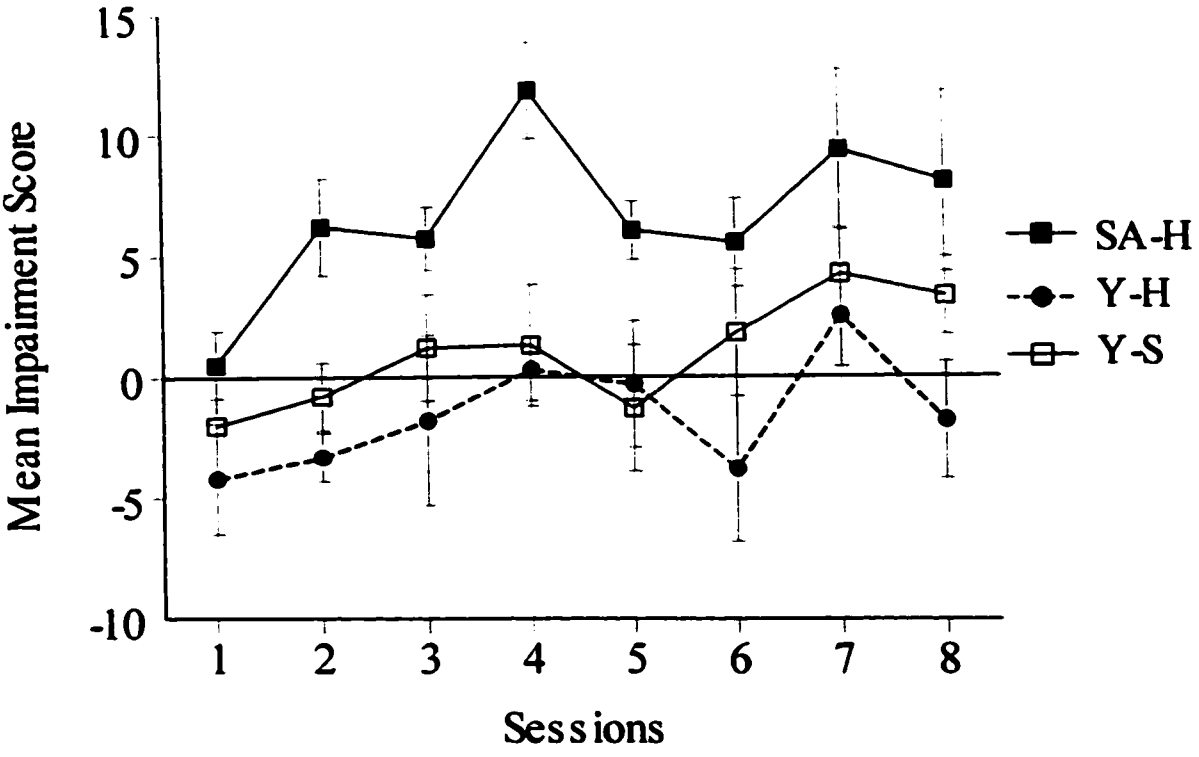
4
5
6
7
8
Sessions
SA-H
Y-H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Mean Impaiment Score
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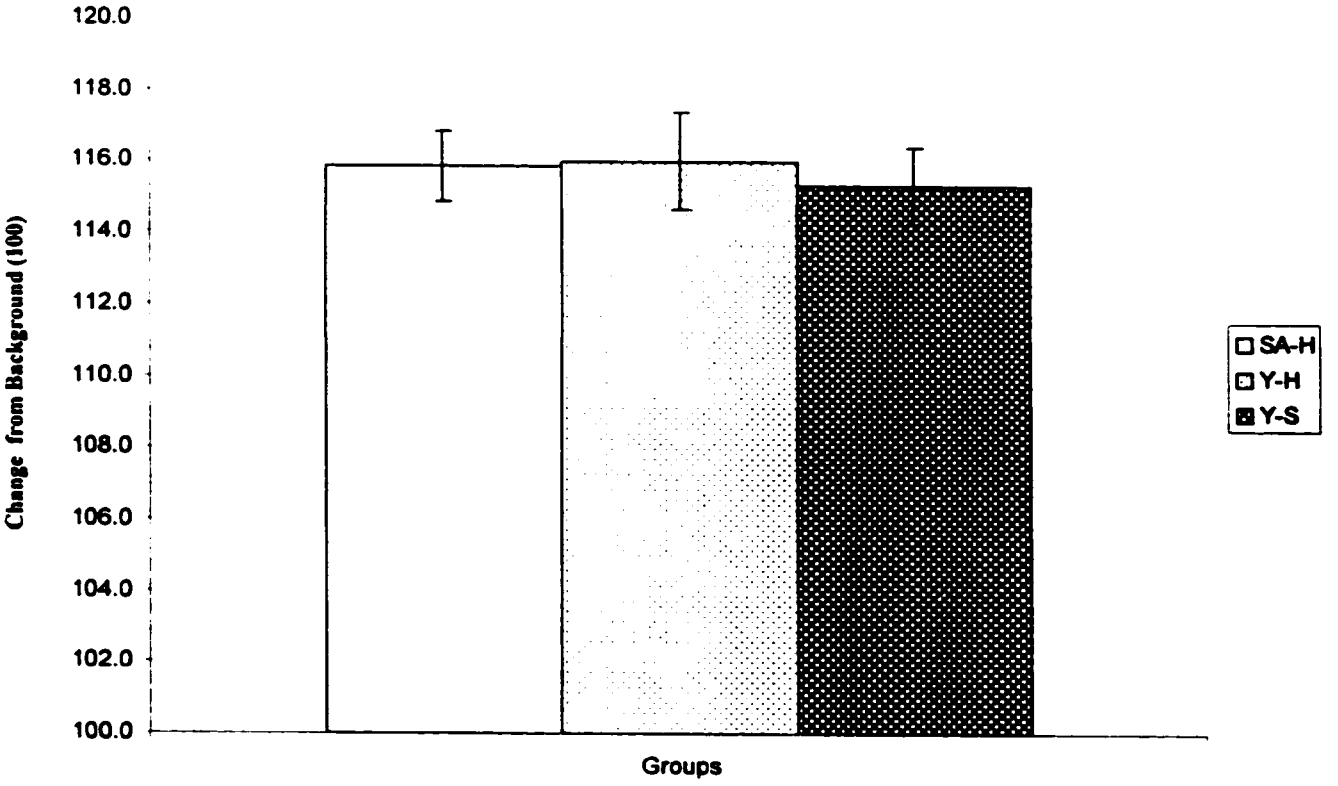
Sessions
SA-H
Y-H
Y-S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Change from Background (100)
Groups
SA-H
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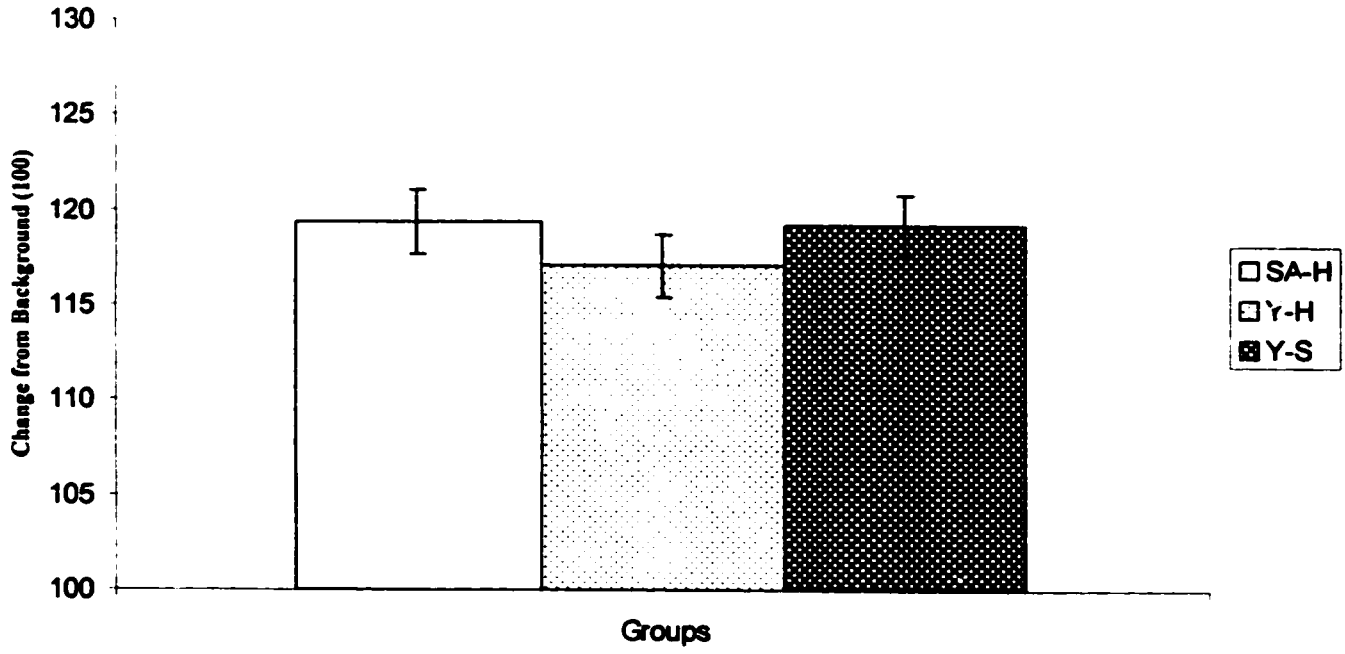
Y-S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Groups
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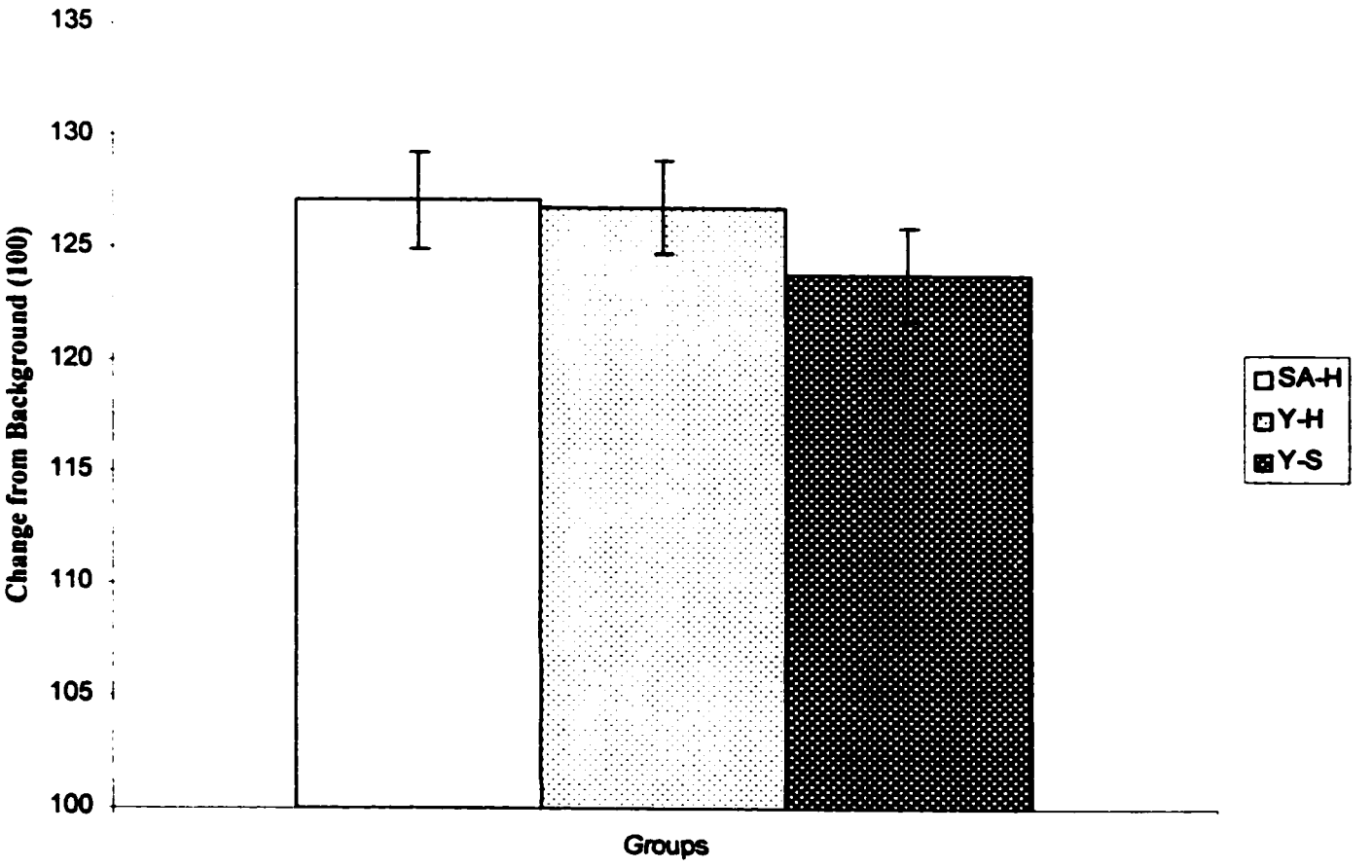
Y-S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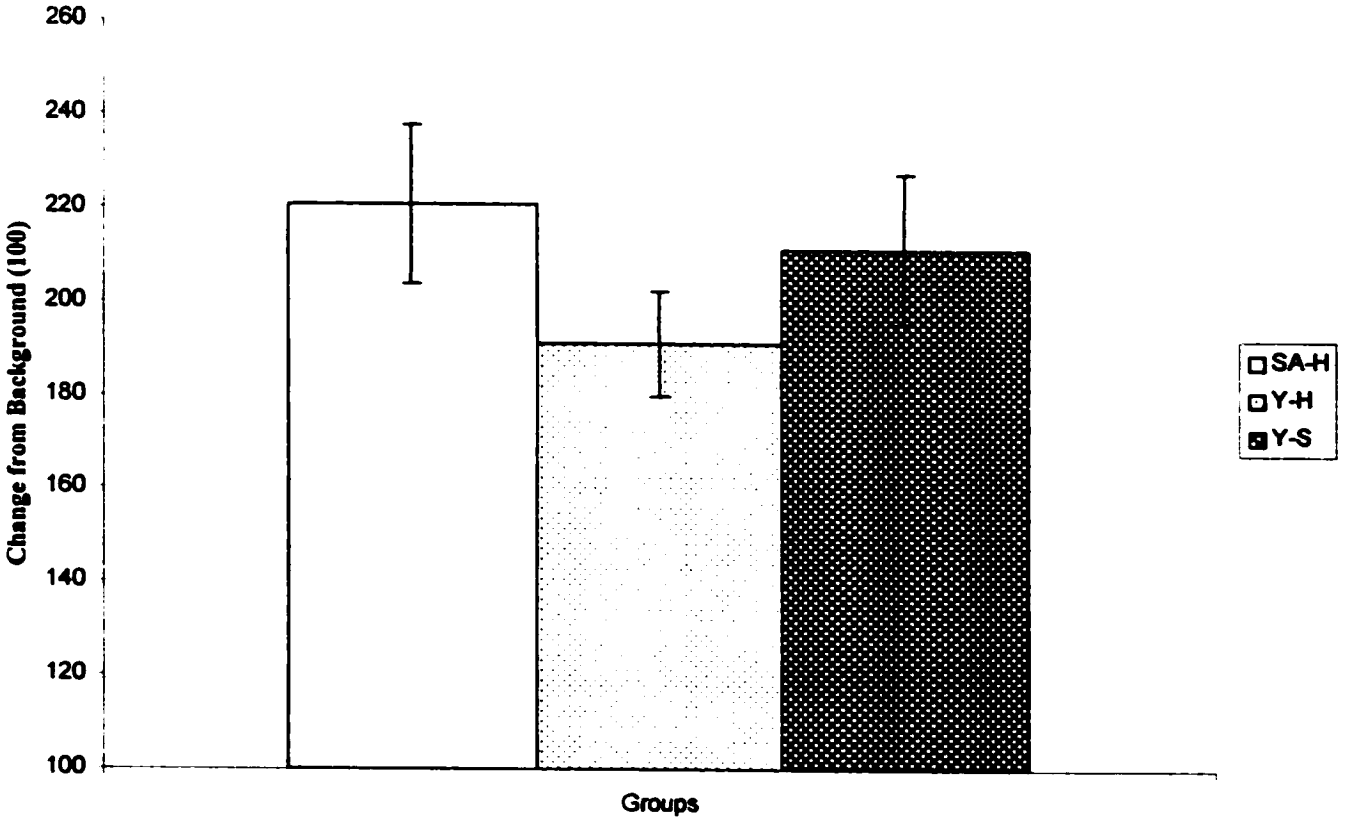
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Groups
SA-H
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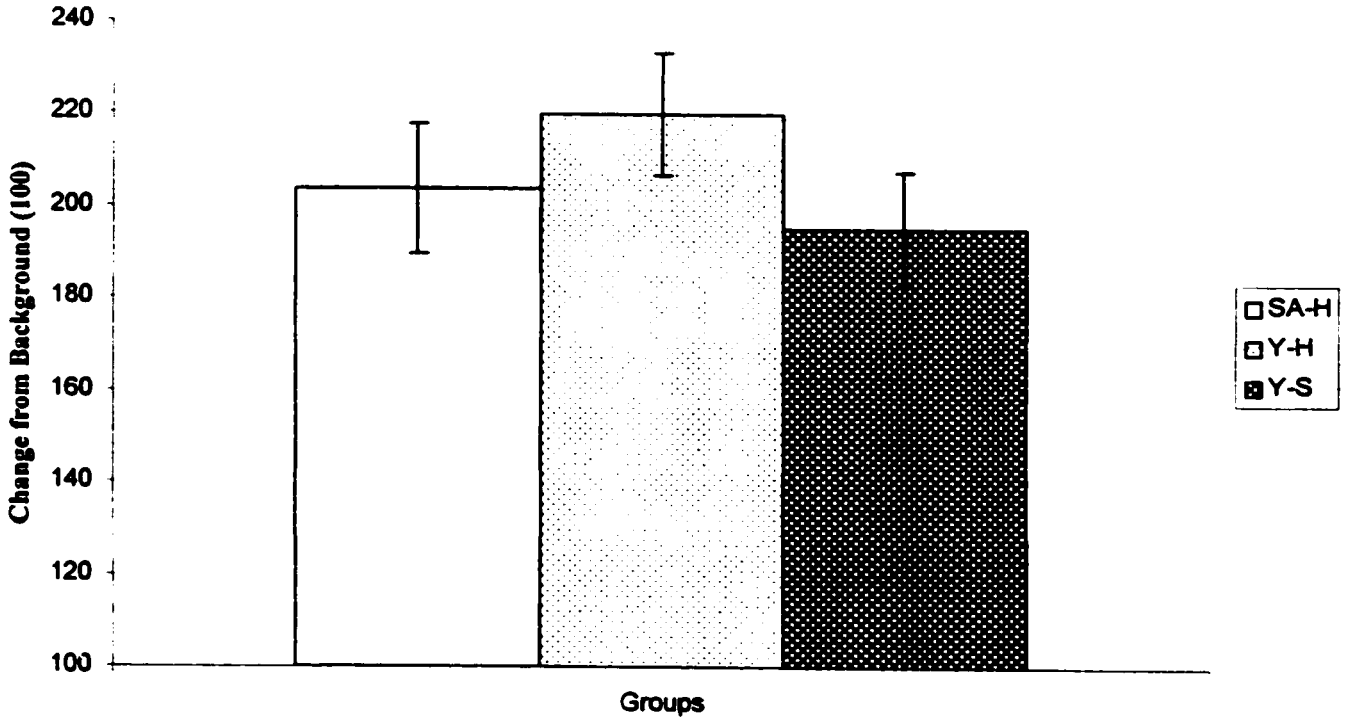
Y-S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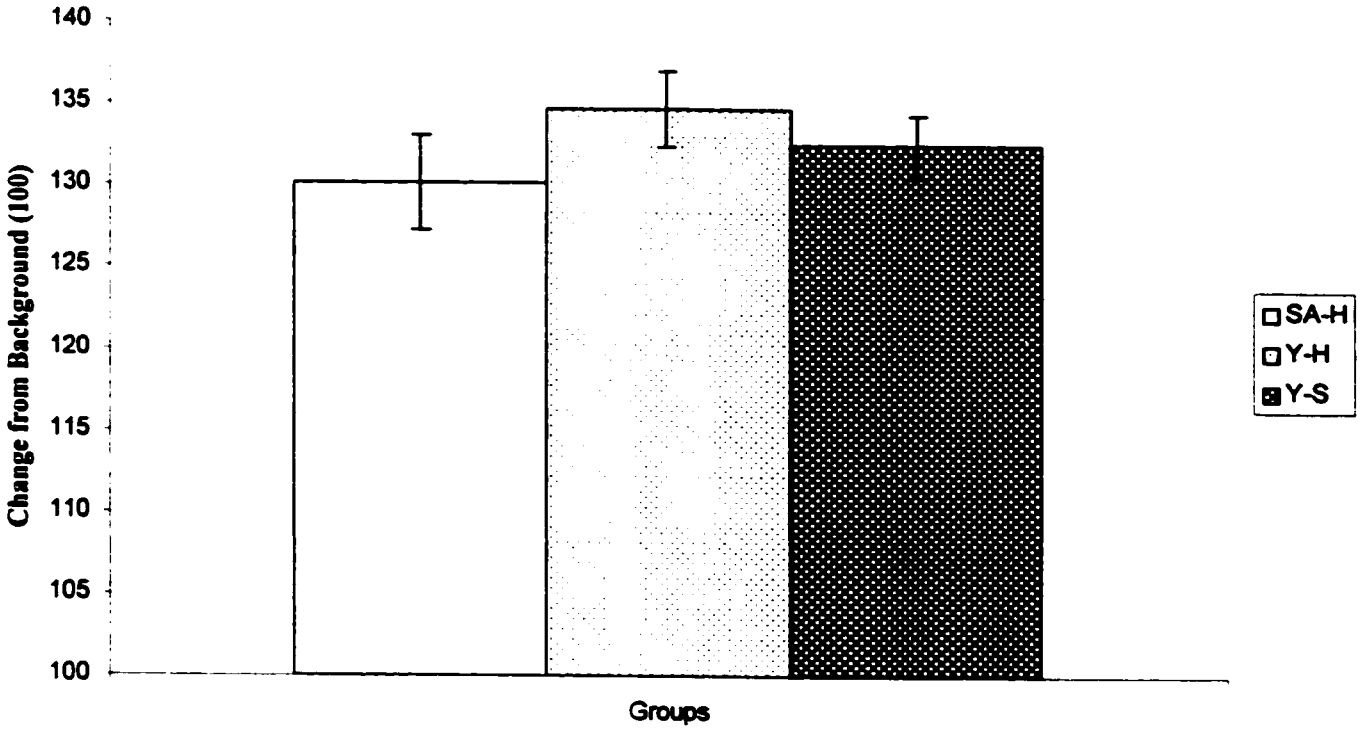
Change from Background (100)
Groups
SA-H
Y-H
Y-S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Change from Background (100)
Groups
SA-H
Y-H
Y-S

Chapter 5

Gene Screening in the NA_{cc} of Heroin Self-Administering and Yoked Rats

Chapter 5: Introduction

While the in situ hybridization experiments of chapter 4 explore the role of only one gene's contribution to associative heroin tolerance (i.e., CCK2 receptor gene), the following experiments take advantage of a new technology, cDNA arrays, to investigate hundreds of genes at once. Although multiple brain areas were examined in chapter 4, we decided to concentrate our gene search with the cDNA array technique to the NA_{cc}. This brain area has been strongly implicated in heroin self-administration. Rats will self-administer opiates directly into the NA_{cc} (Goeders, Lane, & Smith, 1984). Furthermore, lesions of the NA_{cc}, and microinjections of opioid antagonists in the NA_{cc}, selectively impair heroin self-administration (Vaccarino et al., 1985; Zito et al., 1985). Furthermore, GABAergic neurons of the ventral tegmental area (VTA) are disinhibited during opiate administration and thus, release dopamine into the NA_{cc} (Leone et al., 1991). It has been hypothesized that the downstream effect of dopamine receptor activation in the NA_{cc} is early immediate gene activation (Liu et al., 1994).

There is evidence that VTA and NA_{cc} cells respond both to the anticipation and to the administration of heroin. That is, dopaminergic VTA cells will exhibit a profound discharge rate *prior* to self-administration of heroin (Kiyatkin & Rebec, 1997). The VTA cells continue to respond after the infusion and then exhibit inhibition. The firing rate will then gradually return to a high rate prior to the next self-administration. Furthermore, there is evidence that certain sub-populations of NA_{cc} cells respond immediately prior to self-administration of heroin (Chang, Janak, & Woodward, 1998).

The anticipatory cellular response seen in the VTA and the NA_{cc} may not occur in a yoked animal that has no self-administration contingency. Therefore, the NA_{cc} of a SA-H animal may receive an early signal from the VTA that heroin is arriving. In turn, some NA_{cc} neurons of a self-administering animal may fire earlier than NA_{cc} neurons of a yoked animal. It is plausible that interoceptive cues inherent in self-administration may alter gene expression differently in self-administering animals as compared to animals that passively receive the drug. However, there has not been any systematic gene screening conducted in the NA_{cc} after heroin self-administration. In these experiments reported in Chapter 5, Atlas cDNA Expression Arrays (Clontech) were used to determine any differential gene expression between SA-H and Y-H rats.

Each Atlas cDNA Expression Array includes 609 cDNAs spotted in duplicate on a positively charged nylon membrane. Nine housekeeping cDNAs are included as positive controls for normalizing mRNA abundance. Two separate Atlas Expression Array experiments were carried out on SA-H and Y-H rats. In the first experiment, only 1 SA-H rat's NA_{cc} RNA was compared to 1 Y-H rat's NA_{cc} RNA. In a second Atlas cDNA Expression Array experiment, pooled NA_{cc} RNA from 3 SA-H rats and pooled NA_{cc} RNA from 3 Y-H rats were compared.

Methods

Subjects

Long-Evans rats from the Weise-Kelly & Siegel (2001) study, were used in the present study. As described in chapter 3, these animals had been surgically implanted with chronic catheters in the right jugular vein under ketamine and xylazine anaesthesia. A lever press by an SA-H rat resulted in a 3-sec infusion of a .1 mg/ml heroin solution (i.e., .0105 mg of heroin in .105 ml of solution) at a rate of .035 ml/sec, for itself and the Y-H animal. The lever was non-functional in the Y-H chamber. We chose to exclude the Y-S group since only two membranes are used in a cDNA experiment and the important comparison was between those animals that self-administer heroin and those that passively receive the drug. All animals were measured for ataxia with a tilting plane following each self-administration trial.

RNA Isolation

Following the last (8^{th}) session of heroin administration trials, SA-H and Y-H animals were rapidly decapitated and their NA_{cc} dissected. Total RNA was extracted by homogenizing the tissue in 1 ml of Trizol. The samples were incubated for 5 minutes at room temperature, 200 µl of chloroform was added, and the samples were centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase was transferred to fresh tubes and 500 µl of isopropronly alcohol was added. The samples were incubated overnight at -20°C. The following day, the samples were centrifuged at 12,000 g for 30 minutes at

4°C, the supernatant removed, and the pellet washed with 75% ethanol. This was repeated twice and then the RNA was dissolved in DEPC treated water. The concentration of RNA in the samples was measured using an UV spectrophotometer.

DNAase Treatment of Total RNA

A mixture of 100 µl of 10X DNase I buffer, 5 µl of DNase I, and 395 µl de-ionized water was added to each sample and allowed to incubate at 37°C for 1 hour. After the incubation, 100 µl 10X Termination mix and 550 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. The samples were centrifuged at 12,000g for 10 minutes in order to separate the phases. Another 550 µl of chloroform:isoamly alcohol (24:1) was then added to the aqueous layer of each sample, vortexed, and centrifuged at 12,000 g for 10 minutes. The RNA pellets were then washed with ethanol and dissolved in de-ionized water.

cDNA Synthesis from Total RNA

To each sample, 1 µl of 10X CDS primer mix (Clontech) was added and incubated at 70°C for 2 minutes and then at 50°C for another 2 minutes. A master mix of 5X Reaction buffer (Clontech), 10X dNTP mix (Clontech), DTT (100mM),[α-^{32}P]dATP (3,000Ci/nmol, 10mCi/ml), and MMLV reverse transcriptase (50 units/µl) was then added to each sample. The tubes were incubated at 50°C for 25 minutes and the reaction stopped with 1 µl of 10X termination mix. The radio-labelled samples were then purified

through column chromatography. Only the second and third fractions were used as the cDNA probe for each sample.

Hybridization of cDNA probes to Atlas Array

The positively charged nylon membranes containing hundreds of cDNAs spotted in duplicate, were prehybridized in a solution of ExpressHyb (Clontech) and sheared salmon testes DNA at 68°C for 30 minutes with continuous agitation. Afterward, the entire pool of radio-labelled cDNA from the SA-H sample was poured over one membrane and the Y-H sample was poured over the second membrane and allowed to hybridize overnight with continuous agitation at 68°C. After 24 hours, the hybridization solution was removed, discarded, and replaced with wash solutions containing 2X SSC and 1 % SDS detergent. The membranes were again incubated for 30 minutes. This washing procedure was again repeated under a less stringent condition of 0.1X SSC and 0.5% SDS detergent. The membranes were then exposed to x-ray film or phosphorimaged. At the completion of the experiment, the membranes were stripped and stored at – 20°C.

Results

Table 1 lists the 7 genes from the first cDNA array experiment that were up- or down-regulated in the NA_{cc} of a SA-H rat as compared to the NA_{cc} of a Y-H rat. Out of the 7 genes differentially expressed, only 2 were not confirmed in the second cDNA array experiment using NA_{cc} RNA pooled from 3 animals for each group. The gene code in table 1 represents the spatial location of the cDNA on the membrane. Each membrane

consists of 6 quadrants (A-F), each quadrant consists of 7 columns (1-7), and 14 rows (a-n). For example, insulin-like growth factor receptor cDNA is located in quadrant A, column 4, and row l.

Figure 1 shows the two cDNA Atlas Expression Array experiments for the SA-H (top membrane) and Y-H (bottom membrane) rat. The five cDNAs outlined by boxes for both SA-H and Y-H arrays represent those genes that were found to be consistently regulated in both array experiments. Figure 1A shows a cDNA array representing 1 SA-H rat and 1 Y-H rat. Figure1B show a cDNA array representing a replication of the first experiment using RNA pooled from 3 animals for SA-H and Y-H groups. The quality of the second cDNA array experiment is compromised due to the multiple times that these membranes were used. The background signal is higher and several of the housekeeping genes exhibit higher expression in the Y-H group as compared to the SA-H group. Housekeeping genes are used as controls since they are not regulated by any experimental manipulations. Furthermore, there are many more genes differentially expressed between the SA-H and Y-H groups that were not detected in the first cDNA array experiment. However, the majority of those genes that were differentially expressed in the first experiment were also differentially expressed in the same direction in the second replication (5 out of 7).

Discussion

In the yoked-control design, only the SA-H group receives a reliable interoceptive cue signalling the later drug effect. This interoceptive cue inherent in self-administration

may lead to an increase in NA_{cc} dopamine. It has been shown that the downstream result of dopamine receptor activation in the NA_{cc} is gene transcription (Lie et al., 1994). The dopaminergic signal from the VTA to the NA_{cc} encodes "expectations about external stimuli or reward" (Schultz, Dayan, & Montague, 1997, p. 1596). There is evidence suggesting that dopamine neurons are activated by a reward-predicting stimulus and not necessarily by the actual reward (Mirenowicz & Schultz, 1994). For example, dopamine neurons become activated when monkeys receive a small quantity of fruit juice to the mouth as liquid reward. However, after learning the association been a stimulus predicting the delivery of the fruit juice and the arrival of the reward, the dopamine neurons will now only be activated by the predictive stimulus (Mirenowicz & Schultz, 1994). Therefore, prior to learning an association, dopaminergic activity occurs after the US. After an association is learned, dopaminergic activity becomes correlated with the onset of the CS.

Due to the interoceptive cue inherent in self-administration, the dopaminergic activity in the NA_{cc} of the SA-H rat may be occurring earlier and more strongly than the dopaminergic activity occurring in the NA_{cc} of the Y-H rat. These differences in neural activity could be the reason why certain genes are differentially expressed between the two groups. The SA-H rat may have a more predictive neural signal indicating the arrival of the drug. The cDNA array method is an efficient way of narrowing down the search for those genes in the NA_{cc} that are implicated in the learned aspects of opiate tolerance.

Five genes in the NA_{cc} were found to be consistently differentially regulated by the yoked-heroin design. Interestingly, one of the genes that was consistently up-regulated in

the SA-H group was the CCK2 receptor gene. In chapter 4, however, the CCK2 receptor gene was not found to be differentially expressed in the NA_{cc} between SA-H, Y-H, and Y-S groups using in situ hybridization. Other researchers have also reported a failure to confirm some of their cDNA array results using more established molecular techniques (e.g., Nestler et al. 2000). Therefore, the discrepancies in results between the cDNA arrays and the in situ hybridization may reflect some of the shortcomings of cDNA array technology.

Since these experiments, there have been improvements made in cDNA gene screening. The nylon membrane arrays have been replaced with glass surface arrays termed "gene chips". One of the major advances of the gene chip over the nylon membrane is an increase in sensitivity. The smaller area surface of the gene chips reduces the volume of the hybridization solution and thereby, increases the concentration of the labelled probe. Secondly, the glass surface of an array generates less background hybridization signal than the porous membranes of nylon arrays (Johnston, 1998). Furthermore, more genes, up to 10 000, can now be screened at once.

However, these advances do not come without their problems. With gene chips containing more and more genes, it becomes increasingly difficult to interpret the massive amounts of data that can be collected in a single experiment. Decisions have to be made about the criterion as to which differentially expressed genes will be chosen for closer study. It may be wise to narrow down the search to those differentially expressed genes that are simply relevant to opiate tolerance. However, it may be more productive to concentrate on those genes that are most differentially expressed. Furthermore, unlike

prior gene screening methods (e.g., differential display PCR), gene arrays are limited to those oligonucleotide sequences that we already have knowledge about. This will limit the knowledge gained about new genes that may be involved in the learned aspects of opiate tolerance. Caution should be taken so as not to limit the investigation of the molecular basis of associative opiate tolerance with the latest available technology.

Table 1

List of genes that were up- or down-regulated in the SA-H rat

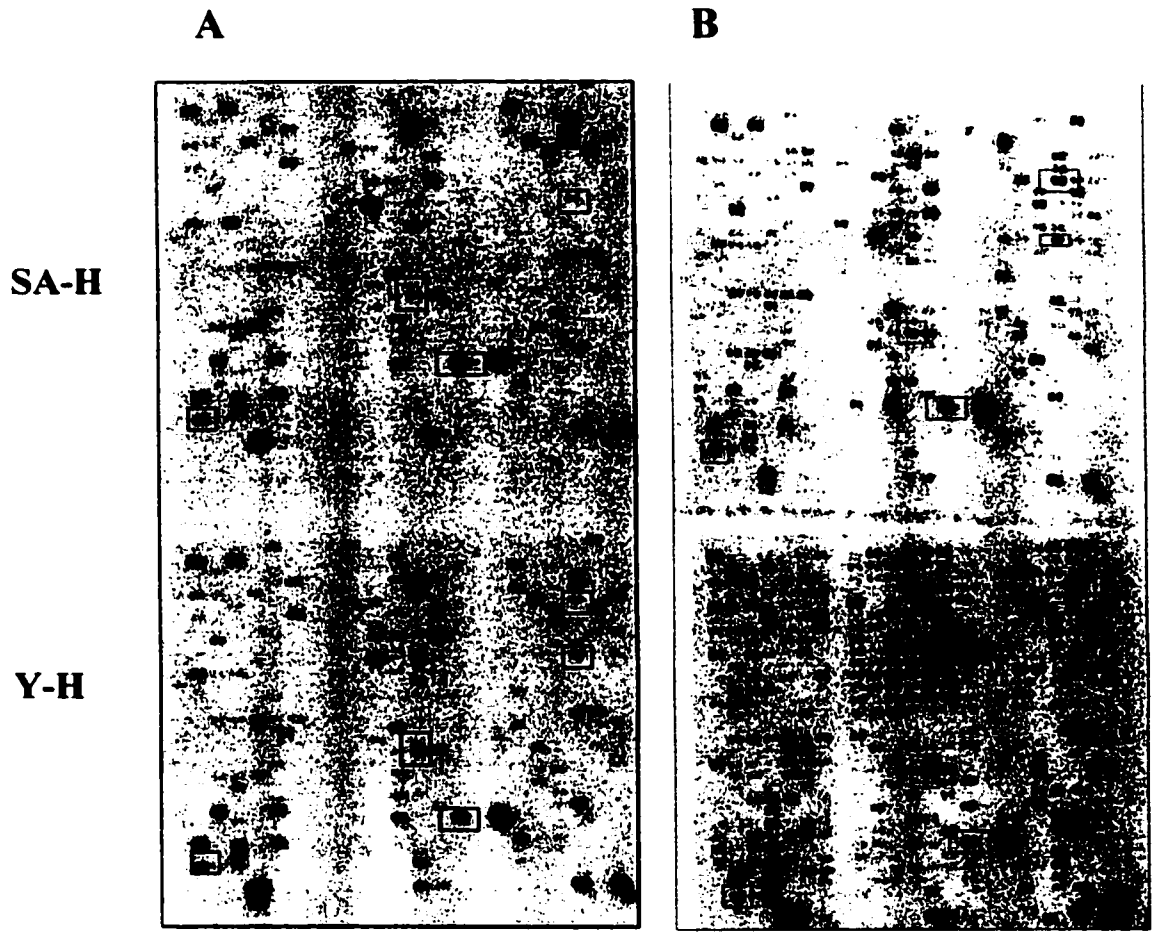
Gene Code	Gene	*Expression
**A4l	Insulin-like growth factor receptor	⇧
C5f	CCK2 receptor	⇧
C5k	Synapsin 2A	⇩
D2n	Steryl-sulfatase precursor	⇧
**D5f	P-selectin precursor	⇧
E5d	Na+/K+ ATPase alpha 1 subunit	⇩
E7j	GABA neuronal transporter	⇧

* SA-H cDNA expression as compared to Y-H control

**Insulin-like growth factor and P-selectin precursor were not differentially regulated in the second replication.

Figure Captions

Figure1. Two cDNA arrays representing RNA expression in the NA_{cc} of SA-H rats (top membranes) and Y-H rats (bottom membranes). (A) Represents a cDNA array of NA_{cc} RNA from 1 SA-H rat and 1 Y-H rat. (B) Represents a cDNA array of NAcc RNA from 3 SA-H rats and 3 Y-H rats.


A
B
SA-H
Y-H

General Discussion

The current experiments challenge the view that chronic drug administration is sufficient to modify gene expression in the striatum and NA_{cc}. Results reported in this thesis have demonstrated that reliable predrug cues (external and internal cues) are important factors contributing to gene expression in the striatum and in the NA_{cc}. Furthermore, several genes were differentially expressed in the NA_{cc} of rats that self-administered heroin versus their yoked partners. Pavlovian conditioning not only plays a role in the acquisition and expression of tolerance, but also modulates the intracellular mechanisms that are hypothesized to mediate tolerance.

The results of experiments in Chapter 2 demonstrated that, just as with morphine analgesic tolerance, striatal c-Fos and AP-1 DNA binding was modulated by external cues signalling morphine. External cues that signal the arrival of morphine induce gene expression in the striatum. Previously, it was assumed that chronic morphine administration alone was sufficient to induce these intracellular changes in the striatum (Nye & Nestler, 1996). The opiate would disinhibit GABAergic neurons via μ receptors in the substantia nigra and cause a dopaminergic influx into the striatum. When this occurs concurrently with the activation of NMDA receptor in the striatum, c-Fos expression occurs. However, it is possible to block the induction of striatal c-Fos by manipulating external predrug cues. The results of experiments presented in Chapter 2 demonstrated that animals that received chronic morphine, but were tested in the presence of an external cue that was unpaired with the drug effects, did not show greater striatal c-Fos expression and AP-1 DNA binding than did saline controls. Therefore,

environmental stimuli modulate not only the expression of tolerance but also the intracellular changes hypothesized to mediate tolerance. Chronic morphine administration does not *ipso facto* induce c-Fos and increase AP-1 DNA binding in the striatum.

Although studies of the associative basis of drug effects have typically manipulated environmental cues (e.g., the room where the drug is administered), there is evidence that interoceptive cues may also become associated with a drug and control the expression of tolerance. Some psychopharmacology researchers investigate effects of drugs that are self-administered (especially the rewarding effects), but most researchers administer the drug to subjects. Thus, much of what we know about the effects of drugs, such as the development of tolerance, is based on results of studies in which the experimenter-not the subject-administered the drug. Recently, Weise-Kelly & Siegel (2001) have demonstrated that the self-administration contingency modulates the ataxic effects of heroin. Rats that self-administer heroin are more tolerant to the ataxic effects of the drug than rats that passively receive the drug. The experiments in Chapter 3 demonstrated that interoceptive cues inherent in self-administration also modulate the intracellular machinery involved in this behaviour. Self-administering rats exhibit higher striatal c-Fos levels than their yoked controls. The induction of c-Fos will occur in GABAergic neurons of the striatum if dopamine binds to D_1 receptors while NMDA receptors are activated. It is plausible that there is no concurrent activation of both D_1 and NMDA receptors in the Y-H rat. Since the Y-H rat is not provided with an interoceptive cue signalling heroin, it is unable to counter the drug effect both behaviourally and

neurochemically. The NMDA receptor may play an important role in this effect. It has have been implicated in memory and may act as a coincidence detector (Swartzwelder et al. 1989). A reliable interoceptive cue may produce a release of cortical glutamate that will bind to NMDA receptors in anticipation of the drug effect. This may explain why rats in the SA-H group have an enhanced ability to maintain their balance compared to their yoked partners. Therefore, both external and internal cues play a role in the regulation of c-Fos in the striatum.

The role of c-Fos is to initiate transcription of genes and the CCK2 receptor gene may be a downstream target. The CCK2 receptor is involved in opiate tolerance (Hoffmann & Weisenfeld-Hallin, 1994; Kellstein & Mayer, 1991) and compensatory conditional responses (Kim & Siegel, in press; Siegel et al., 1999). It has been hypothesized that CCK is released by the central nervous system in response to opiate stimulation and that its binding to CCK2 receptors contributes to tolerance by attenuating the effect of the drug (Rothman, 1994). In the Weise-Kelly & Siegel (2001) study, the interoceptive cue inherent in self-administration appears to elicit a compensatory conditional response in animals that self-administer heroin but not in their yoked partners. In Chapter 4 we investigated, in several rat brain areas, the effect of the heroin self-administration contingency on the distribution of CCK2 receptor RNA. Although no differences were found in CCK2 receptor RNA expression between rats that self-administered heroin and rats yoked to these self-administrators, two cDNA array experiments (Chapter 5) revealed 5 genes (including the CCK2 receptor gene) that were consistently differentially regulated between the groups. Besides the CCK2 receptor gene, none of the other genes

that were differentially expressed have ever been linked to opiate tolerance. The cDNA array technique is a useful tool in discovering the genes that are implicated in certain behavioural processes. These cDNA array experiments have discovered possible genes that are important for learned aspects of opiate tolerance. Although more studies are needed to verify if these genes are simply false positives, the cDNA array approach to gene screening has helped in narrowing down those genes that may be involved in associative tolerance to opiates.

Conclusions

In summary, both external and internal cues contribute in modifying gene expression. Striatal c-Fos is modulated by both audio-visual cues and interoceptive cues inherent in self-administration. Furthermore, it appears that there are several genes that are modulated by interoceptive cues signalling heroin in the NA_{cc}. An initial attempt was made in these experiments to detect those genes that are involved in the associative tolerance to opiates. Although the CCK2 receptor gene was not found to be differentially regulated between rats that self-administer heroin and their yoked partners using in situ hybridization, this gene was consistently found to be up-regulated in the SA-H group using cDNA array technology. With more advanced gene screening techniques (e.g., gene chips), it may be possible to elucidate the molecular mechanisms underlying the learned aspects of opiate tolerance.

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Chapter 2, Experiment 1
Hot-Plate Scores for the Three Test Sessions
(missing values due to subject attrition)

Test session 1

Subject #	PM	UPM	PS	UPS
1	15	30	30	30
2	30	24	30	30
3	16	30	30	30
4	10	30	30	30
5	22	30	30	30
6	26	30	30	30
7	8	30	30	30
8			30	30
9				30
10				30

Test session 2

Subject #	PM	UPM	PS	UPS
1	5	30	30	30
2	9	13	30	30
3	15	24	30	30
4	24	30	30	30
5	16	30	30	30
6	25	9		30
7	8	20		

Test session 3

Subject #	PM	UPM	PS	UPS
1	4	7	30	24
2	7	6	22	30
3	4	14	30	30
4	10	6	30	30
5	7	25	30	30
6	7	8		30
7	10	4		

Chapter 2, Experiment 2
Western Blots: Percent change from Paired Saline

c-Fos

Blot #	PM	UPM	UPS
1	32	-33	-31
2		-38	-38
3	44	47	34
4	30	-72	-10
5	26	-16	10
6	23	-2.5	31
7	13	6	-7
8	2	-39	-32

c-Jun

Blot #	PM	UPM	UPS
1	9	-5	-15
2	-14	-5	-40
3	20	5	3
4	40	1	17
5	-7	-40	-29
6	6	-47	-38
7	-2	-3	1
8	-7	-15	-19
9	1	1	-14

EMSA (AP-1 DNA Binding)
Percent change from Paired Saline

Subject #	PM	UPM
1	21	-40
2	17	-13
3	71	-1
4	54	13
5	78	-11

Chapter 3
Western Blots: Percent Change from Y-S

c-Fos

Triad #	SA-H	Y-H
1	-20	-59
2	34	25
3	-70	-68
4	-10	-86
5	-9	-63
6	-8	-38
7	-16	10
8	1	-71
9	-4	-6

c-Jun

Triad #	SA-H	Y-H
1	-5	28
2	-20	-35
3	25	-64
4	-14	-17
5	-.01	-20
6	-17	-10

Chapter 4
Optical Density of CCK_B in the NA_{cc} of 6 Triads
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
176	196	166	176	159	175
185	194	168	176	165	175
179	195	161	174	168	174
175	195	164	172	164	175
167	195	166	174	158	177
167	195	170	175	162	176
174	195	162	175	164	177
179	193	166	176	162	175
183	194	166	174	162	176
179	194	163	170	165	174

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
156	173	137	151	151	172
154	173	136	138	154	170
152	173	140	145	152	170
154	171	136	141	152	167
150	172	131	146	158	171
156	169	135	157	153	171
145	170	140	151	159	170
157	171	138	154	163	172
151	173	129	149	163	172
148	171	130	144	150	171

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
165	171	155	169	154	177
159	171	146	173	152	177
156	171	145	172	154	176
158	172	151	173	152	175
152	171	146	173	153	175
163	170	155	171	146	175
164	173	152	170	136	176
161	170	159	170	154	173
165	171	154	172	155	172
158	171	152	167	145	174

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
153	167	142	166	156	171
147	168	151	164	161	172
153	165	153	164	161	171
152	166	148	165	153	170
149	163	152	166	160	172
156	168	155	168	157	173
146	167	151	163	155	170
155	169	153	166	158	171
156	166	153	168	161	168
157	167	145	171	160	170

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
154	169	155	171	145	184
159	166	162	172	162	184
161	166	149	171	165	185
160	167	153	172	163	185
166	168	153	171	162	185
157	168	155	171	167	184
157	167	154	172	158	184
158	168	154	172	159	185
160	167	152	169	164	182
153	168	148	171	158	182

Triad 2

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
151	179	164	182	155	171
135	176	161	182	145	171
147	176	145	182	158	172
147	177	160	176	159	168
148	176	161	175	142	163
144	173	170	181	155	163
149	176	167	182	152	165
143	174	165	180	158	171
150	174	170	179	153	163
149	176	167	176	154	167

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	152	170	145	171	136	169
	140	168	157	170	148	170
	146	170	147	171	144	170
	136	170	154	168	154	169
	145	172	149	170	152	169
	150	173	151	169	154	162
	148	168	146	170	143	161
	147	169	153	165	150	170
	148	164	163	168	151	170
	147	169	154	167	150	168

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	135	172	153	172	158	174
	147	172	163	173	152	171
	137	171	159	173	154	173
	145	159	155	172	152	168
	147	159	147	171	144	173
	144	161	156	169	152	169
	148	163	156	169	158	162
	146	169	154	169	142	170
	150	167	162	169	152	165
	144	167	154	165	155	165

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	149	178	156	177	143	166
	140	177	154	177	151	165
	150	178	159	177	150	167
	139	176	158	177	149	166
	141	176	148	177	145	163
	152	163	156	176	141	165
	152	177	161	176	148	164
	146	165	159	176	144	163
	145	164	159	176	149	162
	154	174	165	177	146	168

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
156	171	152	172	158	168
152	173	154	172	157	170
149	169	154	172	157	171
160	167	155	172	156	171
156	176	156	171	155	174
154	176	151	168	160	172
152	169	151	171	145	170
143	166	147	172	156	167
150	171	156	172	151	170
156	167	157	163	153	170

Triad 3

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
129	170	127	161	130	162
131	167	126	170	129	164
131	173	139	163	129	165
129	162	143	152	129	146
130	148	136	147	136	144
131	150	127	144	130	142
127	166	132	169	130	142
131	148	137	145	130	153
132	154	143	140	125	142
128	147	134	169	129	161

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
129	158	132	170	123	155
125	164	141	168	132	149
131	165	135	168	135	156
129	150	135	146	131	155
128	151	130	146	134	157
126	147	131	146	135	150
131	150	136	163	129	157
126	143	133	164	128	155
127	152	142	168	130	154
128	149	142	150	131	151

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	125	162	128	136	139	156
	123	165	133	139	137	157
	122	138	129	138	146	156
	122	139	124	138	137	161
	126	144	135	137	142	161
	136	140	127	155	137	152
	123	141	133	158	137	156
	122	145	135	166	138	155
	118	136	132	166	129	152
	118	142	130	158	135	156

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	125	152	130	136	147	158
	126	150	135	142	148	171
	123	152	131	142	136	166
	122	144	126	148	140	165
	129	147	130	168	148	170
	124	147	141	145	146	167
	122	143	133	143	140	172
	130	146	134	144	141	165
	121	137	135	145	154	165
	124	147	129	147	147	162

Slide 5				
	CCKB	Background	CCKB	Background
	139	147	143	155
	138	144	140	155
	136	144	139	157
	137	155	144	154
	138	153	138	153
	143	139	137	154
	136	142	143	153
	143	165	155	151
	132	147	142	150
	132	157	145	148

Triad 4

Slide 1	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background		
	147	182	161	176		
	127	187	156	169		
	134	189	143	180		
	151	185	154	180		
	160	196	154	171		
	177	194	152	173		
	164	195	153	176		
	163	196	154	176		
	153	194	163	183		
	152	194	162	177		

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
154	182	132	161	145	151
161	184	119	161	149	151
153	183	120	166	141	171
136	181	132	163	144	164
150	178	124	158	140	173
151	172	121	151	152	165
138	177	126	159	143	159
152	184	133	156	143	154
151	167	117	169	145	154
130	180	132	152	131	154

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
122	150	122	155	117	151
120	143	134	151	119	150
105	148	126	165	120	151
130	149	118	153	121	151
119	143	133	166	110	146
127	142	126	149	128	150
117	137	109	143	133	152
115	143	114	147	122	147
124	140	133	149	120	154
115	144	124	148	118	143

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
147	164	119	138	117	153
149	157	107	153	132	145
139	161	113	148	120	159
148	165	113	158	131	140
145	163	113	139	118	141
143	160	113	144	103	148
151	164	120	136	136	147
152	167	120	139	116	139
148	167	114	129	138	152
143	168	113	142	117	144

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
128	140	112	149	142	171
114	139	111	145	150	171
119	138	108	149	148	176
123	144	112	151	145	180
112	136	104	160	130	169
108	144	111	155	144	171
118	150	110	150	143	166
118	150	124	153	139	158
115	144	109	151	136	168
116	142	132	138	137	168

Triad 5

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
120	169	116	157	156	160
130	167	124	159	152	156
135	176	122	156	117	153
132	165	130	158	140	145
138	160	159	161	128	152
131	159	134	156	126	162
131	172	123	157	135	152
147	158	119	168	137	158
129	167	133	163	127	158
129	162	134	155	135	147

Slide 2 SA-H

Y-H		Y-S	
CCKB	Background	CCKB	Background
111	152	123	156
124	158	134	169
102	157	128	161
123	153	124	178
133	149	119	153
122	152	126	176
115	149	122	165
106	146	126	163
114	143	116	162
123	141	126	160

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
117	134	130	160	132	162
123	145	136	157	124	164
121	138	131	169	119	153
116	141	127	161	120	163
134	134	126	161	118	157
99	130	135	157	119	169
142	131	131	156	123	154
130	130	143	149	111	152
121	121	131	153	135	157
125	121	135	157	126	158

Slide 4

CCKB	Background	CCKB	Background	CCKB	Background
134	147	113	151	131	138
128	156	109	147	153	137
132	151	126	142	128	147
128	156	120	155	126	158
128	140	120	154	133	152
120	159	121	156	133	121
143	157	129	153	130	144
148	156	110	151	116	129
129	151	111	152	133	154
160	165	116	147	120	132

Slide 5	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	146	174	139	157	146	148
	145	154	126	156	119	156
	152	176	129	157	120	150
	161	181	139	154	153	162
	138	176	135	159	142	173
	147	163	127	149	137	159
	148	166	137	144	154	173
	136	176	122	148	147	178
	143	181	127	145	135	180
	155	190	133	157	145	149

Triad 6

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
127	154	156	158	148	121
135	159	135	151	125	143
134	159	122	147	97	141
122	161	132	156	116	133
136	157	139	145	111	137
144	157	133	150	133	148
118	153	158	154	112	142
132	148	155	157	130	152
129	157	130	155	125	140
126	153	147	150	119	143

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
140	170	153	175	134	165
139	172	163	179	139	162
155	173	173	180	133	163
149	167	173	185	142	178
147	171	165	187	127	164
158	170	161	195	119	154
152	172	156	194	166	165
149	171	148	186	133	153
150	170	169	192	134	163
158	174	158	189	138	156

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	154	180	145	170	133	155
	171	174	151	173	158	174
	151	177	148	165	125	156
	157	171	143	167	152	154
	168	177	147	167	137	152
	140	169	152	170	131	165
	156	180	156	164	120	159
	154	176	158	166	126	153
	137	183	161	171	135	155
	141	170	139	159	146	151

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	146	177	147	162	126	150
	148	173	148	159	133	143
	150	178	146	157	123	146
	149	170	168	170	120	156
	152	170	156	163	115	157
	153	178	134	163	123	145
	151	183	151	162	133	146
	141	174	159	164	125	157
	131	171	159	154	130	145
	137	167	153	154	119	149

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	110	144	181	184	140	189
	115	143	147	186	152	181
	148	147	153	184	168	176
	124	143	159	173	168	189
	110	148	159	164	157	191
	118	136	155	162	162	186
	119	137	151	192	153	177
	115	145	164	205	139	182
	122	147	158	200	159	169
	126	153	171	207	145	168

Optical Density of CCK_B in the Frontal Cortex of 6 Triads
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
209	221	204	225	204	222	
210	221	210	226	208	222	
208	221	212	226	210	221	
212	220	210	225	213	224	
214	220	204	227	205	224	
216	219	200	227	202	224	
213	220	183	226	200	224	
215	218	200	225	202	223	
204	220	213	225	214	220	
214	220	200	224	200	224	

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
206	228	186	212	221	230
206	227	192	206	221	230
202	229	190	206	225	231
214	225	201	211	215	231
203	227	190	210	223	230
201	227	194	212	214	229
196	229	194	203	217	231
196	226	193	206	216	232
185	227	198	205	216	230
200	231	187	210	213	229

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
214	220	209	228	216	230
206	222	209	229	217	230
210	220	210	230	214	231
208	222	207	230	209	230
209	225	204	231	204	230
209	223	207	229	216	230
206	223	207	230	210	231
208	227	207	230	211	230
202	222	206	231	214	231
205	226	200	232	224	232

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
196	227	195	208	210	227
204	226	197	214	210	226
195	228	200	204	204	223
196	222	201	210	206	226
202	222	205	216	207	225
196	225	193	209	206	224
197	223	199	225	197	225
187	221	202	224	196	225
192	222	206	224	180	227
204	227	197	227	195	227

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
204	222	212	230	222	227
200	222	218	224	212	228
201	222	219	229	209	225
203	223	199	231	214	229
205	222	218	230	207	229
203	221	218	230	205	228
205	222	216	230	203	229
201	223	199	222	206	228
205	219	206	225	204	230
201	221	201	226	210	230

Triad 2

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
191	211	204	205	195	212
188	209	204	201	195	211
195	207	186	204	198	210
195	210	199	204	195	215
193	206	191	204	201	212
172	207	212	206	204	210
187	209	214	203	199	209
197	212	195	199	202	206
184	208	165	200	197	211
187	207	195	199	199	212

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	204	215	191	226	205	225
	205	216	186	224	188	222
	205	217	193	217	204	223
	211	217	196	223	185	213
	208	218	194	226	185	220
	204	216	204	219	204	215
	203	219	194	221	202	216
	209	216	180	223	196	207
	197	217	196	209	193	215
	198	217	197	226	193	215

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	189	218	166	209	195	226
	157	221	197	220	203	220
	170	217	192	206	195	219
	177	220	205	208	208	220
	174	220	207	210	199	217
	159	218	198	206	207	216
	164	210	204	202	213	212
	181	211	185	201	194	214
	175	221	187	206	193	217
	173	220	182	204	196	213

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	184	198	193	204	176	209
	190	201	196	200	198	209
	179	200	176	207	161	206
	191	197	199	203	186	210
	194	197	193	205	177	202
	166	200	204	202	179	225
	175	197	202	205	185	208
	178	203	199	206	184	213
	187	197	195	204	189	211
	184	199	196	198	182	207

Slide 5	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	192	211	208	204	189	224
	197	221	190	206	197	223
	191	205	193	203	195	219
	180	211	187	202	188	217
	186	209	195	201	196	229
	185	211	188	200	176	214
	183	213	206	199	190	210
	186	219	178	201	185	222
	188	214	187	199	185	219
	191	211	188	196	186	222

Triad 3

Slide 1						
	CCKB	Background	CCKB	Background	CCKB	Background
	156	186	166	188	124	192
	162	183	167	187	156	183
	180	184	163	188	167	186
	155	186	156	186	154	177
	154	186	159	183	137	183
	171	187	165	181	139	181
	159	190	171	186	141	182
	163	184	156	187	135	175
	154	187	156	189	145	184
	151	187	161	190	144	182

Slide 2						
	CCKB	Background	CCKB	Background	CCKB	Background
	181	237	187	230	192	217
	186	240	163	216	173	225
	173	235	188	224	192	238
	170	215	188	216	208	234
	189	223	186	230	190	212
	168	214	153	230	177	220
	176	211	162	226	163	226
	194	211	182	219	176	229
	192	227	163	223	184	234
	195	220	148	222	168	225

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	203	225	175	213	176	220
	185	237	176	210	174	219
	176	243	184	210	186	225
	167	227	189	209	178	218
	176	228	178	213	177	220
	168	219	178	220	169	220
	176	235	176	210	176	228
	173	222	164	208	169	226
	143	224	154	219	166	237
	168	234	165	218	176	221

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	135	214	186	207	140	205
	147	213	185	213	186	215
	147	225	179	218	162	214
	184	208	191	221	166	197
	161	201	199	221	175	226
	162	212	187	227	174	229
	172	209	188	217	159	244
	176	226	186	225	150	206
	190	218	169	223	165	224
	171	222	175	213	172	211

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	181	217	181	209	197	213
	193	204	171	196	185	220
	187	214	171	214	189	220
	186	217	159	202	175	214
	188	213	181	221	193	222
	203	215	152	213	187	215
	196	217	174	210	194	220
	192	222	174	204	206	222
	203	226	178	197	192	228
	184	225	166	209	185	223

Triad 4

Slide 1

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
145	169	129	165	144	184
136	168	132	161	132	173
132	168	142	171	152	181
140	171	132	160	127	187
131	169	140	169	138	170
140	164	129	170	133	195
146	169	136	173	154	202
138	165	125	171	133	199
145	171	123	167	133	186
126	168	124	163	125	184

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
156	183	136	171	134	183
159	184	144	169	145	180
146	185	146	171	140	179
154	188	137	176	154	186
154	184	159	177	147	184
148	180	139	175	141	194
159	185	133	172	129	185
147	187	141	163	135	186
155	187	150	168	139	183
146	188	142	178	141	185

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
105	166	128	164	138	167
122	165	128	157	135	163
124	165	119	169	132	159
134	168	119	165	126	169
130	169	132	154	128	161
126	166	125	166	129	167
130	162	117	166	140	166
128	161	129	160	133	166
122	165	124	157	127	166
121	160	111	171	114	169

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
127	167			127	178
138	163			122	175
128	172			127	180
130	168			140	180
130	172			130	176
129	168			133	179
119	162			131	176
130	171			128	177
124	168			134	181
117	162			133	174

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
116	165	132	172	141	187
120	158	117	181	152	191
120	165	125	173	151	183
132	166	122	175	149	184
128	158	127	176	154	180
120	161	124	177	165	185
139	169	126	172	163	188
131	164	147	179	149	188
131	160	121	172	154	188
127	160	130	175	157	187

Triad 5

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
145	165	137	173	136	182
137	165	138	177	142	179
134	173	132	179	152	179
140	171	154	179	153	184
121	165	146	180	155	180
143	175	136	182	155	180
141	170	151	179	157	179
129	165	133	171	143	179
141	173	158	173	164	183
140	169	135	179	153	180

Slide 2	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
120	156	153	184	145	167	
123	153	150	182	154	174	
118	161	159	181	139	175	
120	161	152	181	139	168	
120	156	142	175	141	168	
125	163	149	181	132	169	
114	154	150	179	133	176	
111	171	137	175	139	168	
114	165	155	174	128	169	
112	163	153	184	129	166	

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
114	152	139	190	152	178
110	147	139	190	158	181
113	154	149	185	164	178
112	153	152	184	160	181
113	156	136	187	164	185
111	158	153	183	155	186
112	155	144	188	152	186
121	147	155	189	153	187
112	144	160	177	149	186
108	150	149	183	142	186

Slide 4					
CCKB	Background	CCKB	Background	CCKB	Background
139	165	124	161	144	164
146	162	136	166	143	170
133	169	146	159	127	165
137	168	122	168	136	171
151	161	133	164	131	164
132	166	133	169	136	169
135	170	144	165	143	168
136	162	138	165	126	172
139	164	143	164	131	170
135	170	132	165	127	159

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
129	149	154	189	148	177
128	156	161	192	149	179
128	154	155	187	152	179
128	156	159	183	157	179
126	152	170	190	152	180
140	164	153	183	150	181
130	163	157	183	162	185
135	162	158	183	152	182
126	158	166	187	144	180
132	160	152	184	147	176

Triad 6

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
156	191	157	184	163	186
149	185	158	182	164	182
158	188	166	185	148	183
150	186	158	188	160	182
151	183	168	181	158	185
152	182	163	173	165	189
153	181	159	181	154	182
160	179	173	183	170	181
151	175	167	186	171	184
154	180	176	184	152	186

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
175	188	164	190	134	174
168	189	173	186	142	181
166	190	166	189	135	172
176	187	162	185	150	175
158	187	162	183	143	174
164	188	149	184	147	178
167	190	161	185	150	175
169	184	167	185	131	177
169	182	162	187	141	179
157	185	158	188	141	176

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	148	181	161	181	152	172
	131	180	150	188	150	172
	150	182	166	190	164	172
	158	178	139	184	147	178
	154	178	151	187	142	167
	155	181	149	186	159	174
	142	185	149	187	155	175
	142	180	153	189	152	180
	145	178	163	188	143	175
	137	186	132	182	138	178

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	169	186	143	176	144	166
	160	185	143	174	138	175
	169	184	154	184	148	171
	171	186	158	180	135	170
	155	178	152	185	133	172
	165	186	147	183	162	172
	154	187	159	182	141	174
	168	188	149	175	147	173
	150	187	157	178	141	168
	168	183	158	175	137	171

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	157	186	180	188	157	189
	158	183	171	183	160	191
	169	182	163	188	159	190
	167	187	164	188	163	189
	160	186	173	193	146	188
	156	187	176	193	163	185
	156	181	158	189	170	183
	159	186	160	189	159	186
	160	185	163	193	158	194
	170	187	168	192	161	183

Optical Density of CCK_B in the CA1
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
130	158	143	156	141	155	
143	159	140	155	141	155	
141	160	141	157	134	156	
138	159	143	156	149	154	
137	159	140	156	148	151	
144	157	137	156	145	156	
141	158	140	156	148	153	
136	158	140	156	149	150	
144	158	138	155	150	154	
140	159	132	156	139	156	

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
142	157	150	153	139	154
135	156	152	159	138	152
144	156	153	159	144	155
138	158	150	161	143	153
140	158	148	161	133	156
140	157	144	159	145	157
146	157	156	162	149	158
137	158	152	162	148	158
140	158	148	164	147	159
139	156	150	162	142	153

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
138	154	150	159	148	153
137	157	151	156	151	154
142	156	149	156	140	156
142	158	154	156	147	157
140	156	149	157	143	157
143	156	149	156	156	153
137	156	148	156	146	153
141	156	142	152	149	155
137	157	142	154	145	154
141	156	149	154	148	151

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
148	158	152	157	141	158
139	158	150	155	142	154
132	159	154	153	142	157
145	157	151	153	140	158
139	158	150	155	141	155
141	158	150	150	129	152
142	158	148	154	136	159
145	156	150	151	143	155
145	155	147	153	142	156
139	154	149	151	142	156

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
130	155	145	155	139	160
134	159	145	147	148	157
134	156	144	155	144	157
137	158	147	158	143	161
135	165	138	151	140	160
140	160	143	159	140	162
136	159	135	157	143	162
137	162	128	150	140	157
135	152	141	152	138	158
136	161	140	150	130	158

Triad 2

Slide 1

CCKB	Background	CCKB	Background
99	155	111	159
117	157	119	149
113	153	130	155
119	159	112	150
121	154	110	156
122	156	118	152
114	153	123	143
109	161	129	152
109	158	121	153
107	148	109	159

Slide 2

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
123	158	122	157	119	160
130	153	126	154	123	157
105	151	133	153	133	167
125	159	138	164	101	151
126	155	136	155	121	162
121	158	127	165	124	158
127	158	132	157	139	160
119	152	122	158	128	162
124	149	118	159	129	151
121	153	126	156	111	159

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
119	162	111	155	130	155
109	161	118	162	139	166
103	157	127	158	137	159
106	156	103	160	131	157
111	154	122	163	130	162
118	154	123	160	137	163
111	160	106	147	130	159
112	163	107	153	119	157
107	160	126	163	123	163
102	164	97	145	137	155

Slide 4

CCKB	Background	CCKB	Background	CCKB	Background
124	155	117	147	141	163
121	155	110	156	143	163
110	150	110	156	143	164
118	154	117	161	130	162
117	150	126	166	141	159
119	151	133	157	151	160
120	155	135	154	142	160
123	157	137	156	139	161
117	150	134	150	142	156
124	149	141	147	134	161

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
134	152	130	158	133	161
132	153	129	152	144	156
135	153	109	156	149	159
128	155	124	150	154	158
134	153	126	162	141	159
128	152	137	162	144	157
142	155	129	165	153	161
132	156	128	157	146	157
132	148	132	160	149	151
130	155	135	162	133	159

Triad 3

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
97	147	112	157	140	155
99	152	115	163	143	150
108	148	103	164	142	155
117	176	108	167	139	147
110	152	108	161	140	166
120	156	110	162	139	155
117	156	121	163	136	137
110	151	115	163	142	158
108	144	118	161	139	157
107	151	103	154	127	165

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
104	149	119	146	115	161
123	142	115	147	150	155
118	158	116	152	136	158
135	159	116	161	132	161
116	153	113	160	129	181
111	157	111	151	134	166
109	156	105	162	134	159
118	153	118	167	134	159
120	151	105	167	121	156
106	153	104	156	148	158

Slide 3

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
98	166	103	150	101	155
93	134	105	154	97	155
106	146	102	159	99	142
89	147	122	153	113	156
108	148	89	159	109	160
104	157	110	158	109	159
109	162	109	144	99	144
95	149	124	147	106	155
112	118	111	133	100	156
94	145	106	149	95	156

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
104	154	114	151	123	156
109	151	99	140	127	150
112	146	116	140	131	160
120	145	96	147	129	147
90	146	110	139	134	157
107	145	109	131	130	158
113	143	102	139	123	158
115	138	115	141	138	158
107	146	102	139	125	151
101	154	97	142	131	154

Triad 4

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
129	167	115	134	110	147
133	157	118	150	108	152
130	158	122	148	111	147
121	158	131	154	113	163
122	150	135	146	107	142
116	152	114	154	113	157
117	148	112	151	144	147
118	148	112	152	110	156
125	145	111	152	116	152
118	151	111	144	103	139

Slide 2

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
129	158	109	154	114	156
127	157	132	153	120	155
124	167	110	154	142	153
130	163	119	144	136	140
126	158	107	143	132	138
124	159	118	134	130	151
130	162	110	150	125	158
125	165	131	149	136	152
130	156	117	160	122	150
122	156	115	149	126	151

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
131	156	107	151	119	155
127	155	125	148	127	148
147	146	113	162	123	155
129	153	130	161	129	157
126	158	126	154	130	152
113	152	119	158	125	157
129	143	110	160	125	159
128	146	126	148	135	156
125	155	119	158	137	152
123	158	125	163	136	165

Slide 4

CCKB	Background	CCKB	Background
117	152	116	147
111	145	110	150
101	150	118	155
106	162	124	154
106	155	113	145
103	149	117	160
99	147	129	151
98	137	114	156
100	145	119	141
101	151	116	155

Slide 5	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
		113	151	117	150	
		113	147	135	147	
		120	157	108	153	
		115	150	107	148	
		114	149	122	149	
		130	150	118	151	
		128	153	121	160	
		144	143	118	160	
		117	161	111	162	
		134	150	115	153	

Triad 5

Slide 1					
CCKB	Background	CCKB	Background	CCKB	Background
		123	141	110	149
		101	159	109	166
		117	147	113	154
		112	147	119	170
		113	146	113	163
		104	148	120	148
		109	161	109	143
		106	146	112	136
		109	155	108	149
		122	151	108	144

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
112	154	138	136	121	162
111	152	118	169	111	152
115	164	115	147	111	145
109	164	118	139	112	154
104	175	112	152	122	148
105	162	103	143	124	155
119	158	123	141	119	137
116	159	119	177	115	151
114	158	107	150	120	152
110	154	102	161	118	156

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	115	144	116	139	115	181
	111	132	119	151	103	139
	111	154	116	151	101	171
	109	154	118	140	116	137
	112	135	121	145	108	130
	114	146	103	171	108	153
	112	142	107	146	128	136
	114	136	116	129	104	149
	113	151	116	145	108	153
	123	144	116	153	107	140

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	118	134	123	156	105	146
	116	137	121	153	98	138
	115	129	124	148	102	159
	103	132	116	144	103	149
	111	147	119	141	101	139
	118	138	111	143	112	128
	117	131	118	145	131	152
	122	132	109	140	105	145
	118	132	104	152	97	144
	120	128	115	146	105	160

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	103	132	120	141	107	133
	100	139	114	149	123	148
	105	148	121	141	118	146
	106	141	125	161	114	142
	116	145	122	165	113	131
	110	141	120	147	112	158
	103	151	108	154	112	149
	99	148	120	146	108	145
	102	146	116	148	114	139
	98	145	110	147	121	143

Triad 6

Slide 1

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
107	146	98	151	112	141
107	141	104	140	126	135
107	157	105	147	111	150
109	143	113	142	124	147
115	148	111	144	104	151
114	143	104	131	121	154
102	153	104	143	112	146
115	154	107	130	102	143
112	143	106	146	105	141
112	135	106	141	104	144

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
134	136	128	140	113	136
118	149	123	139	108	140
130	148	112	160	120	149
121	147	118	147	143	143
124	144	114	148	110	147
135	162	105	145	108	151
120	141	121	158	123	141
118	155	109	144	112	148
125	141	118	151	118	143
124	139	114	146	110	152

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
105	151	109	165	102	145
118	155	118	143	97	143
118	144	104	145	116	132
117	153	113	142	108	138
121	152	106	137	105	140
122	146	109	141	103	140
121	159	120	147	108	143
125	145	118	158	119	143
123	141	108	148	108	127
114	137	109	155	102	146

Slide 4	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	129	130	115	142	110	144
	114	138	118	154	117	149
	116	138	119	148	113	149
	133	134	121	142	110	156
	131	154	125	148	117	144
	116	145	120	146	108	153
	115	152	119	151	107	155
	110	145	113	146	107	142
	109	130	122	144	115	152
	112	140	115	145	109	161

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	99	154	107	151	119	155
	115	153	113	148	118	154
	120	154	115	160	124	156
	110	157	119	140	108	155
	114	163	102	142	113	145
	119	145	120	158	117	152
	137	143	108	146	126	153
	121	140	117	155	117	151
	135	150	107	156	115	154
	110	141	113	135	114	148

Optical Density of CCK_B in the CA3
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
139	176	153	201	186	192
136	183	164	202	185	197
132	172	161	192	189	199
136	193	175	188	191	205
142	172	174	190	181	193
141	193	175	198	173	192
129	196	178	202	174	191
126	185	178	200	183	195
137	173	180	200	177	193
128	186	177	200	169	189

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
126	199	142	188	190	216
130	199	158	201	195	233
146	214	179	200	211	217
126	207	180	175	200	218
122	215	169	206	197	208
139	215	153	211	202	215
147	210	168	193	201	219
157	206	172	178	184	215
152	205	157	184	201	235
136	186	169	191	207	202

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
179	190	190	206	187	196
179	192	178	190	165	209
177	203	178	193	168	196
191	196	183	200	191	188
190	200	191	196	183	193
190	207	177	188	173	190
171	196	185	182	175	193
178	199	182	182	190	194
168	194	174	180	182	189
177	206	193	175	177	192

Slide 4

CCKB	Background	CCKB	Background	CCKB	Background
160	195	132	194	157	205
147	185	148	195	150	206
149	189	166	189	154	184
142	189	144	184	125	194
138	183	172	188	148	202
152	181	150	187	156	184
149	190	133	188	165	171
148	183	147	188	162	170
119	176	147	192	162	188
120	187	136	188	157	189

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
88	165	144	186	163	177
99	158	168	179	165	189
77	162	178	181	160	189
110	145	185	186	179	186
114	144	182	167	149	192
81	165	97	178	178	189
88	165	178	184	158	187
72	162	188	178	158	201
103	172	170	182	163	183
83	169	175	174	150	181

Triad 2

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
68	169	71	171	110	165
81	155	114	185	128	175
58	168	93	196	122	175
82	171	69	186	109	171
65	161	80	157	104	177
71	168	73	147	127	168
50	159	91	186	121	176
58	165	123	168	96	172
70	161	94	180	113	178
54	165	93	169	105	170

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	114	179	99	172	112	185
	108	186	65	176	111	172
	130	186	89	192	111	167
	136	179	102	179	103	165
	117	179	133	179	109	187
	107	172	91	170	134	181
	117	174	109	177	112	177
	131	173	131	160	120	180
	138	174	115	166	117	190
	130	175	87	175	128	172

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	31	188	64	176	31	188
	56	190	99	168	38	189
	33	195	123	174	32	188
	68	186	115	180	63	180
	74	190	153	171	28	184
	43	180	104	191	37	183
	44	194	120	197	47	180
	31	181	125	194	68	181
	40	197	95	190	31	183
	31	193	118	174	38	186

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	57	189	89	177	95	166
	69	183	89	170	85	168
	68	194	112	189	81	175
	73	195	102	186	98	174
	63	201	91	151	88	177
	69	191	64	185	98	171
	89	186	109	185	104	170
	73	195	86	181	117	175
	65	190	81	166	111	174
	95	189	113	179	93	172

Slide 5	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
107	176	78	188	119	173	
113	184	59	183	139	178	
94	174	117	179	129	179	
106	181	120	179	147	178	
119	178	125	188	152	182	
110	181	127	183	86	180	
109	164	91	177	96	180	
114	185	98	186	108	179	
95	167	97	182	124	179	
90	173	81	177	117	178	

Triad 3

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
80	209	55	153	124	184
34	210	78	174	110	185
41	193	66	164	103	183
67	204	85	164	142	179
79	196	79	167	124	169
67	178	85	167	99	171
55	194	67	169	110	179
49	198	77	168	104	169
72	184	44	178	122	172
56	156	64	148	115	186

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
46	169	46	171	68	169
55	167	52	166	80	188
70	160	50	144	83	174
85	151	51	169	40	168
78	149	62	151	69	165
73	169	83	182	77	179
94	179	55	154	63	184
61	173	92	182	96	167
87	161	66	157	79	155
81	174	79	160	66	170

Slide 3

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
32	152	59	176	30	174
46	171	51	153	113	178
52	167	68	173	32	148
39	171	66	152	40	150
29	167	67	157	75	158
60	178	77	149	46	163
45	162	48	149	39	182
51	173	59	161	29	166
34	175	60	167	35	174
34	163	46	166	34	177

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
43	148	60	182	70	177
37	154	59	173	90	179
64	150	83	183	104	177
79	161	49	181	101	177
56	139	79	181	112	173
52	129	65	175	107	175
68	163	65	176	114	181
74	158	85	170	89	183
61	157	57	178	84	191
59	150	28	178	75	174

Triad 4

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
90	187	66	204	49	210
107	179	56	207	35	209
94	195	57	202	34	197
114	190	61	207	53	186
107	208	54	207	53	203
92	192	82	201	53	206
83	191	58	188	41	187
89	204	53	207	33	189
85	194	97	195	72	210
79	203	48	202	90	194

Slide 2

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
41	178	76	167	63	184
83	173	80	164	85	182
78	179	78	166	82	191
51	167	101	183	78	186
31	175	107	176	70	176
27	175	79	173	91	182
45	176	67	184	63	183
34	183	69	174	60	185
50	192	65	181	80	184
42	184	83	180	124	178

Slide 3

CCKB	Background	CCKB	Background	CCKB	Background
108	179	52	180	58	175
91	192	85	187	67	166
129	194	67	195	98	173
118	211	72	199	84	183
113	185	68	196	99	189
160	160	69	194	94	193
101	159	83	203	65	176
111	189	71	197	84	168
94	211	80	194	112	172
137	182	67	186	48	175

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
		37	163	43	196
		57	191	57	173
		67	160	91	200
		75	132	70	189
		58	185	52	205
		71	177	54	205
		53	178	63	202
		47	164	80	203
		68	151	93	208
		57	163	55	200

Triad 5

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
		63	163	33	147	
		35	146	57	129	
		42	136	57	153	
		45	112	42	146	
		53	125	65	144	
		61	105	38	134	
		72	90	35	138	
		107	97	43	164	
		78	127	66	126	
		121	123	39	109	

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
55	137	64	134	74	176
71	137	168	109	121	165
55	139	56	134	103	154
64	165	56	87	85	171
73	166	51	120	85	162
83	147	62	105	91	161
46	174	61	126	96	123
53	139	26	108	75	182
43	151	61	121	53	183
49	197	92	105	44	167

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
31	195	55	85	49	140
72	180	47	63	74	150
74	188	57	82	68	125
86	174	35	73	66	128
32	168	63	79	46	147
111	185	64	95	61	161
69	161	99	102	93	150
54	175	87	58	69	114
30	173	51	77	78	119
30	158	26	175	68	130

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
40	67	50	137	103	168
87	91	86	167	89	164
66	93	65	156	89	184
58	133	120	169	54	182
62	114	85	153	78	157
77	88	160	154	75	183
108	120	77	142	115	177
49	136	80	171	61	161
52	124	91	127	84	183
58	119	147	152	81	188

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
64	101	39	122	71	186
106	88	96	146	78	137
42	121	46	85	40	124
85	79	127	149	76	130
82	132	116	114	69	156
95	126	81	159	61	185
74	98	108	126	42	167
47	123	156	131	52	163
82	126	147		60	157
68	142	99		54	147

Triad 6

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
75	144	111	137	74	94
83	158	69	163	76	151
140	153	93	156	41	147
72	142	61	163	50	133
64	150	75	165	32	131
128	222	74	156	86	184
139	192	68	166	31	159
92	218	85	167	63	154
91	133	74	144	53	138
139	193	67	161	56	123

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	109	101	60	153	70	110
	78	130	92	169	107	76
	128	119	139	146	65	105
	113	148	153	166	42	136
	100	181	78	156	48	148
	174	184	127	151	80	128
	116	155	97	133	72	107
	104	108	93	114	65	133
	81	116	95	139	67	99
	108	112	101	114	79	115

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	40	107	53	149	53	131
	75	180	52	167	47	141
	60	136	81	172	51	169
	65	151	133	153	57	153
	73	136	119	146	84	154
	65	129	55	167	67	147
	76	142	88	106	63	124
	103	137	61	116	54	161
	108	126	42	158	65	131
	60	122	67	133	41	87

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	72	213	64	133	60	123
	112	202	46	150	53	139
	62	197	56	134	79	90
	60	195	98	121	68	174
	62	183	62	135	60	131
	74	195	62	122	40	123
	104	194	54	139	50	127
	58	181	88	142	72	134
	41	195	94	119	61	161
	71	179	50	124	79	102

Slide 5	SA-H		Y-H		Y-S	
CCKB	Background		CCKB	Background	CCKB	Background
39	173		54	167	91	148
55	176		67	164	127	156
52	182		70	100	81	174
63	157		63	144	99	182
73	179		51	136	82	129
60	161		61	137	91	188
67	173		72	121	76	172
128	182		70	154	106	94
117	161		68	152	82	155
106	179		60	152	75	151

Optical Density of CCK_B in the Dentate Gyrus of 6 Triads
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
156	222	155	214	127	230	
136	220	159	211	162	235	
115	239	151	186	145	237	
132	225	151	189	134	218	
136	212	141	193	140	198	
116	210	143	175	140	208	
90	219	126	171	139	211	
73	211	122	168	140	182	
124	210	121	164	108	197	
173	211	130	159	126	187	

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
93	167	122	183	138	197
117	179	120	179	161	192
118	189	111	180	167	186
118	224	103	180	152	192
132	224	135	193	164	172
122	210	136	195	157	185
148	211	105	194	163	195
142	205	100	178	150	160
134	204	125	186	163	197
164	199	106	189	160	211

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
105	151	122	190	128	189
145	179	156	195	155	174
139	178	139	203	145	165
156	188	100	191	128	145
145	180	167	204	120	150
144	207	153	185	125	153
137	210	153	193	143	153
94	198	152	216	127	155
109	194	127	217	126	157
99	175	152	206	105	165

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
111	197	110	189	108	157
116	181	128	168	131	150
95	177	134	193	121	146
125	205	98	181	107	137
121	195	115	164	74	133
118	215	145	155	119	132
116	197	122	170	119	142
123	187	107	179	105	124
122	188	125	174	92	137
160	178	107	167	108	145

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
99	140	138	172	171	210
117	145	137	162	131	202
117	162	127	160	160	191
114	165	126	169	161	185
86	163	130	171	219	194
99	161	148	169	194	195
83	145	142	158	164	197
106	175	118	170	150	192
59	166	138	154	128	181
51	165	108	152	147	187

Triad 2

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
48	192	50	195	83	176
86	187	63	210	66	182
85	164	59	195	66	173
64	215	76	205	83	167
43	212	95	194	44	179
41	178	76	198	55	175
30	185	70	205	67	174
37	201	72	214	52	190
39	199	58	216	71	177
34	195	82	203	96	179

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	102	199	65	143	78	156
	100	188	82	148	102	152
	83	190	61	135	90	125
	75	180	65	166	110	167
	77	174	75	167	103	169
	92	193	57	152	109	147
	97	178	65	169	108	141
	96	163	87	150	100	144
	117	188	50	164	109	167
	104	195	50	164	75	189

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	68	169	52	144	81	181
	40	167	66	166	87	185
	53	156	40	159	81	176
	63	151	65	167	101	210
	44	161	79	141	57	177
	67	172	61	122	102	187
	39	149	38	151	60	186
	42	144	43	167	85	181
	50	158	34	130	97	193
	41	149	46	127	88	178

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	96	174	52	127	35	146
	94	170	43	121	36	150
	82	172	60	137	120	162
	80	162	61	116	98	176
	82	174	83	147	127	182
	90	164	48	161	91	189
	67	174	52	139	90	169
	54	198	43	125	104	181
	59	191	72	125	56	179
	73	188	82	132	94	184

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
107	204	69	165	100	189
108	185	51	172	78	176
140	172	84	188	64	171
106	174	91	181	143	170
118	167	96	177	119	164
162	174	103	172	138	188
140	172	84	164	126	182
121	169	61	161	116	188
122	168	77	171	149	202
114	169	92	165	146	196

Triad 3

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
29	167	58	168	72	192
61	132	50	172	103	167
33	117	55	170	76	190
42	133	25	158	78	180
59	151	82	140	91	178
33	136	74	163	92	177
31	145	53	165	73	189
94	119	67	149	106	197
35	179	50	159	119	158
44	186	55	170	84	157

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
51	143	37	161	62	145
80	147	36	172	36	160
102	148	28	170	40	155
70	130	62	165	64	145
76	155	31	155	64	155
37	155	27	150	102	157
77	141	99	173	96	160
77	143	52	126	85	159
51	164	37	155	58	166
70	158	30	150	45	165

Slide 3	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
		54	157	29	133	
		71	177	39	125	
		26	160	41	139	
		31	145	28	127	
		51	135	39	143	
		47	142	53	110	
		43	160	47	118	
		52	148	45	95	
		56	143	33	84	
		27	143	37	118	

Slide 5					
CCKB	Background	CCKB	Background	CCKB	Background
143	165	53	169	79	165
67	164	32	158	66	177
117	166	43	161	67	155
104	116	32	100	68	169
102	182	58	164	91	151
98	129	32	139	77	162
88	118	59	168	91	162
138	97	60	172	72	163
75	132	55	171	61	146
72	133	62	160	97	123

Triad 4

Slide 1					
CCKB	Background	CCKB	Background	CCKB	Background
52	129	91	157	31	132
51	149	87	176	41	158
61	148	67	159	32	125
65	152	67	185	52	144
70	138	73	167	40	93
62	159	81	173	38	106
66	155	47	177	30	106
76	147	39	155	47	112
73	142	69	130	39	125
53	152	74	155	31	141

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	90	195	119	176	99	111
	83	190	76	149	109	134
	97	184	95	159	108	147
	103	171	83	144	84	158
	97	173	119	141	100	156
	87	171	81	131	75	139
	91	191	65	161	93	128
	98	170	79	160	74	174
	75	169	106	148	76	176
	89	177	91	158	77	165

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	60	159	70	138	92	186
	75	143	82	156	105	170
	86	133	76	150	97	164
	115	130	79	119	77	165
	85	128	96	162	71	156
	111	110	95	159	78	169
	61	130	78	130	70	167
	75	131	86	104	88	173
	55	135	59	144	65	176
	84	141	104	161	79	142

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
			73	165	95	182
			76	162	93	186
			88	144	79	166
			70	158	91	171
			67	129	79	141
			55	164	65	118
			32	168	61	177
			38	125	57	151
			34	135	71	146
			32	120	57	118

Slide 5	SA-H		Y-H		Y-S	
CCKB	Background	CCKB		Background	CCKB	Background
			55	124	63	137
			65	148	79	139
			70	164	75	125
			46	160	67	127
			58	105	61	128
			70	126	72	147
			67	122	78	160
			70	106	57	157
			46	130	79	123
			47	152	74	106

Triad 5

Slide 1

CCKB	Background	CCKB		Background	CCKB	Background
			35	119	25	96
			59	116	24	97
			26	149	22	126
			38	126	22	113
			27	142	22	97
			26	132	22	102
			34	135	23	139
			46	130	42	112
			33	142	31	101
			42	120	31	77

Slide 2

CCKB	Background	CCKB		Background	CCKB	Background
75	143		75	113	116	119
58	155		74	118	90	128
77	143		66	107	66	147
76	164		63	94	51	158
70	158		52	61	42	142
62	149		68	87	48	142
65	155		76	100	42	161
80	145		71	123	23	188
80	139		51	135	85	192
101	119		39	125	52	200

Slide 3

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
40	63	27	62	45	117
39	94	38	90	54	122
29	113	26	74	75	125
26	85	25	68	50	91
53	148	25	106	93	125
28	79	23	97	68	74
66	112	22	72	54	141
32	112	21	102	61	141
27	129	22	57	63	109
29	104	22	61	65	116

Slide 4

CCKB	Background	CCKB	Background	CCKB	Background
26	51	90	105	113	140
29	107	96	109	83	142
27	75	84	171	94	117
31	85	80	132	67	111
28	84	94	105	101	164
28	82	104	141	110	133
30	96	29	155	80	114
21	120	38	102	104	147
29	134	72	101	103	118
29	116	65	92	89	140

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
40	86	46	139	58	139
37	98	64	146	93	125
49	94	63	144	49	163
73	118	65	145	52	156
40	90	55	130	58	146
45	75	56	117	84	140
44	61	68	150	57	162
53	53	80	113	55	98
39	63	50	104	59	101
41	123	69	145	47	99

Triad 6

Slide 1	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
			35	119	25	96
	70	148	47	108	96	99
	80	136	65	175	101	149
	80	147	63	153	85	54
	76	105	76	158	77	146
	76	128	104	113	73	107
	104	138	76	138	33	172
	83	124	105	105	48	157
	103	117	68	109	41	148
	109	129	67	121	83	114

Slide 2						
	CCKB	Background	CCKB	Background	CCKB	Background
	122	153	67	133	139	178
	121	158	83	113	147	167
	96	124	77	141	124	198
	109	124	63	112	115	180
	99	134	43	96	96	176
	118	116	68	122	103	176
	112	133	41	84	98	163
	94	151	38	67	93	167
	119	147	38	91	109	149
	125	197	58	96	86	153

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	56	148	34	119	68	174
	42	186	47	96	86	184
	80	147	43	85	81	198
	82	100	47	152	80	145
	49	132	56	78	92	162
	64	132	42	102	79	146
	117	144	39	93	133	161
	106	109	48	104	111	153
	97	128	80	88	126	154
	80	130	54	78	119	177

Slide 4	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	110	147	99	143	133	203
	126	147	94	138	140	204
	113	140	98	140	140	197
	97	132	95	139	138	193
	100	134	93	136	135	196
	105	164	90	127	135	196
	97	177	92	120	126	183
	98	157	84	118	137	187
	100	143	83	120	131	207
	94	150	82	144	149	198

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	115	157	102	181	138	141
	118	166	101	168	122	155
	116	165	107	162	136	176
	92	148	110	157	131	158
	107	161	119	154	109	178
	107	148	123	165	135	190
	98	138	115	163	104	179
	105	150	117	158	112	170
	103	164	115	180	107	170
	102	157	118	178	118	167

Optical Density for CCK_B in the Olfactory Tubercule
(5 slides per triad; 10 counts per section)

Triad 1

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background	
181	198	183	205	167	199	
174	203	179	204	173	202	
172	203	174	201	144	198	
171	205	177	206	144	203	
176	204	169	201	154	207	
175	206	179	201	157	206	
178	205	181	202	164	205	
181	204	178	199	167	202	
187	205	170	200	174	199	
167	204	180	198	167	198	

Slide 2					
CCKB	Background	CCKB	Background	CCKB	Background
151	195	148	203	160	197
156	199	179	205	147	198
168	204	187	209	161	200
148	206	155	208	172	200
155	207	177	207	166	200
162	207	177	217	172	203
153	206	162	216	169	209
155	199	171	214	143	205
166	199	166	215	165	204
162	198	146	214	150	201

Slide 3					
CCKB	Background	CCKB	Background	CCKB	Background
154	197	163	211	148	203
149	205	158	210	168	203
149	213	154	210	148	204
155	211	156	210	163	205
139	205	165	208	171	207
161	204	166	212	153	205
165	201	150	210	143	203
170	198	155	209	155	201
172	209	153	209	155	207
164	204	170	207	160	207

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
183	201	177	208	170	200
186	204	166	207	163	200
183	206	168	205	166	200
172	205	172	205	173	207
162	204	162	203	174	208
188	207	148	202	176	208
181	210	184	199	164	210
175	208	159	206	185	208
190	204	160	209	161	205
198	204	185	208	164	203

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
184	200	151	210	162	198
174	201	152	209	160	197
177	199	150	208	149	195
185	198	147	207	153	196
174	196	155	210	166	201
180	192	149	207	165	202
153	191	145	208	153	201
146	190	156	208	161	202
152	191	165	205	162	199
144	189	160	204	159	195

Triad 2

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
128	192	108	175	129	201
126	190	116	187	143	206
119	189	116	178	122	203
132	190	114	178	137	200
123	190	111	209	121	203
135	193	123	197	139	200
137	189	112	193	146	188
128	181	118	199	136	181
126	168	115	196	138	185
134	191	105	185	153	183

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	146	200	139	195	148	193
	143	193	132	195	153	196
	134	199	132	188	141	188
	133	198	150	185	149	194
	129	196	159	195	152	196
	137	200	144	188	152	195
	139	194	149	199	147	180
	147	195	142	187	145	197
	139	196	138	192	139	190
	140	193	149	181	163	189

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	141	203	138	192	126	188
	140	194	138	195	125	183
	149	199	142	182	109	189
	141	199	162	194	130	181
	147	200	153	186	176	183
	146	196	117	190	153	195
	149	197	155	193	155	192
	160	197	139	192	163	195
	143	203	148	185	169	191
	136	198	148	184	161	206

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	125	178	118	192	128	209
	124	208	132	192	151	204
	157	206	146	196	142	207
	137	220	131	177	143	198
	146	216	126	194	164	209
	140	201	131	183	163	202
	153	180	119	163	156	199
	144	198	150	155	161	201
	154	207	128	194	144	210
	141	205	129	212	135	204

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
139	195	122	183	134	190
140	184	128	185	151	188
144	182	123	194	152	188
145	184	134	192	171	194
150	188	122	190	159	194
160	187	132	190	156	196
143	179	127	193	161	198
149	184	150	185	123	196
165	192	137	189	153	193
145	178	133	189	139	194

Triad 3

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
109	180	137	193	145	204
113	187	142	185	147	196
113	182	132	185	153	200
118	178	139	193	147	201
117	173	127	185	142	200
103	189	111	202	143	198
125	191	114	196	137	212
128	193	123	196	160	200
106	176	117	191	150	198
110	197	110	194	143	213

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
110	196	105	167	114	204
111	189	126	169	146	192
114	182	117	164	113	183
115	192	139	185	122	189
106	189	108	158	121	199
119	190	117	148	108	198
120	185	121	161	114	211
112	183	112	151	104	204
126	202	125	165	108	209
116	189	123	140	107	192

Slide 3	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	106	182	95	155	119	174
	124	178	105	155	130	178
	105	171	102	164	128	172
	108	165	93	167	121	167
	105	162	104	170	114	180
	119	158	98	165	117	171
	119	170	113	170	121	171
	110	184	105	163	129	166
	108	195	109	158	118	155
	112	201	120	169	130	167

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	125	183	128	188	139	191
	114	178	122	183	146	198
	98	176	119	169	162	199
	119	181	126	192	155	192
	126	175	118	182	116	197
	107	186	119	158	130	182
	131	185	128	179	143	182
	106	182	124	178	134	175
	113	167	121	193	129	204
	125	189	133	186	126	182

Slide 5						
	CCKB	Background	CCKB	Background	CCKB	Background
	129	188			145	189
	128	174			164	187
	134	158			164	191
	123	175			161	195
	142	169			160	200
	121	169			142	196
	129	173			165	195
	134	175			161	194
	130	168			189	194
	129	155			161	193

Triad 4

Slide 1	SA-H		Y-H		Y-S	
CCKB	Background		CCKB	Background	CCKB	Background
			150	186	164	197
			144	189	173	188
			149	185	157	188
			150	177	150	193
			137	191	152	195
			150	194	148	192
			143	183	170	187
			147	184	174	192
			152	185	170	198
			136	193	166	198

Slide 2						
CCKB	Background		CCKB	Background	CCKB	Background
169	199		154	182	144	190
170	195		170	194	146	184
163	197		154	183	153	194
151	201		157	182	147	177
149	197		159	187	163	175
150	196		167	187	158	187
180	203		157	195	155	179
176	186		166	176	144	185
162	189		153	194	154	178
169	192		166	205	151	182

Slide 3						
CCKB	Background		CCKB	Background	CCKB	Background
149	198		125	184	140	173
139	174		129	200	145	181
134	177		129	194	162	185
129	203		129	184	143	191
149	195		130	194	160	186
144	201		143	189	131	187
138	198		129	188	146	188
150	183		132	173	141	182
130	162		135	183	168	198
142	192		130	190	155	191

Slide 4

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
192	205	150	180	131	180
191	195	131	196	132	186
159	203	154	199	135	182
154	197	128	177	131	187
182	202	146	157	132	181
159	195	157	158	139	186
177	194	129	173	137	184
166	187	127	184	170	174
177	196	131	172	138	167
179	198	129	165	145	176

Slide 5

CCKB	Background	CCKB	Background	CCKB	Background
137	198	128	176	158	187
130	192	128	177	155	188
137	191	124	159	153	202
139	189	132	181	150	203
145	184	125	185	150	207
174	184	125	173	161	208
145	208	142	186	158	215
121	196	134	165	166	215
117	175	129	172	157	219
155	189	133	184	155	219

Triad 5

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
147	193	160	193	123	202
149	187	152	194	140	167
151	188	150	192	137	203
150	200	147	195	135	210
140	187	139	183	147	206
142	189	139	201	141	194
143	193	149	216	145	205
146	192	119	211	137	195
140	190	144	214	139	177
145	195	132	211	145	198

Slide 2	SA-H		Y-H		Y-S	
	CCKB	Background	CCKB	Background	CCKB	Background
	176	210	175	212	138	201
	174	200	168	207	165	181
	178	200	167	210	140	190
	178	206	161	207	146	195
	175	200	164	204	127	194
	184	204	149	190	147	192
	181	208	146	200	156	200
	161	199	161	189	151	207
	174	188	151	186	154	194
	176	183	150	197	153	193

Slide 3						
	CCKB	Background	CCKB	Background	CCKB	Background
	169	196	130	179	141	200
	184	202	147	168	146	193
	158	210	142	145	153	198
	167	204	133	159	123	190
	171	201	136	167	147	206
	178	200	144	176	150	195
	176	201	150	184	147	192
	173	204	162	151	151	201
	175	200	163	176	139	182
	172	206	136	187	154	203

Slide 4						
	CCKB	Background	CCKB	Background	CCKB	Background
	144	192	150	189	138	187
	165	196	132	209	139	170
	157	192	141	184	141	194
	170	192	154	208	142	181
	165	189	144	209	141	182
	161	194	147	208	131	187
	145	190	146	193	136	182
	159	197	132	203	127	183
	161	197	138	195	139	194
	159	191	144	198	144	193

Slide 5

SA-H		Y-H		Y-S	
CCKB	Background	CCKB	Background	CCKB	Background
140	186	146	215	124	203
147	182	158	218	128	208
158	193	147	215	152	212
147	198	141	206	154	216
153	191	155	209	157	207
155	190	147	207	143	207
150	188	155	205	153	192
159	196	168	199	148	206
153	186	162	197	143	205
162	199	166	195	150	211

Triad 6

Slide 1

CCKB	Background	CCKB	Background	CCKB	Background
162	196	150	163	141	202
190	199	128	154	141	208
165	187	128	138	140	171
179	201	121	147	132	182
169	195	132	173	136	175
171	198	139	184	131	181
163	186	124	173	150	190
169	192	125	158	157	178
172	185	141	161	153	176
176	191	129	162	136	179

Slide 2

CCKB	Background	CCKB	Background	CCKB	Background
152	189	147	232	155	214
156	186	133	193	169	212
157	187	145	181	153	207
168	161	150	181	160	207
154	182	150	192	132	221
153	176	155	166	147	215
152	189	141	168	148	216
148	165	168	165	149	216
157	180	134	175	147	204
154	167	154	171	157	198

Slide 3	SA-H		Y-H		Y-S	
CCKB		Background	CCKB	Background	CCKB	Background
	151	191	104	158	136	189
	132	195	129	172	147	168
	145	192	119	157	138	170
	147	182	118	170	144	174
	157	197	115	187	143	165
	152	193	120	166	135	171
	154	201	116	179	133	173
	153	195	115	161	138	186
	145	201	107	165	126	180
	171	196	111	160	135	170

Slide 4						
CCKB		Background	CCKB	Background	CCKB	Background
	149	164	135	157	138	180
	143	165	153	172	151	179
	154	165	145	188	134	175
	143	170	139	187	147	176
	138	167	151	181	150	190
	147	169	136	190	167	181
	178	163	143	176	162	190
	171	164	144	176	151	184
	166	162	139	174	162	196
	167	181	142	184	160	190

Slide 5						
CCKB		Background	CCKB	Background	CCKB	Background
	143	190	141	193	148	213
	146	193	141	182	146	208
	157	206	141	209	140	206
	155	202	122	204	146	216
	178	199	157	188	143	213
	146	199	149	196	142	210
	144	197	146	208	149	212
	150	191	152	216	154	200
	152	202	137	206	133	208
	138	205	121	198	156	217

Chapter 4
Ataxia Data (Pre- and Post Slip Angles)

Session 1	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad 1	SA-H	35	36	1	2
CRF	Y-H	41	41	0	
	Y-S	35	32	-3	
Triad 2	SA-H	42	45	3	5
CRF	Y-H	42	45	3	
	Y-S	40	41	1	
Triad 3	SA-H	42	45	3	15
CRF	Y-H	41	35	-6	
	Y-S	45	49	4	
Triad 4	SA-H	36	34	-2	7
CRF	Y-H	40	38	-2	
	Y-S	38	41	3	
Triad 5	SA-H	42	40	-2	2
CRF	Y-H	45	33	-12	
	Y-S	47	38	-9	
Triad 6	SA-H	42	36	-6	8
CRF	Y-H	38	30	-8	
	Y-S	43	35	-8	

Session 2	Group	Pre-slip	Post slip	Difference	# of Infusions
Triad 1	SA-H	46	52	6	2
CRF	Y-H	43	42	-1	
	Y-S	55	53	-2	
Triad 2	SA-H	40	55	15	4
CRF	Y-H	37	32	-5	
	Y-S	45	44	-1	
Triad 3	SA-H	39	46	7	15
CRF	Y-H	42	41	-1	
	Y-S	50	48	-2	
Triad 4	SA-H	37	40	3	5
CRF	Y-H	42	40	-2	
	Y-S	42	38	-4	
Triad 5	SA-H	37	42	5	9
CRF	Y-H	35	31	-4	
	Y-S	45	43	-2	
Triad 6	SA-H	42	43	1	10
CRF	Y-H	40	33	-7	
	Y-S	42	36	6	

Session 3	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	48	55	7	3
CRF	Y-H	40	31	-11	
	Y-S	53	55	2	
Triad2	SA-H	46	55	9	4
CRF	Y-H	32	45	13	
	Y-S	45	50	5	
Triad3	SA-H	49	49	0	15
CRF	Y-H	41	41	0	
	Y-S	47	47	0	
Triad4	SA-H	38	45	7	3
CRF	Y-H	48	43	-5	
	Y-S	40	37	-3	
Triad5	SA-H	40	45	5	3
CRF	Y-H	39	40	1	
	Y-S	33	42	9	
Triad6	SA-H	47	53	6	19
CRF	Y-H	42	33	-9	
	Y-S	38	32	-6	

Session 4	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	48	55	7	3
CRF	Y-H	45	45	0	
	Y-S	45	53	8	
Triad2	SA-H	40	58	18	9
CRF	Y-H	49	45	-4	
	Y-S	45	44	-1	
Triad3	SA-H	39	47	8	15
CRF	Y-H	42	41	-1	
	Y-S	41	45	4	
Triad4	SA-H	35	44	9	4
CRF	Y-H	40	44	4	
	Y-S	40	47	7	
Triad5	SA-H	30	48	18	5
CRF	Y-H	40	39	-1	
	Y-S	47	45	-2	
Triad6	SA-H	40	51	11	18
FR3	Y-H	38	42	4	
	Y-S	42	34	-8	

Session 5	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	45	55	10	3
CRF	Y-H	40	40	0	
	Y-S	55	50	-5	
Triad2	SA-H	49	57	8	6
CRF	Y-H	46	46	0	
	Y-S	45	43	-2	
Triad3	SA-H	45	50	5	14
CRF	Y-H	47	51	4	
	Y-S	47	50	3	
Triad4	SA-H	42	43	1	7
CRF	Y-H	41	45	4	
	Y-S	40	43	3	
Triad5	SA-H	43	49	6	4
CRF	Y-H	53	42	-13	
	Y-S	52	42	-12	
Triad6	SA-H	43	49	6	13
FR3	Y-H	33	36	3	
	Y-S	30	35	5	

Session 6	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	50	56	6	3
CRF	Y-H	49	37	-12	
	Y-S	49	45	-4	
Triad2	SA-H	47	61	14	6
CRF	Y-H	49	51	2	
	Y-S	35	49	14	
Triad3	SA-H	45	47	2	37
FR3	Y-H	40	34	-6	
	Y-S	50	49	-1	
Triad4	SA-H	40	45	5	20
FR3	Y-H	52	40	-12	
	Y-S	53	55	2	
Triad5	SA-H	45	48	3	15
FR3	Y-H	35	35	0	
	Y-S	45	45	0	
Triad6	SA-H	43	46	3	27
FR6	Y-H	35	40	5	
	Y-S	33	33	0	

Session 7	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	38	53	15	2
CRF	H-Y	40	41	1	
	S-Y	45	49	4	
Triad2	SA-H	36	57	21	2
FR3	H-Y	35	43	8	
	S-Y	40	50	10	
Triad3	SA-H	40	53	13	36
FR3	H-Y	43	40	-3	
	S-Y	41	49	9	
Triad4	SA-H	39	42	3	10
FR3	H-Y	40	50	10	
	S-Y	47	45	-2	
Triad5	SA-H	47	50	3	9
FR3	H-Y	46	45	-1	
	S-Y	45	48	3	
Triad6	SA-H	47	48	1	23
FR6	H-Y	40	40	0	
	S-Y	37	38	1	

Session 8	Group	Pre-slip	Post-slip	Difference	# of Infusions
Triad1	SA-H	40	60	20	4
CRF	H-Y	41	40	-1	
	S-Y	45	48	3	
Triad2	SA-H	45	63	18	5
FR3	H-Y	43	40	-3	
	S-Y	35	40	5	
Triad3	SA-H	43	42	-1	33
FR3	H-Y	41	36	-5	
	S-Y	46	45	-1	
Triad4	SA-H	47	47	0	12
FR3	H-Y	47	47	0	
	S-Y	40	43	3	
Triad5	SA-H	42	48	6	13
FR3	H-Y	50	40	-10	
	S-Y	35	45	10	
Triad6	SA-H	38	43	5	37
FR6	H-Y	36	44	8	
	S-Y	37	37	0	