ASSOCIATIVE TOLERANCE TO NICOTINE'S ANALGESIC EFFECTS:

STUDIES ON NUMBER OF CONDITIONING TRIALS AND CORTICOSTERONE

A Thesis

by

KRISTINA DAVIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2003

Major Subject: Psychology

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Approved as to style and content by:

Antonio Cepeda-Benito (Chair of Committee)	Thomas Champney (Member)
Paul Wellman (Member)	Steve Rholes (Department Head)

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ABSTRACT

Associative Tolerance to Nicotine's Analgesic Effects: Studies on Number of Conditioning Trials and Corticosterone. (August 2003)

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This study examined the number of conditioning trials necessary to produce associative nicotine tolerance and the changes in corticosterone levels during the procedures. Six independent groups of rats (N = 355) were run through tolerance acquisition procedures for 1, 5, or 10 conditioning sessions. Treatment groups were comprised of animals that received nicotine-environment pairings, animals that received nicotine explicitly unpaired with the drug administration environment, and control groups that received either saline throughout or no treatment. Three of the groups were tested for nicotine-induced analgesia using the tail-flick and hot-plate assays, and three groups were blood sampled after either nicotine or saline injection. Pairing of environment with nicotine produced greater tolerance for rats after 5 conditioning sessions in the tail flick and after 10 conditioning sessions in the hot-plate. Corticosterone levels were elevated in all rats given nicotine. Rats that received the nicotine-environment pairing showed a conditioned release of corticosterone in response to the environment after both 5 and 10 conditioning sessions.

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INTRODUCTION

Drug tolerance (defined as a decrease in a drug's dose effects over continued exposure) is implicated as a key step in drug dependence and addiction (Ramsay and Woods, 1997). Research to elucidate the mechanisms, both physiological and psychological, that control tolerance may, by extension, further the understanding of drug addiction.

Use of tobacco products is widespread, and addiction to nicotine found in tobacco products is well documented. Even in light of serious health risks associated with tobacco use and the fact that most smokers want to quit, becoming a nonsmoker is very difficult. Although smokers become chemically dependent on nicotine, the psychological dependence or reliance on smoking appears to play an important role in smoking and smoking relapse (Cepeda-Benito, 1993). Tolerance may be a key component of nicotine addiction, and associative or conditioned tolerance may play a large in role in human nicotine use. The psychological component of nicotine tolerance seems to be largely due to environmental stimuli that become associated with administration of the drug (Caggiula et al. 1989; Cepeda-Benito et al. 2000; Epstein et al. 1989). Therefore, an important step in understanding the nature of the psychological addiction to nicotine is to first understand the ways in which tolerance may be mediated by environmental cues.

This thesis follows the style of Psychopharmacology.

Previous work, which explored associative tolerance using morphine, investigated the analgesic effects of this drug (Cepeda-Benito and Tiffany 1992; 1996a; 1996b). Nicotine also produces analgesia, therefore, looking at nicotine analgesia, which is an easily measurable effect, enables the investigation of associative tolerance to nicotine. Tolerance to nicotine's analgesic effects is greater in animals that receive nicotine explicitly paired with a specific environment than in animals that receive the same amount of nicotine and exposure to the environment but with the environment and the nicotine explicitly unpaired (e.g., Cepeda-Benito et al. 1998). This contextual effect has been observed also in the development of tolerance to other effects of nicotine, such as nicotine's anorectic effects and nicotine-induced corticosterone (CORT) release (Caggiula et al. 1991). It has been proposed that this associative tolerance occurs through classical conditioning that involves pairing drug administration cues with drug effects, so that physiological mechanisms react "in expectancy" of a drug dose when presented with drug administration cues.

Caggiula and others (1993) compared CORT levels in rats that had received nicotine explicitly paired with a distinctive context with the CORT levels of nicotine-naïve rats never exposed to the distinctive context. These authors took blood samples for CORT assays after exposing both groups to saline injections in the distinctive context. CORT levels were higher in nicotine/context-experienced rats than in nicotine treated rats that were exposed to the distinctive context for the first time on test day. Thus, these authors suggest that the distinctive context functioned as a conditioned stimulus (CS) that elicited a CORT

release or conditioned response (CR) that mimicked the unconditioned response (UR) or nicotine-induced CORT release.

However, Caggiula and colleagues (1991) reported that rats that received repeated nicotine injections in a distinctive environment developed conditioned tolerance to the CORT elevating effects of nicotine. That is, administration of nicotine in a predictive environment resulted in lower CORT release than administration of nicotine in a novel environment. These results could not be explained as stress-induced CORT release by the novel environment because injecting rats with either saline or nicotine in a novel environment did not elevate CORT levels with respect to corresponding controls that were injected in a familiar environment. Thus, Caggiula and colleagues interpreted their findings as evidence that the distinctive context environment was a conditioned stimulus that supported a reduction in CORT release, or that the conditioned response was opposite in direction to the unconditioned response of nicotine induced CORT release. Thus, the findings by Caggiula and colleagues (1991) and the findings by Caggiula and colleagues (1993) appear to contradict each other and warrant further examination of the role of classical conditioning in supporting conditioned-induction and conditioned-suppression of CORT release by an environment associated with repeated administration of nicotine.

Nonetheless, the idea that endocrine responses, especially CORT release, are implicated as mechanisms that affect the physiological reactions (and subsequently behavioral responses) to nicotine is a sound one. With acute doses of nicotine in rats, CORT levels have been shown to rise and stay elevated

for at least some time after injection. Cam and Bassett (1983) found significant increases in CORT starting at 5 min post-injection, and these elevated levels were sustained for 45 min post-injection, with a peak at 20 min post-injection. The initial increase in CORT over the first five minutes post-injection has been shown to follow the increasing plasma nicotine levels over this same time period, however, nicotine plasma levels begin to decline, while CORT levels stay elevated for at least 30 min post-injection (Cam et al. 1979) and up to 1 hour (Weidenfeld 1989; Mellon and Bayer, 1999). In addition, Pauly, Grun, and Collins (1992) purport that elevations of CORT and decrements to nicotine sensitivity do not follow a simple linear relationship.

Chronic nicotine administrations can produce a decrease in the CORT-elevating response to nicotine (tolerance to the CORT-elevating effects). Pauly and others (1992) found that mice given three daily nicotine injections over a 12 day period showed sustained elevated CORT levels. An acute nicotine challenge produced only small increases in CORT while saline treated animals given the same acute challenge showed larger CORT increases. The time course for reduced response in CORT to repeated nicotine exposure has been studied, however, different types of exposure were necessary in each study to see this decrease. Cam and Bassett (1984) found that CORT returned to baseline levels after 30 days of nicotine administration, whereas CORT levels returned to baseline levels as of the 5th treatment day in a study by Benwell and Balfour (1979). These findings indicate that there is a development of tolerance

to the CORT elevating effects of nicotine (nicotine administration no longer produces CORT increases of the same magnitude as the initial exposure).

The most compelling evidence for the role of CORT in the development of nicotine tolerance can be found in adrenalectomy (ADX) studies. That is, ADX has been shown to increase sensitivity to nicotine's effects on physiological (heart rate, body temperature, acoustic startle) and behavioral (locomotion, rearing) systems (Pauly et al. 1988). Moreover, these authors showed that CORT replacement reversed the ADX influence on nicotine's effects, and CORT administrations to intact animals produced subsensitivity to nicotine. Grun, Pauly, and Collins (1992) reported that while chronically nicotine-injected animals became tolerant to the effects of nicotine, tolerance to nicotine was not detected in animals that had been adrenalectomized. Grun, Pauly, Collins. (1992) also found that plasma CORT levels were significantly more elevated in mice repeatedly injected with nicotine than in mice injected with saline. Moreover, Johnson and colleagues (1995) found that ADX prevented sensitization to the locomotor increasing effects of nicotine, and *chronic* CORT replacement reinstated the nicotine sensitization effects commonly seen in intact animals.

Thus, researchers have proposed that conditioned activation of the HPA system and conditioned release of corticosterone may mediate the development of associative nicotine tolerance (Pauly et al. 1992; Caggiula et al. 1993; 1995). While circulating levels of corticosterone have been found to correlate with magnitude of nicotine tolerance, environmental cues associated with nicotine

delivery may elicit a conditioned corticosterone response (Pauly et al. 1992; Buske-Kirschbaum et al. 1996).

The role of CORT in nicotine tolerance is not clear, particularly with regards to cue-dependent tolerance development. For example, saline injections and handling procedures alone can elevate circulating CORT (Pauly et al. 1992). Peck, Disalver, and McGee (1991) demonstrated a reduction in nicotine sensitivity following a period of chronic stress. Therefore, CORT changes appear to be differential depending on the experimental parameters of the drug administration. Additionally, classical conditioning paradigms not involving drug administration have also been shown to influence endocrine function, and this association with endocrine systems is implicated as important for the learning process. More specifically, a conditioned CORT release has been shown in classical CS-US pairings after learning has occurred (Tomie et al. 2002), and, in a taste-aversion paradigm, a conditioned CORT release was seen in animals that received this conditioning (Ader 1976). In addition, a peppermint odor associated with the administration of nicotine was shown to increase CORT levels when the odor was presented alone (Buske-Kirschbaum et al. 1996). This indicates that drug effects can act as part of the CS-US pairings in classical conditioning, and that, even without drug responses, CORT levels can increase in response to situations that produce conditioned responses.

The influences of stress, handling, prolonged exposure to nicotine, and conditioning mechanisms may all lead to changes in CORT levels. The relationship between CORT and associative nicotine tolerance is difficult to

discern given the wide array of influences on the physiological systems governing this tolerance.

Hypotheses

The present research explored the effects of a number of conditioning sessions on the development of associative tolerance to the analgesic effects of nicotine and concurrent CORT level changes. First, the design tested for tolerance to nicotine-induced analgesia after 1, 5, and 10 conditioning sessions. Tolerance was expected to be greater in rats that received repeated administrations of nicotine explicitly paired with a distinctive test-context (DC rats) than in rats that received nicotine explicitly unpaired with the test-context (HC rats). The design also included a baseline control group that received equal exposure to the distinctive context but was injected with nicotine for the first time on testing day (SC rats).

In the second phase of the study, independent groups of DC, HC, and SC rats were also exposed to 1, 5, or 10 conditioning sessions, however, blood samples were taken in lieu of analgesia testing so that CORT levels after either nicotine or saline injection could be examined. It was hypothesized that DC rats would not develop tolerance to the CORT elevating effects of nicotine, but show a contextual release of CORT when injected with saline in the distinctive (drug-paired) context. That is, if the presence of CORT is needed for the development of tolerance to nicotine's analgesic effects (e.g. Caggiula et al. 1993) and reverses hypersensitivity to nicotine produced by ADX (Pauly et al. 1988), it seems unlikely that DC rats would concurrently develop *conditioned* tolerance to

nicotine's CORT elevating effects and conditioned excitation of CORT release. Thus, it was expected that CORT levels after nicotine administration would not be lower for DC rats than HC rats. That is, injecting animals with saline in the distinctive context would produce a CORT elevation in DC rats with respect to HC rats. Likewise, if chronic nicotine injections elevate circulating CORT levels, then greater CORT levels should be seen in HC vs. SC rats.

METHOD

Subjects

The subjects were 355 experimentally naïve, male, Sprague Dawley from Harlan (Houston, TX) rats (approximately 75 days old on test day). They were housed individually in plastic cages with a bed of wood shavings. A total of 216 and 139 rats were used for analgesia and CORT assays, respectively.

Drugs

Nicotine bitartrate was dissolved in physiological saline to produce a nicotine concentration of 1.0 mg/kg. This dose was used for all nicotine injections in all experiments. All nicotine and vehicle solutions were subcutaneously injected in the scruff of the neck (1.0 ml/kg of body weight).

Pre-Habituation Phase

After a week of acclimation to their colony room, the rats were weighed once daily for 3 days, weighed twice daily for an additional 3 days, and then weighed and injected with saline once daily for 8 days. All of these procedures took place in the colony room and were intended to reduce the discriminative salience and stress-inducing effects of injections and other handling procedures (see Cepeda-Benito and Tiffany 1996a).

Tail-Flick Analgesia Assessment (N = 108)

The tail-flick apparatus (IITC, Model 33B) measures the latency for the rat to remove its tail away from a hot beam of light (e.g., Cepeda-Benito and Tiffany 1992). The rat was restrained in an opaque cylinder (6.8 x 22 cm) that had a Plexiglas base (5.5 x 22 cm). Ventilation holes were made on top of and in the

front of the tube. The rat's tail protruded from the back of the tube and was placed in a grooved plate such that the tail was directly under the light source. When the rat moved its tail away from the light-beam, a photo-sensitive cell tripped a timer and the tail-flick latency was automatically recorded. To avoid interactions between tail area stimulated and degree of analgesia (Yoburn et al. 1984), each assessment was the mean of three consecutive trials with the location of the beam varied among the proximal, middle, and distal third of the rat's tail. The beam intensity was adjusted such that undrugged animals flicked their tails at about 4 s. A 15 s limit was used to prevent damage to the tail. The tester was blind to the rat's treatment condition.

Hot-Plate Analgesia Assessment (N = 108)

The hot-plate method measured a rat's latency to lick a paw or jump (e.g., Cepeda-Benito and Tiffany 1996b; Krank 1987). Rats were confined to the hot-plate's surface in a chamber (30 X 30 X 30 cm) with a clear Plexiglas lid. The hot-plate consisted of a metal surface, thermostatically controlled to a constant temperature of 52 C (ITC, Model 35D). Two observers, blind to the rat's treatment condition, timed to the nearest hundredth of a second each rat's latency to the either lick a paw or jump, whichever came first (e.g., Krank 1987). The response latency was the mean of the two observations. The median difference between observers was 1.25 s. Rats were removed from the hot plate as soon as both observers detected either a lick or a jump. Although the observers were aware of each other's timing, they were trained not to stop their watches unless they observed for themselves the rat s response. Animals that

neither licked a paw nor jumped after 60 s were removed from the apparatus to prevent tissue damage.

Blood Sampling Procedures (N = 139)

The blood was collected using the tail snip method: the tail was massaged for 6 to 8 sec and then a small portion of the tail *(about 1 mm*) was clipped off with a razor blade. Approximately 0.5 ml of blood was collected from the tail and centrifuged. Plasma samples were then frozen at –70 ^{o}C until assay. Corticosterone assay was performed using a COAT-A-COUNT solid-phase ^{125}I radioimmunoassay that showed linearity from 4.83 ng/ml (least detectable dose) to 1722.80 ng/ml with an average %CV of 4.42.

Tolerance Development and Testing

Following pre-habituation, each rat was given injections paired with a distinctive context and injections in its home cage environment. The interval between context exposures was 72 hrs and home cage injections were administered 24 and 48 hrs after each distinctive context exposure. For distinctive context exposures, each rat was weighed, individually carried in a small, plastic, bedding-lined container to the distinctive context room, injected with saline or a 1.00 mg/kg nicotine dose, put inside a tube, placed in a dark, scented cabinet, and mock-tested in either the tail-flick or the hot-plate device 4, 8, and 13 min after the injection. The distinctive-context room was set-up to be perceptually different from the colony area using visual, olfactory and auditory changes: the room's light was dimmed (visual), apple-cinnamon air fresheners scented the holding cabinet (olfactory), and white noise was continuously played

(auditory). Each mock tail-flick test consisted of placing the rat on the tail-flick apparatus and going through the motions of conducting three tail-flick tests (i.e., without aiming the light beam at the tail). For each mock hot-plate test, the rat was removed from its tube and placed in a nonfunctional hot plate for 45 s. After each mock test, the rat was returned to the scented cabinets. Rats were returned to their home cage environments 33 min after the last mock test. Rats not tested for analgesia but subjected to blood samplings on test day (CORT rats) were also exposed to tail-flick mock procedures. In addition, rats to be blood sampled had their tail massaged for a few seconds at 6 min after injection to mock blood collection procedures. For home cage injections, the rats were individually weighed, injected with either nicotine or saline, and returned to their home cage.

Animals tested for nicotine's analgesic effects were divided randomly into four conditioning groups, which were further divided into tail-flick and hot-plate assays. DC rats received nicotine in the distinctive context and saline injections in the home cage environment. HC rats received saline in the distinctive context, nicotine for the first and saline for the second home cage injection that followed each context exposure. That is, HC rats received as much nicotine and context exposure as DC rats, but these two stimuli were explicitly unpaired. SC rats received as much exposure to the distinctive context and handling manipulations as DC and HC rats, however, SC animals did not receive nicotine until the test day. A fourth group of animals was never exposed to the experimental procedures until test day (Naïve rats). Animals subjected to blood sampling procedures were randomly divided into DC, HC, and SC groups.

Tail-flick and hot-plate test sessions occurred in the distinctive context 72 hrs after the *first, fifth,* or *tenth* distinctive-context exposure. That is, independent groups of rats at each test session were injected with nicotine (1.00 mg/kg) and tested for analgesia in either the tail-flick or the hot plate in lieu of mock trials, at 4, 8 and 13 min after the injection. The experimental design for analgesia assessments was a 4 (DC, HC, SC and Naïve groups) X 2 (tail-flick and hot-plate test devices) X 3 (1, 5, and 10 tolerance development sessions) design with 9 animals per cell.

Blood sampling procedures occurred between 6 and 7 min after the injection (or 2 min after the first mock tail-flick test) in the distinctive context during the *first* and 72 h after the *fifth* and *tenth* distinctive context exposures, in lieu of blood sampling mock trials. For CORT assays, the design was a 2 (DC & HC groups) X 2 (test after saline or nicotine) X 2 (5 and 10 tolerance development sessions) design with 9 to 10 rats per cell. To examine the effects of handling and exposure procedures on CORT levels in nicotine naïve rats, six additional SC groups were tested with either nicotine or saline during the first, and after the fifth and tenth context exposure sessions.

RESULTS

Analgesia Assessments

The data collected within each test device (tail flick or hot plate) at each of the three test sessions (after 1, 5, or 10 conditioning cycles) were analyzed using repeated measures Analysis of Variance (ANOVA). For each of the six data sets, the between factor was Group (DC, HC, SC and Naïve) and the within factor variable was Time (4, 8, and 13 min post injection). To protect the analyses against violations of the assumption of sphericity, adjusted, Huynh-Feldt degrees of freedom were used for significance testing of within factor and within by between factor effects.

In the presence of significant between group effects, a *priori* planned, repeated, pair-wise contrasts compared the mean response latencies of Naïve vs. SC rats, SC vs. HC rats, and HC vs. DC rats. It was predicted that SC and HC animals would display significantly greater response latencies than HC and DC animals, respectively. Lastly, significant Group by Time interaction effects were followed *post hoc* by comparing HC and DC rats within each assessment time (i.e., at 4, 8, and 13 min post injection). The *p* significance level for *post hoc* comparisons was adjusted using the Bonferroni method (.05 ÷ 3) to $\alpha = .016$. Time and interaction effects were interpreted through visual inspection of latencies across testing and groups.

Tail-flick results. The analyses revealed significant and large Group effects at the tests conducted after the fifth, $\eta^2 = .542$, F (3, 32) = 12.62, $p < .001$, and tenth, $\eta^2 = .755$, F (3, 32) = 32.93, $p < .001$, sessions. Planned contrasts for

significant group effects indicated that DC rats responded with lower latencies than HC animals after both five, $p = .011$, and ten, p = .0001, sessions. However, HC rats were significantly faster than SC rats after five, $p = .047$, but not after ten, $p = .84$, sessions. Saline and Naïve animals did not differ from each other at any of the test sessions, all p's > .39 (see Table 1).

Table 1 Confidence intervals for analgesia tests. Tail-flick and Hot-plate measures for animals tested after 1, 5 for 10 conditioning sessions (1 mg/kg nicotine)

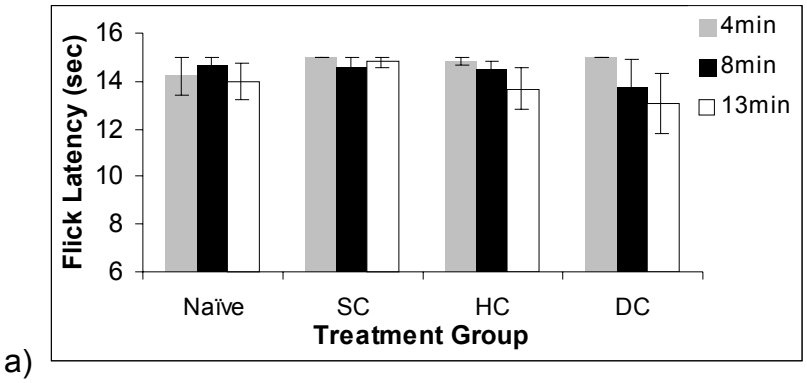
	Tail-Flick Contrasts (95% CI)		Hot-Plate Contrasts (95% CI)	
Session	DC – HC	HC – SC	DC – HC	HC – SC
First	(-)1.98 – 1.21	(-)2.07 – 1.12	(-).24 – 12.67	(-)11.24 – 1.67
Fifth	(-)4.25 – (-).60 *	(-)3.68 – (-).0028*	(-)13.65 – 5.52	(-)10.43 – 8.74
Tenth	(-)4.71 – (-)2.83 *	(-)1.04 - .849	(-)20.11 - .20	(-)22.36 – (-)2.04*

* Significant contrasts at p < .05

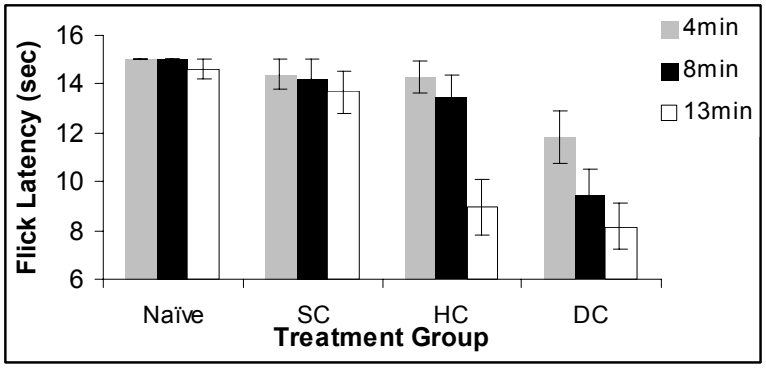
There were significant Time effects at each of the three test sessions, with a medium effect size obtained for the test following the first session, $\eta^2 = .105$, F (2, 59) = 3.77, $p < .05$, and large effect sizes after the fifth, $\eta^2 = .355$, F (2, 57) = 17.59, $p < .001$, and tenth, $\eta^2 = .332$, F (2, 63) = 15.92, $p < .001$, sessions. Similarly, there were two significant Time by Group interaction effects after the fifth, $\eta^2 = .305$, F (5, 57) = 4.68, p < .001, and tenth, $\eta^2 = .517$, F (6, 63) = 11.40, p < .001, sessions. Visual inspection of Figure 1 suggests that latencies

decreased as the time interval between injection and test increased. This effect seems to be largely due to the responses recorded for the DC animals, with the latencies for all other groups remaining largely unchanged throughout the testing times. *Post hoc* contrasts for the sixth session test revealed that DC rats had faster tail-flick latencies than HC rats at 8 min, $p = .002$, but not at 4 min, $p = .018$, or at 13 min, $p = .530$, after injection. However, contrasts for the eleventh session test revealed that DC rats had faster tail-flick latencies than HC rats at 4 min, $p = .010$, 8 min, $p < .000$, and 13 min, $p < .000$, post injection (see Figure 1).

Overall, the tail-flick results confirmed that pairing nicotine administrations with a distinctive context facilitated the expression of nicotine tolerance in that context. In fact, HC animals were relatively more tolerant to nicotine's analgesic effects than SC animals only in one of the three conditions tested, i.e., after five conditioning sessions. The results also show that as few as five conditioning trials are needed for the expression of contextual tolerance to the analgesic effects of nicotine in the tail-flick. However, it appears that the most clear contextual effects occurred after the tenth session, where DC rats were faster than HC rats at each of the three testing times. Regarding time-dose effects, the most reliable interval to test for context tolerance effects was 8 min after injection, as this testing time yielded statistically significant effects both after five and ten conditioning sessions. Visual inspection of Figure 1 indicates that significant Time by Group interaction effects are due to more rapid declines in nicotine's effects for the DC than the other groups.



a)



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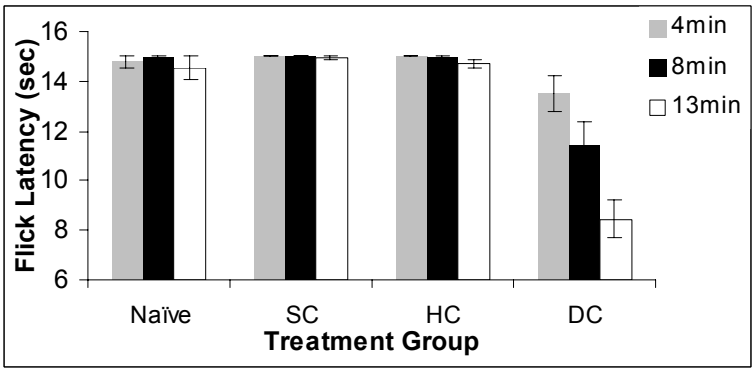


c)

Figure 1 Tail-flick results. Tail-flick latencies for animals tested with 1.00 mg/kg s.c. nicotine at the 4, 8, and 13 min post-injection test times a) after 1 conditioning session, b) after 5 conditioning sessions, and c) after 10 conditioning sessions. During conditioning, DC rats received nicotine explicitly paired with a drug administration environment. HC rats received equivalent amounts of nicotine and context exposure but with the two explicitly unpaired. SC rats received saline during their exposures to the distinctive context, and Naïve rats

received no treatment until test day.

Hot plate results. Unlike the tail-flick results, the analyses only revealed a significant Group effect at the test conducted after the tenth, $\eta^2 = .048$, F (3, 32) = 11.08, $p < .0001$, session. Planned contrasts for this significant group effect indicated that DC rats responded with marginally lower latencies than HC animals, $p = .054$, and HC rats were significantly faster than SC rats, $p = .020$. Like in the tail-flick assay, Saline and Naïve animals did not differ from each other. (See Table 1)

There were significant, large Time effect sizes after the fifth, $\eta^2 = .245$, F (2, 57) = 10.39, $p < .001$, and tenth, $\eta^2 = .522$, F (2, 49) = 34.94, $p < .001$, sessions. However, there was only one significant Time by Group interaction effect, which was obtained after the tenth session, $\eta^2 = .459$, F (5, 49) = 9.06, $p < .001$. Visual inspection of Figure 2 suggests that latencies decreased as the time interval between injection and test increased, particularly for DC and HC animals. A *Post hoc* contrast for the eleventh session test revealed that DC rats had faster paw-lick latencies than HC rats at 8 min, $p = .008$, but not at 4 min, $p = .846$, or at 13 min, $p = .049$, post injection.

Overall, the hot-plate results were not as robust as the tail-flick results. Although, pairing nicotine administrations with a distinctive context facilitated the expression of nicotine tolerance in that context, this effect was clearly observed only at the 8 min post injection test after the tenth session. The results indicate that at least ten conditioning trials are needed for the expression of contextual tolerance to the analgesic effects of nicotine in the hot-plate. This is somewhat congruent with the results obtained with the tail-flick assay, as the most clear

contextual effects in the tail flick also occurred after the tenth session. Moreover, similar to the tail-flick assay, the most reliable interval to test for context tolerance effects in the hot plate was 8 min post injection. Visual inspection of Figure 2 indicates that the significant Time by Group interaction effect at the ten session test was due to faster, nicotine effect declines in DC and HC rats than in SC and Naïve rats.

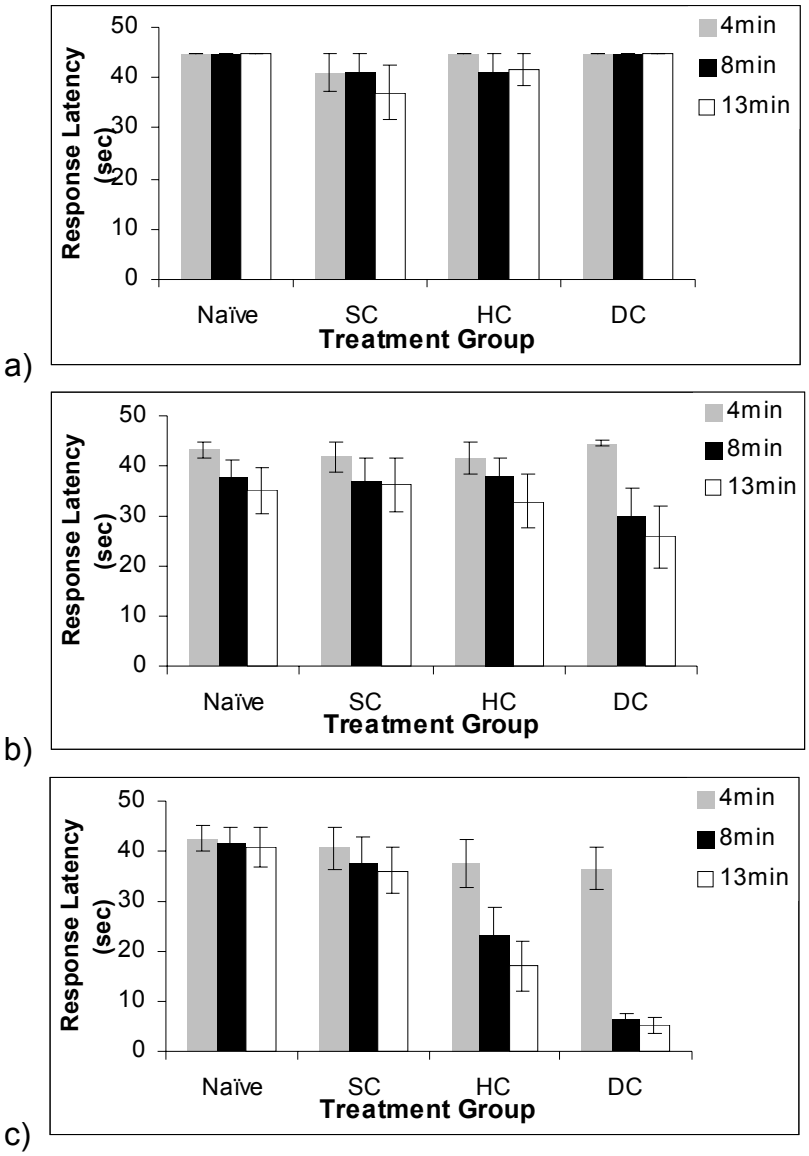


Figure 2 Hot-plate results. Hot-plate latencies to lick or jump at the 4, 8, and 13 min tests (1.00 mg/kg s.c. nicotine) a) after 1, b) after 5, and c) after 10 conditioning sessions. During conditioning, DC rats received nicotine explicitly paired with a drug administration environment. HC rats received equivalent amounts of nicotine and context exposure but with the two explicitly unpaired. SC rats received saline during their exposures to the distinctive context, and Naïve rats received no treatment until test day.

CORT Assays

SC animals. A two-way ANOVA examined the effects of context exposure on CORT levels, with Test Treatment (Nicotine vs. Saline) and Context Exposure (1^{st} vs. 5^{th} vs. 10^{th}) as the two between group factors. The between subjects comparisons yielded significant main effects for both Factors and for the Interaction term. The impact of nicotine on CORT levels was clear as levels of CORT were significantly higher after nicotine than after saline injections, η^2 = *.507*, F (1, 54) = 55.5, $p < .0001$. There also was a significant Context Exposure effect, η^2 = *.145*, F (2, 54) = 4.59, $p < .05$, and a Test by Context interaction, η^2 = *.113*, F (2, 54) = 3.43, $p < .05$. Repeated contrasts between CORT levels at sessions 1 vs. 5, and 5 vs. 10 were analyzed separately for saline and nicotine rats. The results indicated that, in congruence with the Context Exposure main effect, SC rats tested with saline showed an overall decrease in CORT from session 1 to session 5, $p = .005$, and a CORT increase from session 5 to session 10, $p = .04$. Conversely, SC rats tested with nicotine showed no CORT changes from session 1 to session 5, $p = .83$, and a marginal increase from session 5 to session 10, $p = .057$. (See Figure 3)

Overall, the CORT assays conducted on the blood samples collected from SC animals indicated that novelty (or stress) effects increase CORT levels in rats, as observed by a significant decline of CORT levels between session 1 and session 5 in saline tested rats. However, the results also suggested that with prolonged exposure to the experimental procedures CORT levels increased from session 5 to session 10. Likewise, nicotine CORT elevating effects also tended to

increase with level of context exposure from session 5 to session 10 but this effect was only marginally significant.

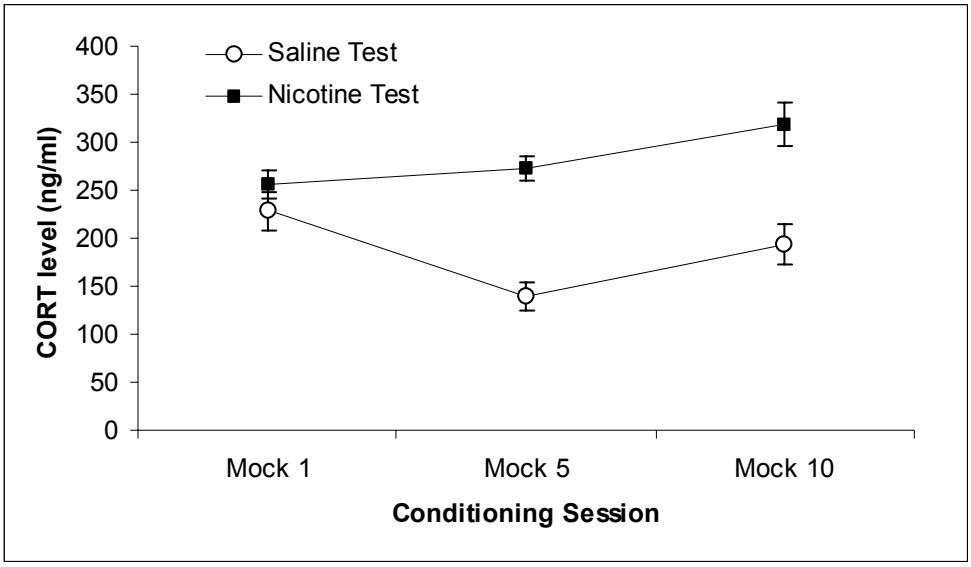


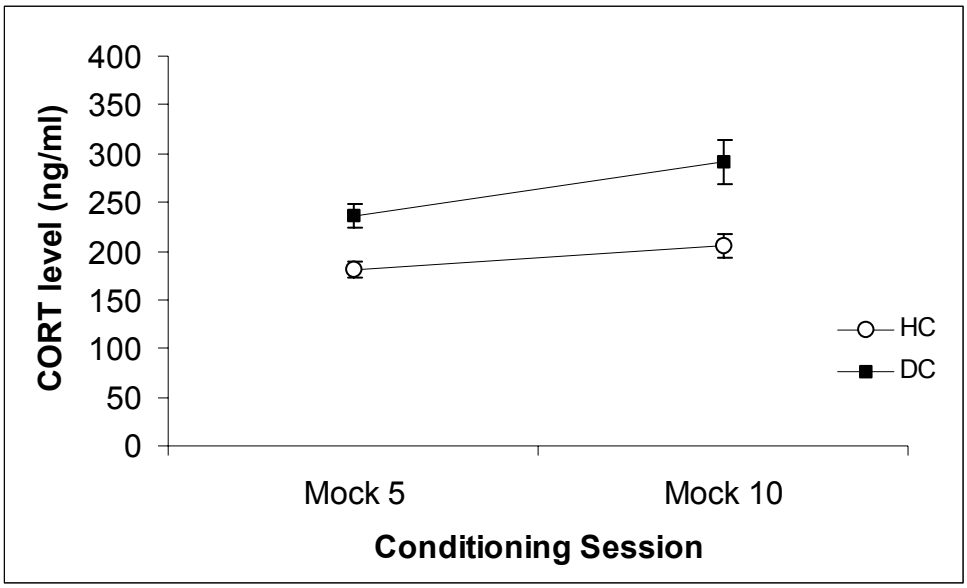
Figure 3 CORT levels for saline controls. Represented here are the CORT levels for SC (saline control) animals tested with saline compared to animals tested with nicotine during the first, after 5 conditioning sessions, and after 10 conditioning sessions.

HC and DC animals. A three-way ANOVA examined the effects of context exposure on CORT levels, with Test Treatment (Nicotine vs. Saline), Context Exposure (5^{th} vs. 10^{th}), and Group (DC vs. HC) as the three between group factors (these results are depicted in Figure 4). The between subjects

comparisons yielded significant main effects for all factors but not for any of the Interaction terms. Levels of CORT were significantly higher after nicotine than after saline injections, $\eta^2 = .276$, F (1, 71) = 27.01, $p < .0001$, after the 10th than after the 5th context exposure, $\eta^2 = .140$, F (1, 71) = 11.51, $p < .005$, and in DC than in HC rats, $\eta^2 = .159$, F (1, 71) = 13.4, $p < .001$.

The CORT elevating effects of nicotine were evident in nicotine experienced animals, or HC and DC rats. That is, CORT levels were higher after nicotine than saline. Tolerance development to nicotine's effects on CORT was not observed, that is CORT levels increased from session 5 to session 10 in nicotine experienced rats (see Figure 3). Moreover, under conditions conducive to the development of contextual tolerance to the analgesic effects of nicotine, DC rats tested with saline showed higher levels of CORT than HC rats (see Figure 4). These results suggest that pairing the context with nicotine resulted in associative learning with the context becoming a conditioned stimulus capable of eliciting a conditioned response or CORT elevation effect.

a)



b)

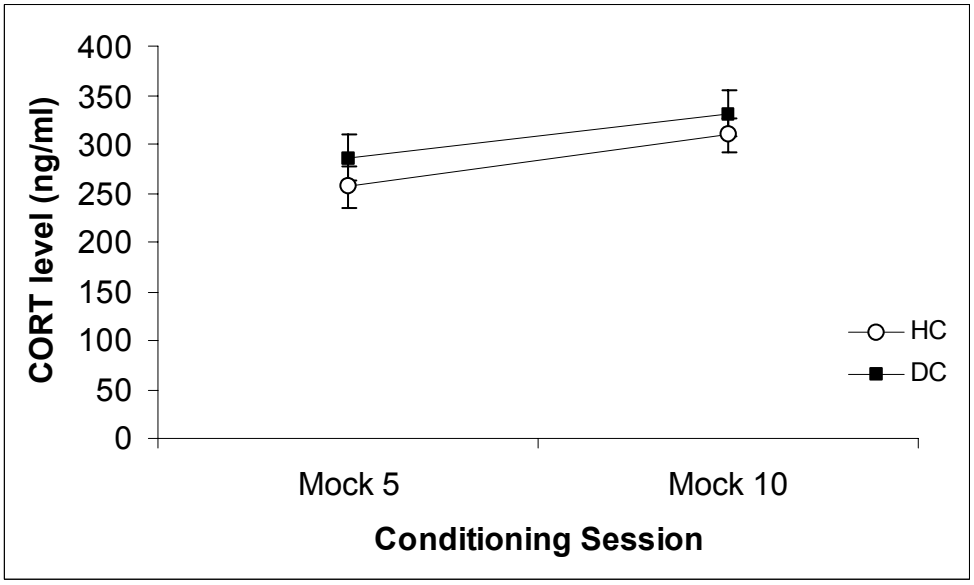


Figure 4 CORT levels for HC and DC rats. DC rat (nicotine explicitly paired with distinctive context) and HC rat (nicotine explicitly unpaired with distinctive context) CORT levels for after 5 and 10 conditioning sessions for both the a) saline tested animals and b) nicotine tested animals.

SUMMARY AND CONCLUSIONS

This investigation shows that associative tolerance to nicotine analgesia as measured by the tail-flick test can develop in as few as 5 conditioning sessions. However, associative tolerance to nicotine's analgesic effects was not observed for the hot-plate assay until after ten conditioning sessions and only at the 8 min post-injection test. This difference between the tail-flick and hot-plate tests may be due to differential effects of nicotine on spinally and supraspinally mediated nociceptive responding.

Several authors have proposed that whereas the tail-flick response is primarily a spinally mediated response, the hot-plate test is both spinally and supraspinally mediated (e.g., Yang et al. 1992). Yang and colleagues found that, with chronic nicotine exposure (extended elevations of nicotine levels in plasma) achieved through mini-pump infusion, tolerance to the analgesic effects of nicotine was only seen for the hot-plate tests. This indicates that, when compared to the results of the current study, chronic nicotine administration gives rise to tolerance to the analgesic effects of nicotine that are mediated by supraspinal mechanisms. However, a drug regime consisting of repeated acute dosing appears more conducive to the development of associative tolerance to the analgesic effects of nicotine that are mediated via spinal mechanisms.

Moreover, Caggiula and others (1995) found that both peripheral (chlorisondamine) and central (mecamylamine) antagonists interrupted nicotine-induced antinociception as measured by the tail-withdrawal method, but only the central antagonist (mecamylamine) produced an interruption in hot-plate

responses to nicotine. Rogers and Iwamoto (1993) showed that both spinal and brain cholinergic systems are responsible for the antinociceptive effects of nicotine and that antagonists to these systems differentially affect tail-flick and hot-plate responses. Their study found that antagonists of nicotinic receptors in the spinal cord do not appear to eradicate antinociceptive responses indicating that the lumbar spinal level uses other neurotransmitter systems to produce antinociception in response to nicotine. However, they also showed that, through antagonism of supraspinal nicotinic receptors, central nicotinic receptors appear to exert effects on NE, 5-HT, and muscarinic receptors in the spinal cord. Therefore, it seems clear that tail-flick and hot-plate responses are not mediated by entirely the same mechanisms, and that manipulations in the present investigation may not have affected these different mechanisms in the same way.

An additional reason why the context effects were clearer in the tail-flick is that the hot-plate assay seems to have a lower dose for optimal response than the tail-flick (Caggiula et al. 1995). That is, Caggiula and colleagues found a contextual effect to a 0.25 mg/kg, and a 0.50 mg/kg nicotine, test-dose, but not to a 0.75 mg/kg test dose. Therefore, it is possible that the 1.00 mg/kg dose produced changes in the spinal cholinergic system but did not affect the supraspinally mediated cholinergic system leading to the hot-plate/ tail-flick discrepancies.

The present investigation contributes to a better understanding of how CORT release may mediate the development of associative tolerance to nicotine's analgesic effects, and is directly related to seemingly contradictory

results in previous investigations. Caggiula and others (1993) demonstrated that following a saline challenge in the distinctive context, animals that had received five nicotine injections paired with the distinctive context at a 48 hr inter-dose-interval (IDI) had higher levels of blood CORT than animals that had received the same drug regime but were exposed for the first time to the distinctive context on test day. That is, exposure to the context had been associated with nicotine-induced CORT level increases. However, in an earlier investigation, Caggiula and colleagues (1991), found that nicotine-induced CORT release in rats that had received eleven nicotine injections (24 hr IDI) in a distinctive context were not higher in the distinctive context than in a novel environment but lower. Thus, together the Caggiula laboratory findings left it unclear whether context-induced CORT release or context-induced CORT suppression was responsible for the development of associative tolerance to nicotine.

The data in the present investigation were consistent with the finding that CORT reduces sensitivity to nicotine effects. It was also consistent with the hypothesis that conditioned corticosterone release may play a large role in the development and expression of associative tolerance to the analgesic effects of nicotine. That is, CORT levels were elevated in animals that received nicotine injections paired with the distinctive context (DC rats) than in animals that received as much nicotine and exposures to the context but with these stimuli explicitly unpaired (HC rats). Moreover, the contextual CORT release was observed when the conditioned stimulus (distinctive environment) was presented with and without the unconditioned stimulus (nicotine). In congruence with the

findings and hypothesis described above, DC animals were less sensitive to nicotine's analgesic effects, while having increased CORT, than HC animals.

The precise mechanisms that may be involved in endocrine pathways to reduce nicotine-induced analgesia in the present experiment are unclear. It has been established that nicotine acts on cholinergic receptors in brain to release corticotropin-releasing factor (CRF) from the hypothalamus. CRF in turn stimulates the release of adrenocorticotropic hormone (ACTH) and B-endorphin (B-end) from the pituitary gland (see Feldman et al. 1997). That is, hypophysectomy eradicates CORT release in response to nicotine while exogenous ACTH administration reinstates CORT releases in response to nicotine – indicating that it is the CRF-ACTH pathway that is necessary for nicotine to induce CORT increases (Cam et al. 1979). Thus, nicotine shares, to some extent, the same pathway that controls stress-induced antinociception (Munck et al. 1984). Given that B-end can act on the central nervous system to reduce the afferent flow of pain information from the peripheral nervous system (see Feldman et al. 1997), it is possible that at least part of nicotine's antinociceptive effects are exerted through the stimulation of B-end. This hypothesis is congruent with previous findings showing that exposure to novel (stressful) stimuli disrupts the expression of associative tolerance to nicotine (e.g., Caggiula et al. 1993)

The current studies showed a decrease in nicotine-induced analgesia after repeated exposures to nicotine paired with the environment, however, CORT levels remained high. Conditioned release of CORT may explain this finding. It

appears that CORT acts as a mediator of B-end by direct actions on the pituitary and by decreasing release of CRF through negative feedback systems (Munck et al. 1984). In addition, CORT acts to downregulate Acetylcholine (ACh) receptors (Munck et al. 1984). Therefore, it is possible that conditioned CORT release acts to decrease nicotine-induced analgesia by inhibiting the CRF-ACTH / B-end pathways and by downregulation of ACh receptors thereby decreasing nicotine's stimulatory effects on these systems (see Figure 5). This conditioned pre-injection release of CORT would lead to high circulating CORT levels but low analgesia in response to nicotine as was found in this investigation.

Additionally, CORT inhibition of ACTH and B-end versus the downregulation of nicotinic receptors by CORT may be differentially affected by the parameters surrounding the acquisition of tolerance (i.e., one pathway may exert more influence in the learning aspects of tolerance while the other may influence the physiological aspects of tolerance). Grun and colleagues (1992) and Pauly, Grun, and Collins (1990) found that tolerance in mice was achieved without any changes in nicotinic receptor number or affinity. When looking at the experimental design of those studies, it seems possible that the procedure used by these authors may have allowed for conditioning to associative cues (although associative cues were not manipulated), therefore, their findings that nicotinic receptors remained unchanged while CORT levels were increased just prior to injection could indicate that associative tolerance is not mediated by nicotinic receptor changes.

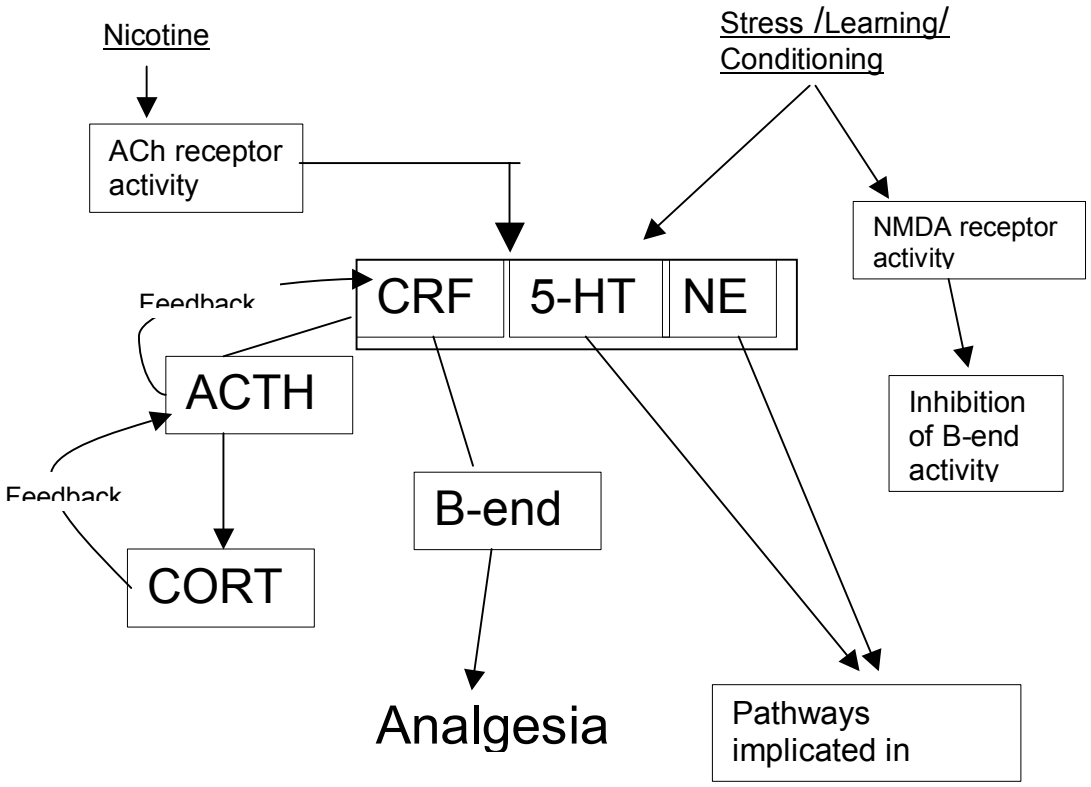


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Figure 5 Model for physiological pathways implicated in analgesia. This model shows possible pathways for analgesia induced by nicotine and stress/ learning/ conditioning as well as the potential convergence of these systems.

The above argument is further supported by Hulihan-Giblin and others (1990) who found, using procedures that would be conducive to measuring non-associative tolerance (subcutaneous daily injections were given and testing occurred with intravenous injections), that changes in nicotinic receptor binding were highly correlated with tolerance to nicotine.

Therefore, it may be postulated that physiological tolerance to nicotine may be mediated by long-term changes in nicotinic receptor activity, and CORT release in response to nicotine may not act efficiently to inhibit B-end thereby producing changes in nicotine responsiveness mostly at the receptor level. Further, CORT increases in response to associative cues *precede* nicotine administration, which may allow for more effective control of B-end thereby producing larger tolerance effects to the analgesic effects of nicotine (see Figure 5).

However, it must be pointed out that stress, conditioning, and nicotine appear to act on norepinephrine (NE), Serotonin (5-HT), and N-methyl-D-aspartate (NMDA) receptor systems (Trujillo 2000; Furst 1999). All of these systems may lead to analgesia through their respective physiological cascades. The implication of so many pathways to nicotine-induced antinociception makes our interpretation of results pertaining to the endocrine mechanisms associated with nicotine analgesia one of many other possibilities. Moreover, because nicotine and stress act on the same systems to produce analgesia, it is clear that the antinociceptive properties of nicotine are confounded with stress-elicited antinociception and is difficult to isolate or control in an associative learning

paradigm. In addition, if physiological responses to learning are factored into the converging pathways of stress and nicotine, the mechanisms involved in associative tolerance to nicotine's analgesic effects are clearly very complex, and it will take extended research to define these pathways.

The next step to elucidating the physiological mechanisms that converge in associative tolerance paradigms may be to explore the role of the other players in the physiological cascades associated with stress, conditioning, and nicotine (B-end, NE, NMDA, CRF, ACTH, and 5-HT). Previous reports have shown that the CRF / ACTH pathway is necessary for nicotine-induced analgesia (Cam et al. 1979). The interruption of nicotine-induced analgesia may be due to a loss of B-end, because hypophysectomy eradicated CRF-stimulated pro-opiomelanocortin (POMC) release, which is the common precursor to ACTH and B-end in intact animals (see Figure 5). Therefore, if B-end is the key player in analgesia within associative tolerance paradigms, then inhibition of B-end should significantly decrease analgesia in response to nicotine. Further, if B-end has a primary role then, all other systems being "equal", associative tolerance should show a decrease in B-end activity while non-associative tolerance should show sustained elevations in B-end in response to nicotine.

It will also be necessary to study NE, NMDA, and 5-HT systems separately in the pathways to the development of associative tolerance to nicotine's analgesic effects. Moreover, all of the physiological systems mentioned seem to be convergent systems in that multiple factors may lead to activation of these systems. Exactly how these systems converge and interact

is unclear, however, elucidating this interaction may be an important step to understanding nicotine addiction. Although the actions of these compounds are known in certain stress-, learning-, or nicotine-induced pathways, associative tolerance provides a situation where stress-, learning-, and nicotine- induced pathways converge. Therefore, the physiological importance that each of these compounds contributes to associative tolerance to nicotine is most likely modified by the converging psychological and physiological mechanisms apparent in associative tolerance paradigms. Additionally, the present investigation only looked at endocrine responses during nicotine-induced analgesia. The endocrine system likely plays a different role in some of nicotine's other effects (e.g., B-end may not be the primary player in the pathways to the anorectic or locomotor effects of nicotine).

If the convergent systems hypothesis (stress, learning, and nicotine) is correct, some degree of cross-tolerance would be expected. Unpublished work in this lab has shown this to occur. Animals conditioned to *nicotine* in the distinctive context (DC animals) showed higher levels of tolerance to *morphine* in that same context than did HC or SC animals. This further supports the idea that a significant part of associative tolerance to nicotine is influenced by the conditioning mechanisms, and, as previously stated, the physiological components within these systems remains to be fully explained in the context of associative tolerance.

Nicotine addiction in humans is very complex, and associative tolerance is just one aspect of continued nicotine use. The implication that learning and

conditioning mechanisms are crucial to nicotine addiction is not new, however, a better understanding of the human condition may be obtained through a close look at the physiological mechanisms that drive learning and conditioning within the context of drug use. It seems likely that the physiological mechanisms involved in nicotine addiction, especially in regards to conditioning, are likely to be involved in other forms of addiction as well.

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