Insular hypocretin transmission regulates nicotine reward

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Damage to the insular cortex can profoundly disrupt tobacco addiction in human smokers, reflected in spontaneous cessation of the tobacco habit and persistently decreased urge to smoke. Little is known concerning the neurobiological mechanisms through which the insula may control the maintenance of the tobacco habit. Emerging evidence suggests that hypocretin (orexin) transmission may play an important role in drug reinforcement processes, but its role in the rewarding actions of nicotine, considered the key addictive component of tobacco smoke, remains largely unexplored. Here we show that blockade of hypocretin transmission at hypocretin-1 (Hcrt-1; orexin-1) receptors decreases i.v. nicotine self-administration in rats and the motivation to obtain the drug. Blockade of Hcrt-1 receptors also abolished the stimulatory effects of nicotine on brain reward circuitries, as measured by reversal of nicotine-induced lowering of intracranial self-stimulation thresholds. In addition, we show that hypocretin-containing fibers innervate the insula, Hcrt-1 receptors are located on insular cells, and blockade of Hcrt-1 receptors in the insula but not in the adjacent somatosensory cortex decreases nicotine self-administration. These data demonstrate that insular hypocretin transmission plays a permissive role in the motivational properties of nicotine, and therefore may be a key neurobiological substrate necessary for maintaining tobacco addiction in human smokers.

craving | orexin | self-administration | intracranial self-stimulation

Cigarette smoking is one of the largest preventable causes of death and disease in developed countries, and accounts for approximately 440,000 deaths and $160 billion in health-related costs annually in the United States (1). Despite the well known negative health consequences of the tobacco smoking habit, only approximately 10% of smokers who attempt to quit annually remain abstinent after 1 year, highlighting the persistence of the smoking habit. The insula is a cortical brain region involved in processing interoceptive information related to emotional and motivational states to facilitate maintenance of physiological homeostasis (2). This brain region may also regulate the experience of conscious urges and cravings (2–4). It was recently reported that damage to the insula in human smokers resulted in a profound disruption of tobacco addiction characterized by spontaneous cessation of the smoking habit and a low urge to smoke thereafter (3). Conversely, abstinence-induced cigarette craving in smokers is highly correlated with activation of the insular cortex (5). The neurobiological mechanisms through which the insula regulates the persistence of the tobacco habit remain unclear.

Nicotine is a major reinforcing constituent of tobacco responsible for the smoking habit in humans (6). In common with other major drugs of abuse, nicotine can directly stimulate reward circuitries in the brain (7), and obtaining the reward-enhancing effects of nicotine may contribute to the persistence of the tobacco habit in human smokers (8, 9). Hypocretin peptides 1 and 2, also known as orexin A and B, are lateral hypothalamic (LH) neuropeptides that are emerging as important regulators of reward and motivation. Chemical activation of LH hypocretin neurons reinstates extinguished morphine seeking behavior in rats (10), an effect blocked by the selective Hcrt-1 receptor antagonist SB-334867 (10). Blockade of Hcrt-1 transmission also decreases alcohol self-administration (11) and cue-induced reinstatement of extinguished alcohol (11) and cocaine (12) seeking, and attenuates stress-induced reinstatement of extinguished cocaine (13) and alcohol (14) seeking. These findings support an important role for hypocretin transmission in drug-seeking and drug-taking behaviors. However, the role of hypocretin transmission in nicotine reward remains largely unexplored. Here, we tested the hypothesis that hypocretin transmission in the insula may contribute to the motivational properties of nicotine.

Results

Hypocretin Transmission Regulates Nicotine Reinforcement. First, we investigated the role of hypocretin transmission at Hcrt-1 receptors in regulating nicotine consumption. Rats responding for intravenously self-administered nicotine infusions (0.03 mg/kg per infusion) under a fixed-ratio five time-out 20-sec (FR5TO20 sec) schedule of reinforcement were treated with the selective Hcrt-1 receptor antagonist SB-334867 (0, 1, 2, and 4 mg/kg i.p.), and nicotine intake was assessed according to a Latin-square design. To identify possible non-specific actions of SB-334867 on operant performance, we also assessed the effects of the drug on responding for food pellets in hungry rats under an FR5TO20 sec schedule. One-way repeated-measures ANOVA demonstrated that SB-334867 significantly decreased nicotine intake ($F_{[3, 23]} = 6.5$, $P < 0.005$; Fig. 1*A*), but did not alter responding for food reinforcement ($F_{[3, 27]} = 0.8$; Fig. 1*B*). Two-way repeated-measures ANOVA was used to directly compare the effects of SB-334867 on nicotine and food consumption, expressed as percent change from baseline intake. This analysis demonstrated significant main effects of reinforcer ($F_{[1, 33]} = 15.2$, $P < 0.005$) and dose ($F_{[3, 33]} = 6.4$, $P < 0.005$), and a significant reinforcer × dose interaction ($F_{[3, 33]} = 3.6$, $P < 0.05$). Bonferroni post-tests among means demonstrated that nicotine intake was significantly lowered compared with food intake at the 4-mg/kg dose of SB-334867 ($P < 0.01$; Fig. 1*C*). SB-334867 did not alter inactive lever responses (see *Materials and Methods*) in the nicotine or food rats [supporting information (SI) Fig. S1].

Next, we investigated the role of Hcrt-1 receptors in regulating the motivation to obtain nicotine, as measured by responding for the drug under a progressive ratio (PR) schedule of reinforcement. Direct comparison of the effects of SB-334867 on nicotine or food intake by two-way repeated-measures ANOVA demonstrated significant main effects of reinforcer ($F_{[1, 57]} = 30.5$, $P < 0.0001$) and dose ($F_{[3, 57]} = 23.6$, $P < 0.0001$), and a significant reinforcer × dose interaction ($F_{[3, 57]} = 12.9$, $P < 0.0001$). Bonferroni post-tests demonstrated that SB-334867 significantly

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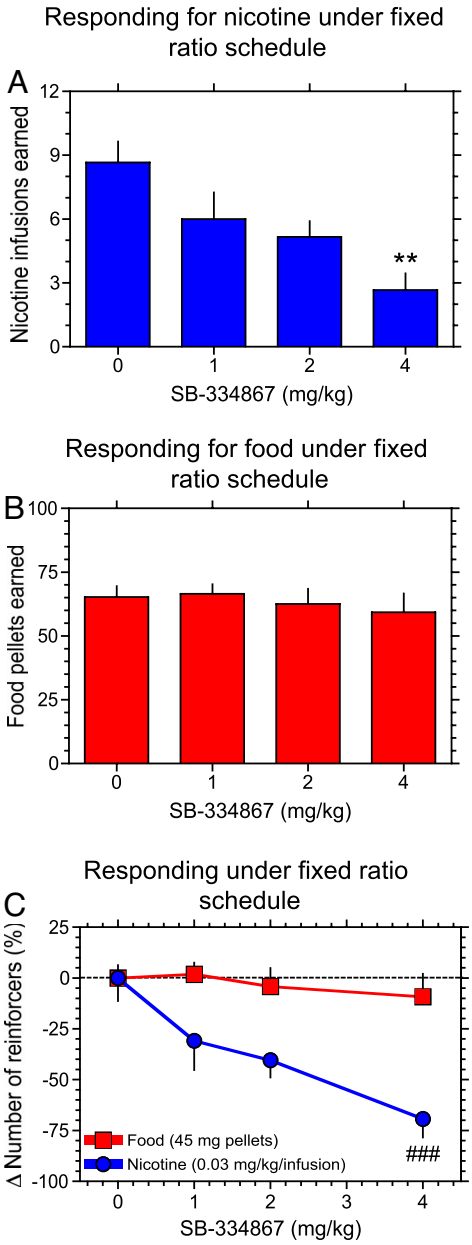

Fig. 1. Hypocretin transmission at Hcrt-1 receptors regulates nicotine intake. The effects of systemically administered SB-334867 on responding for nicotine or food rewards were tested under an FR schedule of reinforcement (see *Materials and Methods*). (*A*) Mean (±SEM) number of nicotine reinforcers earned under an FR5TO20 sec reinforcement schedule. **, $P < 0.01$ compared with vehicle treatment; *post-hoc* test after a significant main effect in one-way repeated-measures ANOVA. (*B*) Mean (±SEM) number of food reinforcers earned under the same reinforcement schedule. (*C*) Direct comparison of the effects of SB-334867 on responding for nicotine or food rewards, expressed as percent change from baseline intake. ###, $P < 0.001$ nicotine rewards earned compared with food rewards after treatment with the same dose of SB-334867; Bonferroni post-tests after significant interaction effect in two-way repeated-measures ANOVA.

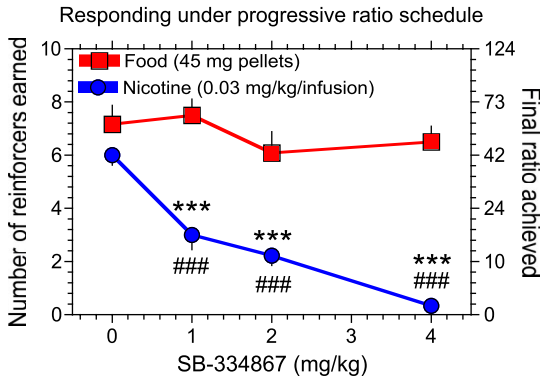

Fig. 2. Hypocretin transmission at Hcrt-1 receptors regulates the motivation to consume nicotine. The effects of SB-334867 on responding for nicotine or food rewards were tested under a PR reinforcement schedule. Data are presented as mean (±SEM) number of nicotine or food reinforcers earned (*Left*, ordinal axis) or the corresponding final ratio achieved (i.e., break points; *Right*, ordinal axis) in rats responding under a PR reinforcement schedule. ***, $P < 0.001$ compared with vehicle treatment; *post-hoc* test after a significant main effect in one-way repeated-measures ANOVA. ###, $P < 0.001$ nicotine rewards earned compared with food rewards after treatment with the same dose of SB-334867; Bonferroni post-tests after significant interaction effect in two-way repeated-measures ANOVA.

decreased the number of nicotine rewards earned compared with the number of food rewards at the 1- to 4-mg/kg doses of SB-334867 ($P < 0.001$ in each case; Fig. 2). One-way repeated-measures ANOVA demonstrated that SB-334867 significantly decreased the number of nicotine rewards earned ($F_{[3, 35]} = 32.9$, $P < 0.0001$; Fig. 2). *Post-hoc* comparisons among means revealed that nicotine intake was significantly decreased by each dose of SB-334867 compared with vehicle treatment ($P < 0.001$ in each case; Fig. 2). In contrast, SB-334867 had no statistically significant effects on food responding ($F_{[3, 47]} = 1.2$; Fig. 2). SB-334867 did not alter inactive lever responses in the nicotine or food rats (Fig. S1). Taken together, these self-administration data demonstrate that hypocretin transmission at Hcrt-1 receptors regulates ongoing nicotine self-administration behavior and the motivation to seek and obtain the drug.

Hypocretin Transmission Regulates the Reward-Enhancing Effects of Nicotine. Nicotine lowers intracranial self-stimulation (ICSS) thresholds in rats (15). This property of nicotine reflects enhanced activity of brain reward systems in response to the drug, resulting in increased sensitivity to the reward value of ICSS. Obtaining the reward-enhancing effects of nicotine may provide an important source of motivation that establishes and maintains nicotine self-administration behavior (15). One mechanism by which hypocretin transmission may control nicotine intake and the motivation to obtain the drug is by gating the stimulatory actions of nicotine on brain reward systems. Indeed, blocking the stimulatory effects of nicotine on brain reward circuitries, as measured by nicotine-induced lowering of ICSS thresholds, decreases nicotine intake in rats (15). To test this hypothesis we assessed the effects of SB-334867 (0, 1, 2, 4, and 6 mg/kg i.p.) on nicotine-induced lowering of ICSS thresholds in rats. Mean absolute ICSS threshold before SB-334867 treatment was 113.5 ± 14.1 μA. Two-way repeated-measures ANOVA demonstrated a significant main effect of nicotine ($F_{[1, 45]} = 11.65$, $P < 0.01$), but no effect of SB-334867 ($F_{[4, 45]} = 1.31$), nor a nicotine × SB-334867 interaction ($F_{[4, 45]} = 1.0$). Based on our *a priori* hypothesis that SB-334867 would block nicotine-induced lowering of ICSS thresholds, we proceeded to analyze the effects of the nicotine/SB-334867 treatment conditions on ICSS thresholds using pre-planned *post-hoc* comparisons. As expected, we found that reward thresholds were significantly lowered by vehicle-nicotine ($P < 0.001$) treatment. Pretreatment with the lowest dose of SB-334867 (1 mg/kg) did not alter the threshold-lowering effects of nicotine ($P < 0.01$ vs. vehicle-saline treatment; Fig. 3). However, pretreatment with higher doses of SB-334867 (4–6 mg/kg) blocked the threshold-lowering effects of nicotine. This was reflected in reward thresholds being

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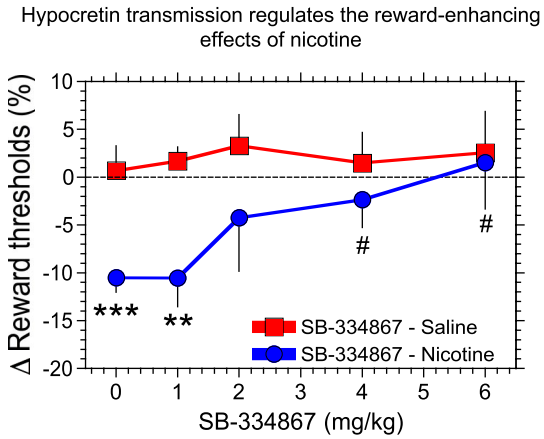

Fig. 3. Hypocretin transmission at Hcrt-1 receptors regulates the reward-enhancing effects of nicotine. The effects of SB-334867 on nicotine-induced lowering of ICSS thresholds were tested. Rats were pretreated with SB-334867 or its vehicle, and subsequently received nicotine (0.25 mg/kg) or saline solution injections. Data are expressed as mean (SEM) percentage change from baseline reward thresholds. **, $P < 0.01$, ***, $P < 0.001$, nicotine treatment compared with saline solution treatment at the same dose of SB-334867; #, $P < 0.05$ compared with the vehicle-nicotine treatment group; pre-planned *post-hoc* comparisons among means after significant main effect of nicotine in two-way repeated-measures ANOVA (see *Results*).

significantly lowered after vehicle-nicotine treatment compared with thresholds when nicotine was delivered in combination with SB-334867 (4–6 mg/kg; $P < 0.05$ in each case; Fig. 3). Response latencies were not altered by SB-334867 at any dose (data not shown). These data demonstrate that hypocretin transmission at Hcrt-1 receptors plays a permissive role in the stimulatory effects of nicotine on brain reward systems, and in this manner may regulate the motivation to consume the drug.

To verify that the low doses of SB-334867 we used in the aforementioned nicotine self-administration and ICSS experiments were sufficient to penetrate into brain tissues, we assessed the pharmacokinetic and brain penetration properties of SB-334867 (4 mg/kg i.p.). As seen in Table 1, we observed detectable levels of SB-334867 in blood even 2 h after i.p. administration, with detectable levels of the drug also found in brain tissues at this time point.

Insular Hypocretin Transmission Regulates Nicotine Reinforcement. Next, we tested the hypothesis that hypocretin transmission at Hcrt-1 receptors in the insular cortex (Fig. 4*A*) may regulate nicotine reinforcement. First, we observed dense innervation of Hcrt-1 peptide-containing neurons into the insula (Fig. 4*B*). We also found expression of Hcrt-1 receptors on cells within the insula (Fig. 4*C*). Moreover, we found that direct infusion of SB-334867 (0.04–5 μg/side; 0.125–15.6 nmol/side) into the insula decreased nicotine self-administration ($F_{[5, 83]} = 7.0$, $P < 0.0001$; Fig. 4*D*). *Post-hoc* tests among means demonstrated that nicotine intake was significantly lowered compared with vehicle at the 0.2 μg/side ($P <$ 0.05), 1 μg/side ($P < 0.01$), and 5 μg/side ($P < 0.001$) doses. Importantly, when the highest dose of SB-334867 (5 μg/side) was infused 2 mm above the insula into the somatosensory cortex, a *post-hoc* test demonstrated that there was no statistically significant effect compared with vehicle infusion (Fig. 4*D*). However, intake after somatosensory cortex infusion was significantly higher compared with intake after insular infusion of the same SB-334867 dose

Table 1. Mean (±SEM) pharmacokinetic and brain penetration properties of SB-334867 (4 mg/kg i.p.)

Time, min	Plasma concentration, μM	Brain concentration, μM
5	3.32 ± 0.1	—
15	6.84 ± 2.2	—
30	6.44 ± 1.1	—
60	2.69 ± 0.7	—
120	0.25 ± 0.1	0.05 ± 0.02

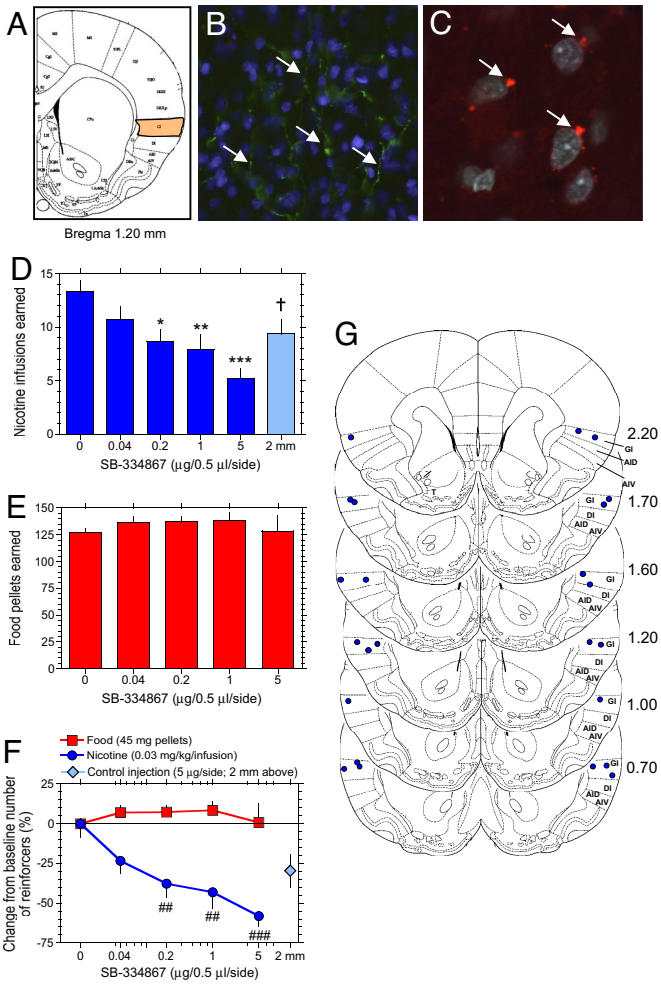

Fig. 4. Insular hypocretin transmission at Hcrt-1 receptors regulates nicotine reinforcement. The effects of micro-infusing SB-334867 into insular cortex on responding for nicotine or food rewards were tested under an FR reinforcement schedule. (*A*) Graphical representation of the region of the insula within which we assessed innervation by Hcrt-1-positive neurons (green-stained fibers) (*B*), and expression of Hcrt-1 receptors on insular cells (red-stained punctate receptor clusters) (*C*). Cell nuclei are highlighted by DAPI staining (blue). (*D*) SB-334867 (0.05–5 μg/side; 0.125–15.6 nmol/side) administered into the insula, but not 2 mm above into the somatosensory cortex (5 μg/side), significantly decreased nicotine self-administration, but did not alter operant responding for food (*E*). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ compared with vehicle treatment; †$P < 0.05$ compared with SB-334867 (5 μg/side) administered 2 mm below into the insula; *post-hoc* tests after significant interaction effect in two-way repeated-measures ANOVA. (*F*) Direct comparison of the effects of SB-334867 on responding for nicotine or food rewards, expressed as percent change from baseline intake. ##, $P < 0.01$, ###, $P < 0.001$: nicotine rewards earned compared with food rewards after treatment with the same dose of SB-334867; *post-hoc* test after significant main effects in two-way repeated-measures ANOVA. (*G*) Histological reconstruction of the injection sites in the insula. Blue dots indicate locations of injector tips from the animals that were included in statistical analysis. The number beside each reconstructed image indicates the distance (in mm) from Bregma. GI, granular insula; DI, dysgranular insula; AID, anterior agranular insula; AIV, ventral agranular insula.

($P < 0.05$). Insular infusions of SB-334867 did not alter responding for food rewards ($F_{[4, 24]} = 0.5$; Fig. 4*E*). Direct comparison of the effects of SB-334867 on responding for nicotine or food rewards, expressed as percent change from baseline, demonstrated statistically significant main effects of reinforcer (nicotine or food; $F_{[1, 60]} = 21.1$, $P < 0.001$) and dose ($F_{[4, 60]} = 2.9$, $P < 0.05$), and a significant reinforcer × dose interaction ($F_{[4, 60]} = 3.4$, $P < 0.05$). Bonferroni post-tests demonstrated that SB-334867 significantly decreased the number of nicotine rewards earned compared with the number of food rewards earned at the 0.2- to 5-μg/0.5 μl/side doses of SB-334867 (Fig. 4*F*). Intra-insular SB-334867 did not alter inactive lever responses in the nicotine or food rats (Fig. S1).

Discussion

This report demonstrates that hypocretin transmission regulates the motivational properties of nicotine and plays a permissive role in the stimulatory effects of nicotine on brain reward circuitries. We also show that neurons containing Hcrt-1 peptide densely innervate the insular cortex, that cells within this structure express Hcrt-1 receptors, and that blockade of hypocretin transmission at Hcrt-1 receptors in the insula decreases nicotine intake in rats. These findings suggest that insular hypocretin transmission may be a key substrate necessary for maintaining the tobacco habit in human smokers.

Hypocretin 1 and 2 (orexin A and B) neuropeptides are produced almost exclusively in the LH and posterior hypothalamus areas (16). The LH has long been associated with reward and motivation (17), and emerging evidence strongly implicates hypocretin transmission in drug dependence processes. Exposure to environmental cues that were repeatedly paired with cocaine, morphine, or food reward robustly activated hypocretin-positive LH neurons, as measured by Fos immunostaining (an index of neuronal activation) (10). The rewarding effects of morphine were absent in mice deficient in the prepro-hypocretin gene tested in a place conditioning procedure (18). Lesions of hypocretin-enriched areas of the LH blocked a conditioned place preference for morphine (19). Conversely, chemical activation of hypocretin neurons, achieved by infusing the neuropeptide-Y Y4 receptor agonist rat pancreatic polypeptide into the LH, reinstated extinguished morphine seeking behavior in rats (10), an effect blocked by SB-334867 (10). Finally, hypocretin transmission at Hcrt-1 receptors plays a key role in cue- and stress-induced reinstatement of extinguished cocaine- and alcohol-seeking behaviors (11–14). Importantly, the role of hypocretin transmission in the motivational properties of nicotine has remained largely unexplored.

In the present study low doses of SB-334867, shown to effectively penetrate into brain tissues, decreased nicotine intake and the motivation to obtain the drug in rats, as measured under fixed ratio (FR) and PR schedules of reinforcement, respectively. Importantly, SB-334867 did not alter responding for food pellets in hungry rats tested under the same reinforcement schedules. It should be noted that responding for food rewards under the FR schedule was far higher than that observed for nicotine rewards, which may raise concerns related to rate-dependent effects of SB-334867 on behavior and whether food responding under this schedule served as an appropriate control for operant performance. Importantly, however, responding for food and nicotine rewards was similar under the PR schedule, in which SB-334867 selectively decreased nicotine intake without altering responding for food. Previously, it was shown that low doses of SB-334867 similar to those used in the present study did not alter arousal (20), sleep-wake cycles (20), food intake (21), or behavioral satiety (21) in rats. However, at higher doses (≥10 mg/kg), SB-334867 decreased consumption of palatable high-fat food (22) and baseline food intake (21). These observations suggest that the inhibitory effects of low doses of SB-334867 (1–4 mg/kg) on responding for nicotine rewards were not related to deficits in operant performance, nor secondary to drug-induced alterations in circadian, feeding, or satiety state. Instead, this action likely reflects a selective disruption in the maintenance of nicotine-taking behavior and decrease in the motivation to consume the drug. It was previously shown that acute nicotine administration increases Fos expression in LH hypocretin neurons (23), supporting the notion that nicotine can enhance hypocretin transmission in the brain. Taken together, the present data suggest that nicotine-enhanced hypocretin transmission plays a central role in regulating the motivational properties of the drug.

Experimenter-administered or self-administered nicotine infusions lower ICSS thresholds in rats (15), reflecting nicotine-enhanced activity of brain reward systems. We found that SB-334867 decreased nicotine self-administration and also abolished the stimulatory effects of nicotine on brain reward systems, as measured by reversal of nicotine-induced lowering of ICSS thresholds. Obtaining the reward-enhancing effects of nicotine is hypothesized to provide an important source of motivation that drives nicotine self-administration in rats (9, 15) and perhaps the tobacco habit in human smokers. Hence, these findings suggest that hypocretin transmission may regulate the stimulatory effects of nicotine on brain reward circuitries, and in this manner control the motivation to consume the drug. An important caveat to this hypothesis is the fact that the lower doses of SB-334867 (1–2 mg/kg) decreased nicotine self-administration without modifying the ICSS threshold-lowering effects of nicotine. Thus, it is possible that obtaining the reward-enhancing effects of nicotine is only partly responsible for maintaining nicotine self-administration behavior, and that SB-334867 may block other behavioral actions of nicotine (e.g., anxiolytic effects) that also contribute to nicotine reinforcement. It is important to note that SB-334867 administered alone did not modulate ICSS thresholds, suggesting that hypocretin transmission does not tonically regulate the baseline sensitivity of brain reward systems. This supports the rationale of developing hedonically inert Hcrt-1 receptor antagonists as novel smoking cessation therapeutic agents, as compounds with constitutive ICSS threshold-lowering effects are likely to have intrinsic abuse potential, whereas compounds that elevate reward thresholds may have anhedonia-like side effects that reduce compliance.

Recently it was reported that human smokers with damage to the bilateral posterior (i.e., granular) and right anterior (i.e., agranular) insula cortex were more likely to experience a disruption in tobacco addiction than those with damage to tissues that did not include the insula cortex (3). Disruption of tobacco addiction in these individuals was characterized by increased likelihood to spontaneously quit smoking, and concomitantly reduced conscious urges to smoke (3). Intriguingly, it was also recently shown that Fos immunoreactivity was simultaneously increased in both the insular cortex and also in LH hypocretin neurons in rats exposed to amphetamine-paired environmental stimuli that were actively seeking the drug (25). This observation may represent the mechanistically unrelated yet temporally coincident activation of two neurobiological substrates that are involved in drug seeking behavior. Alternatively, it is possible that drug-induced activation of hypocretin inputs to the insula may contribute to the motivational properties of drugs of abuse. Based on this, we tested the hypothesis that hypocretin transmission in the insular cortex may regulate the reinforcing effects of nicotine. In the rat brain, the insula is comprised of a dorsally located granular region, a ventral agranular region, and the dysgranular region located between these areas. Previously it was shown that the dorsolateral striatum, a brain area heavily implicated in the expression of habitual responding for drugs of abuse (26), receives dense innervation from the granular and dysgranular portions of insula (25). Moreover, temporary inactivation of the granular insula revers-

ibly disrupted amphetamine seeking in rats tested in a place conditioning procedure (26). Based on these data, and the fact that bilateral disruption to the posterior (i.e., granular) insula disrupted tobacco addiction in human smokers, we targeted the granular region to test the hypothesis that insular hypocretin transmission may regulate the motivational properties of nicotine. Consistent with this hypothesis and with previous reports (27, 28), we found dense innervation of Hcrt-1 peptide-containing neurons throughout the insular cortex of rats. In addition, we also observed expression of Hcrt-1 receptors on cells in this brain region. Furthermore, direct administration of SB-334867 into the insula, but not into the adjacent somatosensory cortex (as described later), dose-dependently decreased nicotine self-administration but not responding for food rewards in rats. Taken together, these data suggest that insular hypocretin transmission may be a key substrate regulating the reinforcing effects of nicotine. Intriguingly, even the highest dose of intra-insula SB-334867 (5 μg/side; 15.6 nmol/side) did not completely abolish nicotine self-administration behavior, but instead reduced intake by $\approx$60%. It is therefore likely that hypocretin transmission in other brain regions may also contribute to this process. For example, the shell region of the nucleus accumbens, a brain area critically involved in the reinforcing effects of addictive drugs, receives afferent input from the agranular and dysgranular regions of the insular cortex (29). Further, the agranular insula receives innervation from reward-relevant brain regions including the ventral tegmental area (29), amygdala, and prefrontal cortex (30). Thus, it is an interesting possibility that hypocretin transmission in other portions of the insula, particularly the agranular insula, or in other reward-relevant brain regions such as the ventral tegmental area (10, 31), may also regulate the motivational properties of nicotine. Finally, as noted earlier, infusion of SB-334867 (5 μg/side) 2 mm above the insula into the somatosensory cortex did not significantly decrease nicotine self-administration. Nevertheless, there was a clear trend for nicotine intake to be diminished. We observed innervation by hypocretin-containing neurons and expression of Hcrt-1 receptors in the somatosensory cortex, albeit at lower densities than observed in the insular cortex (data not shown). Thus, it is possible that hypocretin transmission in the somatosensory cortex may also play a role in the detection or experiencing of nicotine reinforcement, but to a lesser degree than the insula.

Collectively, our findings demonstrate that hypocretin transmission at Hcrt-1 receptors in the insular cortex is a critical target necessary for the expression of the motivational properties of nicotine. Hence, destruction of insular hypocretin transmission in smokers who suffer damage to this brain region may explain the profound disruption of tobacco addiction observed in these individuals.

Materials and Methods

Animals. Male Wistar rats ($N = 67$; Charles River Laboratories) weighing 300 to 320 g were housed in groups of two per cage in a temperature-controlled vivarium on a 12-h reverse light/dark cycle (lights off at 1200 hours) with *ad libitum* access to food and water. Behavioral testing occurred during the dark portion of the light/dark cycle. All procedures were conducted in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of The Scripps Research Institute.

Drugs. (-)-Nicotine hydrogen tartrate (Sigma) was dissolved in sterile saline solution (0.9% wt/vol), and delivered in a volume of 1 ml/kg body weight by s.c. injection. SB-334867 (Tocris Bioscience; or synthesized by T.K.) was dissolved in 10:10:80 DMSO:Tween-80:water (vol:vol:vol) and delivered in a volume of 10 ml/kg body weight by i.p. injection. Aversive behavioral effects were not observed using this injection volume. For intra-insular administration, SB-334867 was dissolved in a 50:50 sterile saline solution:DMSO vehicle. SB-334867 concentrations for intra-insular infusion were prepared by serial dilution such that the final concentration of DMSO was the same for each dose of SB-334867 tested. DMSO has vascular and metabolic actions in the brain (32), but we did not observe any deleterious behavioral effects of insular vehicle injections in rats. All SB-334867 treatments were delivered according to a Latin-square design, with 3 to 5 intervening treatment-free days. For self-administration, nicotine was dissolved in sterile saline solution (pH adjusted to $\approx$7). Each infusion earned resulted in the delivery of 0.03 mg/kg nicotine free base, in a volume of 0.1 ml and delivered over a period of 1 sec.

Nicotine Self-Administration. Animals were trained to respond on an ''active'' lever for food pellets (45 mg; 30-min sessions) or nicotine infusions (0.03 mg/kg per infusion; 1-h sessions) under an FR5TO20 schedule of reinforcement, as described previously (15). Rats were also presented with an ''inactive lever,'' responding on which was recorded but was without scheduled consequence. A second group of rats responded for nicotine or food rewards under a PR reinforcement schedule in which the response requirements were increased according to the exponential progression ($5e^{0.25 \times [\text{infusion number} + 3]} - 5$], with the first two values replaced by 5 and 10 (i.e., 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc.). Rats responded each day under the PR schedule until they did not receive a reward for $>$1 h (i.e., break-point was achieved). Treatments with SB-334867 commenced after stable responding for nicotine under the FR or PR schedules was achieved, defined as $<$20% variation in the number of infusions earned per session over three consecutive sessions, and requiring between seven and 14 self-administration sessions. To directly compare the effects of SB-334867 on responding for nicotine or food rewards, nicotine and food intake data from each animal were expressed as percentage change from baseline number of rewards earned, where baseline was defined as the mean number of rewards earned by animals after vehicle treatment.

Intracranial Self-Stimulation. Animals were surgically prepared with an ICSS stimulating electrode and trained in a rate-free, discrete-trial, current-threshold procedure described previously (15). Percentage change from baseline thresholds was calculated by expressing the drug-influenced threshold scores as a percentage of the baseline threshold, where baseline was defined as the mean ICSS thresholds obtained over the 3 days before each drug testing session.

Cannula Implantation and Intracerebral Injection Procedure. Rats prepared with intracerebral cannulae were first anesthetized by inhalation of 1% to 3% isoflurane in oxygen and positioned in a stereotaxic frame (Kopf Instruments). Bilateral stainless steel guide cannulae (23 gauge, 14 mm in length) were implanted 2.5 mm above the insula (antro-posterior, 1.20 mm from bregma; medio-lateral, $\pm$2.70 mm; dorso-ventral, $-$4.50 mm from dura; 20° angle toward midline; flat skull position). Four stainless steel skull screws and dental acrylic held the cannulae in place. SB-334867 injections were administered bilaterally in a volume of 0.5 μl per side over a period of 60 sec. Injectors were subsequently removed and replaced with 14-mm stylets. Only animals with injector tips verified to be located within the insula were included in statistical analyses.

Immunochemistry. Rats were anesthetized and perfused trans-cardially with cold 4% paraformaldehyde in PBS solution, pH 7.4. Brains were postfixed in 4% paraformaldehyde overnight and stored in 30% sucrose in PBS solution. Sections (30 μm) were collected on a freezing microtome in the coronal plane. Immunohistochemistry was performed on free-floating sections using the following primary antibodies: rabbit anti-hypocretin-1 peptide (Chemicon International) or goat anti-hypocretin-1 receptor (Santa Cruz Biotechnology). Primary antibodies were incubated overnight at 4 °C in 1% BSA in PBS solution at 1:1,000 (Hcrt-1 peptide) and 1:100 (Hcrt-1 receptor) dilutions. This was followed by incubation for 1 h with the following fluorescent-tagged secondary antibodies: Alexa Fluor 488 goat anti-rabbit (Invitrogen) or Alexa Fluor 660 donkey anti-goat IgG (Invitrogen), both at a 1:100 dilution. To visualize cell nuclei, sections were treated with 300 nM DAPI for 30 min. Sections were mounted on Superfrost Plus slides (Fischer Scientific), dehydrated, and coverslipped. Sections were visualized by using a fluorescence microscope (BX61; Olympus) at magnifications of $\times$20 and $\times$40.

Pharmacokinetics and Brain Penetration of SB-334867. Rats were injected with SB-334867 (4 mg/kg i.p.; $n = 6$) and blood was collected 5, 15, 30, 60, and 120 min after injection. Brains were collected at 120 min after injection. Concentrations of SB-334867 in blood samples were quantitated by liquid chromatography/tandem MS. Peak areas of the m/z 320$\rightarrow$146 product ion of SB-334867 (DP = 40, CE = 24) were measured against the peak areas of the internal standard (i.e, sunitinib) m/z 399$\rightarrow$283 product ion (DP = 86, CE = 41) of the internal standard. Data were fit using WinNonLin. Similar conditions were used to determine brain levels of SB-334867 except the brain samples were frozen upon collection.

Statistical Analyses. Data were analyzed by one- or two-way repeated-measures ANOVA. Significant main or interaction effects were followed by Bonferroni post-tests or Newman-Keuls *post-hoc* tests as appropriate. All statistical analyses were performed using GraphPad Prism software. In all cases, the level of significance was set at 0.05.

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