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Nicotine stimulates secretion of corticosterone via both CRH and AVP receptors

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Abstract

Corticosterone–releasing hormone (CRH) and arginine vasopressin (AVP) are crucial components of the hypothalamic-pituitary-adrenal (HPA) axis that stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary and mediate the stress response. CRH binds to two subtypes of CRH receptors (CRH-R1 and CRH-R2) that are present in both central and peripheral tissues. We used the CRH-R1 specific antagonist, antalarmin (ANT), the CRH-R1 and CRH-R2 peptide antagonist, astressin (AST), and the CRH-R2 specific peptide antagonist, astressin2b (AST2b), to determine which CRH receptor is involved in the nicotine-stimulated secretion of corticosterone. Male C57BL/6 mice were administered ANT (20 mg/kg, i.p.), AST (0.3 mg/kg, i.p.), AST2b (0.3 mg/kg, i.p.) or vehicle prior to administration of nicotine (1.0 mg/kg, s.c.), CRH (10 µg/kg, s.c.), AVP (10 µg/kg, s.c.) or saline (SAL) (s.c.), sacrificed 15 min later and trunk blood collected and assayed for corticosterone plasma levels. We found that CRH enhanced corticosterone release, and this response was blocked by both AST and ANT. Nicotine also increased corticosterone secretion, but this effect persisted in the presence of either CRH antagonist. Furthermore, AST but not ANT or AST2b decreased corticosterone levels associated with stress of handling and injection. We also assessed the role of AVP V_{1b} specific receptor antagonist, SSR149415 alone and in combination with AST and AST2b. Although the AVP antagonist did not alter basal or nicotine-stimulated corticosterone secretion, it attenuated the AVP-induced stimulation of corticosterone and its combination with AST but not AST2b completely abolished nicotine-mediated stimulation of corticosterone secretion. Our results demonstrate that the nicotine-induced stimulation of the hypothalamic-pituitary adrenal axis (HPA) is mediated by both the CRH-R and the AVP V_{1b} receptor and when the CRH receptor is blocked, nicotine may utilize the AVP V_{1b} receptor to mediate secretion of corticosterone. These results argue in favor of the development of specific antagonists that block both AVP and CRH receptors to decrease the pleasurable component of nicotine, which may be mediated by corticosterone.

Keywords

Nicotine; CRH; AVP, Astressin; Antalarmin; Corticosterone; Mouse

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the stress response and functions to maintain basal and stress-related homeostasis (Chrousos 1995, Chrousos & Gold 1992). Corticotrophin-releasing hormone (CRH), a 41-amino acid hypothalamic neuropeptide, and its homologues urocortin (Ucn), Ucn II and Ucn III are critical coordinators of the HPA axis (Bale *et al.* 2002, McCarthy *et al.* 1999, Miragaya & Harris 2008). CRH, along with arginine vasopressin (AVP) stimulates adrenocorticotrophic hormone (ACTH) release from the pituitary, leading to increases in cortisol (corticosterone in rodents) secretion from the adrenal glands. CRH binds to two G-protein coupled receptors: CRH-R1 and CRH-R2 with CRH-R2 presenting with three splice variants of the same gene (CRH-R2 α , CRH-R2 β and CRH-R2 γ) (Bale *et al.* 2002, Chen *et al.* 1993, Grigoriadis *et al.* 1996, Lovenberg *et al.* 1995, Perrin *et al.* 1993). CRH-R1 is abundantly expressed in the cerebral cortex, cerebellum, medial septum and anterior pituitary (Chen *et al.* 1993), CRH-R2 α is present in subcortical regions including the lateral septum and paraventricular and ventromedial nuclei of the hypothalamus, CRH-R2 β is the predominant receptor expressed in the peripheral tissues, such as the heart, gastrointestinal tract, arterioles and muscle. CRH-R2 γ has recently been identified in the human brain in septum and hippocampus, with weaker but detectable expression in the amygdala, nucleus accumbens, midbrain, and frontal cortex (Chen *et al.* 1993, Gallagher *et al.* 2008, McCarthy *et al.* 1999, Kostich *et al.* 1998).

Knockout and anti-sense experiments suggest that CRH-R1 mediates the effects of CRH in regulating the HPA axis and anxiety-related behaviors (Heinrichs *et al.* 1997, Preil *et al.* 2001, Smith *et al.* 1998). Mice deficient for CRH-R1 display a severe impairment of CRH- and stress-induced ACTH release from pituitary corticotropes, marked glucocorticoid deficiency, and reduced anxiety-like behaviors (Muller *et al.* 2001, Smith *et al.* 1998, Timpl *et al.* 1998). Analyses of CRH-R2-deficient mice demonstrate that CRH-R2 plays a modulatory role in the stress response involving the maintenance of ACTH release during stress exposure and the return to basal levels of plasma corticosterone following the removal of stressors (Bale *et al.* 2002, Coste *et al.* 2000).

CRH antagonists have been developed that inhibit CRH binding to its receptors and antagonize its actions *in vivo* and *in vitro* (Curtis *et al.* 1994, Gulyas *et al.* 1995). Astressin (AST), a non-selective CRH-antagonist that blocks both CRH-R1 and CRH-R2 and is particularly potent at inhibiting the HPA axis (McCarthy *et al.* 1999, Spina *et al.* 2000, Tu *et al.* 2007, Zorrilla *et al.* 2003). AST has been found to be particularly effective in reducing the stress response in relatively severe stressful situations (Spina *et al.* 2000). A non-peptide CRH receptor antagonist, specific for CRH-R1, antalarmin (ANT), penetrated the blood-brain barrier and inhibited CRH binding in rat pituitary and brain regions (Webster *et al.* 1996). When used acutely, ANT inhibited CRH-stimulated ACTH release (Webster *et al.* 1996), and the conditioned fear response in rats (Deak *et al.* 1999) as well as stressed-related behaviors in rhesus monkeys (Habib *et al.* 2000), but not the ACTH or corticosterone response to inescapable shock (Deak *et al.* 1999). CRH-R2-selective peptide antagonists, antisauvagine-30 (Ruhmann *et al.* 1998) and astressin-2B (Rivier *et al.* 2002) have both been used to characterize the effects mediated specifically by the CRH-R2.

Three subtypes of vasopressin receptors (V_{1a}, V₂ and V_{1b} or V₃) have been cloned (de Keyser *et al.* 1996, Lolait *et al.* 1992, Morel *et al.* 1992). V_{1a} receptors are present in vascular and hepatic tissues and mediate the pressor response of AVP. V₂ receptors are present in the kidney and mediate the anti-diuretic properties of AVP. The pituitary vasopressin receptor, V_{1b} (also called V₃), is related to, but distinct from the V_{1a} receptor and mediates the AVP-induced secretion of ACTH at the corticotroph. An investigational compound produced by Sanofi-Aventis, SSR149415 (SSR), is a V_{1b} AVP receptor

antagonist (Serradeil-Le Gal *et al.* 2002) and has been found to be effective in rodent models of anxiety and depression (Griebel *et al.* 2005, Hodgson *et al.* 2007).

Some of the behavioral and addictive properties of nicotine appear related to its activation of the stress response that is mediated by the HPA axis (Gilbert 1979). Acute nicotine administration elevates plasma ACTH and corticosterone in laboratory animals (Andersson *et al.* 1981, Cam *et al.* 1979, Lutfy *et al.* 2006). When rats self-administer nicotine in both response-contingent and response-independent paradigms, a rise in corticosterone is seen (Donny *et al.* 2000). Additionally, cigarette smoking increases circulating cortisol in humans (Pomerleau & Pomerleau 1990, Seyler *et al.* 1984). Nicotine acts centrally to stimulate ACTH release, rather than directly at the level of the pituitary (Matta *et al.* 1987) and CRH has been implicated as a mediator of this response, although this notion has not been proven (Matta *et al.* 1998, Rosecrans & Karin 1998). Therefore, in this paper, we used selective and non-selective CRH-R antagonists to elucidate the role of each CRH receptors in the nicotine-induced activation of the HPA axis, as measured by increases in plasma corticosterone secretion. We also used SSR, an AVP V_{1b} antagonist, to study the role of AVP receptors in the nicotine-induced stimulation of corticosterone secretion. Furthermore, we determined the effect of a combination of an AVP V_{1b} antagonist with selective CRH R2 or non-selective CRH antagonists to elucidate interactions between these receptors that might mediate the ability of nicotine to stimulate corticosterone secretion. For comparison, the effect of CRH-R and AVP-R antagonists was studied on CRH- and AVP-induced corticosterone secretion, respectively.

Materials and methods

Chemicals

(-)-Nicotine ([-]-1-Methyl-2-[3-pyridyl]-pyrrolidine), AST, AST2b and CRH (human, rat) were purchased from Sigma/Aldrich (St. Louis, MO) and dissolved in saline (SAL) 1 h prior to intraperitoneal (i.p.) or subcutaneous (s.c.) administration. Antalarmin hydrochloride was a generous gift from George P. Chrousos, MD (University of Athens, previously at the National Institutes of Health) and dissolved in 70% ethanol which was then diluted 100-fold with SAL so that the final ethanol concentration was 0.7%. Injection of this concentration of ethanol compared to SAL did not affect corticosterone levels (Aimiwu and Friedman, unpublished data). SSR149415 was generously supplied by Sanofi-Aventis and dissolved in DMSO and was then diluted with SAL so the final DMSO concentration was 10%. Doses of ANT (Deak *et al.* 1999, Navarro-Zaragoza *et al.* 2010), AST (Gulyas *et al.* 1995) and AST2b (Broadbear *et al.* 2004, Rivier *et al.* 2002) were based on the literature. There was no difference in corticosterone secretion following administration of SAL or 10% DMSO solution so the vehicle groups were combined, wherever appropriate.

Animals

Eight-week-old male C57BL/6J mice (25-28 g), obtained from The Jackson Laboratory (Bar Harbor, ME), were group-housed in cages (n = 4 mice per cage) with bed o' cob ¼ inch rodent bedding (Newco Laboratory, Rancho Cucamonga, CA, USA) and kept under a 12-h light/12-h dark cycle (lights on at 0700 and off at 1900) with free access to water and food. All experimental procedures were conducted in accordance with the guidelines of the National Institutes of Health for the proper use of laboratory animals and approved by the Institutional Animal Care and Use Committee at Charles R. Drew University.

Experimental Procedures—Mice were brought to a soundproof testing room at 0800 and left undisturbed for 3 h to acclimatize to the procedure room. Injections (described below) began between 1100 and 1200 h and were staggered so the time of sacrifice occurred

at 1300 for each mouse. The time points and doses of each inhibitor (described below) were based on our pilot time-course and dose-response studies and our previous study (Lutfy et al., 2006). Mice were euthanized by decapitation in a separate room from the mice receiving injections. Trunk blood was collected in ice-cold 1.5-ml vials containing 50 μ l of 7% EDTA. Samples were then centrifuged at 4°C (12,000 rpm for 5 min), supernatants (300 to 600 μ l) were collected and frozen at -80°C until assayed for corticosterone.

Experiment 1: The effect of ANT and AST on CRH-induced release of corticosterone:

Mice were treated with SAL or ANT (20 mg/kg, i.p.) followed by SAL or CRH (10 μ g/kg, s.c.) 90 min later. In separate experiments, mice were treated with SAL or AST (0.3 mg/kg, i.p.) followed by SAL or CRH (10 μ g/kg, s.c.) 30 min later. For all treated groups, mice were sacrificed 15 min after SAL or CRH administration and trunk blood collected and processed as described above. We used this dose of CRH that was on the linear portion of the dose-response curve so that any increase in the level of corticosterone could be easily detected.

Experiment 2: The effect of SSR on AVP-induced secretion of corticosterone: Mice were treated with SAL or SSR (10 mg/kg, i.p.) followed by SAL or AVP (10 μ g/kg, i.p.) 20 min later. Mice were then sacrificed 15 min after SAL or AVP administration, trunk blood collected and processed as described above.

Experiment 3: The effect of ANT and AST on nicotine-induced secretion of corticosterone: Mice were treated with SAL or ANT (20 mg/kg, i.p.) followed by SAL or nicotine (1.0 mg/kg, s.c.) 90 min later. In separate experiments, mice were treated with SAL or AST (0.3 mg/kg, i.p.) followed by SAL or nicotine (1.0 mg/kg, s.c.) 30 min later. For all treated groups, mice were sacrificed 15 min after SAL or nicotine administration, trunk blood collected and processed as described above. We selected the time and dose of nicotine from our prior paper in which we performed time-course and dose-response studies examining the effect of nicotine on corticosterone secretion (Lutfy et al. 2006).

Experiment 4: The effect of SSR alone and in combination with AST on nicotine-induced secretion of corticosterone: Mice were treated with SAL, SSR (10 mg/kg, i.p.), AST (0.3 mg/kg) or a combination of SSR and AST followed by SAL or nicotine (1.0 mg/kg, s.c.) 30 min later. Naïve mice did not receive any injections. Mice were sacrificed 15 min after SAL or nicotine administration, trunk blood collected and processed as described above. The time of drug administration and the dose of each drug were based on our pilot and previous studies (Lutfy et al. 2006).

Experiment 5: The effect of AST2b alone or with SSR on nicotine-stimulated corticosterone secretion: Mice were treated with SAL, SSR (10 mg/kg, i.p.), AST2b (0.3 mg/kg) or a combination of SSR and AST2b followed by SAL or nicotine (1.0 mg/kg, s.c.) 30 min later. Mice were sacrificed 15 min after SAL or nicotine administration, trunk blood collected and processed as described above. The time of drug administration and the dose of each drug were based on our pilot and previous studies (Lutfy et al. 2006).

Plasma hormone assays

All plasma hormone concentrations were measured in duplicate by radioimmunoassay (RIA) as previously described (Lutfy et al. 2006) and batch-processed, i.e., all samples collected were assayed at once. All hormone values were on the linear portion of the standard curve for the assay. Plasma corticosterone levels (5 μ l of serum per assay) were measured by rat/mouse corticosterone 3 H kit (MP Biomedicals, Costa Mesa, CA). Cross-reactivity, variance and assay range is given in our prior paper (Lutfy et al. 2006).

Statistical Analyses

Data are expressed as the mean $\pm$ standard error of the mean (S.E.M). The data were analyzed using one- or two-way analysis of variance (ANOVA) followed by the Newman-Keuls or Bonferroni *post-hoc* test for one- and two-way ANOVA, respectively, to reveal significant differences between various groups. A value of $p < 0.05$ was considered statistically significant.

Results

The effect of ANT or AST on CRH-induced corticosterone secretion

Fig. 1 depicts the effect of ANT pretreatment on CRH-induced release of corticosterone. A two-way ANOVA revealed a significant effect of pretreatment, i.e., ANT vs. vehicle (VEH) ($F_{1,27} = 15.2$; $p < 0.0001$), a significant effect of treatment, i.e., CRH vs. SAL ($F_{1,27} = 16.8$; $p < 0.0001$) and a trend toward a significant interaction between the two factors ($F_{1,27} = 3.30$; $p = 0.08$). *Post-hoc* testing showed that CRH stimulated the secretion of corticosterone in VEH-pretreated animals ($p < 0.001$; compare VEH-CRH vs. VEH-SAL), an effect that was blocked by ANT ($p < 0.001$; compare ANT-CRH vs. VEH-CRH group). This result suggests that the CRH-induced secretion of corticosterone is likely mediated by the same receptor that is blocked by ANT, which is most likely CRH-R1.

Fig. 2 illustrates the effect of AST pretreatment on CRH-stimulated secretion of corticosterone. A two-way ANOVA revealed a significant effect of pretreatment, i.e., AST vs. SAL ($F_{1,27} = 28.2$; $p < 0.0001$), a significant effect of treatment, i.e., CRH vs. SAL ($F_{1,27} = 18.8$; $p < 0.0002$) and a significant interaction between the two factors ($F_{1,27} = 4.38$; $p < 0.05$). *Post-hoc* testing showed that CRH increased corticosterone secretion in VEH-pretreated animals ($p < 0.0001$; VEH-CRH vs. VEH-SAL group), an effect that was blocked in AST-pretreated mice ($p < 0.001$; AST-CRH vs. VEH-CRH group). This result suggests that blockade of both receptors may be essential for the complete inhibition of CRH-mediated corticosterone secretion.

The effect of SSR on AVP-stimulated secretion of corticosterone

Fig. 3 shows the effect of SSR pretreatment on AVP-stimulated secretion of corticosterone. A two-way ANOVA revealed a significant effect of treatment ($F_{1,20} = 11.0$; $p < 0.005$), but no significant effect of pretreatment ($F_{1,20} = 2.76$; $p > 0.05$) and no significant interaction between the two factors ($F_{1,20} = 0.51$; $p > 0.05$). *Post-hoc* testing showed that AVP increased corticosterone secretion in VEH-pretreated mice ($p < 0.05$, compare VEH-SAL and VEH-AVP) and this response was attenuated by SSR pretreatment (compare SSR-SAL and SSR-AVP). The reduction in corticosterone levels by SSR following AVP did not reach significance compared to vehicle plus AVP ($p > 0.05$). SSR did not alter basal level of corticosterone (compare SSR-SAL vs. VEH-SAL group; $p > 0.05$). These results suggest that the V_{1b} AVP receptor may mediate the stimulatory action of AVP on corticosterone secretion.

The effect of ANT or AST on nicotine-stimulated secretion of corticosterone

Fig. 4 shows the effect of ANT pretreatment on nicotine-stimulated secretion of corticosterone. A two-way ANOVA revealed a significant effect of treatment ($F_{1,22} = 68.6$; $p < 0.0001$), but no significant effect of pretreatment ($F_{1,22} = 0.04$; $p > 0.05$) and no significant interaction between the two factors ($F_{1,22} = 0.04$; $p > 0.05$). *Post-hoc* testing showed that nicotine increased corticosterone secretion in both VEH- and ANT-pretreated mice ($p < 0.001$; compare VEH-NIC vs. VEH-SAL group and ANT-NIC vs. ANT-SAL group). ANT did not alter basal level of corticosterone (compare ANT-SAL vs. VEH-SAL group;

$p > 0.05$). This result suggests that CRH-R1 receptor alone does not mediate the stimulatory action of nicotine on corticosterone secretion.

Fig. 5 demonstrates the effect of AST pretreatment on nicotine-stimulated secretion of corticosterone. A two-way ANOVA revealed a significant effect of pretreatment ($F_{1,54} = 66.4$; $p < 0.0001$), a significant effect of treatment ($F_{1,54} = 37.2$; $p < 0.0001$) and a trend toward a significant interaction between the two factors ($F_{1,54} = 3.7$; $p = 0.06$). *Post-hoc* testing indicated that nicotine increased corticosterone secretion in both VEH- and AST-pretreated mice (compare VEH-NIC vs. VEH-SAL group and AST-NIC vs. AST-VEH group; $p < 0.01$ or better). Examining this figure carefully, although the level of corticosterone was lower in the AST-treated group following by nicotine compared to mice receiving SAL followed by nicotine (compare AST-NIC vs. VEH-NIC group; $p < 0.01$), AST alone also reduced basal corticosterone secretion (compare AST-SAL vs. VEH-SAL group; $p < 0.001$). Furthermore, the difference in corticosterone secretion, following nicotine in mice receiving AST (76 ng/mL obtained by subtracting the basal from nicotine-stimulated corticosterone levels for the AST-pretreated mice) was greater than the increase in corticosterone level following nicotine in mice receiving SAL (39 ng/mL, obtained by subtracting the basal from nicotine-stimulated corticosterone levels for the VEH-pretreated mice). Together, these results indicate that AST may alter the level of nicotine-stimulated secretion of corticosterone via an inhibitory action on basal corticosterone level, but does not block the nicotine-stimulated secretion of corticosterone *per se*.

The effect of SSR alone and in combination with AST on nicotine-induced corticosterone secretion

Figure 6 depicts the effect of AST and SSR pretreatment either alone or in combination on nicotine-stimulated secretion of corticosterone in mice. A one-way ANOVA revealed a significant effect of treatment ($F_{8,65} = 20.6$; $p < 0.001$). *Post-hoc* testing revealed that nicotine significantly increased corticosterone secretion in VEH-pretreated mice (compare VEH-NIC vs. VEH-SAL group; $p < 0.001$). AST significantly ($p < 0.001$) reduced the nicotine-stimulated secretion of corticosterone (compare AST-NIC vs. VEH-NIC group; $p < 0.001$). However, as observed earlier (Fig. 5), the effect of AST was likely due to its ability to reduce corticosterone secretion induced by stress of injection (compare AST-SAL vs. VEH-SAL and notice the effect of stress: VEH-SAL vs. naïve group). On the other hand, the AVP antagonist SSR did not alter the response to injection (compare SSR-SAL vs. VEH-SAL group; $p > 0.05$) or the action of nicotine (compare SSR-NIC vs. VEH-NIC group; $p > 0.05$). However, the combination of SSR with AST significantly reduced the nicotine-stimulated corticosterone secretion and this response was significantly greater than the effect of AST or SSR alone (compare AST-SSR-NIC vs. AST-NIC or SSR-NIC; $p < 0.001$). This result suggests that nicotine exerts its stimulatory action on corticosterone secretion via both CRH and AVP receptors.

In pilot data, we found that DMSO (10%) alone did not significantly affect the level of corticosterone compared to saline-treated controls. Therefore, the data for the DMSO-SAL and SAL-SAL groups were combined (Fig. 6). Corticosterone levels following DMSO +AST +NIC were similar to AST-NIC (data not shown).

The effect of AST2b alone or with SSR on nicotine-stimulated corticosterone secretion

Fig. 7 illustrates the effect of the CRH-R2 specific antagonist, AST2b, pretreatment alone or with SSR on nicotine-stimulated secretion of corticosterone. One-way ANOVA revealed a significant effect of treatment ($F_{6,31} = 13.6$; $p < 0.0001$). *Post-hoc* testing showed that nicotine increased corticosterone secretion in VEH-treated mice (compare VEH-NIC vs. VEH-SAL group, $p < 0.05$); however pretreatment with AST2b and AST2b+SSR did not block the

stimulation (compare VEH-NIC with AST2b-NIC and VEH-NIC with AST2b-SSR-NIC; $p=NS$). This result suggests that blocking both the CRH-R2 receptor and the V_{1b} AVP receptor is not sufficient to block the stimulatory action of nicotine on corticosterone secretion.

The effect of ANT, AST or AST2b on vehicle-stress-stimulated secretion of corticosterone

ANT pretreatment did not reduce corticosterone secretion compared to VEH-treated mice in Fig. 1 and 4 indicating that the secretion of corticosterone induced by stress of handling/injection does not proceed via CRH-R1. Similarly, AST2b pretreatment did not affect this response (Fig. 7; $p=NS$). In contrast, AST pretreatment reduced corticosterone secretion compared to VEH-treated mice (Figs. 5 and 6) but it did not achieve statistical significance level in one experiment although clear reduction was evident (Fig. 2). When the data were combined, AST pretreatment significantly reduced corticosterone levels ($p < 0.001$). Overall, these results suggest that the stimulation of corticosterone secretion induced via the stress of handling and injection most likely proceeds via activation of both CRH-R1 and CRH-R2 receptors.

Discussion

One of the many physiological effects of nicotine is the activation of the HPA axis with a resultant increase in secretion of ACTH and glucocorticoids. In rodents, acute nicotine administration elevates plasma ACTH and corticosterone (Andersson *et al.* 1981, Cam *et al.* 1979). Chronic nicotine administration given by repeated injections leads to an increase in corticosteroid production, albeit at a lower level than acute administration (Caggiula *et al.* 1991, Cam & Bassett 1984, Fuxe *et al.* 1989). Furthermore, nicotine withdrawal leads to anxiety and stress, both of which are powerful incentives to continue smoking or to trigger relapse in former smokers; this effect is likely mediated by CRH (Benowitz 2010). In humans, cigarette smoking increases circulating cortisol (Pomerleau & Pomerleau 1990, Seyler *et al.* 1984, Wilkins *et al.* 1982). We have previously shown that nicotine administration acutely increases the level of corticosterone in C57Bl/6 mice (Lutfy *et al.* 2006). However, the underlying mechanisms of nicotine-stimulated secretion of corticosterone are not fully defined. Accordingly, in the current study we used CRH antagonists to define the mechanism of this stimulatory action of nicotine on corticosterone secretion. Given that activation of the HPA axis is primarily mediated by the release of CRH, we first assessed the role of CRH receptors in the action of nicotine.

Our results revealed that pretreatment with ANT [a non-peptide CRH-R1 receptor antagonist (Webster *et al.* 1996)] blocked the CRH-evoked secretion of corticosterone. However, ANT pretreatment did not block the nicotine-induced secretion of corticosterone, suggesting that nicotine increased corticosterone secretion via a receptor other than CRH-R1. We then used AST, which is known to antagonize both CRH-R1 and CRH-R2 (Gulyas *et al.* 1995, McCarthy *et al.* 1999, Spina *et al.* 2000, Zorrilla *et al.* 2003) and found that it blocked the ability of CRH to increase corticosterone secretion, but failed to block the stimulatory action of nicotine on corticosterone secretion. Although the level of nicotine-stimulated corticosterone secretion was significantly lowered in mice pretreated with AST, a closer observation of the data suggest that this was due to AST decreasing the rise in corticosterone secretion associated with the stress of injection and therefore, AST-sensitive receptors do not mediate the nicotine-induced stimulation of corticosterone secretion. Furthermore, the CRH-R2 antagonist, AST2b also failed to alter nicotine-induced stimulation of corticosterone secretion. We then used the AVP V_{1b} receptor antagonist, SSR, and found that this drug, which reduced AVP-mediated corticosterone secretion, did not block nicotine-induced corticosterone secretion. However, in the presence of AST, SSR completely blocked nicotine-induced corticosterone secretion. We interpret these results as

that when either the CRH receptor(s) or the AVP V_{1b} receptor is blocked, a compensatory pathway using the other receptor is utilized by nicotine to stimulate corticosterone secretion. Nevertheless, when both the CRH antagonist, AST, and the AVP V_{1b} receptor antagonist, SSR, were used together, nicotine-induced corticosterone secretion was completely blocked.

We are familiar with the work of Okada and colleagues (Okada *et al.* 2003) who administered CP-154,526, a CRH-R1 antagonist closely related to ANT, intracerebroventricularly to anesthetized rats and found that CP-154,526 blocked the nicotine-induced stimulation of corticosterone. However in that paper, the basal corticosterone levels were 247 ng/mL which went up to over 400 ng/mL following nicotine administration. These levels were superphysiologic and thus the role of CRH-R1 in regulating more physiological levels of corticosterone was unclear. However, the failure of CRH antagonists in our study to alter the nicotine-stimulated secretion of corticosterone was not due to the choice of the dose of these antagonists because both antagonists clearly blocked the effect of CRH and these doses have been used previously (Deak *et al.* 1999, Navarro-Zaragoza *et al.* 2010, Gulyas *et al.* 1995, Broadbear *et al.* 2004, Rivier *et al.* 2002). Additionally, the lack of the response is not due to central vs. peripheral effects of ANT since there is good evidence that ANT readily crosses the blood-brain barrier (Webster *et al.* 1996).

The adrenal cortex expresses both CRH (Muller *et al.* 2001) and nicotinic acetylcholine (Davila-Garcia *et al.* 2003) receptors. Thus, it is possible that nicotine stimulates hypothalamic CRH that then travels systemically to stimulate secretion of corticosterone from the adrenal cortex or that nicotine directly stimulates corticosterone release from the adrenal gland. We consider this unlikely as elegant experiments conducted over 30 years ago showed that hypophysectomy completely blocked the effect of nicotine on corticosterone (Cam *et al.* 1979), suggesting that the nicotine-mediated secretion of corticosterone is mediated by the pituitary. CRH-R2, in addition to CRH-R1 is expressed in the pituitary (Muller *et al.* 2001), and thus, we suspect that the site of action of the nicotine-induced CRH is at the level of the pituitary. Our results are complicated by the finding that CRH-R1 null mice do not respond to CRH in terms of ACTH and corticosterone secretion (Muller *et al.* 2001). It is possible that nicotine-induced endogenous CRH has different biological actions compared to exogenous CRH and may have different sensitivity profile to CRH1 and CRH2 antagonism.

We found that AST, but not ANT or AST2b blocked the increase in corticosterone induced by handling and injection. Thus, it appears that CRH-R1 mediates the CRH-induced activation of corticosterone, while both CRH-R1 and CRH-R2 play an important role in corticosterone secretion induced by stress of handling and injection.

In summary, AST, but not ANT completely abolished CRH-evoked release of corticosterone. Neither AST nor ANT blocked nicotine-induced-corticosterone secretion. Similarly, SSR alone was without effect on nicotine-stimulated corticosterone secretion. However, the combination of AST and SSR completely abolished this action of nicotine. We conclude that nicotine-induced corticosterone secretion involves both CRH receptors as well as the AVP V_{1b} receptor since when the CRH receptor(s) is blocked, nicotine may induce its action via the alternative pathway utilizing the AVP V_{1b} receptor. Our findings suggest that pharmacological intervention to block the pleasurable effect of nicotine, which may be partially mediated by corticosterone, could involve the development of drug(s) that block both CRH and AVP V_{1b} receptors, while drugs that block one system alone are unlikely to be effective. Further studies are needed to determine whether the combination of these antagonists would alter the rewarding and addictive actions of nicotine.

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Fig. 1.

The effect of ANT on CRH-stimulated corticosterone secretion. Mice ($n = 7-8$ per group) were pretreated with ANT ($20 \mu\text{g/kg}$, i.p.) or VEH (i.p.) (pretreatment is shown in the legend) and 90 min later injected with CRH ($10 \mu\text{g/kg}$, s.c.) or SAL (s.c.) (treatment is shown on the x-axis). Mice were sacrificed 15 min later and trunk blood was collected and used for the measurement of corticosterone. *** $p < 0.001$ compared to VEH-SAL.

Fig. 2.

The effect of AST on CRH-mediated secretion of corticosterone. Mice ($n = 7-10$ per group) were pretreated with AST (0.3 mg/kg, i.p.) or VEH (i.p.) and 15 min later were injected with CRH (10 μ g/kg, s.c.) or SAL (s.c.). Mice were sacrificed after an additional 15 min. Trunk blood was collected and used for the measurement of corticosterone. *** $p < 0.001$ compared to compared to VEH-SAL.

Fig. 3.

The effect of SSR149415 (SSR) on AVP-mediated secretion of corticosterone. Mice ($n = 6$ per group) were pretreated with SSR (10 mg/kg, i.p.) or VEH (i.p.) and 20 min later were injected with AVP (10 μ g/kg, s.c.) or SAL (s.c.). Mice were sacrificed after an additional 15 min. Trunk blood was collected and used for the measurement of corticosterone. * $p < 0.05$ compared to VEH-SAL.

Fig. 4. The effect of ANT on nicotine-induced corticosterone secretion. Mice ($n = 5-8$ per group) were treated with ANT ($20 \mu\text{g/kg}$, i.p.) or VEH (i.p.) followed, 90 min later, by nicotine (NIC; 1 mg/kg , sc) or SAL (s.c.). Mice were sacrificed 15 min later and trunk blood was collected and used for the measurement of corticosterone. *** $p < 0.001$ compared to their respective SAL group.

Fig. 5.

The effect of AST on nicotine-stimulated corticosterone secretion. Mice ($n = 14-15$ per group) received AST (0.3 mg/kg, i.p.) or VEH (i.p.) followed, 15 min later, by nicotine (NIC; 1.0 mg/kg, sc) or SAL (s.c.). Mice were sacrificed 15 min later and trunk blood was collected and used for measurement of corticosterone. # $p < 0.05$ compared to VEH-SAL group; ** $p < 0.01$ vs. VEH-SAL group; *** $p < 0.001$ vs. AST-SAL group; ++ $p < 0.01$ vs. VEH-NIC group.

Fig. 6.

The effect of AST or SSR alone or in combination on nicotine-stimulated corticosterone secretion. Mice ($n = 6 - 8$ per group) received AST (0.3 mg/kg, i.p.), SSR (10 mg/kg, i.p.) either alone or in combination. Controls were injected with VEH ($n = 8$ mice; i.p.) or left untreated (naïve; $n = 4$ mice). Fifteen min later, mice received nicotine (NIC; 1 mg/kg, s.c.) or SAL (s.c.) and were sacrificed 15 min later. Trunk blood was collected and used for the measurement of corticosterone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. VEH-SAL treated control group. ++ $p < 0.01$ and +++ $p < 0.001$ vs. VEH-NIC; # $p < 0.01$ vs. SSR-SAL.

Fig. 7.

The effect of AST2b alone or with SSR on baseline and nicotine-stimulated corticosterone secretion. Mice ($n = 4 - 8$ per group) received AST2b (0.3 mg/kg, i.p.) with and without SSR (10 mg/kg, i.p.) or vehicle. Fifteen min later, mice received nicotine (NIC; 1 mg/kg, s.c.) or SAL (s.c.) and were sacrificed 15 min later. Trunk blood was collected and used for the measurement of corticosterone. * $p < 0.05$ vs. VEH-SAL treated control group..