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Deficits in Substance P mRNA Levels in the CeA are Inversely Associated with Alcohol-Motivated Responding

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Abstract

In the present study, *in vitro* and *in vivo* studies were conducted to determine the relationship between innate substance P [SP] levels and alcohol-motivated behavior in alcohol-preferring [P] and nonpreferring [NP] rat lines. In experiment 1, *in situ* hybridization and quantitative autoradiography were used to detect and measure SP mRNA levels in discrete brain loci of the P and NP rats. The results indicated significantly lower SP mRNA levels in the central nucleus of the amygdala [CeA] of P compared with NP rats. Experiment 2 evaluated the effects of SP, microinfused into the CeA, on alcohol [10% v/v] and sucrose [2% w/v] motivated responding in the P rat. The results revealed that, when infused into the CeA [1 – 8 µg], SP reduced alcohol responding by 48 – 85% of control levels, with no effects on sucrose responding. Neuroanatomical control infusions [1 – 8 µg] into the caudate putamen [CPu] also failed to significantly alter alcohol or sucrose-motivated behaviors. Given the selective reductions on alcohol [compared to sucrose] responding by direct intracranial infusion of SP, the data suggest that deficits in SP signaling within the CeA [an anxiety regulating locus] are inversely associated with alcohol-motivated behaviors. Activation of SP receptors in the CeA may reduce anxiety-like behavior in the P rat and contribute to reductions on alcohol responding. The SP system may be a suitable target for the development of drugs to reduce alcohol-drinking behavior in humans.

Keywords

Substance P mRNA; Central Nucleus of the Amygdala; Alcohol Preference; Alcohol Intake; Anxiety

INTRODUCTION

SP is an undecapeptide of the CNS and the PNS and belongs to a family of chemically-related neuropeptides referred to as the tachykinins [TKs] (Kramer et al., 1998). SP participates in neurotransmission by itself, or often as a neuromodulator in the monoamine,

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acetylcholine, and GABA systems in nerve terminals (Acsady et al., 1997; Chen et al., 2003; Marksteiner et al., 1996); its primary receptor is the NK1 receptor (Nakanishi 1991). Because the SP-NK1 system is localized in the regions of the extended amygdala and hippocampus (Dam et al., 1990; Emson et al., 1978; Levita et al., 2003), the activity of the central tachykinergic pathway is conceived to be mechanistically related to psychiatric conditions such as depression and anxiety (for review see Kramer et al., 1998; Kushner et al., 2000).

The most direct evidence of a role for SP in the control of fear-related behavior comes from reports that injections of SP agonists into the lateral ventricles, amygdala, or periaqueductal gray exert anxiogenic effects, whereas injections of SP antagonists produce anxiolytic-like effects in different tests of anxiety (File 1997; Kramer et al., 1998; Nikolaus et al., 1999a, b; 2000; Teixeira et al., 1996). Thus, these data suggest that anxiety is increased by excessive SP and decreased by reductions in SP. More recent findings from our laboratory have also linked stress/anxiety to SP in the central amygdala nucleus [CeA] by demonstrating that SP receptors in the CeA are upregulated by restraint-induced stress in outbred rats (Hwang et al., 2005b). Moreover, SP has also been reported to regulate states of anxiety in humans, particularly via the amygdala (Carletti et al., 2005). Working with a population of recently detoxified human alcoholics, Heilig and his colleagues (George et al., 2008) demonstrated that administration of a LY686017, an NK1 receptor antagonist, suppressed craving for alcohol and blunted cortisol release, lending further support to the link between neuronal stress systems and alcohol dependence. Thus, SP appears to mediate states of anxiety in rodents and humans, particularly within the amygdala.

Given the substantial clinical findings of comorbidity between anxiety and alcoholism in humans (Kushner et al., 2000; Schuckit and Hesselbrock, 1994), investigators have attempted to determine whether SP's anxiolytic regulatory mechanisms might also be salient in predisposing subjects to initiate alcohol drinking on the one hand (Hwang et al., 2005a; Slawecki et al., 2001), or, on the other, influence alcohol drinking behaviors upon direct pharmacological manipulation of SP itself (Bychkov et al., 2001; Hwang et al., 2005a; Nikolaev et al., 2002, 2003). Indeed, the relationship of other neuronal peptides [e.g., CRF, NPY] and anxiety to alcohol drinking behavior is well established (Funk and Koob, 2007; Primeaux et al., 2006). It is possible that the establishment of links between SP, anxiety, and alcoholism might be helpful in the development of putative pharmacotherapies to treat the comorbid disorder of anxiety and alcoholism.

One of the initial studies to evaluate the effects of SP on alcohol intake was conducted by Nikolaev et al. (2002, 2003), who reported that intracerebroventricular [i.c.v.] doses of 3 nmol/kg of SP were effective in lowering home cage alcohol preference in a chronic model of forced alcohol consumption, and resulted in a concomitant, albeit modest, elevation of both dopamine [DA] and DA metabolites [e.g., DOPA, HVA]. However, it is nearly impossible to interpret the significance of this study insofar as a dose-response analysis and fluid control procedures were not incorporated into its design. Thus, additional studies are warranted to further examine the role of SP in regulating alcohol-drinking behavior following direct administration into the CNS.

In the present study, we hypothesized that deficits in SP signaling within the CeA [an anxiety regulated locus (Funk and Koob, 2007; Pandey et al., 2005b, c)] might be associated with alcohol-motivated responding/preference in the innately anxious P rat (Pandey et al., 2005b, c; Stewart et al., 1993). To evaluate this hypothesis, *in situ* hybridization with quantitative autoradiography was used to determine the SP mRNA levels in brain loci associated with anxiety (Pandey et al., 2005 b, c) in the P and NP rat lines. Following confirmation of significantly lower SP mRNA levels in the CeA of the P compared with the

NP rats, *in vivo* studies were then conducted to determine if direct activation of SP receptors in the CeA were effective in modulating alcohol and sucrose-motivated behaviors. Reinforcer and neuroanatomical control studies with SP were also conducted.

MATERIALS AND METHODS

Animals

Male P and NP rats were obtained from the Indiana University Alcohol Research Center. For the *in situ* hybridization study, naïve P and NP rats from the 54th generation of selective breeding were used. For the alcohol/sucrose self-administration studies, adult male P rats from the 55th through 57th generations were used. In both studies, rats had *ad libitum* access to standard rat chow [from Harlan Teklad] and water; however, during the alcohol/sucrose initiation phases, P rats were water deprived for 3 – 5 days (June et al., 2001; June et al., 2002). At the time of euthanasia for the *in vitro* studies, young adult P and NP rats weighed between 282 – 302 g and were approximately 8 weeks old. At the beginning of the alcohol and sucrose self-administration studies, adult P rats weighed between 397 – 458 g. All behavioral data were collected between 9 am – 4 pm during the dark phase. The treatment of all subjects was approved by the IACUC of the University of Maryland School of Medicine and the School of Medicine of Indiana University.

In Vitro Studies

Tissue Sectioning for In Situ Hybridization—To evaluate innate differences in SP mRNA levels, naïve P and NP rats [n = 6/line] were decapitated, and the brains carefully removed from the skull and quickly trimmed to isolate a block extending out from the amygdala at a level that also contained the dorsal hippocampus as described in previous studies (Hwang et al., 2004b, 2005a; Suzuki et al., 2004). The brains were kept in vials and stored in a deep freezer until sectioning. Each tissue block containing the dorsal hippocampus was cut into 18 µm frozen sections using a Leica cryostat [model CM 1850]; sections were collected, quickly dried and stained with toluidine blue (Hwang and Guntz, 1997) for examination under a microscope to determine the appearance of the CeA. The location was verified using the Paxinos and Watson (1986) atlas. After verification, sequential tissue sections were cut and collected alternatively on 9 vectabond-coated slides. Specifically, a one-in-nine serial series of 6 sections/slide was collected. Eventually, 54 tissue sections [6 × 9 = 54] were collected on 9 slides.

In Situ Hybridization of SP mRNA—The antisense 48-mer SP oligodeoxynucleotide [ODN] sequence for studying SP mRNA [5'-TCG GGC GAT TCT CTG AAG AAG ATG CTC AAA GGG CTC GGG CAT TGC CTC-3'] is complementary to nucleotide sequences 124 – 171 of the preprotachykinin mRNA that encodes the SP biosynthesis (Krause et al., 1987), and has been used by Young et al. (1986) to examine SP mRNA expression. The probe was labeled with [³³P]-dATP [Perkin Elmer, Boston] using the terminal transferase recombinant labeling kit [Roche, Indianapolis]. [³³P]-probe was eluted using a Mini Quick Spin column [Roche] and used immediately following the *in situ* hybridization procedure. Fixed tissue sections were hybridized with 250 µL of [³³P]-labeled ODN probe in *in situ* hybridization buffer containing 10% dextran sulfate, 4× SSC, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.4, salmon sperm DNA [100 µg/mL], yeast t-RNA [100 µg/mL], and 50% formamide to detect mRNAs as previously reported (Hwang et al., 2004a, b, c). Tissue sections were incubated with [³³P]-labeled probe plus 1 mM cold SP for the nonspecific background control signals. These background control signals were not different from those produced using NEN [³³P]-randomer or sense probe as demonstrated from previous experience. SP mRNA signals in six brain regions including the CeA, interstitial nucleus of

the posterior limb of anterior commissure [IPAC], caudate putamen [CPu], and medial habenular nucleus were measured as described in the next section.

Quantitative Autoradiography in Conjunction with AIS Image Analysis System for the Measurements of SP mRNA

—After *in situ* hybridization for SP mRNA, the radiolabeled tissue sections on slides were lined up on cardboard and apposed to BioMax MS films for autoradiographic exposure to produce autoradiograms. Specific signals of autoradiograms detected on BioMax MS films were determined by the following formula: specific signals per unit area = [total binding of a discrete region per unit area] – [nonspecific signals per unit area of its own section]; such subtraction was used to eliminate potential variability in tissue thickness (Kunkler and Hwang, 1995). Signals were measured using a computer-assisted image analysis system (Hwang and Guntz, 1997; Hwang et al., 2004a, b) and expressed in relative optical density [ROD] per unit area. The ROD for the imaging analysis system was calibrated using Kodak's Standards, as previously reported (Hwang et al., 2004a; 2005a).

In Vivo Studies

Ethanol and sucrose self-administration—In some experiments, rats [$n = 10$] were trained to lever press for EtOH [10% v/v] using a modified version of the sucrose fading-technique (June, 2002). These rats were employed to evaluate centrally-administered SP in the CeA. In brief, animals were water-deprived on the first 3 – 5 days of training for 23 h daily. They were then presented with a sucrose solution [10% w/v] under a fixed-ratio 1 [FR1] schedule for 5 – 7 days. Each lever press delivered 0.1 mL of reinforcer. During the second phase of the training, animals lever-pressed for an EtOH + sucrose cocktail mixture under an FR1 schedule. Subsequently, the rats responded under an FR4 schedule for EtOH [10% v/v] on both right and left levers until their responses stabilized, defined as having daily responses within $\pm 20\%$ of the average responses for five consecutive days (June, 2002; June and Eiler, 2007). Finally, two additional sets of rats [$n = 5/\text{locus}$] were trained to lever press for sucrose at the 2% [w/v] concentration such that reinforcer specificity in the experimental locus [i.e., CeA] and in the neuroanatomical control locus [i.e., CPu] could be evaluated at response rates which were similar to those for EtOH in CeA-microinfused animals. All operant testing was performed during the dark phase.

Intracerebral Implantation of Cannula for Microinfusions

Surgery—P rats were anesthetized via isoflurane/oxygen gas inhalation and placed in a stereotaxic apparatus to allow for bilateral implantation of 22-gauge guide cannulae into the CeA or the neuroanatomical control locus, the CPu. The cannulas were anchored to the skull by stainless steel screws and UV-sensitive dental acrylic. A stylet was inserted into each cannula to maintain its viability and was only removed during infusion times. The coordinates were based on the rat brain atlas of Paxinos and Watson (1986) and were as follows: CeA: AP -2.0 , ML ± 3.6 , DV -8.5 from bregma; CPu: AP $+1.5$, ML ± 2.5 , DV -4.2 from bregma. 13 rats were implanted in the CeA, while 10 rats were implanted in the CPu. Each cannula was placed 1.0 mm above the intended target. This allowed the injector tip to extend below the cannula tip. The animals were given a seven day recovery period before restabilization on the operant self-administration sessions.

Microinfusion procedure—SP was prepared prior to each infusion. The drug was mixed into 1 mL of sterile saline and then bilaterally infused into the CeA at a rate of $0.1 \mu\text{L}/\text{min}$ for 5 min using a Harvard infusion pump. The total injection volume was $1.0 \mu\text{L}$ or $0.5 \mu\text{L}$ per hemisphere. The injector tip was left in the cannula for an additional minute to facilitate the diffusion of all injected drug from the injector tip into the CeA and CPu. Immediately following each microinfusion, rats were placed in the operant chamber for a 30 min session

to lever press for EtOH [10% v/v] or sucrose [5% w/v or 2% w/v]. A first group of CeA-implanted rats [$n = 5$] randomly received injections of 0, 1, 4, and 8 μg of SP prior to their EtOH consumption. The second group of CeA-implanted rats [$n = 5$] randomly received injections of 0, 1 and 8 μg of SP prior to their sucrose [2% w/v] consumption. The neuroanatomical control CPu rats [$n = 9$] randomly received injections of 0, 1, 4, 8, and 16 μg of SP prior to their EtOH consumption, and injections of 0, 1, 4, and 8 μg of SP prior to their sucrose [2% w/v] consumption. A minimum of 72 – 96 h were allocated between drug infusions (see June, 2002).

Operant Chambers—Animals were tested in 10 standard operant chambers [Coulbourn Instruments, Inc, Lehigh Valley, PA], each equipped with two levers and two dipper assemblies. Each reinforced response delivered 0.1 mL of the reinforcer. The duration of each operant session was 30 min. Other specific details of the chamber have been previously published (June, 2002; Foster et al., 2004).

Drugs and Solutions—The EtOH [USP] [10% v/v] and sucrose solutions [Fisher Scientific] [2 and 5%] were prepared as previously reported (June et al., 2001; June 2002). Similarly, SP [Sigma Chemical Company, St. Louis, MO] was mixed immediately before the experiments in sterile saline [0.90% NaCl, Fisher Scientific] for microinfusions as described above (also see June, 2002).

Histology—After completion of the behavioral testing, animals were sacrificed by CO₂ inhalation. Cresyl violet acetate [0.20 μL] was injected into the infusion site, and the brains were removed and frozen. The frozen brains were sliced on a microtome at 50 μm sections, which were stained with cresyl violet acetate. Infusion sites were examined under a light microscope and indicated on drawings adapted from the rat brain atlas of Paxinos and Watson (1986); rats with improper placements were excluded from the final data analysis.

Statistical Analyses

Data Analyses for mRNA—To examine innate differences of SP mRNA in the CeA between P and NP rats, analyses were performed using the Student's *t-test*. Signals for SP mRNA from 4 – 6 sections of each rat were collected.

Data Analyses for Alcohol and Sucrose Self-Administration—The EtOH and sucrose self-administration data were collected using Graphic State Notation computer software [Coulbourn Instruments, Inc., Lehigh Valley, PA]. The dependent variables were EtOH- or sucrose-maintained responding [i.e., lever presses] collected during the 30 min operant sessions. Data were analyzed by single factor repeated-measures ANOVA with drug treatment [i.e., dose] as the independent factor; each dependent variable was analyzed separately. *Post-hoc* comparisons between the saline/vehicle conditions against the SP treatment conditions were made using the Newman Keuls test in all experiments. *All* microinjection data were analyzed following correct histological verification under a light microscope. Data analysis was performed with the StatMost statistical package [Dataxiom Software Inc., Los Angeles, CA].

RESULTS

The Substance P [SP] mRNA in the CeA

Using [³³P]-labeled 48-mer SP antisense ODN probes, significantly lower SP mRNA signals in the CeA of the P relative to the NP rats were observed [Figure 1]. Figure 1c shows that the quantitative data [i.e., ROD per unit area] for the SP mRNA signals in the CeA of the P, relative to the NP, rats was also significantly lower [$p < 0.01$]. Quantitative data for all of

the six brain areas investigated are shown in Table 1. These data show that significantly lower SP mRNA levels were also detectable in the interstitial nucleus of the posterior limb of anterior commissure [IPAC] of P, relative to NP, rats [$p < 0.02$]. In contrast, significantly higher SP mRNA levels were detected in the medial habenular nucleus [Ham] of P, relative to NP, rats [$p < 0.01$]. SP mRNA levels were similar in the medial amygdaloid nucleus [MA], CPu, and ventromedial nucleus of the hypothalamus [VMH] in the P and NP rats [$p > 0.05$].

Histology

Figure 2A shows a reconstruction of serial coronal sections of the rat brain, illustrating the location of the bilateral microinjection cannulas in the CeA for the P rats in the alcohol/sucrose groups [$N = 10$]. The cannula tracks were well localized in the CeA. Figure 2B illustrates the location of the bilateral microinjection cannulas in the CPu for the P rats in the alcohol/sucrose groups [$N = 10$]; again, the cannula tracks were well localized in the CPu.

Intracerebral Microinfusions of SP into the CeA on Alcohol and Sucrose-Motivated Responding in P Rats

Given the deficits in SP mRNA levels in the CeA of P rats, we examined the effect of intracerebral microinfusions of SP into the CeA on alcohol-motivated responding. Figure 3A shows the effects on alcohol responding of direct microinfusion of SP [$1 - 8 \mu\text{g}$] into the CeA. SP produced both a marked and dose-related reduction in alcohol-motivated responding, resulting in profound effect of drug treatment [$F(3, 9) = 9.08, p < 0.01$]. Post-hoc tests revealed that the $1 - 8 \mu\text{g}$ doses significantly reduced lever pressing by 63 to 98% of control levels [$p \leq 0.01$]. Figure 3B shows the effect of microinjected SP on sucrose-motivated responding in a second cohort of P rats. As noted above in the methods section, the sucrose concentration was reduced from 5 to 2% in an attempt to evaluate reinforcer specificity in the CeA at a reward efficacy lower than or nearly equal to that of the alcohol [10% v/v] reinforcement. The importance of alternative reinforcers of similar/near similar efficacy in examining the positive reinforcing properties of drugs of abuse has been discussed previously (June, 2002; Meisch et al., 1993). Nevertheless, in contrast to alcohol-motivated responding, microinfusion of the lowest and highest SP doses [$1 - 8 \mu\text{g}$] was ineffective in altering sucrose-motivated responding, producing a nonsignificant effect of drug treatment [$F(2, 12) = 1.28, p > 0.319$].

Intracerebral Microinfusions of SP into the CPu on Alcohol and Sucrose-Motivated Responding in P Rats

As previously discussed by June and colleagues (June, 2002; June and Eiler, 2007), microinjection studies purporting to delineate CNS loci in regulating alcohol-motivated behaviors should employ neuroanatomical control loci. Given the high levels of SP mRNA in the CPu in both P and NP rats [see Table 1] and the markedly high SP levels reported in the CPu in outbred rats (Harlan et al., 1989), combined with the relative proximity of the CPu to the CeA, the CPu is well-suited as a neuroanatomical control locus in the present study. Figure 4A shows the effects of direct microinfusion of SP [$1 - 16 \mu\text{g}$] into the CPu on alcohol-motivated responding in P rats. The $1 - 8 \mu\text{g}$ doses of SP which were highly effective in the CeA [see Figure 3A] were completely ineffective in altering EtOH-motivated responding in the CPu; however, the $16 \mu\text{g}$ dose significantly reduced EtOH-motivated responding relative to the $1 \mu\text{g}$ dose condition and nonsignificantly reduced EtOH-motivated responding relative to the saline control condition. These effects resulted in a significant effect of drug treatment [$F(4, 24) = 9.11, p < 0.01$]. *Post-hoc* analyses confirmed the effects of the $16 \mu\text{g}$ SP dose relative to the $1 \mu\text{g}$ and saline control conditions [$p < 0.01, p < 0.09$, respectively]. Figure 4B shows the effects of direct microinfusion of SP [$1 - 8 \mu\text{g}$] into the CPu on sucrose-motivated responding. SP failed to significantly alter

sucrose-motivated responding following microinfusion into the CPu [$F(4, 12) = 0.843, p > 0.05$].

DISCUSSION

Deficits in SP mRNA levels within the CeA are inversely associated with alcohol-motivated responding

The role of SP in regulating alcohol-motivated behavior is not clearly understood. Here, we provide evidence for the first time that an innate difference in SP mRNA levels exists in the CeA of P, compared with NP, rats. The CeA is a pivotal structure of the brain reward circuitry (Koob and Bloom, 1988) associated with emotionality (Funk et al., 2006; Pandey et al., 2005a; Richter et al., 2000; Yilmazer-Hanke et al., 2002) and alcohol-seeking behavior (Hwang et al., 2001; Koob et al., 1998; McBride, 2002; Pandey et al., 2005a). We hypothesized that this deficit of SP mRNA in the CeA is associated with a high alcohol drinking preference in the P rats. Our hypothesis is supported by the current findings that direct activation of SP receptors within the CeA was observed to reduce alcohol-motivated responding. These results suggest that augmentation of SP neuronal activity markedly decreases alcohol drinking in P rats, which have been consistently shown to display an innate anxiogenic-like behavioral phenotype (Stewart et al., 1993; McBride, 2002; Pandey et al., 2005a).

Interestingly, Slawecki et al. (2001) reported a select SP neuronal deficiency in the hypothalamus and cortex in P rats when compared with NP rats; however, differences were not observed in the amygdala. It is not clear what could account for these discrepant results; however, different methodological procedures between the two studies and the failure of Slawecki et al. (2001) to specifically evaluate the CeA could both be factors which may explain the discrepant results. It should be noted, however, that, through use of immunocytochemical procedures to evaluate protein levels (Hoffman and June, unpublished data), our preliminary results strongly support the *in situ* data of the present study showing that the P rats indeed have lower SP levels in the CeA compared with NP rats.

In addition, it should be noted that Pompei et al. (1998) demonstrated that brain mRNA levels for preprotachykinin-A [PPT-A], which codes for SP, were 50% lower in the bed nucleus of the stria terminalis [BST] of Sardinian alcohol-preferring [P] rats than in the non-preferring [NP] rats. Unfortunately, these researchers did not investigate the CeA, and our study did not investigate the BST. However, given that both the BST and CeA have been associated with alcohol reward (June and Eiler, 2007), both studies may suggest that SP deficits in these loci may contribute to increased alcohol consumption. However, unlike the Indiana animals, the Sardinian P and NP rats do not possess the innate anxiety phenotype. Hence, the association between anxiety reduction by alcohol consumption via a SP deficient mechanism in the CeA appears more applicable to the Indiana, rather than the Sardinian, P rats.

Finally, it should be noted that both the present and the Pompei et al. (1998) studies observed similar SP and PPT-A mRNA brain levels, respectively, in the medial amygdala. Clearly, a comparison of similar brain loci using identical methods to evaluate mRNA/protein levels is warranted to determine if any of these differences may be explained via differences in the two alcoholic rat strains.

Activation of SP receptors selectively reduce alcohol-motivated responding in P rats

A second major finding of the present study was that intracerebral microinfusions of SP into the CeA selectively reduced alcohol-motivated responding in P rats, which have been proposed as an optimal model of oral alcohol consumption in humans (McBride and Li,

1998; Li et al., 1998). The SP reduction of alcohol responding following intracerebral microinfusions was highly reinforcer-specific when compared against a palatable sucrose [5% w/v] reinforcer. Thus, unlike alcohol-motivated behaviors, at the doses tested in the present study, SP did not appear to be directly involved in the regulation of general ingestive behaviors in P rats. Moreover, the present study has further demonstrated that even when the palatability/reinforcing efficacy of the sucrose reward was reduced to levels below or similar to the alcohol reinforcer [see Figure 3B], SP still failed to significantly attenuate the alternative ingestive behavior.

Our findings in the CeA are consistent with the ICV infusion studies reported by Nikolaev et al. (2002, 2003). However, unlike those studies (Nikolaev et al., 2002, 2003), a more optimal animal model of alcohol initiation and clear dose-response analyses were employed in the present study. Thus, our data suggest that the CeA is a specific locus in which SP mediates alcohol consumption. Moreover, the findings of the present study, along with prior reports from our laboratory (Hwang et al., 2004a, b; 2005b) and others (see Funk et al., 2006; Funk and Koob, 2007), strongly support the hypothesis that deficits in SP neuronal peptide signaling within the CeA alone, or interactions with other neuronal peptides within the CeA [e.g., CRF, NPY], may function to regulate maintenance of excessive alcohol-motivated behaviors, and possibly high anxiety, in the P rat.

Alternative hypotheses

While the data of the present study provide substantial evidence that a reduced SP neuronal input predisposes P rats to initiate alcohol intake, it is important to note that, in addition to the lower SP mRNA levels being found within the CeA, markedly lower levels were also observed in the IPAC of P relative to NP rats [see Table 1]. The *in situ* quantitative data sections of the CeA were generally collected at bregma levels of -1.60 to -1.88 mm from bregma. The IPAC sections, although dorsolateral to the CeA, were also collected at relatively similar bregma levels. Unfortunately, the role of the IPAC in alcohol drinking or anxiety is not known. It should also be noted that some researchers have implicated the medial amygdala in neuronal regulation of high anxiety and excessive alcohol-drinking behavior in the P rat (Pandey et al., 2005a). However, the SP mRNA levels in the medial amygdala were similar in the present study; hence, it is unlikely that medial amygdala SP mRNA levels contribute to alcohol drinking or anxiety in the P rat. Further, as noted above, the BST has also been suggested to be an important locus in the regulation of anxiety and alcohol-drinking behavior in outbred rats and rats selectively bred to initiate alcohol drinking, although the primary neurotransmitters of interest in some of these studies have been dopamine (Eiler et al., 2003) and GABA (Koob, 2004), and not solely neuronal peptides (Koob et al., 1998; Pompei et al., 1998).

As noted previously, the P rat is an animal model of high-drinking behavior that exhibits an innately anxiogenic-like phenotype (Pandey et al., 2005b, c; Stewart et al., 1993). However, given our hypothesis that deficits in SP signaling within the CeA might be associated with alcohol-motivated responding/preference in the P rat, a seeming paradox arises: how can decreased signaling of a predominately anxiogenic peptide lead to an "anxious" rat that drinks more alcohol? This may be explained by recent reports from Huston and his colleagues (Nikolaus et al., 1999a; 2000), which have provided evidence that SP can be both anxiogenic and anxiolytic depending on both the dose and brain locus. Within the VP, Huston and his colleagues reported that not only can SP exhibit anxiolytic properties, it can also display reinforcing actions following direct VP injections using the CPP model (Hasenohrl et al., 1998; Nikolaus et al., 1999b). The systemic NK1 antagonist WIN51, 708 was effective in attenuating the VP SP-mediated actions. Huston and colleagues suggested a possibility that the SP-mediated anxiolytic actions may be mediated via the NK1-receptor induced inhibition of other neuronal systems within the amygdala-ventral pallidum circuitry.

Nevertheless, these findings are intriguing in so far as they indicated for the first time that both increases and reductions of the SP-NK1 circuitry may produce anxiolytic effects.

It would also be salient to comment on the similarity between SP signaling and that of CRF, another neuropeptide that has been implicated in anxiogenesis. Like SP, CRF levels are demonstrably lower in the CeA of Indiana P rats, compared to their NP counterparts (Hwang et al., 2004b, Ehlers et al., 1992). Interestingly, a set of studies employing the Sardinian P [sP] rat, another line exhibiting alcohol preference and spontaneous anxiety-like behavior, reported a profound *elevation* in basal dialysate CRF levels in the CeA when compared to Sardinian non-preferring [sNP] animals (Richter et al., 2000). These seemingly disparate results may be reconciled if one postulates that the decrease in peptide tissue content in the Indiana rats follows a similar upregulation in extrasynaptic release and signaling of the molecule, resulting in negative feedback of CRF gene expression. A similar mechanism may provide an alternate explanation for the low SP levels in the anxious P rats of the present study. Systemic CRF injection has been shown to decrease ethanol drinking (Bell et al., 1998), an effect which may be mediated by CRF2 receptors (Funk and Koob, 2007). It should be noted that CRF mediates contrasting effects on anxiety depending on the subtype of CRF receptor activated (Zhao et al., 2007). A similar case might someday be made for [multiple] neurokinin receptors, including those involved in SP signaling.

Finally, it is important to note that the CeA is a rather heterogeneous structure. Specifically, it contains a number of intrinsic and extrinsic connections to other components of the extended amygdala loci on the one hand (Sun and Cassell, 1993; Sun et al., 1994), as well as colocalized neuronal peptide and GABA neurons (Veinante et al., 1997) on the other. Thus, it is likely the CeA may work in conjunction with other loci and multiple neuronal systems in regulating anxiety and alcohol drinking (Heilig et al., 1994; Koob, 2000; Koob and Le Moal, 2005).

The results of the present study have shown a significantly lower level of SP mRNA in the CeA of P rats relative to NP rats. We hypothesize that this inherent deficit of SP in the P rats is associated with a high alcohol-drinking preference. Our study has further suggested that the SP suppression of alcohol responding is regulated via the CeA, a substrate purported to play a role in both the reinforcing and anxiolytic actions of alcohol (for review, see Koob and Le Moal, 2005). Hence, deficits in SP signaling within the CeA appear to be associated with excessive alcohol-motivated responding, and likely the high phenotypic expression of anxiety, in the P rat (McBride and Li., 1998), while activation of SP receptors in the CeA or augmentation of SP levels in the brain can markedly reduce alcohol intake and may perhaps reduce anxiety in the P rat. We propose that the establishment of links between SP, anxiety and alcoholism may be helpful in the development of putative pharmacotherapies to treat the comorbid disorder of anxiety and alcoholism.

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SP mRNA in the CeA

Figure 1.

Substance P [SP] mRNA signals were strong in different parts of the brain as shown in the profiles of forebrain sections from a P rat [B] and an NP rat [A] at -1.88 mm from bregma. Two horizontal arrows point to the central nucleus of the amygdala [CeA], whose SP mRNA signals are much less in P rats than in NP rats. The images suggest that there is a deficit of SP mRNA in the CeA of P rats, as compared with NP rats.

Figure 1c. This histogram showed a significantly lower level [relative optical density per unit area] of SP mRNA signals in the CeA of P rats [$n = 6$] than in NP rats [$n = 6$]. For relative optical density of SP mRNA signals, please refer to the top line of Table 1. They were statistically different between P and NP rats at the $p < 0.01$ level, as evaluated using un-paired two-tailed Student's *t*-test. Error bars represent standard error of the mean [SEM].

Figure 2.

Reconstruction of serial coronal sections of the rat brain illustrating the bilateral guide cannula tips of the [A] central nucleus of the amygdala [$n = 10$] and [B] caudate putamen [$n = 10$]. Each rat is represented by two open circles: one in the left hemisphere and one in the right. CeA = central nucleus of the amygdala; CPu = caudate putamen. Coronal sections are adapted from the rat brain atlas of Paxinos and Watson (1986) for this figure.

Figure 3. Total responding for [A] 10% [v/v] ethanol [n = 5] and [B] 5% [v/v] sucrose [n = 5] following i.p. injections of SP into P rats. Statistical comparisons with the Newman Keuls' test indicated that there were no significant differences in sucrose responding between the saline control and SP conditions. However, a significant difference in ethanol responding was found between the saline control and each of the experimental conditions [treated SP in the dose of 0.25, 0.50, 1.0, and 2.0 mg], with $**p < 0.01$ compared with saline control conditions. Error bars represent standard error of the mean [SEM].

A **B** **Figure 4.**

Total responding for [**A**] 10% ethanol [$n = 5$] and [**B**] 2% sucrose [$n = 5$] following microinfusions of SP into the CPu of P rats. Statistical differences [$*p < 0.05$] were found between the 16 μg condition compared with the saline control and 1 μg condition in the ethanol-drinking animals using the Newman-Keuls' test. Error bars represent standard error of the mean [SEM].

Table 1

Substance P mRNA [relative optical density per unit area] in different discrete brain regions of NP and P rat. Signals of SP mRNA were measured using a computer-assisted image analysis system. Four to six sections of each region were read to represent an animal. CeA = central nucleus of the amygdala; IPAC = interstitial nucleus of the posterior limb of anterior commissure; VMH = ventromedial hypothalamic area; HAm = medial habenular nucleus; MA = medial amygdaloid nucleus.

	NP (n= 6)	P (n=6)
CeA	0.212 ± 0.020	0.103 ± 0.015**
MA	0.174 ± 0.014	0.152 ± 0.010
CPu	0.272 ± 0.021	0.284 ± 0.017
IPAC	0.422 ± 0.010	0.368 ± 0.030*
VMH	0.213 ± 0.006	0.222 ± 0.019
HAm	0.245 ± 0.015	0.327 ± 0.012*

*
P < 0.02

**
P < 0.01 when compared with the NP values from the same structure, with two-tailed unpaired Student's t-test