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Angiotensin II and CRF Receptors in the Central Nucleus of the Amygdala Mediate Hemodynamic Response Variability to Cocaine in Conscious Rats

Mari A. Watanabe, Sarah Kucenas, Tamara A. Bowman, Melissa Ruhlman, and Mark M. Knuepfer

Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St. Louis, Missouri 63104

Abstract

Stress or cocaine evokes either a large increase in systemic vascular resistance (SVR) or a smaller increase in SVR accompanied by an increase in cardiac output (designated vascular and mixed responders, respectively) in Sprague-Dawley rats. We hypothesized that the central nucleus of the amygdala (CeA) mediates this variability. Conscious, freely-moving rats, instrumented for measurement of arterial pressure and cardiac output and for drug delivery into the CeA, were given cocaine (5 mg/kg, iv, 4-6 times) and characterized as vascular (n=15) or mixed responders (n=10). Subsequently, we administered cocaine after bilateral microinjections (100 nl) of saline or selective agents in the CeA. Muscimol (80 pmol), a GABA_A agonist, or losartan (43.4 pmol), an AT₁ receptor antagonist, attenuated the cocaine-induced increase in SVR in vascular responders, selectively, such that vascular responders were no longer different from mixed responders. The corticotropin releasing factor (CRF) antagonist, α -helical CRF₉₋₄₁ (15.7 pmol), abolished the difference between cardiac output and SVR in mixed and vascular responders. We conclude that greater increases in SVR observed in vascular responders are dependent on AT₁ receptor activation and, to a lesser extent on CRF receptors. Therefore, AT₁ and CRF receptors in the CeA contribute to hemodynamic response variability to intravenous cocaine.

Keywords

central nucleus of the amygdala; losartan; α -helical CRF₉₋₄₁; muscimol; systemic vascular resistance; cardiac output

1. Introduction

The central nucleus of the amygdala (CeA) plays a critical role in integrating sympathetic and behavioral responses to stress (Bohus et al., 1996; Davis, 2000; Gray, 1993; Saha, 2005). Stimulation of the CeA produces increases in blood pressure and heart rate (Hilton and

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Corresponding author: Mark M. Knuepfer, Ph.D., Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104, (314) 977-6463, (314) 977-6411 (FAX), knuepfmm@slu.edu.

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Zbrożyna, 1963; Iwata et al., 1987; Schlör et al., 1984; Stock et al., 1978). Conversely, ablation of the CeA attenuates the increase in blood pressure and heart rate to conditioned stress in rats (Iwata et al., 1987; Sananes and Campbell, 1989). The CeA is necessary for learning increased alertness to conditioned fear (Davis, 2000). There are extensive and often reciprocal projections between the CeA and nuclei in the hypothalamus and medulla that regulate autonomic and cardiac functions (Gray et al., 1989; Jhamandas et al., 1996; Pitkänen, 2000; Veening et al., 1984; Volz et al., 1990). These observations underscore the importance of the CeA in modulating the hemodynamic and behavioral responses to stress.

Several neurotransmitters and receptors have been localized in the CeA. The CeA contains GABA receptors (Marowsky et al., 2004) that have been shown to inhibit hemodynamic and behavioral responses to stress (Saha, 2005). The CeA also contains angiotensin II (Ang), angiotensin converting enzyme and angiotensin receptors (Brownfield et al., 1982; von Bohlen und Halbach and Albrecht, 1998). In addition, CRF-like immunoreactivity exists in the CeA (Sakanaka et al., 1986; Uryu et al., 1992). Microinjection of Ang in the CeA elicits a pressor response, whereas CRF evokes both an increase in plasma catecholamines and arterial pressure (Brown and Gray, 1988; Ku et al., 1998). Cocaine and acute stress increase CRF and/or its mRNA in the amygdala (Gardi et al., 1997; Hsu et al., 1998; Makino et al., 1999; Sarnyai, 1998). Therefore, multiple studies suggest that Ang and CRF are key neurotransmitters in the CeA involved in regulation of sympathetic and hemodynamic responses to stress.

It has been reported that acute stress produces a pressor response dependent on an increase in systemic vascular resistance in some humans (vascular responders), and an increase in cardiac output (cardiac responders) in others (Brod, 1963; Turner et al., 1992). Vascular responders are more likely to develop hypertension and heart disease (Eliot, 1992; Turner et al., 1992). Our laboratory has identified a rodent model of a similar inter-individual hemodynamic response variability. We have demonstrated that intravenous cocaine administration or behavioral stressors evokes a pressor response due solely to an increase in systemic vascular resistance in some rats, whereas in other rats they evoke a smaller increase in systemic vascular resistance accompanied by an increase in cardiac output (Knuepfer and Mueller, 1999; Knuepfer et al., 2001). We named these rats vascular and mixed responders, respectively (Knuepfer and Mueller, 1999). Vascular responders are more susceptible to cocaine-induced cardiomyopathies and toxicity than mixed responders (Knuepfer et al., 1993; Williams et al., 2003). They also display a sustained elevation of arterial pressure after exposure to chronic stress (Muller et al., 2001) or repeated cocaine administration (Branch and Knuepfer, 1994; Knuepfer and Mueller, 1999). Therefore, vascular responder rats resemble vascular responders in humans both with regard to their hemodynamic response profile, and their predisposition to cardiovascular disease.

Recently, we found that intracerebroventricular administration of Ang or CRF receptor antagonists reduced the greater increase in systemic vascular resistance observed in vascular responders during behavioral stress or cocaine administration (Knuepfer et al., 2005; Rowe et al., 2006). However, intracerebroventricular administration of a drug affects broad areas of the brain. We hypothesized that we might be able to further localize the difference between vascular and mixed responders to the CeA, and determine the role of AT₁ and CRF receptors in the difference, based on the literature described above. We believed there could be a greater sympathetic reaction to stress in vascular responders. To test our localization hypothesis, we microinjected muscimol, a GABA_A receptor agonist, into stereotaxic coordinates for the CeA. To identify the neurotransmitters responsible, we also microinjected losartan, an AT₁ receptor antagonist, and α -helical CRF₉₋₄₁, a CRF antagonist, into the same area. We used cocaine as our stressor. We expected all three drugs to prevent the greater vascular response of vascular responders. Finally, we chemically ablated the same area using ibotenic acid to prevent all synaptic transmission. Our results suggest that stimulation of

GABA_A and AT₁ receptors in the CeA facilitates increases in systemic vascular resistance in vascular responders, whereas stimulation of CRF receptors contributes to differences in hemodynamic responses between vascular and mixed responders.

2. Results

Histological analysis of cannula placement and dye locations revealed that 16 rats had received bilateral placement of the cannulae in the CeA (8 mixed and 8 vascular responders) while 9 rats had only unilateral placement in the CeA (Figure 1). In 5 rats, we missed the CeA bilaterally and 2 others had at least one cannula in the lateral ventricle. These 7 rats were excluded from further analysis. There was no significant tissue disruption at the microinjection sites despite repeated drug administration.

Cocaine (5 mg/kg, iv) evoked a pressor response in all rats. In some rats (n=10), the increase in arterial pressure was due to a small increase in cardiac output (4.7 $\pm$ 0.8 %) and an increase in systemic vascular resistance (Figure 2). In the remaining 15 rats, the pressor response was dependent on a substantial increase in systemic vascular resistance and occurred despite a decrease in cardiac output (-8.4 $\pm$ 1.0 %), during the first minute after cocaine administration. As described in the introduction, we classified these rats as mixed and vascular responders, respectively. There was no difference in baseline cardiac output values between the vascular and mixed responders, nor in baseline arterial pressure or heart rate (Table 1). Cocaine produced a significantly greater increase in systemic vascular resistance and decrease in cardiac output (both $p < 0.0001$) and a greater reduction of stroke volume in vascular responders compared to mixed responders ($p = 0.0101$). Heart rate and arterial pressure responses were not statistically different. A lower dose of cocaine (0.5 mg/kg, iv) evoked a pressor response and varying hemodynamic responsiveness (data not shown) similar to responses observed with the greater dose of cocaine as previously reported (Branch and Knuepfer, 1994).

Since most saline trials were performed in the morning, we compared responses to cocaine alone in the morning and a minimum of 3 hours later, similar to the protocol for testing responses before and after CeA drug administration. There were no significant differences in hemodynamic response patterns between morning and afternoon cocaine administrations verifying that tachyphylaxis does not occur with this dosing regimen (data not shown).

Effects of Muscimol

The effects of CeA injection of muscimol (80 pmol) were studied in 10 vascular and 7 mixed responders (Figure 3). Ten minutes after muscimol injection (before cocaine administration), there were no significant changes in hemodynamic variables (Table 2) from pre-muscimol values. Muscimol attenuated the arterial pressure increase ($F_{1,15} = 8.9$, $p = 0.0093$) but not the decrease in stroke volume ($p = 0.068$) evoked by cocaine. All other comparisons had highly significant interactions ($p < 0.002$) due to opposing effects in vascular and mixed responders. These were resolved by analyzing each group independently with a two way analysis of variance. Mixed responders had a significant reduction in heart rate ($p = 0.037$) and cardiac output ($p = 0.0002$) responses. In contrast, vascular responders had an increase in cardiac output ($p = 0.0003$) and stroke volume ($p = 0.019$) responses and a decrease in systemic vascular resistance ($p = 0.002$) in response to cocaine administration.

When the integrated response (geometric area under the curve) to cocaine over the first 60 sec was compared by analysis of variance (ANOVA) and post hoc tests (Figure 4), the attenuation of increase in arterial pressure by muscimol ($p = 0.0177$) was found to be due to a significant drug effect in vascular responders ($p < 0.05$). Since there were significant interactions in the ANOVA for heart rate, systemic vascular resistance and cardiac output responses due to opposite changes in mixed and vascular responders, data from these groups were analyzed

separately. In mixed responders, the heart rate ($p=0.012$) and cardiac output ($p=0.0075$) responses were significantly attenuated by muscimol. In vascular responders, the decrease in cardiac output ($p=0.0087$) and stroke volume ($p=0.0204$) and the increase in systemic vascular resistance ($p=0.022$) were attenuated by muscimol pretreatment.

Effects of Losartan

The effects of intra-amygdalar injection of losartan (43.4 pmol) were studied in 9 vascular and 8 mixed responders. After losartan injection and before cocaine administration, cardiac output and stroke volume were reduced ($F_{1,15} = 6.04$, $p = 0.0266$ and $F_{1,15} = 8.27$, $p = 0.0115$, respectively) and systemic vascular resistance was increased ($F_{1,15} = 8.48$, $p = 0.0107$) from pre-losartan values compared to responses with vehicle administration (Table 2). The effect of losartan to reduce cardiac output and increase systemic vascular resistance was due to significant changes in vascular responders since post-hoc analysis demonstrated a significant change only in this subset.

Losartan pretreatment attenuated the decrease in stroke volume after losartan pretreatment ($F_{1,15} = 7.67$, $p = 0.0143$) but did not affect arterial pressure or heart rate (Figure 5). Changes in cardiac output and systemic vascular resistance responses were complicated by significant interaction terms ($p < 0.003$). To avoid significant interactions, mixed and vascular responders were analyzed independently. Mixed responders had a significant reduction in the tachycardia in response to cocaine. In vascular responders, the decreases in cardiac output ($p < 0.0001$) and stroke volume ($p=0.0086$) were prevented and the increase in systemic vascular resistance was attenuated ($p=0.0011$).

Analysis of integrated data with ANOVA revealed no effect of losartan treatment on arterial pressure or heart rate responses to cocaine but significant interactions for both cardiac output and systemic vascular resistance. There was a significant attenuation of the integrated cardiac output ($p=0.001$) and systemic vascular resistance ($p=0.0017$) responses to cocaine after losartan administration (Fig. 4).

Effects of CRF antagonist

Effect of intra-amygdalar injection of α -helical CRF₉₋₄₁ (15.7 pmol) was studied in 8 vascular and 8 mixed responders (Figure 6). After α -helical CRF₉₋₄₁ injection and before cocaine administration, values of hemodynamic parameters did not change from pre-injection values (Table 2). When responses to cocaine were studied, there were significant interactions for the cardiac output, systemic vascular resistance and stroke volume responses. When groups were analyzed separately, mixed responders had a significant increase in the systemic vasoconstrictor response ($p=0.026$) and a decrease in the cardiac output ($p=0.0043$) response after treatment. In contrast, vascular responders had a smaller reduction in cardiac output ($p=0.0009$) and a smaller increase in systemic vascular resistance ($p=0.019$).

Analysis of integrated data over 60 sec demonstrated significant interactions for the arterial pressure, cardiac output, systemic vascular resistance and stroke volume responses after α -helical CRF₉₋₄₁ administration (Figure 4). When groups were analyzed separately, mixed responders had a significant increase in the pressor response ($p=0.019$) and the systemic vasoconstrictor response ($p=0.026$) and a decrease in the cardiac output ($p=0.022$) response. In comparison, vascular responders had a significant reduction in the systemic vasoconstrictor response ($p=0.013$) and an increase in the cardiac output ($p=0.0025$) response (Figure 4).

Effects of Ibotenic acid

Ibotenic acid (0.06 M) was used to ablate the CeA chemically. Six vascular responders were tested after a minimum 3 day recovery period. Although arterial pressure and heart rate

responses were not altered, ibotenic acid attenuated the cocaine-induced decreases in cardiac output ($F_{1,11} = 19.96$, $p = 0.0012$, significant interaction, $p = 0.0358$) and stroke volume ($F_{1,11} = 12.17$, $p = 0.0058$) and the increase in systemic vascular resistance ($F_{1,11} = 15.06$, $p = 0.0031$) in vascular responders (Figure 7).

3. Discussion

Our results demonstrate that cocaine elicits specific cardiovascular response patterns that are dependent on neurotransmission through the CeA. We have proposed that hemodynamic response patterns to cocaine in rats are very similar to those observed with acute stress (Knuepfer and Mueller, 1999; Knuepfer et al., 2001). Therefore, our data support the proposed role of the amygdala in integrating hemodynamic responses to stress (Baklavadzhyyan et al., 2000; Saha, 2005) and implicate specific peptide neurotransmitters. The CeA was important for the greater vasoconstriction, since muscimol administration or chemical ablation of the CeA attenuated the cocaine-induced increase in systemic vascular resistance and decrease in cardiac output in vascular responders selectively. In addition, we demonstrated that Ang receptor activation in the CeA was necessary for the greater vasoconstrictor response to cocaine and that CRF receptor activation in the CeA may contribute to differences in hemodynamic responses to cocaine to a lesser extent.

Role of the CeA in hemodynamic responses to cocaine

The lateral and basolateral amygdala receive different modalities of sensory input that are relayed to the CeA to generate appropriate neurohumoral and autonomic responses to stress (Bohus et al., 1996; Saha, 2005; Sarnyai, 1998). Single neurons in the amygdala are sensitive to a wide variety of sensory inputs in addition to baroreceptor and chemoreceptor input (Jhamandas et al., 1996; Knuepfer et al., 1995). The CeA in turn, projects to a number of CNS sites that regulate arterial pressure including the preoptic and paraventricular nuclei in the hypothalamus, the locus coeruleus, and the rostral ventrolateral medulla, nucleus ambiguus and dorsal motor nucleus of the vagus in the brain stem (Volz et al., 1990). Electrical stimulation of the CeA increases blood pressure and heart rate (Hilton and Zbrożyna, 1963; Iwata et al., 1987; Stock et al., 1978) and suppresses baroreflex function (Schlör et al., 1984). These studies indicate that the CeA modifies arterial pressure and other cardiovascular parameters via the autonomic nervous system.

Furthermore, the CeA is important in mediating cardiovascular responses to stress in particular. Behavioral stress elicits increases in arterial pressure and sympathetic nerve activity that are dependent on the CeA (Koepke et al., 1987). Ablation of the CeA attenuates hemodynamic, behavioral and autonomic responses to conditioned and unconditioned stressors (Iwata et al., 1987; Kapp et al., 1979; LeDoux et al., 1988; Roozendaal et al., 1991; Sananes and Campbell, 1989). In humans, electrical stimulation of the amygdala produces feelings of stress and anxiety in addition to increases in heart rate and blood pressure (Chapman et al., 1954). Our current results extend these observations by implicating the CeA as a mediator of individual-specific hemodynamic response patterns to cocaine, a pharmacological stressor. Specifically, our results suggest that the CeA is necessary for the vascular type of response pattern to stress that is usually exhibited by about two thirds of the rats we study, regardless of whether the stressor is behavioral (Knuepfer et al., 2001) or pharmacological (cocaine).

In this study, we demonstrated that activation of GABA_A receptors in the CeA with muscimol selectively attenuated the cocaine-induced increase in systemic vascular resistance observed in vascular responders. GABA_A receptors that inhibit synaptic transmission have been shown to be widely distributed in the amygdala (Marowsky et al., 2004). Microinjection of the GABA antagonist, bicuculline, in the CeA elicited increases in arterial pressure and heart rate (Gören et al., 1996) suggesting that these receptors play a role in regulation of arterial pressure.

Therefore, we propose that the CeA is, at least in part, responsible for greater vascular responsiveness to pharmacological stress (cocaine) observed in vascular responders (Knuepfer and Mueller, 1999).

This observation was corroborated by chemical ablation of the CeA with ibotenic acid. Using a neurotoxic dose of ibotenic acid (Schwarcz et al., 1979) in vascular responders only, we noted a significant attenuation of both the decrease in cardiac output and the increase in systemic vascular resistance after CeA ablation. It has been reported that inhibition of the CeA with muscimol reduced amphetamine self-administration in a subset of rats that prefer psychostimulants (Cain et al., 2008). These data further support the concept that the CeA plays an important role in mediating differences in responsiveness to psychostimulants.

Muscimol alone or ibotenic acid in the CeA did not alter resting arterial pressure, heart rate or cardiac output suggesting that the amygdala is not necessary for tonic maintenance of arterial pressure. This is consistent with the proposed role of the CeA in modulating arterial pressure only during stressful or emotional crises (Bohus et al., 1996; Gray, 1993; Saha, 2005) although this may not be true in the spontaneously hypertensive rat (Folkow et al., 1982; Galeno et al., 1982).

The role of Ang receptors in the CeA

Ang receptors in the CNS play an important role in autonomic and neurohumoral responses to behavioral and pharmacological stress (Jezova et al., 1998; Knuepfer et al., 2005; Rowe et al., 2006; Saiki et al., 1997). Ang and AT₁ receptors exist in the amygdala and, in particular, in the CeA (Brownfield et al., 1982; von Bohlen und Halbach and Albrecht, 1998). Microinjection of Ang in the amygdala of the rat increases the discharge rate of amygdalar neurons and the increase can be blocked by either AT₁ or AT₂ receptor antagonists (Albrecht et al., 2000). Although studies of the specific role of Ang in the amygdala are limited, it has been shown that Ang microinjection into the CeA produces an increase in blood pressure and bradycardia (Brown and Gray, 1988). In our study, intra-amygdalar injection of losartan attenuated the cocaine-induced rise in systemic vascular resistance selectively in vascular responders such that their hemodynamic response pattern was no longer different from that of mixed responders. Therefore, we conclude that activation of AT₁ receptors in the CeA is necessary for the greater vascular response observed in vascular responders. This is consistent with previous observations demonstrating that intracerebroventricular administration of Ang receptor antagonists or converting enzyme inhibitors attenuated vasoconstrictor responses to cocaine or cold stress in vascular responders selectively whereas administration of Ang enhanced these responses (Knuepfer et al., 2005; Rowe et al., 2006). These data suggest that Ang signaling in the CeA may differ between vascular and mixed responders resulting in varying responses to cocaine in different populations.

Losartan administration in the CeA produced an increase in baseline values of systemic vascular resistance and a decrease in cardiac output which was highly variable (Table 2). Although this finding suggests that there could be tonic release of Ang in the CeA acting on AT₁ receptors that suppress vascular resistance, the general lack of effect of drugs on baseline parameters supports the hypothesis that the CeA is integral to modifying the hemodynamic response pattern to cocaine, rather than to directly mediating the response to cocaine administration.

These observations suggest that AT₁ receptors in the CeA may also be necessary for the development of experimental hypertension since vascular responders are more susceptible to a sustained elevation in arterial pressure in response to cocaine or stress (Branch and Knuepfer, 1994; Muller et al., 2001). In fact, it has been reported that ablation of the amygdala attenuates the development of hypertension in the spontaneously hypertensive rat (Folkow et al., 1982;

Galeno et al., 1982) and may prevent stress-induced hypertension (Baklavadzhyan et al., 2000). The specific role of AT₁ receptors in the CeA in long term regulation of arterial pressure and hypertension is not understood.

The role of CRF receptors in the CeA

CRF-like immunoreactivity has been observed in the CeA (Sakanaka et al., 1986). Moreover, CRF activity in the CeA is sensitive to a variety of stressors since psychological or restraint stress induces CRF mRNA and CRF-like immunoreactivity in the CeA (Hsu et al., 1998; Makino et al., 1999). In a similar manner, cocaine administration increases CRF like-immunoreactivity in the CeA (Gardi et al., 1997; Sarnyai, 1998). Investigators have reported that microinjection of CRF into the CeA increases arterial pressure and circulating catecholamines (Brown and Gray, 1988; Ku et al., 1998). Activation of CRF receptors in the CeA increases heart rate by inhibiting parasympathetic tone (Wiersma et al., 1993). Our results demonstrate that CRF receptor antagonism in the CeA prevented the difference in cardiac output and vascular resistance responses to cocaine (Figure 6). These data suggest that CRF receptors in the CeA may contribute to response variability but possibly to a lesser extent than AT₁ receptors. Alternatively, a greater dose or more selective CRF receptor antagonist may have demonstrated significant effects. In any case, our data support a role for CRF in the CeA mediating hemodynamic response variability to cocaine.

The role of CRF receptors in individual variability in responsiveness to cocaine is not unique since CRF receptors have been reported to mediate variable locomotor responses to cocaine. Male rhesus monkeys can be classified as high or low responders to cocaine administration according to their locomotor responses to cocaine (Sarnyai, 1998). Low responders have a decrease in ACTH and cortisol levels whereas high responders have increased ACTH and cortisol responses (Sarnyai, 1998). Although this response could be due to effects in the hypothalamus, CRF receptor antagonism reduces cocaine-induced hyperactivity particularly in high responders. Therefore, there is evidence that central CRF may play a role in response variability to cocaine.

These data do not fully support previous studies from our laboratory. We reported that intracerebroventricular administration of α -helical CRF₉₋₄₁ prevents the greater increase in SVR in vascular responders in response to cocaine (Dong et al., 2001) or to startle with cold water (Tan et al., 2003). In the present study, we also prevented differences between vascular and mixed responders. We believe that CRF may play a role in enhancing vasoconstrictor responses to cocaine at other sites, particularly the paraventricular nucleus of the hypothalamus (Vetter et al., 2007). We hypothesize that lateral ventricular administration of drugs has a greater effect on downstream hypothalamic sites.

Limitations of our study

We included all 25 rats who had received at least unilateral CeA cannula placement for data analysis although only 16 of the 25 rats had received bilateral CeA cannula placement. We included all 25 for two reasons. Others investigators have examined structure-function relationships from unilateral microinjection (diMicco and Monroe, 1998; Soltis and diMicco, 1991). We also believe if the erroneous location of the second cannula site is randomly distributed as we revealed in Figure 1, it will not contribute a consistent trend to the results.

Summary

In conclusion, the CeA conducts critical information regulating the pattern of cardiovascular responses to acute cocaine administration but is not necessary for the pressor response. AT₁ receptor blockade in the central nucleus of the amygdala reduced the greater increase in systemic vascular resistance characteristic of the vascular response to cocaine and made the

vascular responders indistinguishable from mixed responders. CRF receptor blockade also prevented differences in cardiovascular responses by a somewhat different mechanism. Therefore, the CeA plays an important role in mediating hemodynamic and autonomic response variability to cocaine administration.

4. Experimental Procedures

General Surgical Instrumentation

All surgical and experimental procedures were approved by the St. Louis University Institutional Animal Care and Use Committee, and adhered to guidelines described in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996). Specific pathogen-free, male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 275 - 350 g were used.

All surgical procedures were performed using aseptic technique. Rats were anesthetized with isoflurane (1.8-2%) and intubated for artificial respiration with 100% oxygen. They were surgically instrumented with bilateral intracerebral cannula guides (C232G-8.6 Cann Guide Dbl, Plastics One, Inc., Roanoke, VA) stereotactically placed 1 mm above the CeA. A miniaturized pulsed Doppler flow probe (Iowa Doppler Products, Iowa City, Iowa) was placed around the ascending aorta for measurement of cardiac output (Branch and Knuepfer, 1994), and its leads were externalized at the base of the skull and affixed to the surface of the skull with dental cement. Rats were given buprenorphine (0.05 mg/kg i.m.) for pain and closely monitored for several hours after surgery. After 1-3 weeks and recovery to pre-surgical weight, rats were reanesthetized for placement of intravascular cannulae (vinyl, 0.5 mm OD) into the left femoral artery and vein. The cannulae were threaded subdermally and externalized between the scapulae. Rats were allowed to recover for 2-4 days before testing.

Experimental Protocol

Cardiac output, arterial pressure and heart rate were recorded using a polygraph (Model 7D Grass Instruments, Quincy, Mass.) and digitized at 1000 Hz (WINDAQ Pro+ software, DATAQ Instruments, Dayton, Ohio). Cardiac output was measured using a 20 MHz pulsed Doppler flowmeter with anti-aliasing circuitry (Bioengineering Dept, University of Iowa, Iowa City, Iowa). The moment of the initial increase in arterial pressure due to cocaine was considered the onset (zero time) of the response in order to synchronize responses in multiple trials. If the change in cardiac output averaged over the first minute after cocaine was negative, the rat was categorized as a vascular responder. In contrast, if the change in cardiac output was positive, the rat was designated a mixed responder. The first minute was used to determine changes because the greatest differences in responses between vascular and mixed responders are apparent during this period as described in previous reports (Branc and Knuepfer, 1994; Knuepfer and Mueller, 1999; Knuepfer et al., 2001).

The change in systemic vascular resistance was numerically computed as the change in mean arterial pressure divided by change in cardiac output as previously described (Branch and Knuepfer, 1994). The change in stroke volume was numerically computed as change in cardiac output divided by change in heart rate. Changes in mean arterial pressure and heart rate were expressed as absolute differences, while changes in cardiac output, systemic vascular resistance, and stroke volume were expressed as percent change from baseline.

Experiments were begun at least 2 days after cannulation to allow sufficient recovery from surgery. On the first day, a low dose (0.5 mg/kg body weight) of cocaine was given via the femoral venous cannula. Rats were categorized as vascular or mixed responders according to their hemodynamic response to at least four doses of a higher dose (5 mg/kg) of cocaine, each

delivered intravenously over 45 s. No more than two doses of 5 mg/kg cocaine were administered per day, separated by a minimum of 3 hours to avoid tachyphylaxis or sensitization as previously described (Branch and Knuepfer, 1994).

After categorizing individual rats as vascular or mixed responders, rats were given 5 mg/kg cocaine, 10 minutes after pretreatment of the CeA with vehicle (saline) or a specific agent. The vehicle or agent was given in volumes of 100 nl bilaterally over two minutes into the CeA area using a 32 awg stainless steel cannula that protruded 1 mm beyond the permanently placed cannula guide. The specific agents used included 80 pmol muscimol, a GABA_A agonist (doses are given as injection amount per site), 60 ng (15.7 pmol) α -helical CRF₉₋₄₁, a CRF antagonist, (Sigma Chemical Co., Inc., St. Louis, Missouri), and losartan (20 ng, 43.4 pmol), a selective AT₁ receptor antagonist (kindly provided by Merck & Co., Inc., Whitehouse Station, New Jersey). The 80 pmol dose of muscimol used has been found to be effective in preventing heart rate responses to stress after hypothalamic administration (Stotz-Potter et al., 1996). The 15.7 pmol CRF antagonist dose was similar to that used by others to block projection fields of the CeA (80 ng, Wu et al., 1999). A lower dose of losartan (20 pmol) has been reported to be effective in preventing Ang inhibition of sexual behavior after injections in the medial amygdala (Breigeiron et al., 2002). Cocaine hydrochloride was obtained from the National Institute on Drug Abuse and dissolved in saline (5 mg/ml). The three centrally delivered agents were given in random order with a minimum of 24 hours between delivery.

After the conclusion of the agonist/antagonist experiments, the CeA was ablated with bilateral injections of 100 nl of 0.06 M of ibotenic acid into the CeA. At least 3 days later, hemodynamic responses to cocaine were measured a final time. This was followed by bilateral injection of 100 nl of 2% Chicago sky blue (Sigma Chemicals, St. Louis, Missouri) to mark the injection sites. Rats were euthanized with pentobarbital (60 mg/kg) 10 minutes later. Brains were fixed in 10% formalin and cut on a cryostat (Cryo-Cut II, American Optical, Buffalo, New York) in 40 μ m sections for histological confirmation of the amygdalar injection sites using the rat brain atlas of Swanson (2004). If one or both microinjection sites were in the CeA, the data were included.

Data Analysis

The differences in hemodynamic response and time course between vascular and mixed responders before and after amygdala injections were assessed using a three way analysis of variance (ANOVA). We compared differences between mixed and vascular responders, control vs. drug, and effect of time (first 60 sec). Two way analysis of variance was also applied to integrated values for responses during the first 60 sec followed by a post hoc (Newman Keul's) test. We integrated by calculating the geometric area under the time response curve. For all analyses, we often obtained a significant interaction because drug treatments had opposite effects on responses to cocaine in vascular and mixed responders especially on cardiac output and systemic vascular resistance responses. In these cases, we analyzed groups independently (2 way ANOVA for timed data and Students paired t test for integrated data) to compensate for opposite drug effects in each group. Statistical significance was assumed at a p value of < 0.05. Values are expressed as mean $\pm$ S.E.M.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SVR	systemic vascular resistance
CeA	central nucleus of the amygdala

Ang angiotensin II

Figure 1.

Location of cannula guide tips in frontal sections. Gray circles indicate the positions and approximate spread of Chicago Sky Blue staining in the central nucleus of the amygdala in the 25 rats used in the study. Gray squares indicate positions of sites of staining that were outside the central nucleus of the amygdala. From top to bottom, locations are depicted on sections 1.8, 2.1, and 2.3 cm caudal to bregma using the atlas of Swanson (2004). Two sites that were more rostral and two that were more caudal than these sections but within the boundaries of the CeA have been marked in the closest section displayed. In some cases, sites from different rats appeared identical and were marked with a single gray circle. Abbreviations: CeA, central nucleus of the amygdala; C, M, L, capsular, medial and lateral sections of the central nucleus

of the amygdala; GP, globus pallidus; CP, caudate putamen; SI, substantia innominata; LA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; IA, intercalated amygdalar nucleus; BMA, basomedial nucleus of the amygdala.

Figure 2.

Hemodynamic responses to cocaine (5 mg/kg, iv) in vascular (n=12, solid lines, filled squares) and mixed (n=9, dashed lines, filled circles) responders. Responses shown include mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR) and heart rate (HR). Pound signs denote significant differences ($p < 0.05$, ANOVA) between vascular and mixed responders during the first 60 sec. Error bars indicate s.e.m.

Figure 3.

Hemodynamic changes in response to cocaine (5mg/kg, iv) in rats after microinjection of saline (control) or 80 pmol muscimol into the amygdala. Vascular responses (n=10) are shown using square symbols and mixed responders (n=7) using circles. Responses to cocaine after saline injection are shown with filled symbols; responses after muscimol injection are shown with open symbols. M indicates significant differences ($p < 0.05$) between responses to saline and muscimol in mixed responders and V indicates differences between responses to saline and muscimol in vascular responders as determined by a two way analysis of variance. Abbreviations are described in Figure 2. Error bars indicate s.e.m.

Figure 4.

Hemodynamic responses integrated (geometric area under the curve) over the first 60 sec after cocaine administration comparing control (saline microinjection, Coc) to drug administration for muscimol (+Mus), losartan (+Los) and α -helical CRF₉₋₄₁ (+CRFa) in mixed responders (solid bars) and vascular responders (shaded bars). A two way analysis of variance was used to determine differences due to drug pretreatment (asterisks) or between vascular and mixed responders (pound sign). When significant differences due to drug treatment were only observed in one subset of rats (e.g. vascular responders), the asterisk was placed over that particular bar. Abbreviations are described in Figure 2. Error bars indicate s.e.m.

Figure 5.

Hemodynamic changes from baseline in response to cocaine (5 mg/kg, iv) in rats after microinjection of saline (control) or 43.4 pmol losartan into the amygdala. Vascular responders ($n=9$) are shown using square symbols and mixed responders ($n=8$) using circles. Responses to cocaine after saline injection are shown with filled symbols; responses after losartan microinjection are shown with open symbols. M indicates significant differences ($p<0.05$) between responses to saline and losartan in mixed responders and V indicates differences between responses to saline and losartan in vascular responders as determined by a two way analysis of variance. Abbreviations are described in Figure 2. Error bars indicate s.e.m.

Figure 6.

Hemodynamic changes from baseline in response to cocaine (5 mg/kg, iv) in rats after microinjection of saline (control) or 15.7 pmol α -helical CRF₉₋₄₁ into the amygdala. Vascular responders (n=8) are shown using square symbols and mixed responders (n=8) using circles. Responses to cocaine after saline injection are shown with filled symbols; responses after CRF antagonist injection are shown with open symbols. M indicates significant differences ($p < 0.05$) between responses to saline and α -helical CRF₉₋₄₁ in mixed responders and V indicates differences between responses to saline and α -helical CRF₉₋₄₁ in vascular responders as determined by a two way analysis of variance. Abbreviations are described in Figure 2. Error bars indicate s.e.m.

Figure 7. Hemodynamic changes from baseline in response to cocaine (5 mg/kg, iv) in rats during characterization (filled square symbols, solid lines) and several days after microinjection of 0.06 M ibotenic acid into the CeA (open square symbols, dashed lines). Only the responses of the vascular responders (n=6) are shown. Asterisks indicate significant differences (p<0.05) between control and ablation with ibotenic acid. Abbreviations are described in Figure 2. Error bars indicate s.e.m.

Table 1

Hemodynamic parameters before and during the first minute of cocaine (5 mg/kg, iv).

	Baseline values		Change with cocaine (60 sec)		p
	Mixed (n=10)	Vascular (n=15)	Mixed (n=10)	Vascular (n=15)	
MAP (mmHg)	111 ± 3	111 ± 3	19.7 ± 1.8	22.0 ± 1.6	ns
HR (beats /min)	379 ± 13	366 ± 11	6 ± 6	-8 ± 7	ns
CO (kHz shift) *	9.6 ± 0.9	9.5 ± 0.7	4.7 ± 0.8	-8.4 ± 1.0	<0.0001
SVR *			16.7 ± 2.3	34.5 ± 2.0	<0.0001
Stroke Volume *			2.1 ± 1.3	-6.1 ± 1.9	0.0101

* Change with cocaine in these parameters is shown as % change from baseline.

Table 2
Resting hemodynamic variables and effects of drug pretreatment before cocaine treatment.

Drug	Class (n)	MAP (mmHg)	Chg. MAP (mmHg)	HR (b/min)	Chg. HR (b/min)	CO (kHz)	Chg. CO (%)	Chg SVR (%)	Chg. SV (%)
Muscimol	Mixed (7)	110 ± 5	3.1 ± 2.8	381 ± 24	17 ± 11	10 ± 1.3	0.8 ± 2.4	2.8 ± 4.1	-2.7 ± 4.4
	Vascular (10)	116 ± 3	-2.9 ± 2.1	384 ± 12	2 ± 10	10.2 ± 0.6	-1.6 ± 2.6	0 ± 1.0	-2.2 ± 1.8
Losartan	Mixed (8)	115 ± 5	-0.4 ± 1.0	393 ± 17	5 ± 6	11.2 ± 1.2	-5.6 ± 2.7*	6.2 ± 3.2*	-6.8 ± 3.2
	Vascular (9)	115 ± 4	2.4 ± 1.7	376 ± 19	8 ± 12	10.4 ± 0.7#	-1.7 ± 2.4	4.3 ± 3.0*	-3.6 ± 3.9
α -helical CRF ₉₋₄₁	Mixed (8)	118 ± 2	-0.6 ± 1.5	394 ± 16	3 ± 10	10.2 ± 1.1	6.1 ± 5.0	-5.0 ± 4.1	5.1 ± 5.2
	Vascular (8)	112 ± 3	0 ± 1.1	348 ± 11#	15 ± 10	9.0 ± 0.8	3.6 ± 3.1	-2.8 ± 3.8	-0.2 ± 3.6

* p<0.05 for drug effect compared to vehicle (saline) as determined by analysis of variance.

p<0.05 for difference between vascular and mixed responders as determined by analysis of variance.

NOTE: number of rats in parenthesis under Class.