LOCUS COERULEUS NORADRENERGIC INNERVATION OF THE AMYGDALA
FACILITATES ALERTING-INDUCED CONSTRUCTION OF THE RAT TAIL ARTERY

Mohammed M, Kulasekara K, Ootsuka Y, Blessing WW

Centre for Neuroscience, Department of Human Physiology, Flinders University, Adelaide, SA, Australia.

Address for correspondence
Dr William Blessing
Centre for Neuroscience, Department of Human Physiology,
Flinders University, Adelaide SA, AUSTRALIA
Tel +061 8 8204 4736
Fax +061 8 8204 5768
Email: w.w.blessing@flinders.edu.au

Copyright © 2016 by the American Physiological Society.
Abstract

The amygdala, innervated by the noradrenergic locus coeruleus, processes salient environmental events. Alpha-2 adrenoceptor-stimulating drugs (clonidine-like agents) suppress the behavioral and physiological components of the response to salient events. Activation of sympathetic outflow to the cutaneous vascular bed is part of the physiological response to salience-mediated activation of the amygdala. We have determined whether acute systemic and intra-amygdala administration of clonidine, and chronic immunotoxin-mediated destruction of the noradrenergic innervation of the amygdala, impairs salience-related vasoconstrictor episodes in the tail artery of conscious freely-moving Sprague-Dawley rats. After acute i.p. clonidine (10, 50, 100 µg/kg) there was a dose-related decrease in the reduction in tail blood flow elicited by alerting stimuli, an effect prevented by prior administration of the alpha-2 adrenergic blocking drug idazoxan (1 mg/kg i.p. or 75 nmol bilateral intra-amygdala). A dose-related decrease in alerting-induced tail artery vasoconstriction was also observed after bilateral intra-amygdala injection of clonidine (5, 10, 20 nmol in 200 nl), an effect substantially prevented by prior bilateral intra-amygdala injection of idazoxan. Intra-amygdala injection of idazoxan by itself did not alter tail artery vasoconstriction elicited by alerting stimuli. Intra-amygdala injection of saporin coupled to antibodies to dopamine-β-hydroxylase (immunotoxin) destroyed the noradrenergic innervation of the amygdala and the parent noradrenergic neurons in the locus coeruleus. The reduction in tail blood flow elicited by standardized alerting stimuli was substantially reduced in immunotoxin-treated rats. Thus inhibiting the release of noradrenaline within the amygdala reduces activation of the sympathetic outflow to the vascular beds elicited by salient events.
Introduction

After Kluver and Bucy (35) reported that monkeys with extensive temporal lobe lesions exhibit “psychic blindness”, the amygdaloid nuclear complex, within the temporal lobe, was shown to be a component of the forebrain circuitry regulating emotional, behavioral and autonomic responses (9, 27, 33, 34, 69, 70, 72). Modern studies in experimental animals (12, 43, 62) and in humans (32, 40, 58) have extended the evidence for this view, so that the amygdaloid complex is considered important for the processing of “salience, significance, ambiguity, unpredictability and other aspects of biological value” (53).

Subnuclei within the amygdala receive an extensive noradrenaline-synthesizing innervation, deriving principally from the locus coeruleus (23, 46). Consistent with this anatomical arrangement, evidence from diverse experimental models suggests that the locus coeruleus contributes substantially to the processing of salient events (2-8). The adrenergic receptors associated with the noradrenaline-synthesizing input to the amygdala include the alpha-2 subtype, located principally presynaptically on the noradrenaline-synthesizing nerve terminals (37, 57, 59, 63, 64, 66, 67, 75). Activation of these alpha-2 adrenoceptors inhibits release of noradrenaline (26, 36, 50, 68).

When ambient temperature is within the thermoneutral range, salient stimuli powerfully activate the brain centers controlling sympathetic outflow to the thermoregulatory cutaneous vascular beds, resulting in sudden intense vasoconstriction in the thermoregulatory vascular beds (reviewed in (10)). These episodes may occur apparently spontaneously or they may be evoked by experimentally-controlled alerting stimuli. The episodes are referred to as “sympathetic cutaneous vasoconstrictor alerting responses” or SCVARS. We have demonstrated that non-selective inhibition of neuronal function in the amygdala substantially inhibits the occurrence of SCVARS (43, 72, 74).
Both systemic and intra-amygdala application of alpha-2 adrenergic agonists like clonidine inhibit behavioral and physiological processes that are normally part of the animal’s response to salient, stressful or fear-inducing environmental events (18, 22, 24, 25, 41, 48, 60, 61). In the present paper we have tested the hypothesis that release of noradrenaline within the amygdala is important for the occurrence of SCVARS. In the first series of experiments we used acute systemic and intra-amygdala administration of clonidine and idazoxan (alpha-2 adrenergic agonist and antagonist respectively), and tested the effect on SCVARs occurring in the tail artery of conscious freely moving rats. In the second series of experiments, also in rats, we used intra-amygdala injections of saporin coupled to antibodies against dopamine-B-hydroxylase (saporin anti-DBH) to destroy the noradrenergic neurons innervating the amygdala, including those in the locus coeruleus (11, 56, 71). At least one week after intra-amygdala administration of the immunotoxin we assessed the occurrence of SCVARS in conscious freely moving animals.

**Materials and Methods**

*Animals and surgical procedures*

Experiments, approved by the Animal Welfare Ethical Committee of Flinders University, were carried out on male Sprague-Dawley rats (300-400 g). For implantation of measuring devices rats were anaesthetized with 2% isoflurane (Veterinary Companies of Australia Pty, Ltd., NSW, Australia) in 100% oxygen. An ultrasonic Doppler blood flow probe (Iowa Doppler Products, IA, USA) was implanted around the base of the tail artery (28, 43). In animals in which only systemic injections of drugs were planned, subcutaneous flow probe wires were connected to a head-piece which was then attached to the skull. In animals to be used for intra-amygdala injections of drugs, intracerebral cannulae were implanted (see below) and flow probe wires were then connected to a head-piece and the head-piece attached to the skull.
Preparation for acute injection of clonidine or idazoxan into the amygdala

The procedure is fully described in Mohammed et al. (43). Rats were anesthetized as above and stainless steel guide cannula (26 gauge, Plastics One, Roanake, VA) were positioned bilaterally, 1 mm dorsal to the central nucleus in the amygdaloid complex (AP -2.3 mm; ML +4.8 mm; DV 7.0 mm, (51)) and fixed to the skull. Stainless steel stylets (33 gauge), with plastic caps, were inserted into the guide cannulae. Rats were treated post-operatively with analgesic (Caprofen, 5 mg/kg s.c, Norbrook Laboratories, Melbourne) and antibiotic (Baytril, 15 mg/kg s.c, Bayer Australia, Sydney). Animals recovered in the Animal House for at least 1 week.

For intra-amygdala injections, the stylet was removed from the guide cannula and replaced with an injection cannula (Hypotube S/S 304-RW stainless steel 33 gauge, Small Parts, Inc. Miramar, FL, USA), with the ventral tip protruding 1 mm from the tip of the guide cannula. The proximal end of the injection cannula was already attached to plastic tubing (Polypropylene Dural Plastics & Engineering, Auburn, NSW, Australia) connected to a calibrated 5 μl glass micropipette (Clay Adams, Division of Becton, Dickinson and Company, Parsippany, NJ). Either drug or vehicle was pressure injected. Movement of a fluid-air meniscus in the micropipette was used to monitor the volume of the injection.

Drugs

Clonidine hydrochloride and idazoxan hydrochloride (Sigma Chemical Co, St Louis, MO) were dissolved in Ringer solution. For systemic administration clonidine (10-100 μg/kg) and idazoxan (1 mg/kg) were dissolved in 0.5 ml of Ringer. For intra-amygdala administration we used clonidine (10-100 nmol in 200 nl Ringer) or idazoxan (75 nmol in 200 nl Ringer) injected bilaterally over a period of approximately 1 min. The injection cannula was removed after an additional 1 min, and replaced by the stylet. The doses of
clonidine and idazoxan were obtained from the literature (21, 48, 60, 61) and from our pilot experiments. We performed a dose-response pilot study of the effects of i.p. idazoxan (0.1, 1 and 10 mg/kg) on the SCVAR index. There was a significant log-dose regression (P<0.001, \( R^2 =0.73 \)). The values of the SCVAR index for the increasing dose were 80±3%, 65±9% and 12±6% respectively. We chose the 1mg/kg i.p. dose for our study (see Discussion).

**Experimental protocol for intraperitoneal and intra-amygdala injection of drugs**

On the day of the experiment the rat was transferred to a wooden box (40 x 40 x 40 cm, temperature 24-26°C) with a swivel device that could be connected by a flexible cable to the head socket. The tail artery Doppler blood flow signal was continually recorded for 20 min (see below) with the animal left undisturbed.

In an intra-peritoneal dose-response study, clonidine (10, 50 or 100 µg/kg) was injected i.p and after 20 min the alerting stimuli were administered and the SCVAR index assessed. In separate experiments, idazoxan (1mg/kg) was injected i.p., followed after 20 min by clonidine 100µg/kg i.p.. After an additional 20 min the alerting stimuli were administered and the SCVAR index assessed. In further separate experiments, idazoxan (75 nmol) was injected bilaterally into the amygdala, followed after 20 min by clonidine 100 ug/kg i.p. After an additional 20 min the alerting stimuli were administered and the SCVAR index assessed.

For the bilateral intra-amygdala administration of drug studies we established the effect of bilateral injection of vehicle (200 nl) and, in separate experiments, bilateral administration of idazoxan (75 nol in 200 nl), with the alerting stimuli administered 20 min after the injections. An intra-amygdala dose response study of clonidine (5, 10 and 20 nmol in 200 nl bilaterally) was performed, with the alerting stimuli administered 20 min after administration of clonidine.

In separate experiments, vehicle was injected into the amygdala bilaterally, followed
after 20 min by bilateral intra-amygdala clonidine (20 nmol in 200 nl). After an additional 20 min the alerting stimuli were administered and the SCVAR index assessed. In further separate experiments, idazoxan (75 nmol) was injected bilaterally into the amygdala, followed after 20 min by bilateral intra-amygdala clonidine (20 nmol in 200 nl). After an additional 20 min the alerting stimuli were administered and the SCVAR index assessed.

At the conclusion of each experiment the animal was then returned to the Animal House. Each rat received no more than 3 intraperitoneal or bilateral amygdala injections in counterbalanced order, with at least 2 days between injections. Intracerebral injection sites were marked by including fluorescent beads (FluoSpheres, Molecular Probes, Oregon) or a crystal of Horseradish Peroxidase (Sigma V1, St Louis) in the injectate of the final bilateral injection for each rat.

**Preparation of rats with chronic immunotoxin lesions of the noradrenergic neurons innervating the amygdala**

Rats were anesthetized and placed in the stereotaxic apparatus. Unilateral or bilateral burr holes were made in the skull. A small cut was made in the dura mater. A long-shanked 5 µl glass micropipette (Accu-Fill 90, Micropet, Clay Adams, NJ), calibrated in 100 nl steps, was filled with vehicle or immunotoxin (saporin coupled to anti-DBH, Advanced Targeting, San Diego, CA). The tip of the micropipette was lowered into the amygdala (AP -2.3 mm; ML +4.8 mm; DV 8.0 mm) (51). Immunotoxin (5 µg in 250 nl) or vehicle was injected during approximately 1 min, and the pipette left in place for an additional 1 min. Further surgery for implantation of a Doppler flow probe around the base of the tail artery and a head-piece attached to the skull was then performed, as described above. Animals were returned to the Animal House. Experiments were performed between 1 and 3 weeks after injection of immunotoxin. Rats were transferred to the wooden box in the experimental room (see above). The tail artery Doppler blood flow signal was continually recorded for 1 hour.
with the animal left undisturbed and then the standardized alerting stimuli (see below) were
administered.

Alerting stimuli

All experiments were conducted during the rat’s 12 hour dark phase. Standardized alerting stimuli were administered at least 5 min apart, at times when the tail artery blood flow was at a high level. A flexible metal rod was released from a restraint so that it suddenly tapped the side of the wooden box. A 0.5 s, 90 dB, 100 Hz sound was made outside the box. The box was dropped 1.5 cm. The box was vigorously moved to and fro 2-3 times. A small window (15x15 cm) in the front of the box was suddenly opened. The door of the box was opened and a single pinprick over the thigh was administered using a 23 G sterile needle.

Data recording and statistical analysis

The tail artery Doppler signal was transmitted via the subcutaneous wires to the head piece and then, via a flexible cable and a swivel device to a System 6 Model 200 signal processor (Triton Technology, San Diego). The output voltage signal was calibrated in cm/s using the Triton internal calibrator. The signal was transferred to a MacLab/s device and analysed using Chart 7 (ADinstruments Inc, Castle Hill), IgorPro software (Wavemetrics, Lake Oswego) and Statview (SAS institute, Carey, NC, USA).

We calculated the percentage coefficient of variation (pCV) of 20 min segments of Doppler flow signal as an index of spontaneous reductions in tail artery blood flow. As an index of experimenter-controlled reductions in tail artery blood flow we calculated the percentage reduction in the Doppler signal (SCVAR index) elicited by the standardized alerting stimuli. The SCVAR index formula was 100-[(pre-stimulus mean flow + pre-stimulus mean pulse amplitude)/(post-stimulus mean flow + post-stimulus mean pulse amplitude)* 100]. The pre-stimulus flow and pulse amplitude (3s sample) and post-stimulus
flow and pulse amplitude (3s sample at lowest flow level in the 10 s after the stimulus onset) were used. The SCVAR index for each of the 6 alerting stimuli were averaged to obtain a single SCVAR index for each rat in each experimental condition.

Group data are shown as mean±sem unless otherwise indicated. SCVAR and pCV values for each condition were analysed using factorial ANOVA. If the overall analysis was significant at P<0.05 groups were compared using Fisher’s protected least significance difference test. In addition, for both IP and intra-amygdala experiments, we used linear regression to determine the relation between log dose of clonidine and both pCV and SCVAR index.

Histological examination of clonidine-idazoxan intracerebral injection sites, and examination of brain after injection of immunotoxin into the amygdala

After completion of experiments rats were anesthetized with pentobarbital (100 mg/kg i.p.). Brains were perfused transcardially with 4% formaldehyde fixative in 0.5M phosphate buffer, removed and left in the fixative overnight with 30% sucrose. Serial coronal sections (50 µm) were cut using a freezing microtome.

After intracerebral injections of clonidine or idazoxan forebrain sections processed for HRP reaction product were visualized with the diaminobenzidine (Sigma, St Louis) hydrogen-peroxide reaction and counter-stained for Nissl substance using Neutral Red. The fluorescent beads were examined in an Olympus AX50 fluorescence microscope.

After intra-amygdala administration of immunotoxin, animals were maintained for up to one month before anesthetization and fixation of the brain as described above. In these animals, 50 µm coronal sections were cut through the forebrain and the hindbrain. Free-floating brain sections were processed immunohistochemically using a monoclonal anti-DBH raised in mouse (Millipore-Chemicon Temecula, CA) 1/5000, biotinylated anti-mouse (1/200) raised in goat (Sigma-Aldrich, Castle Hill NSW, Australia) using the avidin-biotin-
peroxidase procedure. Sections were examined in an Olympus BH-2 microscope using either
darkfield or lightfield illumination.

RESULTS

Systemic administration of clonidine

Before injection of clonidine the tail artery blood flow record displayed rapid
fluctuations between peak levels and near zero levels (Fig. 1). After i.p. administration of
clonidine both the variability of tail artery blood flow and the acute reductions in flow
elicited by alerting stimuli were substantially reduced, as can be seen in the records of
individual experiments shown in Fig. 1. After the higher dose of clonidine these fluctuations
were much less evident, and the acute reductions in flow normally elicited by alerting stimuli
were substantially decreased or entirely prevented, as shown for an individual animal in Fig.
1B. Confirmation of the dose-responsive nature of these changes is provided in the group
data presented in Fig. 2 and the substantial reduction in the SCVAR index is reflected in the
$R^2$ value (0.80). of the log dose-response regression The pCV was reduced from 80±9% to38±2% and the combined SCVAR index is reduced from 83±4% to 7±2%, with
significance values and confirmation of dose-responsiveness documented in the legend to
Fig. 2.

After pretreatment with idazoxan (1 mg/kg i.p or 75 nmol bilaterally into the
amygdala) and subsequent injection of clonidine (100 µg/kg i.p), the SCVAR index was
substantially increased in comparison with the value observed for clonidine 100 µg/kg i.p.
without idazoxan pretreatment (Fig. 2). The idazoxan pre-treatment (i.p. and intra-
amygdala) did not entirely restore the SCVAR index to the baseline (clonidine 10 µg/kg i.p.)
level (Fig. 2). The pCVs after injection of clonidine 100 µg/kg i.p. were not significantly
increased by pretreatment with either i.p. or intra-amgydala administration of idazoxan (Fig.
2).
Bilateral injection of clonidine into the amygdala or into the striatum dorsal to the amygdala

After bilateral injection of vehicle into the amygdala, the tail artery blood flow record displayed the normal rapid fluctuations between peak levels and near zero levels. Records of individual rats are shown in Fig. 3 and group results are presented in Fig. 4. After injection of vehicle the pCV was 81±7% and the SCVAR index was 80±4%. After bilateral intrastriatal (2 mm dorsal to the amygdala) injection of 20 nmol clonidine the pCV was 79±7% and the SCVAR index was 95±2%. After bilateral injection of idazoxan (75 nmol) into the amygdala, without subsequent injection of clonidine, the pCV was 49±4% and the SCVAR index was 82±3% (see Fig. 3B for an individual record).

After bilateral intra-amygdala injection of clonidine (5, 10, and 20 nmol) there was a dose dependent reduction in both the pCV and the SCVAR index. The pCV was decreased from 80±9% to 38±2% and the combined SCVAR index was decreased from 83±4% to 7±2%, with significance values documented in the legend to Fig. 2.

After pretreatment with vehicle bilaterally into the amygdala and subsequent injection of clonidine (20 nmol) bilaterally into the amygdala neither the pCV nor the SCVAR index were changed from the corresponding clonidine 20 nmol intra-amygdala values obtained with no vehicle pretreatment (see Fig. 4 and legend to Fig. 4 for statistical documentation).

After pretreatment with idazoxan (75 nmol) bilaterally into the amygdala and subsequent injection of clonidine (20 nmol) bilaterally into the amygdala, the SCVAR index was substantially increased in comparison with the corresponding value after bilateral intra-amygdala injection of vehicle followed by bilateral intra-amygdala injection of clonidine (20 nmol), although the SCVAR index value (65±4%) was still less than the value (81±7%) recorded after bilateral injection of vehicle into the amygdala without any other treatment, as documented in Fig. 4.

Bilateral injection of anti-DBH saporin immunotoxin into the amygdala
After unilateral or bilateral intra-amygdala injections of immunotoxin, the pCV and the SCVAR index were substantially reduced compared to the corresponding values obtained at least one week after similar injection of vehicle. Individual records are presented in Fig. 5 and group results are presented in Fig. 6. Bilateral administration of immunotoxin caused a greater reduction in the SCVAR index in comparison with unilateral administration (Fig. 6).

**Histological Findings**

Fig. 7 is a representative coronal section at the level of the amygdala complex of nuclei, stained with the DAB reaction after HRP was included in the clonidine-containing cannula directed at the amygdala. The reaction product is present in the amygdala sub-nuclei, with spread to portions of the surrounding temporal lobe.

Immunohistochemical staining with antibodies to DBH after unilateral intra-amygdala injections of immunotoxin documented virtually complete destruction of noradrenergic axonal processes in virtually the whole of the ipsilateral telencephalon, including the amygdaloid complex of nuclei and the surrounding temporal lobe (Fig. 8 A,C). In contrast, after unilateral injection of vehicle there were normal DBH-positive axonal processes in these regions (Fig. 8 B,D). After unilateral intra-amygdala injection of immunotoxin the rostral two-thirds of the ipsilateral locus coeruleus was substantially destroyed, with preservation of the contralateral locus coeruleus (Fig. 8, E and F). After bilateral intra-amygdala injections of immunotoxin, neurons in the rostral two-thirds of both locus coerulei were destroyed but the ventrolaterally situated A7 neuronal perikarya were intact, as were the A5 neurons (not shown). The A1 and A2 noradrenergic neurons in the caudal medulla were also affected after intra-amygdala injections of immunotoxin but the degree of loss was difficult to quantify.

**DISCUSSION**
After either systemic or intra-amygdala administration, clonidine reduced both spontaneous reductions in tail artery blood flow and those induced by alerting stimuli controlled by the experimenter. The clonidine-elicited reductions were dose-dependent for both routes of administration. The $R^2$ value for the regression between intra-amygdala log dose of clonidine and SCVAR index was 0.77 and the $R^2$ value for the regression between IP log dose of clonidine and SCVAR index was 0.80, indicating a substantial SCVAR-inhibiting effect after both routes of administration. The effect of clonidine on the falls normally observed in response to the controlled administration of alerting stimuli was especially substantial, so that the percentage decrease in flow (the SCVAR index) was reduced from approximately 80% to approximately 7% after i.p. injections and to approximately 15% after intra-amygdala injections. When the maximum dose of clonidine was injected into the striatum dorsal to the amygdala there was no reduction in the SCVAR index.

Pre-treatment with the alpha-2 adrenergic antagonist idazoxan, administered systemically or via direct injection into the amygdala, significantly prevented the SCVAR inhibitory effects of systemic clonidine, and pre-treatment with intra-amygdala idazoxan substantially prevented the inhibitory effects of subsequent intra-amygdala clonidine. The intraperitoneal dose of idazoxan used in our experiments (1 mg/kg) is at the upper end of the range used by other investigators (eg (21)) and this dose of the drug, administered by itself, did reduce the SCVAR index. However the intra-amygdala dose of idazoxan (75 nmol), by itself, had no effect on the SCVAR index, but it substantially prevented the inhibitory effect of subsequently administered clonidine (both i.p and intra-amygdala). We appreciate that idazoxan also interacts with imidazole receptors (31). However, taken together, the idazoxan results in our experiments strongly support the view that the SCVAR-inhibitory action of
Clonidine is mediated by actions at alpha-2 adrenergic receptors, including receptors in the amygdala.

Clonidine, via its agonist action at alpha-2 adrenergic receptors, inhibits the release of noradrenaline from noradrenergic nerve terminals (26, 50, 63, 64, 68). Clonidine, administered systemically or into specific brain regions, also hyperpolarizes and decreases the discharge of noradrenergic perikarya, as first demonstrated in Aghajanian’s laboratory (1, 17, 65). Thus in the present experiments systemically administered clonidine might also inhibit amygdala neurons by decreasing the discharge of locus coeruleus neurons, thereby reducing noradrenergic excitation of amygdala neurons mediated via alpha-1 adrenergic or beta adrenergic receptors. Our saporin anti-DBH experiments support this view. The retrogradely transported immunotoxin substantially destroyed the rostral two thirds of the locus coeruleus, virtually eliminating the noradrenergic innervation of the amygdala as well as the remainder of the telencephalon. After unilateral intra-amygdala injections of immunotoxin, neuronal destruction was observed principally in LC, consistent with the known principally unilateral projection of locus coeruleus axons to the telencephalon (29, 42, 46, 49). We also observed damage to the A1 and A2 groups of neurons in the medulla oblongata that also provide noradrenergic input to the amygdala (49, 54) but quantification of this damage was difficult.

Clonidine, after both systemic and intra-amygdala administration in doses generally similar to those used in the present study has been shown to inhibit a number of behavioral and physiological processes that are normally part of the animal’s response to salient, stressful or fear-inducing environmental events (14, 18, 20, 22, 24, 25, 47, 48, 52, 60, 61). We have demonstrated in both rabbits and rats that acute vasoconstriction in thermoregulatory beds elicited by alerting stimuli is immediately preceded by a sudden increase in the proportion of theta rhythm in the hippocampal EEG (19, 73), a reliable
indication that the animal is suddenly directing attention to a possible threat in the external
environment (15, 16). Thus our present study adds to the evidence that the noradrenergic
innervation of the amygdala, deriving principally from the locus coeruleus, contributes to the
autonomic responses associated with the processing of salient, and possibly threatening,
environmental stimuli.

Madden and colleagues (41) demonstrated that administration of clonidine, systemically
and directly into the rostral raphe pallidus region, inhibits brown adipose tissue (BAT)
thermogenesis and lowers body temperature. The authors propose that clonidine acts on post-
synaptic alpha-2 receptors located on bulbospinal neurons known to regulate the sympathetic
outflow to BAT (30). Bulbospinal neurons in the rostral raphe pallidus, including serotonin-
synthesizing neurons, also control the sympathetic outflow to the tail artery (10), so that
systemically applied clonidine could contribute to inhibition of SCVARs via actions on
alpha-2 receptors in the rostral raphe pallidus region. Presynaptic actions of clonidine on
terminals of catecholamine-synthesizing neurons innervating the rostral raphe pallidus [see
review in (41)] could also contribute to these actions. Increased body temperature via brown
adipose tissue thermogenesis and cutaneous vasoconstriction is part of the autonomic
response to emotional arousal (10, 44), so that the present results complement those of
Madden and colleagues (41).

We appreciate that the size of our clonidine injections and the widespread destruction
of noradrenergic terminals after our intra-amygdala injections of immunotoxin means that
our study does not allow us to make conclusions concerning specific subnuclei within the
amygdala. Nevertheless, taken together, our clonidine and immunotoxin results provide
strong evidence that inhibiting the release of noradrenaline within the region of the
amygdaloid complex of nuclei inhibits the CNS processing of salient events and the resultant
activation of the sympathetic outflow to the thermoregulatory vascular beds.
PERSPECTIVES AND SIGNIFICANCE

Pharmacological manipulation of alpha-2 adrenoceptors in the CNS has contributed to the therapeutic management of medical conditions related to emotional arousal including hypertension (55) and major neuropsychiatric disorders (13, 38). The wide range of behavioral and physiological variables affected by clonidine-like agents presumably reflects the widespread distribution and axonal projections of noradrenergic neurons in the brain. In particular, the locus coeruleus has been implicated in many aspects of emotional arousal (2), so that functional inhibition of the extensive locus coeruleus-derived noradrenergic innervation of centers known to be important in emotional arousal, including the amygdala, is likely to contribute to the therapeutic actions of clonidine-like agents. The locus coeruleus also has major reciprocal connections with the orexin-synthesizing neurons in the hypothalamus (76), and rats with genetically lesioned orexin-neurons have reduced emotional arousal as reflected in reduced SCVAR responses to alerting stimuli, reduced thermogenic responses to an intruder rat, and in a reduced amplitude of the increases in a number of physiological variables associated with the ultradian basic rest-activity cycle (45). Clonidine has similar effects on these physiological variables (39, 41) so that it may well be that the noradrenergic input to the orexin neurons is also inhibited by clonidine. Clozapine and clozapine-like drugs, antipsychotic agents that powerfully reduce the behavioral and physiological aspects of emotional arousal, interact with alpha-2 adrenergic receptors (13), so that the neural mechanisms whereby clonidine reduces emotional arousal may overlap with those mediating the therapeutic effects of clozapine-like antipsychotic agents.
Acknowledgements: We thank Pam Simpson and Jessi Moore for technical assistance.

Grants: Our research was supported by NHMRC Grants APP535025 and APP105826
Figure Legends

Figure 1
Tail artery blood flow Doppler signals before and after IP injection of clonidine at low (10 µg/kg, A) and high (100 µg/kg, B). Administration of alerting stimuli is indicated by the vertical arrow heads. The lower panels in both A and B show a shorter time base record of the Doppler signal just before and after the relevant alerting stimulus (marked by a rectangle in the upper panels, and by the long slanted arrow extending from the upper to the lower panels of A and B).

Figure 2
Group data (mean±sem) showing (slanting pattern bars) the percentage coefficient of variation of flow (pCV) and (black filled bars) percentage reduction in tail artery blood flow in response to standardized alerting stimuli (SCVAR index) 30 min after i.p. injection of clonidine (10, 50 and 100 µg/kg) and clonidine 100 µg/kg i.p. after pretreatment with i.p. idazoxan (1 mg/kg) or bilateral intra-amygdala idazoxan (75nmol in 200nl). N=6 rats in each experimental condition.

** significant linear regression between log dose of clonidine and percentage coefficient of variation, P<0.0001, R^2=0.62..

•• significant linear regression between log dose of clonidine and SCVAR index, P<0.0001, R^2=0.80.

ns not significantly different from pCV after injection of clonidine 100 µg/kg i.p. without prior pretreatment, P=0.2124 for IP idazoxan and P=0.3726 for intra-amygdala idazoxan.

¶¶ significantly greater than SCVAR index after injection of clonidine 100 µg/kg i.p.,
P<0.0001 for i.p. idazoxan and P<0.0001 for intra-amygdala idazoxan.

†† significantly less than SCVAR index after i.p. injection of clonidine 10 µg/kg without pretreatment, P=0.0002 for i.p. idazoxan and P=0.0037 for intra-amygdala idazoxan.

Figure 3
Tail artery blood flow Doppler signals before and after bilateral intra-amygdala injection of vehicle (A), bilateral intra-striatal injection of 20 nmol clonidine (B) and bilateral intra-amygdala injection of 20 nmol clonidine (C). at low (10 µg/kg, A) and high (100 µg/kg, B). Administration of alerting stimuli is indicated by the vertical arrow heads. The lower panels in A-C show a shorter time base record of the Doppler signal just before and after the relevant alerting stimulus, (marked by a rectangle in the upper panels, and by the long slanted arrow extending from the upper to the lower panels of A, B and C).

Figure 4
Group data (mean±sem) showing (slanting pattern bars) the percentage coefficient of variation of flow (pCV) and (black filled bars) percentage reduction in tail artery blood flow in response to standardized alerting stimuli (SCVAR index) 30 min after bilateral injections of vehicle, clonidine (dose-response), or clonidine (20 nmol) after pretreatment either with bilateral injection of vehicle into the amygdala or bilateral injection of idazoxan into the amygdala. N=6 rats in each condition.

** significant linear regression between log dose of clonidine and percentage coefficient of variation, P=0.0008, R²=0.52.

•• significant linear regression between log dose of clonidine and SCVAR index, , P<0.0001, R²=0.77.

ns not significantly different from pCV after injection of clonidine 20 nmol into amygdala.
following bilateral injection of vehicle into the amygdala, $P=0.068$

¶¶ significantly greater than SCVAR index after injection of clonidine 20 nmol into amygdala following bilateral injection of vehicle into the amygdala, $P<0.0001$.

†† significantly less than SCVAR index after bilateral injection of vehicle into amygdala, $P=0.0058$.

Figure 5
Tail artery blood flow Doppler signals at least one week after bilateral intra-amygdala injection of vehicle (A) and bilateral intra-amygdala injection of saporin immunotoxin (B). Administration of alerting stimuli is indicated by the vertical arrow heads. The lower panels in A and B show a shorter time base record of the Doppler signal just before and after the relevant alerting stimulus, (marked by a rectangle in the upper panels, and by the long slanted arrow extending from the upper to the lower panels of A and B).

Figure 6
Group data (mean±sem) showing (slanting pattern bars) the percentage coefficient of variation of flow (pCV) and (black filled bars) percentage reduction in tail artery blood flow in response to standardized alerting stimuli (SCVAR index) at least one week after either unilateral (A) or bilateral (B) injection of vehicle or anti-DBH saporin immunotoxin into the amygdala. Numbers of rats in each condition are shown at the base of each bar.

$$\text{significantly less than pCV index 1 week after corresponding intra-amygdala injection of vehicle, } F(3,20) =8.030, P=0.0016 \text{ for unilateral injection (A) and } P=0.0081 \text{ for bilateral injection (B).}$$
significantly less than SCVAR index 1 week after corresponding intra-amygdala injection of vehicle. $F(3,20) = 30.638$, $P=0.0002$ for unilateral injection (A) and $P<0.0001$ for bilateral injection (B).

NS not significantly different from corresponding pCV after unilateral injection of immunotoxin.

** significantly different from corresponding SCVAR index after unilateral injection of immunotoxin $P=0.0002$.

**Figure 7**

Coronal section through the region of the amygdala after injection of inclusion of HRP in the vehicle and tissue processing for the DAB-peroxidase reaction product (see dashed outline). The area dorsal to the amygdala, showing the cannula tract, also indicates the intra-striatal control region used for anatomical control injections of clonidine.

BLA; basolateral amygdala nucleus
BMA; basomedial amygdala nucleus
CEA; central amygdala nucleus
f; fornix
LA; lateral amygdala nucleus
LV; lateral ventricle
mtt; mammillothalamic tract
ot; optic tract

**Figure 8**

Each panel shows coronal sections of brain immunohistochemically staining with antibodies to DBH after administration of saporin immunotoxin or vehicle into the amygdala. (A) Dark
field photomicrograph of a coronal section through the region of the amygdala on the ipsilateral side after unilateral intra-amygdala injection of saporin immunotoxin, demonstrating the absence of noradrenergic nerve terminals. (B) Dark field photomicrograph of a coronal section through the region of the amygdala on the ipsilateral side after unilateral intra-amygdala injection of vehicle, demonstrating abundant noradrenaline-synthesizing nerve terminals. (C) Light field photomicrograph of the area indicated by the white rectangle in A. (D) Light field photomicrograph of the area indicated by the white rectangle in B. (E) Ipsilateral and (F) contralateral light-field photomicrographs of the locus coeruleus after unilateral injection of saporin immunotoxin into the amygdala.

BLA; basolateral amygdala nucleus
BMA; basomedial amygdala nucleus
CEA; central amygdala nucleus
LC; locus coeruleus
sep; superior cerebellar peduncle
st; stria terminalis
subC; subcoeruleus
ot; optic tract

References


17. **Cedarbaum JM, and Aghajanian GK.** Noradrenergic neurons of the locus coeruleus: inhibition by epinephrine and activation by the alpha-antagonist piperoxane. *Brain Res* 112: 413-419, 1976.


Mohammed M, Ootsuka Y, and Blessing WW. Brown adipose tissue thermogenesis contributes to emotional hyperthermia in a resident rat suddenly confronted with an intruder rat. *Am J Physiol Regul Integr Comp Physiol* 2014.


Zitnik GA. Control of arousal through neuropeptide afferents of the locus coeruleus. *Brain Res* 2015.
A

clonidine 10 µg/kg i.p.

Tail Doppler flow signal (cm/s)

clonidine 10 µg/kg i.p.

alerting stimuli

alerting stimulus (Drop cage 1 cm)

B

clonidine 100 µg/kg i.p.

Tail Doppler flow signal (cm/s)

clonidine 100 µg/kg i.p.

alerting stimuli

alerting stimulus (Tap on cage)
Intraperitoneal administration of clonidine

Percentage coefficient of variation (pCV)
Percentage reduction in tail blood flow (SCVAR Index)

<table>
<thead>
<tr>
<th>Dose (µg/kg)</th>
<th>10 µg/kg</th>
<th>50 µg/kg</th>
<th>100 µg/kg</th>
<th>100 µg/kg</th>
<th>100 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>80%</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>60%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After pretreatment with idazoxan 75 nmol to amygdala bilaterally.
A

Bilateral injection of Ringer into amygdala

Tail Doppler flow signal (cm/s)

Alerting stimulus (move cage sideways)

5 min

2 s

B

Bilateral injection of idoxazan (75 nmol in 200nl) into amygdala

Tail Doppler flow signal (cm/s)

Idoxazan injection

Alerting stimuli

5 min

2 s

C

Bilateral injection of clonidine (20 nmol in 200nl) into amygdala

Tail Doppler flow signal (cm/s)

Clonidine injection

Alerting stimuli

5 min

2 s
Intra-amygdala administration of clonidine

Percentage coefficient of variation (pCV)
Percentage reduction in tail blood flow (SCVAR Index)

- Bilateral injection of vehicle into amygdala
- Bilateral injections of clonidine into the amygdala

After pretreatment with idazoxan 75 nmol to amygdala bilaterally
After pretreatment with vehicle to amygdala bilaterally

Coeff of Variation
SCVAR index

**
††
ns
A. One week after bilateral injection of Ringer into amygdala

Tail Doppler flow signal (cm/s)

alerting stimulus (Tap on cage)

alerting stimuli

5 min

2 s

B. One week after bilateral injection of anti_DBH saporin into amygdala

Tail Doppler flow signal (cm/s)

alerting stimulus (Tap on cage)

alerting stimuli

5 min

2 s
Intra-amygdala injections of vehicle or immunotoxin

Percentage coefficient of variation (pCV)
Percentage fall in tail blood flow (SCVAR index)

A UNILATERAL

B BILATERAL