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European Journal of Pharmacology 464 (2003) 127–133
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The CRF_1 receptor antagonist, DMP695, abolishes activation of locus coeruleus noradrenergic neurones by CRF in anesthetized rats

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Received 16 September 2002; received in revised form 22 January 2003; accepted 28 January 2003

Abstract

Corticotropin-releasing factor $(CRF)_1$ receptors have been implicated in the excitatory influence of CRF upon noradrenergic perikarya of the locus coeruleus. This study thus characterized the influence of the novel CRF_1 receptor antagonist, DMP695 (*N*-(2-chloro-4,6-dimethylphenyl)-1-[1-methoxymethyl-(2-methoxyethyl]-6-methyl-1*H*-1,2,3-triazolo[4,5-*c*]pyridin-4-amine mesylate), upon the electrical activity of noradrenergic perikarya in the locus coeruleus of anesthetized rats. Intracerebroventricular injection of CRF dose-dependently (0.05–4.0 μg) enhanced the firing rate of noradrenergic cell bodies and transformed their firing pattern into a burst mode. This action was dose-dependently abolished by i.v. administration of DMP695 (0.125–2.0 mg/kg i.v.), which did not itself modify the electrical activity of noradrenergic neurones. These data demonstrate antagonist properties of DMP695 at central CRF_1 receptors excitatory to ascending noradrenergic neurones, an action which may contribute to its distinctive profile of anxiolytic properties.

Keywords: Corticotropin-releasing factor (CRF); Locus coeruleus; DMP695; Unit activity

1. Introduction

Cerebral corticotropin-releasing factor (CRF)-containing neurones play an important role in the modulation of mood and in the response to stress. Correspondingly, antagonists at CRF_1 receptors, which are enriched in corticolimbic structures and implicated in the actions of CRF, are targets for the treatment of stress-related disorders (De Souza, 1995; Smith et al., 1998; Steckler and Holsboer, 1999; Gilligan et al., 2000; Grigoriadis et al., 2001; Millan et al., 2001). There is particular interest in the influence of CRF upon noradrenergic pathways originating in the locus coeruleus in view of their implication in depressive and anxious states (Charney et al., 1995; Sullivan et al., 1999; Millan et al., 2000b; Harro and Oreland, 2001). The locus coeruleus is innervated by CRF-containing fibers arising in diverse regions of the central nervous system (Valentino et al., 1992; Sawchenko et al., 1993). With only a few exceptions, neurochemical and electrophysiological studies indicate that CRF exerts a *facilitatory* influence upon locus coeruleus-derived noradrenergic projections, an action mediated via CRF_1 receptors. These CRF_1 sites are probably localized in the locus coeruleus itself (Conti et al., 1997; Curtis et al., 1997; Page and Abercrombie, 1999; Chen et al., 2000; Jedema and Grace, 2000; Palamarchouk et al., 2000) though their precise location and relationship to noradrenergic perikarya/dendrites, local interneurones and locus coeruleus afferents remain under study (Borsody and Weiss, 1996; Bundey and Kendall, 1999; Chen et al., 2000; Van Bockstaele et al., 2001; Sauvage and Steckler, 2001; Valentino et al., 2001). Further, actions of CRF at sites outside the locus coeruleus may indirectly excite noradrenergic neurones (Smagin et al., 1995; Borsody and Weiss, 1996; Van Bockstaele et al., 2001; Palamarchouk et al., 2000). Underlying the physiological and therapeutic pertinence of this interaction, CRF_1 receptor antagonists attenuate the enhancement of noradrenergic output provoked by stress (Emoto et al., 1993; Smagin et al., 1997; Mar Sanchez et al., 1999; Van Bockstaele et al., 2001; Griebel et al., 2002). Such actions may be involved in their anxiolytic and/or antidepressive properties (Swiergiel et al., 1992; Mitchell, 1998; Steckler and Holsboer,

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doi:10.1016/S0014-2999(03)01383-9

1999; Valentino and Van Bockstaele, 2001; Harro and Oreland, 2001).

For novel ligands, then the demonstration of antagonist properties at CRF_1 receptors excitatory to locus coeruleus noradrenergic pathways is of particular importance. Though such actions have been shown for peptidergic antagonists, such as antalarmin, α-helical-CRF-(9–41) and D-phe-CRF-(12–41) (Borsody and Weiss, 1996; Curtis et al., 1997; Zhang et al., 1998; Menzaghi et al., 1994; Page and Abercrombie, 1999), comparatively little information is available for non-peptidergic ligands. Nevertheless, systemic administration of CRA1000 (*N*-ethyl-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydro-1-pyridinyl]-*N*-[4-isopropyl-2-(methylsulfanyl) phenyl]-6-methylpyrimidin-2-amine) and CP154,526 (*N*-butyl-*N*-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-*N*-ethylamine) attenuated the CRF-induced increase in electrical activity of locus coeruleus neurones (Schulz et al., 1996; Okuyama et al., 1999), while both CP154,526 (perfused locally) and SSR125543A (4-(2-chloro-4-methoxy-5-methylphenyl)-*N*-[2-cyclopropyl-1(*S*)-(3-fluoro-4-methylphenyl)ethyl]-5-methyl-*N*-(2-propynyl)thiazol-2-amine hydrochloride), upon systemic administration, suppressed the stress-induced activation of the locus coeruleus as monitored by release of noradrenaline in the frontal cortex (Kawahara et al., 2000; Griebel et al., 2002).

DMP695 is a novel, high affinity and selective antagonist at both rat and human CRF_1 receptors, which displays marked activity in rodent models of anxiolytic properties (Bakthavatchalam et al., 1998; He et al., 2000; Gilligan et al., 2000; Millan et al., 2001). In light of the above comments, employing an electrophysiological approach in anesthetized rats, the present study evaluated its potential antagonist properties at CRF_1 receptors excitatory to noradrenergic projections.

2. Materials and methods

2.1. Animals

This study employed male Wistar rats (Iffa Credo, L'Arbresle, France) weighing 275–325 g, housed in sawdust-lined cages with unrestricted access to food and water, with lights on from 0730 to 1930 h. Animals were adapted for at least 1 week to laboratory conditions prior to use.

2.2. Recording from noradrenergic neurones

Techniques detailed previously (Millan et al., 2000a; Lejeune and Millan, 2000) were employed for determination of the influence of drugs upon the electrical activity of noradrenergic perikarya localized in the locus coeruleus. Briefly, rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.), the femoral vein was catheterized, and the animal was placed in a stereotaxic apparatus with the incisor bar set for flat skull. The needle of a 5-μl microsyringe, monitored by an electronic microinjecter (Unimecanique, Epinay/Seine, France), was positioned in the lateral ventricle (AP = − 1.0 from bregma, H = − 3.5 from dura and L = 1.4) for CRF infusion and a tungsten microelectrode (10 MΩ, HSE) was lowered by an electronic microdrive (Unimecanique) into the ipsilateral locus coeruleus (AP = − 0.8/ − 1.2 from zero, L = 1.0/1.2, H = − 5.5/ − 6.5 from the sinus surface). Noradrenergic neurones were identified according to their waveform and typical response induced by a contralateral paw pinch. Baseline recording was undertaken for at least 5 min. One cell was recorded in each animal. At the end of the experiments, electrode-tip placements were verified by injection of methylene blue (10 nl, saturated solution) at the exact position at which noradrenergic cells has been recorded.

2.3. Drugs and treatments

The actions of CRF upon i.c.v. administration require 10–12 min for their full expression and may last as long as 45 min (Curtis et al., 1997). However, it would be virtually impossible to continue recording from a single neurone for a period of time sufficient to perform a dose–response study if successive doses were injected at intervals of 45 min. Thus, since the effects of CRF are well established by 4–5 min (see figures), this interval was selected for the dose–response study. CRF (Neosystem, Strasbourg, France) was dissolved in distilled water (1 μg/μl) and infused at a constant rate of 0.01 μl/s. DMP695, synthesized by Servier chemists (P. Casara), was dissolved in diluted lactic acid and the pH adjusted to as close as possible to neutrality with NaOH; it was slowly administered by the i.v. route (0.5 ml/kg over 1 min). For antagonist studies, DMP695 (2 mg/kg, i.v.) or vehicle was administered 4–5 min before infusion of CRF (2.5 μg, i.c.v.) or vehicle. Following completion of the recording, to confirm the identity of noradrenergic neurones, the α_2-adrenoceptor agonist, clonidine (Sigma, St. Quentin-Fallavier, France), dissolved in sterile water, was injected i.v. at a dose of 10 μg/kg.

2.4. Recording and analysis

The extracellular unit activity of locus coeruleus neurones was monitored with a digital storage oscilloscope and simultaneous fed into a computer. Acquisition and off-line analysis were performed by Spike2 software (CED, Cambridge, UK). Drug effects were measured over 60-s bins at the time of peak action. Data are means ± S.E.M.. For data normalisation, changes in firing rates were expressed relative to mean baseline values which were defined as 100%. The beginning of a burst was defined as an inter-spike interval of less than 100 ms and the end of a burst corresponded to an interval of more than 160 ms. The quantification of bursts corresponds to the ratio between

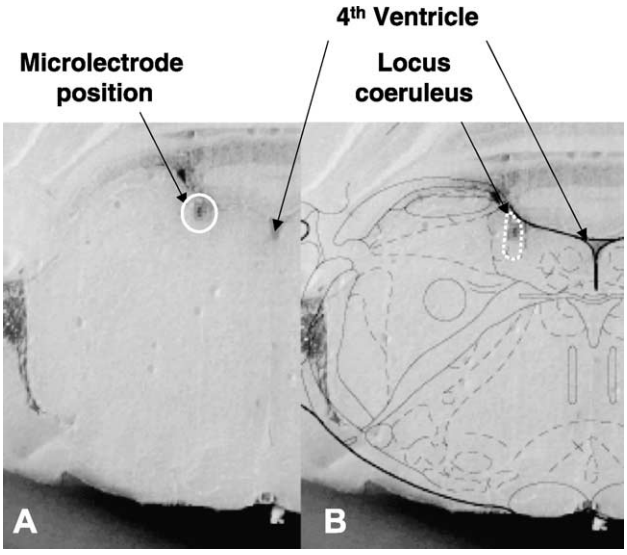

Fig. 1. Histological control of the microelectrode tip in the locus coeruleus. (A) Photograph of a whole brain section with the blue spot showing the position of the electrode tip. (B) Superposition of the coronal plane of the atlas (Paxinos and Watson, 1986) corresponding to the level presented in (A) showing that the electrode tip was located in the locus coeruleus.

spikes in burst and the total number of spikes, expressed as a percentage.

3. Results

3.1. Identification of locus coeruleus noradrenergic neurones

Noradrenergic neurones in the locus coeruleus were identified by (1) stereotaxic criteria, (2) histological controls (Fig. 1), (3) their distinctive waveform characterized by a notch on the last ascending limb of the biphasic potential, (4) a biphasic excitatory–inhibitory response to a contralateral paw pinch and (5) following completion of experiments, the complete suppression of firing by clonidine (0.01 mg/kg, i.v.), an agonist at inhibitory α_2-adrenoceptors localized on dendrites of locus coeruleus cell bodies (Aghajanian et al., 1977; Millan et al., 2000a,b).

3.2. Influence of CRF

I.c.v. administration of vehicle failed to modify the spontaneous electrical discharge of noradrenergic neurones (data not shown). The maximal variation observed after vehicle treatment was $93.5 \pm 6.6\%$ (1.28 ± 0.16 Hz, Student's t-test, $p>0.05$) as compared to baseline values (1.37 ± 0.14 Hz = 100%). In contrast, CRF elicited a pronounced increase in firing rate with a maximal effect of $190.5 \pm 6.9\%$ (2.67 ± 0.21 Hz, Student's t-test, $p<0.01$) at the dose of 4.0 μg, i.c.v. The influence of CRF upon firing rate was expressed dose-dependently. However, as mentioned in Materials and methods, successive doses were administered at intervals *shorter* than the duration of action of CRF. This point should be born in mind in interpreting the quantitative effects of doses reported herein. The enhancement of discharge rate elicited by CRF was accompanied by an augmentation of the number of bursts (though not the number of spike in burst) from $2.0 \pm 1.1\%$ to $11.9 \pm 2.3\%$ (Student's t-test, $p<0.01$). These effects of CRF were seen following a delay of several minutes, reflecting its diffusion from the anterior ventricle to the locus coeruleus via the cerebrospinal fluid (Fig. 2).

3.3. Influence of DMP695

In comparison to pretreatment with vehicle, i.v. administration of DMP695 prior to CRF dose-dependently blocked the excitatory influence of an invariant dose (2.5 μg, i.c.v.) of CRF upon the firing rate of noradrenergic neurones (Fig. 3). This blockade was complete at the dose of 2.0 mg/kg, i.v. of DMP695 ($108.6 \pm 8.3\%$). In addition, DMP695 antagonized the ability of CRF to elicit burst firing (Fig. 4). These actions of DMP695 were expressed in the absence of any intrinsic influence upon noradrenergic neurons ($92.1 \pm 7.8\%$ at 2.0 mg/kg, i.v.).

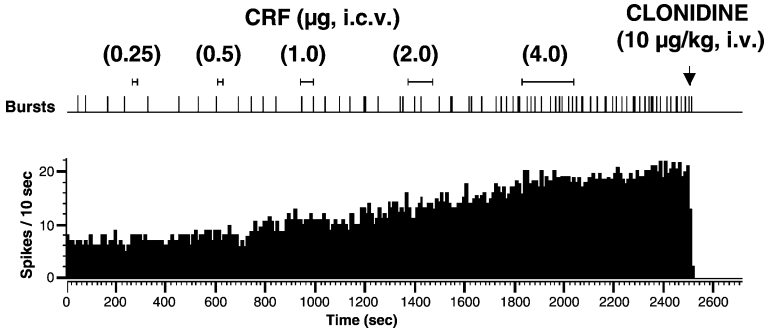

Fig. 2. Influence of i.c.v. administration of cumulative doses of CRF upon the electrical activity of a representative locus coeruleus neuron. Following administration of CRF, activity was abolished by the α_2-adrenoceptor agonist, clonidine (10 μg/kg, i.v.). In the upper part of the figure, each vertical bar represents one burst.

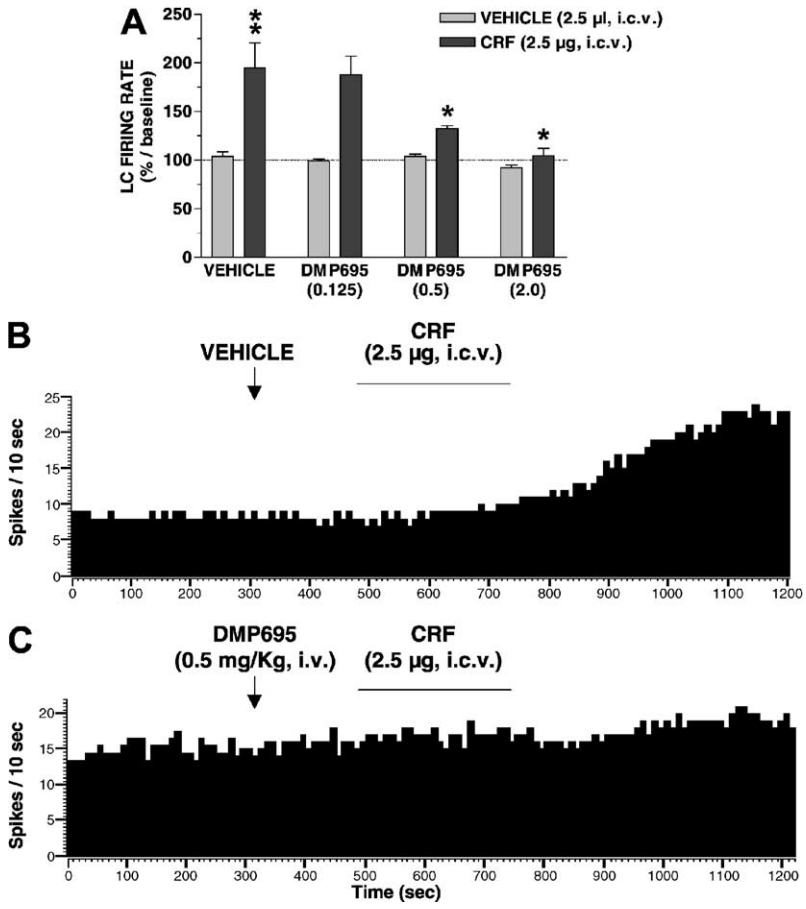

Fig. 3. Influence of DMP695 upon the increase in firing rate of noradrenergic neurones in the locus coeruleus elicited by i.c.v. administration of CRF. (A) shows the dose-dependent blockade of the action of CRF in the absence of any intrinsic effect of DMP695 itself. Basal firing rates were as follows: CRF + vehicle, 1.42 ± 0.26 Hz; CRF + DMP695, 1.46 ± 0.30 Hz (Student's *t*-test, $p>0.05$). Data are means ± S.E.M. $n \geq 5$ per value. ** indicates significance of difference between vehicle (i.v.) + CRF (i.c.v.) and vehicle (i.v.) + vehicle (i.c.v.) values in Student's *t*-test ($t = 4.2$, $p < 0.01$). * indicates significance of difference between DMP695 (i.v.) + CRF (i.c.v.) and vehicle (i.v.) + CRF (i.c.v.) values in Dunnett's test following ANOVA, $F(3,19) = 6.9$, $p < 0.01$. (B) and (C) illustrate the action of DMP695 (0.5 mg/kg, i.v.) against CRF (2.5 µg, i.c.v.) for individual neurones.

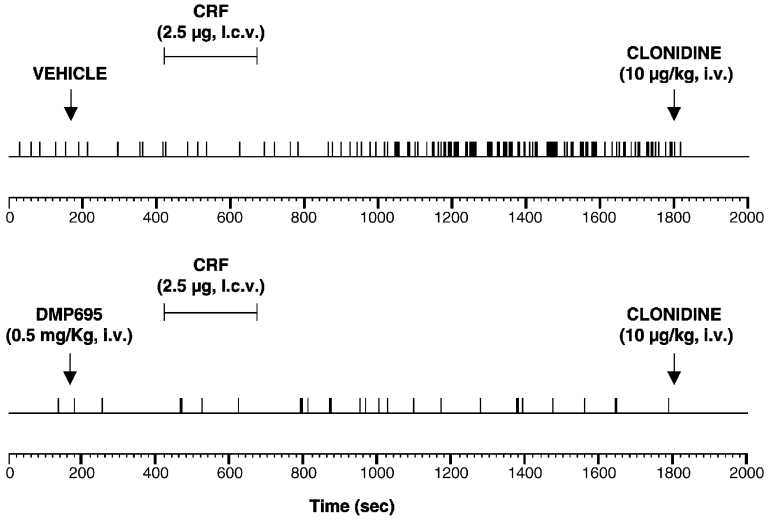

Fig. 4. Influence of DMP695 upon the induction of burst firing in noradrenergic neurones of the locus coeruleus by i.c.v. administration of CRF. Each vertical bar represents the detection of one burst (Spike2). Data are shown from two representative neurones.

4. Discussion

4.1. Influence of CRF upon locus coeruleus neurones

Though the majority of studies (Schulz et al., 1996; Page and Abercrombie, 1999; Palamarchouk et al., 2000; Conti et al., 2001) have examined the actions of only a *single* dose of CRF (generally 3 μg), the present study demonstrates that CRF dose-dependently enhances the firing rate of locus coeruleus noradrenergic cell bodies over a dose range of 0.5–4.0 μg, i.c.v. These observations correspond well to those of Curtis et al. (1997) who observed an increased in electrical discharges of a similar magnitude over a comparable dose range with i.c.v. administration of CRF (0.2–10 μg). As previously observed (Conti et al., 1997), the magnitude of the effect of CRF was less pronounced ($151.8 \pm 4.1\%$ at 2 μg) when injected in *cumulative* doses ($0.25 + 0.25 + 0.5 + 1.0$ μg) as compared to a *single* dose of 2.5 μg ($194.9 \pm 25.9\%$), reflecting the great latency (5–10 min) necessary to attain peak effect and, possibly, the occurrence of mild tachyphylaxis. A further observation herein was that CRF reduced the inter-spike interval, thereby increasing the occurrence of bursts in the discharge pattern. Similar observations were made by: (1) Page and Abercrombie (1999), upon pressure injection of CRF directly into the locus coeruleus of anesthetized rats; and (2) Jedema and Grace (2000), employing direct application of CRF to locus coeruleus neurones in an in vitro preparation. That CRF induces burst firing is of significance since this firing pattern is associated with an enhanced transmitter release by noradrenergic pathways and other classes of neurones (Suaud-Chagney et al., 1990; Gobert et al., 2000). Indeed, activation of noradrenergic neurones by CRF is reflected in an increase of noradrenaline output in the frontal cortex, hippocampus and other cerebral regions (Curtis et al., 1997; Zhang et al., 1998; Page and Abercrombie, 1999; Van Bockstaele et al., 2001). Moreover, Florin-Lechner et al. (1996) have observed that the enhancement of noradrenaline release in the frontal cortex induced by electrical stimulation of the locus coeruleus was dependent not only upon pulse frequency but also upon pulse pattern, noradrenaline increase being greater after burst stimulation.

4.2. Blockade of the actions of CRF by DMP695

As mentioned in Introduction, several studies have demonstrated the specificity of the influence of CRF upon noradrenergic neurones in showing that its actions can be blocked by central administration of peptidergic antagonists at CRF_1 receptors (Curtis et al., 1997; Zhang et al., 1998; Page and Abercrombie, 1999). Upon systemic administration, the first-generation non-peptidergic antagonist, CP154,526, dose-dependently (Schulz et al., 1996; 1–5.6 mg/kg, i.v.) attenuated the influence of CRF (3 μg, i.c.v.). However, it only submaximally reduced the action of CRF, possibly since it possesses weak partial agonist actions at CRF_1 receptors (Grosjean-Piot et al., 1997). Moreover, as acknowledged by the authors, a possible, mild excitatory effect of CP154,526 alone upon locus coeruleus firing could not be properly characterized owing to the transient facilitatory influence of vehicle injection. Evaluated against a similar dose of CRF (2.5 μg, i.c.v.), DMP695 likewise dose-dependently suppressed the excitatory influence of CRF herein. Notably, inasmuch as DMP695 is a pure antagonist of CRF_1 receptors (Bakthavatchalam et al., 1998), it abolished the excitatory influence of CRF, an action analogous to that of the structurally distinct, pure antagonist, CRA1000 (Okuyama et al., 1999). At the maximal dose used, DMP695 was inactive alone indicating that the level of tonic activity of "spontaneously released" CRF at CRF_1 receptors controlling locus coeruleus activity is low, a finding paralleling the majority of electrophysiological and dialysis studies with other antagonists (Curtis et al., 1997; Zhang et al., 1998; Page and Abercrombie, 1999; Isogawa et al., 2000; Millan et al., 2001). DMP695 expressed its actions over a dose range comparable to that of CP154,526 in line with their similar affinities at CRF_1 receptors and their similar in vivo potencies upon direct comparison in models of potential anxiolytic activity (Schulz et al., 1996; Bakthavatchalam et al., 1998; Gilligan et al., 2000; He et al., 2000; Millan et al., 2001). These observations support the specificity of the action of DMP695 against CRF_1. This is also underlined by its pronounced selectivity for CRF_1 receptors (preceding citations) as well as its lack of influence upon locus coeruleus neurones alone.

4.3. Functional significance

In view of the implication of locus coeruleus-derived noradrenergic projections in anxious states (Charney et al., 1995; Sullivan et al., 1999; Millan et al., 2000b), the inhibitory influence of DMP695 upon the excitation of noradrenergic neurones by (endogenous pools of) CRF may participate in its anxiolytic properties (He et al., 2000; Millan et al., 2001). Consistent with this notion, CP154,526 and a further non-peptidergic CRF_1 receptor antagonist, SSR125543A, inhibit the excitation of noradrenergic neurones induced by *stress* at doses exerting anxiolytic properties (Griebel et al., 2002).

4.4. Conclusions

The present study demonstrates that central administration of CRF results in a dose-dependent increase in the firing rate and a change in the firing pattern (induction of bursts) of noradrenergic neurones. These actions are dose-dependently abolished by the novel, non-peptidergic CRF_1 receptor antagonist, DMP695, which itself does not modify firing rate or pattern. These observations support the excitatory influence of CRF_1 receptors upon ascending noradrenergic projections, the activity of which is implicated in the induction of anxious states. They thus corroborate the potential

utility of DMP695 and other non-peptidergic CRF_1 receptor antagonists in the clinical treatment of anxious states. Finally, the present data support the use of DMP695 as a tool for the exploration of the functional significance of CRF_1 receptors.

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