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CAPTODIAMINE, A PUTATIVE ANTIDEPRESSANT, ENHANCES HYPOTHALAMIC BDNF EXPRESSION IN VIVO BY SYNERGISTIC 5-HT2C RECEPTOR ANTAGONISM AND SIGMA-1 RECEPTOR AGONISM.

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Abstract.

The putative antidepressant captodiamine is a 5-HT_{2c} receptor antagonist and agonist at sigma-1 and D_3 dopamine receptors, exerts an anti-immobility action in the forced swim paradigm, and enhances dopamine turnover in the frontal cortex. Captodiamine has also been found to ameliorate stress-induced anhedonia, reduce the associated elevations of hypothalamic corticotrophin releasing factor (CRF) and restore the reductions in hypothalamic BDNF expression. Here we demonstrate chronic administration of captodiamine to have no significant effect on hypothalamic CRF expression through sigma-1 receptor agonism however both sigma-1 receptor agonism or 5-HT_{2c} receptor antagonism were necessary to enhance BDNF expression. Regulation of BDNF expression by captodiamine was associated with increased phosphorylation of transcription factor CREB and mediated through sigma-1 receptor agonism but blocked by 5-HT_{2c} receptor antagonism. The existence of two separate signalling pathways was confirmed by immunolocalisation of each receptor to distinct cell populations in the paraventricular nucleus of the hypothalamus. Increased BDNF induced by captodiamine was also associated with enhanced expression of synapsin, but not PSD-95, suggesting induction of long-term structural plasticity between hypothalamic synapses. These unique features of captodiamine may contribute to its ability to ameliorate stress-induced anhedonia as the hypothalamus plays a prominent role in regulating HPA axis activity.

Key words.

5-HT_{2c} receptor, sigma-1 receptor, BDNF, synapsin, hypothalamus, plasticity.

Introduction.

Brain-derived neurotrophic factor (BDNF) has been described as a potential biomarker of depression and is consistently reported to be reduced in both the plasma and post-mortem brain tissue of patients with depression (Chen et al., 2001; Karege et al., 2002; Grønli et al., 2005; Karege et al., 2005; Gass and Hellweg, 2010; Takebayashi et al., 2011). Many clinically effective antidepressants are known to enhance BDNF expression, mainly in the cortex and hippocampus, and this is believed to contribute, in part, to their therapeutic effects (Nibuya et al., 1995; De Foubert et al., 2004; Alboni et al., 2010). In marked contrast to this regional specificity attributed to most antidepressant drugs, we recently demonstrated the putative antidepressant captodiamine (2-[(4-butylsulfanylphenyl)-phenyl-methyl]-*N*,*N*-dimethylethanamine) to specifically increase BDNF protein in the mouse hypothalamus and to be without effect on its expression in either the frontal cortex or hippocampus. This relatively unique feature of captodiamine may be important in its ability to ameliorate stress-induced anhedonia as the hypothalamus plays a prominent role in regulating both HPA axis activity (Kageyama and Suda, 2009) and the ability to evaluate and respond appropriately to reward (Nocjar et al., 2012).

Captodiamine was originally identified in a systematic re-evaluation of neurotherapeutics removed from the market by the Food and Drug Administration (FDA) in an administrative process known as the Drug Efficacy Study Implementation (DESI) (Shorter, 2002; Greene and Podolsky, 2012). Previously, captodiamine, an anti-histamine derivative of diphenhydramine, was marketed as a sedative and anxiolytic under trade names such as Covatine, Covatix and Suvren. More recently, captodiamine has been suggested to be useful in controlling withdrawal symptoms in patients being weaned from the habitual use of

benzodiazepines (Mercier-Guyon et al., 2004). The DESI process, in essence, arose from the Kefauver-Harris Amendments (1962) to the Federal Food, Drug, and Cosmetic Act (1938) which required the clinical efficacy of all marketed pharmaceuticals to be more rigorously demonstrated despite being generally regarded as safe in humans. The drug-like qualities of captodiamine were identified using web-based data sources and its psychotropic actions evaluated using a modified SHIRPA protocol. A combination of *in vitro* and *in vivo* procedures employing ligand-binding, cell signalling in receptor-transfected cells and the forced swim paradigm, using a dose range of 3-5 mg/kg, demonstrated captodiamine to be polyvalent, acting as an antagonist at the 5-HT_{2c} receptor antagonist and as an agonist at the sigma-1 and dopamine D_3 receptors (Ring et al., 2013). The anti-immobility actions of captodiamine were mediated by its agonist action at the sigma-1 receptor and antagonist action at the 5-HT_{2c} receptor, actions previously associated with clinically effective antidepressants (Clenet et al., 2001; Urani et al., 2002; Dekeyne et al., 2008).

A unique feature observed with chronic administration of captodiamine (5 mg/kg, 7 days) was its ability to ameliorate stress-induced anhedonia and the associated increases hypothalamic corticotrophin releasing factor (CRF) (Ring et al., 2013). Stress-induced anhedonia is a core symptom of depression (Nestler and Carlezon, 2006) and the hypothalamic release of CRF plays a central role in controlling the stress response by regulating the activity of the HPA axis (Kageyma et al., 2011). Captodiamine not only attenuated hypothalamic CRF signalling but also enhanced BDNF protein expression. This effect was only observed in the hypothalamus and captodiamine had no effect on BDNF protein expression in the frontal cortex or hippocampus (Ring et al., 2013).

The ability of captodiamine to attenuate CRF hyperactivity in the HPA axis and stress-induced deficits in BDNF expression may prove to be an important aspect in its putative antidepressant actions as these two parameters are the most commonly reported pathological features in patients with depression (Karege et al., 2005; Hashimoto, 2010; Piwowarska et al., 2012). It is perhaps not surprising, therefore, that captodiamine acts to reduce CRF and increase BDNF in the same brain region as it has been previously reported that chronic HPA axis hyperactivity can negatively influence growth factors such BDNF (Smith et al., 1995). Many other 5-HT_{2c} receptor antagonists with clinical antidepressant properties are known to increase BDNF expression in the hippocampus and frontal cortex (Dekeyne et al., 2008; Ladurelle et al., 2012) and similar effects are observed with sigma-1 receptor agonists (Kikuchi-Utsumi and Nakaki, 2008). However, the mechanism by which captodiamine mediates this hypothalamus-specific neuroplastic action remains to be established. Here we provide evidence to suggest that captodiamine enhances hypothalamic BDNF protein expression by acting on a direct sigma-1 receptor signalling pathway and through an indirect mechanism involving the 5-HT_{2c} receptor both actions ultimately resulting in CREB phosphorylation which regulates BDNF transcription (Conti et al., 2002; Tabuchi et al., 2002). Secondly, we show the action of BDNF to be correlated with enhanced synapsin expression, a marker of presynaptic plasticity (Cesca et al., 2010), but to have no influence on postsynaptic density protein-95 (PSD-95) expression suggesting it unlikely to increase synaptic complement.

Materials and methods.

Source and maintenance of C57Bl6 mice.

C57Bl6 male mice, 5 weeks old, were purchased from Harlan Laboratory (UK) and were housed in UCD Biomedical Facility in groups of three to five animals in a holding cage supplemented with a cardboard tube shelter. Animals were maintained at 22±2 °C and 40±5% humidity in a 12-h light-dark cycle with *ad libitum* access to food and water and allowed to acclimate to the Biomedical Facility for 3 weeks prior to any experiment. The experimental procedures were approved by the Animal Research Ethics Committee of University College Dublin, conformed to the EU Council Directive 86-609-EEC, and were carried out by individuals retaining the appropriate license issued by the Irish Department of Health.

Drugs administration schedules.

Drugs and vehicle were administered via the peritoneal cavity (i.p.) route at the same time daily for 7 days and all animals were drug free for 24 hours prior to sacrifice. Captodiamine was custom synthesised (Almac, UK; TopChem, IRL) and administered at a dose of 5 mg/kg which, in preliminary studies, had been demonstrated to yield maximal effects in behavioral paradigms (Ring et al., 2013). Rimcazole (5 mg/kg) was purchased from Tocris Biosciences (UK) and ritanserin (4 mg/kg) was purchased from Sigma Aldrich (UK). The drugs were dissolved in saline (0.9% NaCl in distilled water) except for ritanserin which was dissolved in aqueous solution containing dimethyl sulfoxide (1% v/v; DMSO). Drugs and vehicle were administered in a final injection volume of 10 ml/kg. all animals were randomly assigned to the treatment groups (n=4-6).

Analysis of brain tissue BDNF and CRF levels.

Tissue preparation and protein extraction.

Animals, drug free for 24 hours, were sacrificed by cervical dislocation and hippocampus, hypothalamus and frontal cortex were quickly dissected, snap frozen in cryotubes using liquid nitrogen, and stored at -80°C until use. When required, the samples were thawed and homogenised in 10% w/v stock lysis buffer (20mM Tris, pH 8, containing 150 mM NaCl, 1% v/v Nonidet-P40, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride inhibitor and 0.5 mM sodium vanadate, the latter two being included as protease inhibitor and phosphatase inhibitors, respectively. The samples were homogenised by sonication for 3 seconds using a probe sonicator (Soniprep-150; Sanyo, Japan), centrifuged at 10,000 rpm for 10 minutes at 4°C to pellet nuclear material and other cellular debris, and the supernatant was stored at -20 °C in 25 µl aliquots until needed. Protein concentrations were determined using the BCA™ assay, according to manufacturer's instructions (Pierce, USA).

Corticotrophin releasing factor (CRF) and brain-derived neurotrophic factor (BDNF) immunoassays.

The concentration of CRF in hypothalamic protein extracts was determined by ELISA (Phoenix Pharmaceuticals Inc., USA) (Feng et al., 2007) using a standard curve ranging from 0.01 ng/ml to 100 ng/ml CRF. BDNF concentrations were also determined by ELISA (Promega, USA) (Angelucci et al., 2003) and a standard curve ranging from 7.8 to 500 pg/ml BDNF.

Analysis of CREB phosphorylation status and PSD-95 and synapsin expression.

Protein samples were boiled for 3 minutes in 70 mM Tris–HCl, pH 6.8 containing 33 mM NaCl, 1 mM EDTA, 2% w/v SDS, 0.01% (w/v) Bromophenol Blue, 10% glycerol and 3% v/v dithiothreitol reducing agent, separated using pre-prepared 10% polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Biorad, UK). Remaining reactive groups on the nitrocellulose sheet were then

inactivated using Tris-buffered saline solution (TBS-T) blocking buffer (10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 0.05% v/v Tween-20, and 5% w/v non-fat milk powder or bovine serum albumin) and the membrane was subsequently incubated overnight (20 hours) at 4 °C in blocking buffer (5% v/v) containing the appropriate antibody. These included anti-phospho-CREB(ser133) (rabbit monoclonal IgG; Cell Signalling Technology, USA; diluted 1:10,000 v/v), anti-CREB (rabbit polyclonal IgG; Cell Signalling Technology, USA; diluted 1:10,000 v/v), anti-PSD-95 (rabbit polyclonal IgG; Cell Signalling Technology, USA; diluted 1:5000 v/v). The membrane was then incubated for 1 in hour in TBS-T containing a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Novagen, UK; 1:10,000 dilution), reacted for 5 min with a chemiluminescent peroxidase substrate (Pierce, USA) and exposed to X-ray film (Fuji, UK). The X-ray films were scanned, converted into a digital format and the immunostained band density analysed using ImageJ software (http://rsb.info.nih.gov/ij/docs/index.html). The staining density of phospho-CREB was expressed as percentage of total CREB and those of synapsin and PSD-95 as a percentage of total protein as measured by incubation of the nitrocellulose membrane with Napthol Blue (0.1% w/v; Sigma, UK).

Immunolocalisation of CRF, sigma-1 receptor and 5-HT_{2c} receptor in the hypothalamic paraventricular (PVN) nucleus.

Frozen brain sections (12µm) were taken throughout the rostro-caudal extent of the PVN (bregma -0.82mm to -0.92mm) (Paxinos and Watson, 2007), thaw-mounted onto glass slides, immersion fixed for 30 min in 70% (v/v) ethanol and incubated overnight with either goat polyclonal anti-5-HT_{2c} receptor antibody (diluted 1:100; Abcam, UK), rabbit polyclonal anti-CRF polyclonal antibody (diluted 1:500; Chemicon, UK) or rabbit polyclonal anti-OPRS1 (Sigma-1 receptor) antibody (diluted 1:200; Abcam,

UK). The sections were washed and subsequently exposed for 3h to donkey anti-goat Alexa-488 (diluted 1:500; Invitrogen, UK) or donkey anti-rabbit Dylight-594 (diluted 1:500; Pierce, USA) and counterstained with 0.5 μg/ml propidium iodide (Sigma, UK). For dual-immunolabelling sections were initially incubated with goat polyclonal anti-5-HT_{2c} receptor antibody (diluted 1:100; Abcam, UK) for 3 hours and subsequently incubated overnight with either rabbit polyclonal anti-CRF antibody (diluted 1:500; Chemicon, UK) or rabbit polyclonal anti-OPRS1 (sigma-1 receptor) antibody (diluted 1:200; Abcam, UK). The second layer antibodies were employed as described above. All antibodies were diluted in 0.1 M PBS containing 1% (w/v) bovine serum albumen (BSA) and 1% (v/v) normal goat serum (NGS). Images were acquired using a LSM Pascal V confocal microscope (Zeiss, Germany).

Statistical analysis.

Analysis of immunoassay data and densitometric scans for statistical significance employed the Student's t-test for two variables and a one-way ANOVA, followed by a Bonferroni post-test, for more than two variables. A p value < 0.05 was deemed to be significant in all cases.

Results.

Modulation of hypothalamic CRF and BDNF expression by captodiamine.

As reported previously, captodiamine significantly reduced CRF and increased BDNF protein expression in the hypothalamus of C57Bl6 mice following its chronic administration (5 mg/kg, 7 days) (Ring et al., 2013). CRF protein expression became significantly reduced ($p=0.03$, Student's t-test) and this effect could not be reversed by the sigma-1 receptor antagonist rimcazole (4 mg/kg; $p<0.05$, one-way ANOVA)

and was not further reduced by co-administration of the 5-HT_{2c} antagonist ritanserin (5 mg/kg; $p<0.05$, one-way ANOVA) (Figure 1 A). These data indicated that the effect of captodiamine in reducing hypothalamic CRF could not be attributed to its actions at the sigma-1 or 5-HT_{2c} receptors. In contrast, the ability of captodiamine to enhance BDNF expression was blocked by co-administration of rimcazole and significantly enhanced by co-administration of ritanserin ($p=0.03$, Student's t-test; n=6) (Figure 1 B). Captodiamine was without effect on BDNF expression in the hippocampus ($p=0.16$, Student's t-test; n=6 or frontal cortex ($p=0.78$, Student's t-test; n=6) of C57Bl6 mice (data not shown), as we have reported previously.

Influence of captodiamine on CREB phosphorylation status.

Given that BDNF expression is thought to be mediated by the transcription factor CREB (Conti et al., 2002; Tabuchi et al., 2002) we sought evidence for captodiamine-mediated regulation of CREB by activation of its phosphorylation state. Chronic administration of captodiamine was found to significantly increase hypothalamic CREB phosphorylation state ($p=0.03$, Student's t-test; n=4), an effect not observed in the hippocampus ($p=0.8$, Student's t-test; n=4) or the frontal cortex ($p=0.4$, Student's t-test; n=4) (data not shown). The ability of captodiamine to enhance CREB phosphorylation was blocked by co-administration of rimcazole ($p>0.05$, Bonferroni's, post-hoc test; n=3) (Figure 2 A and B) but, significantly, co-administration of ritanserin also blocked the increase in CREB phosphorylation ($p>0.05$, Bonferroni's, post-hoc test; n=3) and the expected summation by both 5-HT_{2c} receptor antagonists was not observed (Figure 2 C and D). Such an effect has been observed previously in the hippocampus but not in the hypothalamus (Ladurelle et al., 2012).

Immunolocalisation of captodiamine signalling pathways.

The ability of captodiamine to induce BDNF protein expression through both 5-HT_{2c} receptor antagonism and sigma-1 receptor agonism implied the possibility of a common pathway or two separate signalling mechanisms. This was further investigated using immunocytochemistry to resolve the cellular location of both receptors in the paraventricular nucleus (PVN) of the hypothalamus. Sigma-1 receptor immunostaining gave the appearance of being specifically associated with dendrite-like structures (Figure 3 B), as has been previously reported for the neuronal subcellular localisation of this receptor (Alonso et al., 2000). In contrast, 5-HT_{2c} receptor immunoreactivity was associated in the perinuclear area of cells primarily located adjacent to the third ventricle although some were also located in the parenchyma of the PVN (Figure 3 C). Cells immunopositive for CRF showed a similar distribution to that observed for the 5-HT_{2c} receptor immunoreactive cells including the localisation of the immunostaining to the perinuclear region (Figure 3 D). Dual labelling of the CRF immunopositive cells with those expressing the 5-HT_{2c} receptor demonstrated both to be co-localised (Figure 4 A and B), as has been reported in previous studies (Heisler et al., 2007). No such co-localisation was observed between 5-HT_{2c} receptor and sigma-1 receptor immunoreactivities (Figure 4 C and D) as these two populations of cells were separate and located to different regions of the PVN. These latter observations suggest the regulation of CRF secretion to be mediated by the 5-HT_{2c} receptor.

Modulation of hypothalamic synapsin and PSD-95 by captodiamine.

The ability of captodiamine to enhance BDNF protein expression also implies the potential of this putative antidepressant to induce long-term structural modifications within the hypothalamus. This idea was explored by analyzing the effect of

captodiamine on the expression of synapsin and PSD-95 which are pre- and post-synaptic markers of structural neuroplasticity. Captodiamine was found to significantly increase the expression of hypothalamic synapsin ($p= 0.001$, Student's t-test; n=4) but had no effect on PSD-95 expression ($p=0.63$, Student's t-test; n=6). (Figure 5 A and B). Given that captodiamine was without effect on BDNF expression in the hippocampus or frontal cortex, we also determined its effect on synapsin and PSD-95 expression in these brain regions. Captodiamine was found to have no effect on the expression of either synapsin ($p=0.61$, Student's t-test; n=4) or PSD-95 ($p=0.13$, Student's t-test; n=6) expression in the hippocampus or the frontal cortex.

Discussion.

In this study we have presented findings that support a dual mechanism by which captodiamine induces hypothalamic BDNF through the synergistic action of 5-HT_{2c} receptor antagonism and activation of the sigma-1 receptor (see Figure 6). Individually sigma-1 receptor agonism and 5-HT_{2c} receptor antagonism have been separately shown to induce BDNF expression within the hippocampus and frontal cortex (Kikuchi-Utsumi and Nakaki, 2008; Ladurelle et al., 2012). However, the combined 5-HT_{2c} receptor antagonism and sigma-1 receptor agonism provided by captodiamine appears to exert a brain region-specific effect on hypothalamic BDNF expression which may relate to its modulation of activity within the HPA axis. The brain region-specific effect of captodiamine in the hypothalamus is consistent with its antagonism of the 5-HT_{2c} receptor as activation of this receptor on the CRF neurons in the paraventricular nucleus (PVN) of the hypothalamus is known to result in increased CRF expression (Heisler et al., 2007). Furthermore, the antagonistic action of captodiamine at the 5-HT_{2c} receptors in the PVN may also account for its ability to

modulate BDNF as CRF can negatively regulate BDNF expression (Smith et al., 1995).

Captodiamine regulates CREB phosphorylation in the hypothalamus.

The BDNF gene contains a CREB regulatory element (CRE) in its promoter region and its transcription is regulated by CREB (Conti et al., 2002; Tabuchi et al., 2002). Antidepressants that increase BDNF expression also increase CREB phosphorylation (Nibuya et al., 1996; Alboni et al., 2010) and, in keeping with this general observation, captodiamine was found to induce hypothalamic CREB phosphorylation thereby suggesting a role for CREB in mediating its brain region-specific transcription of BDNF. The effect was also consistent with captodiamine activating the sigma-1 receptor as its antagonist rimcazole inhibited CREB phosphorylation. Thus captodiamine by activating the sigma-1 receptor on the endoplasmic reticulum results in mobilisation of calcium (Hayashi and Su, 2007) and the activation of calcium calmodulin kinases (CaMK) that leads to CREB phosphorylation (Moriguchi et al., 2011).

Captodiamine-induced CREB phosphorylation was inhibited by the 5-HT_{2c} receptor antagonist ritanserin. This effect was unexpected as the 5-HT_{2c} receptor is a G_q protein-coupled receptor which when activated increases calcium mobilisation via inositol trisphosphate (IP_3) production (Backstrom et al., 1999; Hannon and Hoyer, 2008). Hence inhibition of the 5-HT2c receptor should lead to reduced calcium mobilisation and an attenuation of CREB phosphorylation. These opposing effects of 5-HT_{2c} receptor antagonism and sigma-1 receptor agonism on hypothalamic CREB phosphorylation suggested a divergence in signalling pathways employed by captodiamine and predicted the existence of distinct populations of cells separately

expressing the 5-HT_{2c} and sigma-1 receptors. Consistent with previous studies, we could demonstrate the 5-HT_{2c} receptor to be located on CRF containing neurons in the PVN of the hypothalamus where its activation is known to induce CRF expression via CREB-mediated transcription through the CRE in the CRF gene promoter (Mayr and Montminy, 2001; Yao et al., 2007; Heisler et al., 2007). It is likely, therefore, that captodiamine inhibits CRF expression via 5-HT_{2c} receptor inhibition and subsequent reduction in CREB phosphorylation. This is further supported by the fact that the captodiamine-induced reduction in CRF expression was not reversed by the sigma-1 receptor antagonist rimcazole.

In addition to directly increasing hypothalamic BDNF expression through activation of the sigma-1 receptor, captodiamine may also indirectly increase its expression through inhibition of CRF expression. This is part suggested by the observation that the 5-HT_{2c} receptor antagonist ritanserin potentiates captodiamine-induced BDNF expression and also the substantial evidence that elevated levels of stress hormones significantly reduce BDNF expression (Smith et al., 1995; Murakami et al., 2005; Grønli et al., 2006).

Influence of captodiamine on hypothalamic plasticity.

The ability of captodiamine to increase the expression of hypothalamic BDNF also implies it may significantly enhance synaptic plasticity within this brain region. In *in vitro* studies, for example, BDNF has been shown to increase hippocampal dendritic spine number and length and the expression of synaptic proteins such as PSD-95, synapsin, SNAP-25, synaptogamin and synaptophysin (Tartaglia et al., 2001; Liao et al., 2007; Kumamaru et al., 2008). The effects of BDNF on synaptic protein expression *in vitro* are similar to those observed following antidepressant treatment *in*

vivo (Chen et al., 2008, 2010; O'Leary et al., 2009) indicating that they may be mediated by increased BDNF expression and that this effect may be a characteristic of clinically effective antidepressants.

Consistent with these ideas we found chronic captodiamine treatment to significantly increase protein expression of the synapsin synaptic marker within the hypothalamus but not in the hippocampus or frontal cortex. Unexpectedly, captodiamine had no effect on the postsynaptic marker PSD-95 in any brain region examined suggesting it unlikely to increase hypothalamic synaptic complement. This, nevertheless, remains consistent with the therapeutic potential of captodiamine as post-mortem studies of patients with depression have not shown hypothalamic atrophy (Rajkowska et al., 1999; Cotter et al., 2002; Stockmeier et al., 2004).

That captodiamine significantly increases hypothalamic synapsin expression suggests it more likely to modulate existing synapse plasticity. Synapsins play a crucial role in tethering synaptic vesicles to the actin cytoskeleton thereby facilitating synaptic vesicle docking in pre-existing synapses and the promotion of activity between synapses and synaptic stabilisation (Cesca et al., 2010). The captodiamine-induced increases in synapsin expression are likely mediated by BDNF signalling as as these are observed only in the hypothalamus. Furthermore, there is substantial evidence to suggest BDNF, which is released from the postsynaptic element, preferentially affects TrkB receptors on the pre-synapse and induces an increase in the number of docking vesicles (Pozzo-Miller et al., 1999; Tyler et al., 2006; Cohen-Cory et al., 2010).

Conclusion.

This study delineates mechanisms by which captodiamine induces BDNF expression in the hypothalamus and demonstrates its capacity to induce changes in synaptic

plasticity within the hypothalamus. These findings may contribute to explaining the significant ability of captodiamine to ameliorate stress-induced anhedonia (Ring et al., 2013). The prominence of anhedonia and reduced motivation in depression highlights their importance in its symptomatology and may even suggest an involvement in its pathophysiology and etiology (Nestler and Carlezon, 2006). Further, disinhibition of the HPA axis, as in glucocorticoid receptor-heterozygous mutant mice (GR+/-), is associated with depressive behaviours, a dysregulation of serotoninergic neurotransmission and a reduction in BDNF expression (Ridder et al., 2005; Schulte-Herbrüggen et al., 2007). Thus the specific actions of captodiamine on the HPA axis may provide a fundamentally new mechanism to enable the development of antidepressant medications for the treatment of specific subtypes of depression that are dominated by states of anhedonia.

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Conflict of Interest Statement

CMR is a Director and Shareholder of Berand Neuropharmacology which holds a license to the rights to captodiamine.

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Figure legends.

Figure 1: Influence of captodiamine on CRF and BDNF expression in the hypothalamus. The effect of chronic administration of captodiamine (5 mg/kg, 7 days) on CRF and BDNF protein levels is shown in Panels A and B, respectively, (filled columns) and values significantly different ($p<0.05$) to the vehicle control (open column) are indicated with an asterisk. The consequence of co-administration of rimcazole (5 mg/kg; hatched columns) or ritanserin (4 mg/kg; stippled columns) with captodiamine on CRF and BDNF expression is also demonstrated and values significantly different ($p<0.05$) to captodiamine alone are indicated with a double asterisk. All values are expressed as mean±SEM (n=4-6).

Figure 2: Influence of sigma-1 receptor agonism and 5-HT_{2c} receptor antagonism on captodiamine-induced CREB phosphorylation in the hypothalamus. Immunoblots illustrating the effect of captodiamine, alone (filled column) and co-administered with rimcazole (4 mg/kg, hatched column), on CREB and its phosphorylated protein product (pCREB) are shown in Panel A and their semi-quantitative densitometric analysis is shown in Panel B. Similarly, the effect of captodiamine, alone and co-administered with ritanserin (5 mg/kg; stippled column), is shown in Panels C and D. All values are expressed as mean±SEM (n=4-6) and those significantly different ($p<0.05$) to the vehicle control (open column) are indicated with an asterisk.

Figure 3: Cellular localisation of sigma-1 receptor, 5-HT_{2c} receptor and CRF in the hypothalamic paraventricular nucleus. Coronal sections were imaged bilateral to the 3rd ventricle as illustrated in propidium iodide staining in Panel A and the distribution of immunopositive cells is indicated by the filled arrowheads in Panels B-

D. CRF immunopositive cells in the parenchyma are indicated with open arrowheads (Panel D). Immunofluoresence is shown in green and the red fluorescence arises from the propidium iodide counterstaining of cell nuclei.

Figure 4: Co-localisation of immunopositive sigma-1 receptor and 5-HT_{2c} receptor cells with CRF immunopositive cells in the hypothalamic paraventricular nucleus. Cells expressing CRF co-localised with immunopositive 5-HT_{2c} receptor cells (Panels A and B; hatched circles) whereas regions immunopositive for the sigma-1 receptor (filled arrowheads) were found to contain no immunopositive 5-HT_{2c} receptor cells (Panels C and D).

Figure 5: Immunoblots illustrating the effect of captodiamine (filled column) on synapsin and its semi-quantitative densitometric analysis is shown in Panel A. Similarly, the effect of captodiamine on PSD-95 expression is shown in Panel B. All values are expressed as mean±SEM (n=4-6) and those significantly different ($p<0.05$) to the vehicle control (open column) are indicated with an asterisk.

Figure 6: Proposed signalling cascade induced by captodiamine in the hypothalamus.

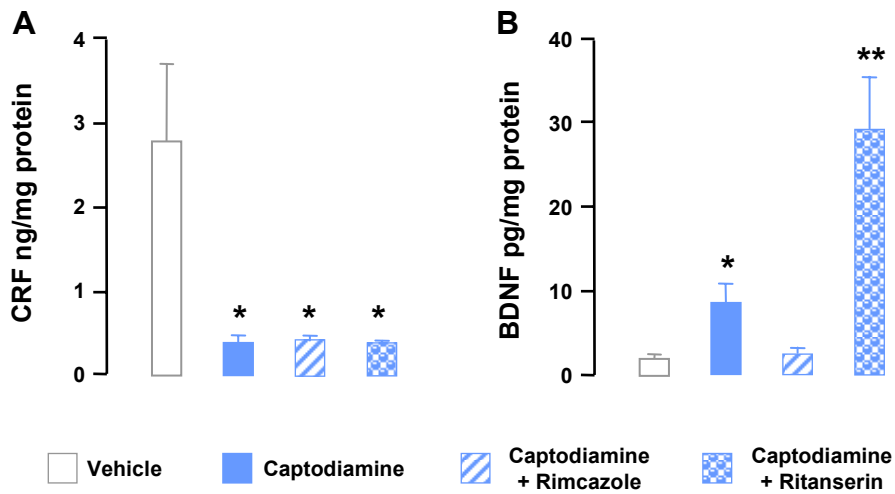


Figure 1, Ring and Regan

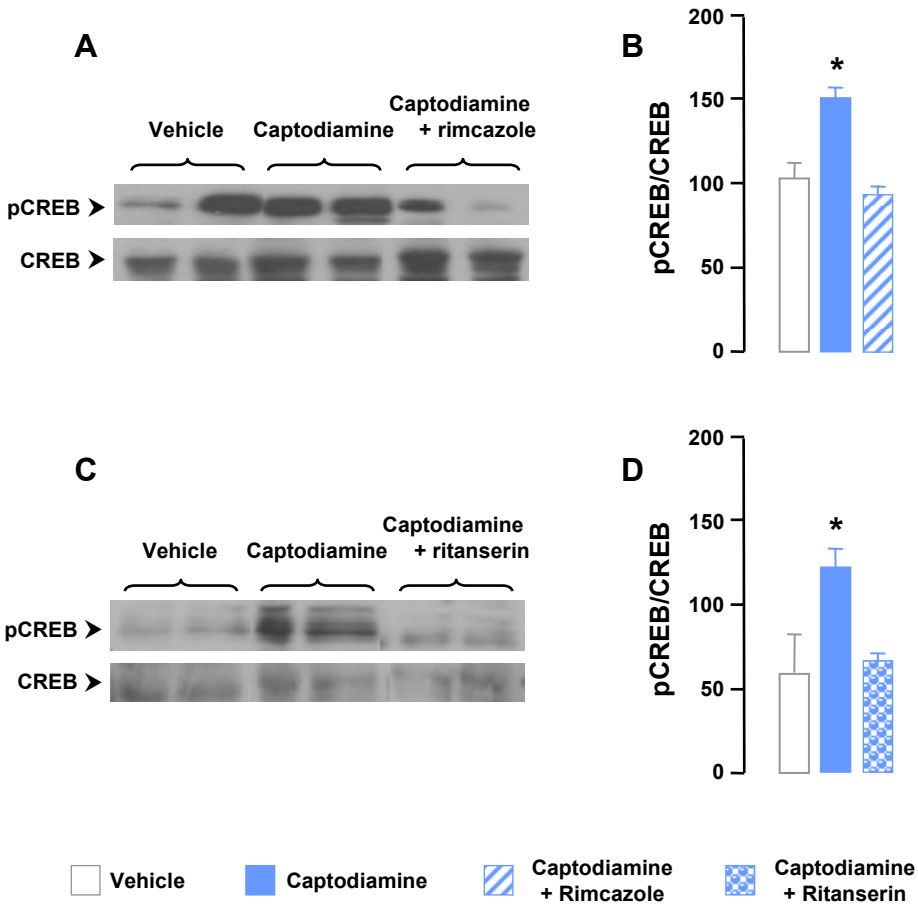
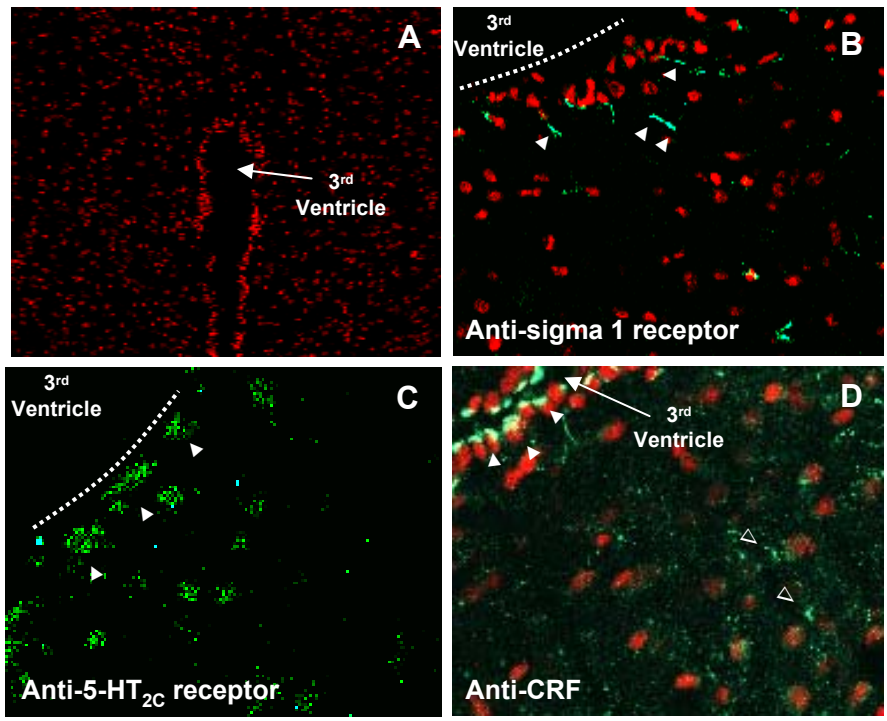


Figure 2, Ring and Regan



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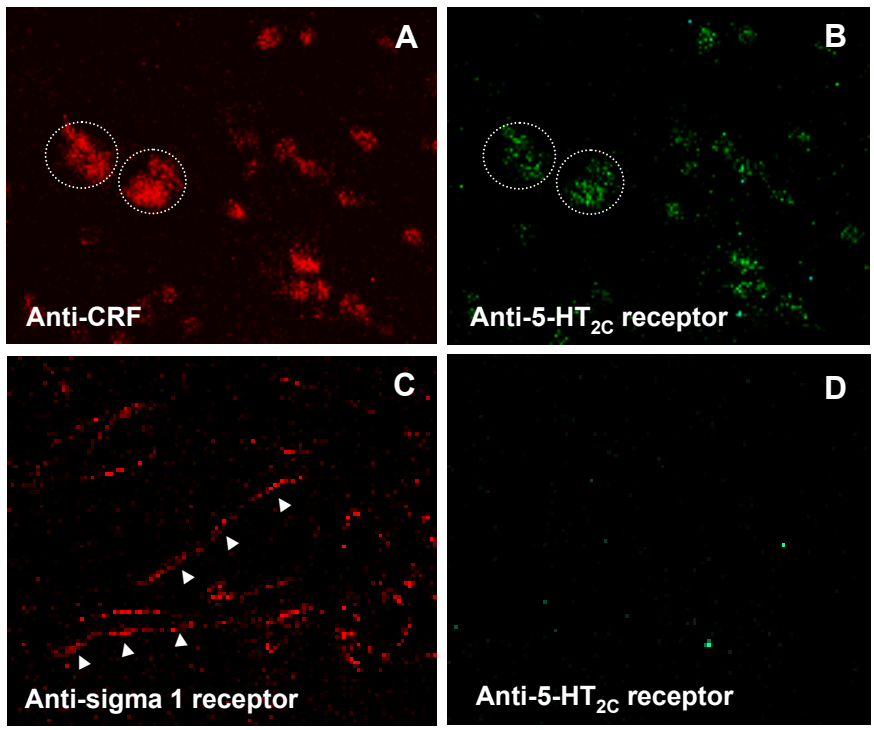

Figure 4, Ring and Regan

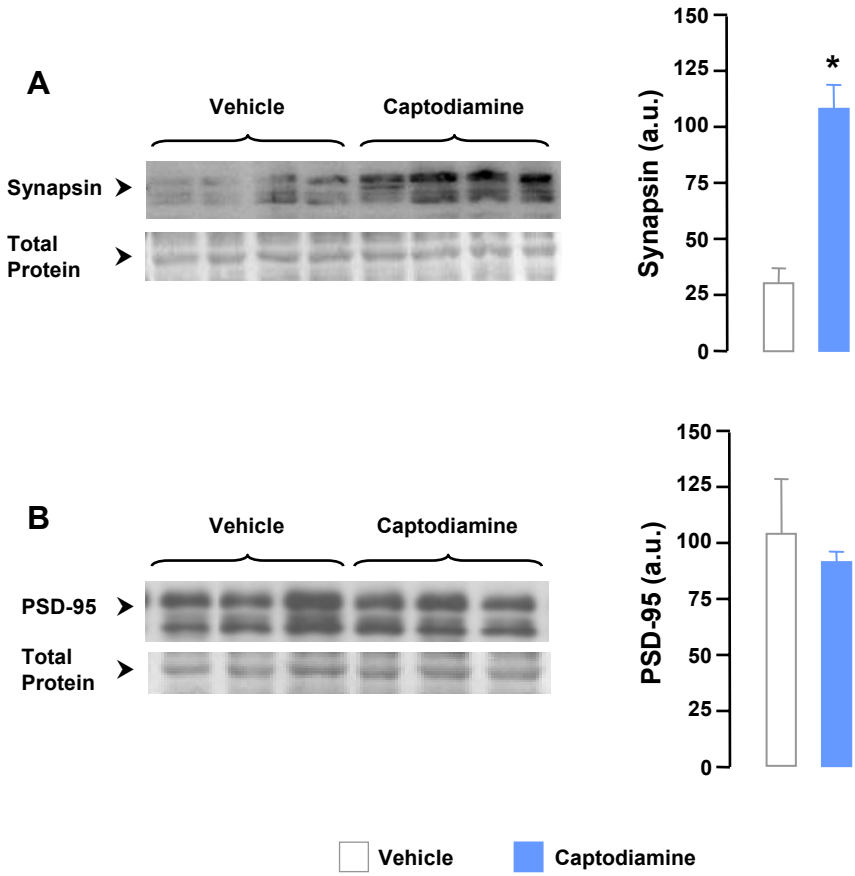

Figure 5, Ring and Regan

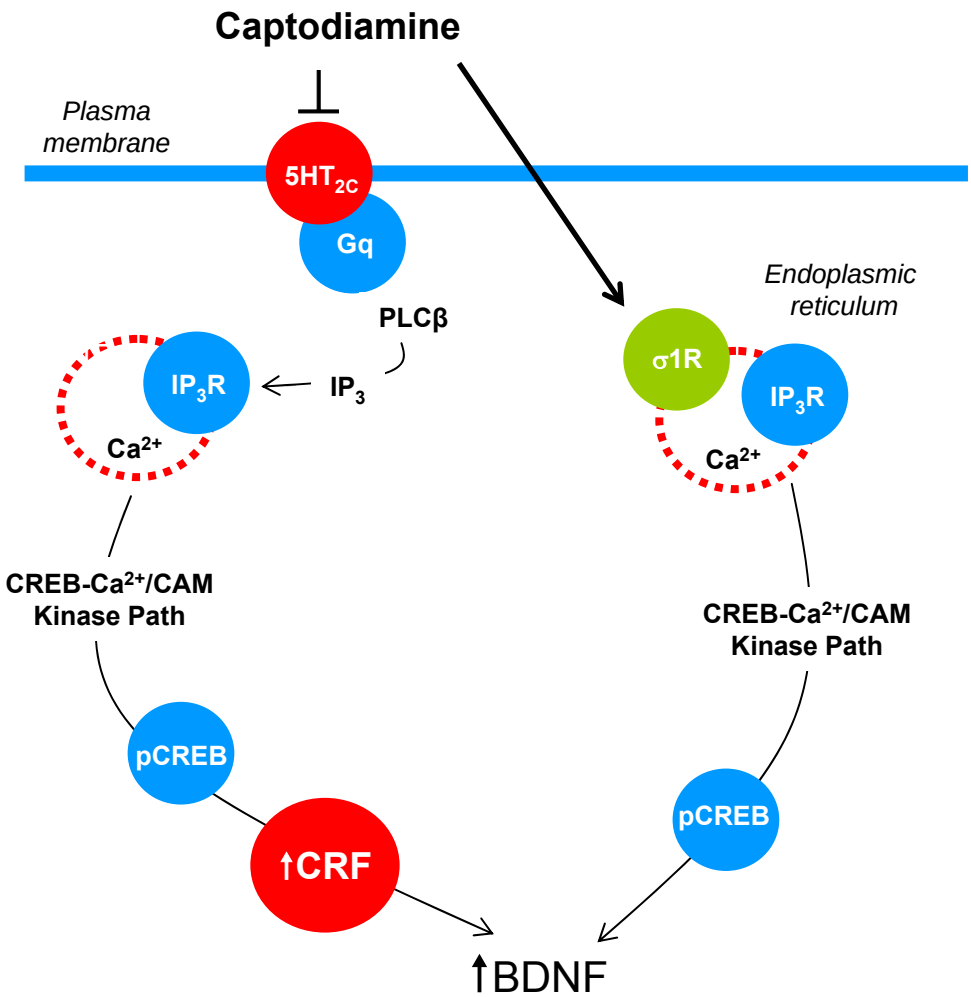

Figure 6, Ring and Regan