Panicolytic-like effect of BDNF in the rat dorsal periaqueductal grey matter: the role of 5-HT and GABA

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Abstract

A wealth of evidence suggests a role for brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) in the aetiology of depression and in the mode of action of anti-depressant drugs. Less clear is the involvement of this neurotrophin in other stress-related pathologies such as anxiety disorders. The dorsal periaqueductal grey matter (DPAG), a midbrain area rich in BDNF and TrkB receptor mRNAs and proteins, has been considered a key structure in the pathophysiology of panic disorder. In this study we investigated the effect of intra-DPAG injection of BDNF in a proposed animal model of panic: the escape response evoked by the electrical stimulation of the same midbrain area. To this end, the intensity of electrical current that needed to be applied to DPAG to evoke escape behaviour was measured before and after microinjection of BDNF. We also assessed whether 5-HT- or GABA-related mechanisms may account for the putative behavioural/autonomic effects of the neurotrophin. BDNF (0.05, 0.1, 0.2 ng) dose-dependently inhibited escape performance, suggesting a panicolytic-like effect. Local microinjection of K252a, an antagonist of TrkB receptors, or bicuculline, a GABA_A receptor antagonist, blocked this effect. Intra-DPAG administration of WAY-100635 or ketanserin, respectively 5-HT_1A and 5-HT_2A/C receptor antagonists, did not alter BDNF’s effects on escape. Bicuculline also blocked the inhibitory effect of BDNF on mean arterial pressure increase caused by electrical stimulation of DPAG. Therefore, in the DPAG, BDNF-TrkB signalling interacts with the GABAergic system to cause a panicolytic-like effect.

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Introduction

Since the observation that the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus is decreased by stressful stimuli/situations and that antidepressant drugs counteract this effect, the role played by this neurotrophin and its receptor tropomyosin-related kinase B (TrkB) in the pathophysiology of stress-related disorders such as depression has been increasingly investigated (for review see Martinowich et al. 2007). Earlier studies on the subject also showed that BDNF may exert antidepressant-like effects in other brain areas apart from the hippocampus, such as the midbrain (Siuciak et al. 1997). Accordingly, it has been shown that injection of BDNF in an area comprising the periaqueductal grey matter (PAG) and dorsal raphe nucleus (DRN) decreases the behavioural deficits (i.e. escape failure) caused by exposure of rats to inescapable electric footshocks and reduces immobility time in the forced swimming test (Siuciak et al. 1997). Interestingly, this antidepressant effect was associated with an augmentation of serotonergic activity locally and in brain areas such as hippocampus, striatum and cortex (Siuciak et al. 1994, 1996). Later study with RT–PCR indicated that BDNF injection in the PAG/DRN area may increase 5-HT synthesis by directly enhancing tryptophan...
hydroxylase (the rate-limiting enzyme in 5-HT synthesis) mRNA levels (Siuciak et al. 1998).

Of interest to the present paper, the aforementioned studies by Siuciak and co-workers reveal that BDNF exerts a modulatory effect on the serotonergic system in brain loci (i.e. PAG and DRN) critically associated with the pathogenesis and treatment of another psychiatric pathology, panic disorder. PAG, more specifically its dorsal aspect (DPAG), has long been associated with panic. In experimental animals, electrical or chemical stimulation of this structure, which has high levels of TrkB and BDNF receptor mRNAs and proteins (Conner et al. 1997; Numan & Serogy, 1999; Yan et al. 1997), evokes escape responses accompanied by autonomic changes that are similar to those observed in markedly aversive situations such as confrontation with predators (Bandler et al. 1991; Graeff et al. 1993; Olds & Olds, 1962; Schenberg et al. 2001). In neurosurgical patients, stimulation of this structure evokes strong feelings of fear, impending death or non-localized pain, and prominent autonomic changes, as in a panic attack (Amano et al. 1978; Nashold et al. 1969). Based on the similarities between the autonomic and behavioural effects of DPAG stimulation and the symptoms of panic attacks, it has been suggested that DPAG is involved in the genesis of panic disorder in humans and that stimulation of this midbrain area in animals can model panic attacks (Graeff, 1990; Graeff & Zangrossi, 2002; Jenck et al. 1995; Lovick et al. 2000; Schenberg et al. 2001).

Using the electrical stimulation of DPAG as a model of panic, Graeff & co-workers (Nogueira & Graeff, 1995; Schütz et al. 1985) showed that intra-DPAG injection of 5-HT- or GABA-enhancing drugs inhibits the escape response generated in this structure, indicating a panicolytic-like action. More recently, it has been shown that chronic but not acute treatment with antidepressants clinically effective in the treatment of panic disorder, such as the non-selective 5-HT/ noradrenaline reuptake inhibitor imipramine, or the selective 5-HT reuptake inhibitor fluoxetine, enhance the inhibitory effect of 5-HT on escape, implicating this mechanism in the mode of action of panicolytic compounds (de Bortoli et al. 2006; Jacob et al. 2002; Mongeau & Marsden, 1997; Zanoveli et al. 2005).

Given the above background and scant information on the role of BDNF in anxiety disorders, in particular panic disorder, in the present paper we investigated the effect of intra-DPAG injection of BDNF on the escape response evoked by the electrical stimulation of the same midbrain area. We also examined whether 5-HT or GABA can mediate putative BDNF effect on escape behaviour and the relevance of TrkB receptors for this process. Finally, it was of interest to verify if BDNF may also interfere with an autonomic component of the escape reaction induced by stimulation of DPAG, i.e. the rise in mean arterial pressure (MAP) (Krieger & Graeff, 1985).

Material and methods

Animals

Male Wistar rats weighting 300–330 g were housed in groups of 4–6 per cage under a 12-h light/dark cycle (lights on 07:00 hours) at 22 ± 1 °C, with food and water available ad libitum, except during testing. All experiments were performed in accordance with the Brazilian Society of Neuroscience and Behaviour for the care and use of laboratory animals and were approved by the Experimental Animal Ethical Committee of University of São Paulo (protocol number: 114/2007). All efforts were made to minimize animal suffering.

Drugs

The following drugs were used in the study: recombinant human BDNF (rh-BDNF; Promega, USA), K252a (Sigma-Aldrich, USA), ketanserin tartrate (Tocris, USA), WAY-100635 maleate (Sigma-Aldrich), 5-hydroxytryptamine creatinine sulphate (5-HT; RBI, USA), bicuculline methiodide (Sigma-Aldrich). The drugs were dissolved in Dulbecco’s PBS (DPBS: 2.7 mM KCl, 137 mM NaCl, 1.47 mM KH2PO4, 8.1 mM Na2HPO4, 0.5 mM MgCl2, 0.9 mM CaCl2; pH 7.4), except ketanserin which was suspended in 0.2% Tween-80 solution in sterile saline and K252a, solubilized in 0.2% DMSO in DPBS solution.

Surgery

Rats were anaesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.) followed by local anaesthesia (2% lidocaine with a vasoconstrictor; Harvey, Brazil) and fixed in a stereotaxic frame (David Kopf, USA) for implantation of a chemitrode in the DPAG. The chemitrode was made of stainless-steel guide cannula (outside diameter 0.6 mm, 13 mm long) glued to a brain electrode made of stainless-steel wire (diameter 250 µm), enamel insulated, except at the cross-section of the tip, reaching 1 mm below the lower end of the cannula. The electrode wire was connected to a male pin, parallel to the outer end of the cannula that could be plugged into an amphenol socket at the end of a flexible electrical cable and used for brain stimulation. The chemitrode was implanted using coordinates...
according to the atlas of Paxinos & Watson (2007). Holding the incisor bar 3.3 mm below the interaural line, the chemitrode was introduced 1.7 mm lateral to lambda, at an angle of 22° with the sagittal plane, until the electrode tip was 5.0 mm below the surface of the skull. The chemitrode was attached to the skull by means of acrylic resin and two stainless-steel screws. A stylet of the same length as the guide cannula was introduced inside the cannula to prevent obstruction. At the end of surgery, all animals were injected (i.m.) with an antibiotic preparation (Pentabiotic®, Brazil; 1.0 ml/kg) to prevent possible infections. In addition, flumixin meglumine (Schering-Plough, Brazil; 2.5 mg/kg), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia. The animals were left undisturbed for 5–7 d after surgery, except for normal handling for cage cleaning.

**Intracerebral injections**

For drug injection into DPAG, a needle (outside diameter 0.3 mm) was introduced through the guide cannula until its tip was 1 mm bellow the cannula end. A volume of 0.2 µl was injected for 120 s using a 10 µl microsyringe (Hamilton, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. The needle was removed 60 s after the injection was finished.

**Escape threshold determination**

Escape behaviour induced by DPAG electrical stimulation was evaluated in a circular arena (40 cm in diameter) with 40 cm high walls made of transparent Plexiglas. Brain stimuli were generated by a sine-wave stimulator. The stimulation current (peak to peak) was monitored on an oscilloscope screen (Minipa, Brazil). The brain electrode was connected to the stimulator by means of an electromechanical swivel and a flexible cable, allowing ample movement of the animal inside the experimental cage.

On test day, the animals were placed into an experimental cage, and the escape threshold was determined through electrical stimuli (AC, 60 Hz, 10 s) presented through the implanted chemitrode. The interstimulus interval was 10 s. The current intensity started at the level of 20 µA and was increased by 4-µA steps until the rat started running around the circular arena, characterizing the escape behaviour. In most cases, this vigorous reaction was accompanied by vertical jumps. When these behaviours were observed, application of electrical stimulation to DPAG was interrupted by the experimenter. The basal escape threshold was defined as the lowest current intensity that evoked escape in three successive trials of electrical stimulation. Animals with basal thresholds above 150 µA were excluded from the study.

**Expt 1: BDNF effect on the escape threshold**

Following basal escape threshold determination, independent groups of animals received an intra-DPAG injection of BDNF (0.05, 0.1 or 0.2 ng; n = 5) or vehicle (n = 6) and the escape threshold was re-analysed 30 min later. The variation in escape threshold (Δ) was then calculated for each animal and refers to the difference between escape threshold values (µA) obtained post- and pre-DPAG injection of BDNF or vehicle. An increase in this value was taken as a panicolytic-like effect and a decrease, the opposite.

**Expt 2: K252a microinjection and BDNF effect**

After determination of the basal escape threshold, previously naive animals received an injection of K252a (10 pmol, i.e. 4.67 ng) or its vehicle into the DPAG. Ten minutes later, they were microinjected in the same brain area with BDNF (0.2 ng) or its respective vehicle. The following groups were formed: vehicle/vehicle (n = 5), K252a/vehicle (n = 5), vehicle/BDNF (n = 5) and K252a/BDNF (n = 6). Escape threshold was re-determined 30 min after the last drug or vehicle injection. The K252a dose was chosen based on previous behavioural studies (Shirayama et al. 2002).

**Expts 3 and 4: ketanserin or WAY-100635 microinjection and BDNF effect**

The involvement of 5-HT1A/1C (expt 3) or 5-HT1A (expt 4) receptors for the effect of BDNF on escape performance was investigated. In expt 3, variation in escape threshold was determined in animals that received an intra-DPAG injection of BDNF (0.2 ng) or vehicle 10 min before local injection of ketanserin (10 nmol, i.e. 5.63 µg) or its respective vehicle. The following groups were formed: vehicle/vehicle (n = 6), BDNF/vehicle (n = 5), vehicle/ketanserin (n = 5) and BDNF/ketanserin (n = 5). Escape thresholds were re-determined 20 min after the last drug or vehicle injection.

In expt 4, microinjection of BDNF (0.2 ng) or vehicle preceded (10 min before) local injection of WAY-100635 (0.37 nmol, i.e. 0.2 µg) or its vehicle. The groups formed were (n = 7 for each group): vehicle/vehicle,
BDNF/vehicle, vehicle/WAY and BDNF/WAY. As in expt 3, post-drug escape threshold was assessed 20 min after the last drug injection.

Three days after testing, animals from expts 3 and 4 were submitted to another experimental session in order to assess if ketanserin or WAY-100635 could block the effect of locally injected 5-HT on escape threshold. After randomly allocating rats to new groups, the basal escape threshold of each rat was re-determined. Rats previously tested in expt 3 were injected into DPAG with ketanserin (10 nmol) or vehicle and animals from expt 4 with WAY-100635 (0.37 nmol) or vehicle. Ten minutes later, half of the animals of each group were injected with 5-HT (10 nmol, i.e. 3.87 μg) and the other half with its respective vehicle. Escape thresholds were reassessed 10 min after the last drug or vehicle injection. The doses of 5-HT, ketanserin and WAY-100635 were chosen based on previous studies with the elevated T-maze (de Paula Soares & Zangrossi, 2004) or the electrical stimulation of DPAG (Nogueira & Graeff, 1995; Schütz et al. 1985).

Expt 5: bicuculline microinjection and BDNF effect

In this experiment, the role of GABA_A receptors for the effect of BDNF on escape performance was investigated. To this end, we initially examined the effect of intra-DPAG injection of bicuculline (5, 10 or 20 pmol, i.e. 2.5, 5 or 10 ng, respectively; n = 5) or its vehicle (n = 5) on escape performance. In this study, post-drug escape threshold was determined 10 min after injections. After establishing a dose-response curve for this GABA_A antagonist, in a subsequent study, using naive rats randomly allocated in independent groups of animals, we calculated the variation in escape threshold of animals microinjected with BDNF (0.2 ng) or its vehicle 10 min before the injection of bicuculline (5 pmol) or its vehicle. The groups formed were (n = 5 for each group): vehicle/vehicle, BDNF/vehicle, vehicle/bicuculline and BDNF/bicuculline. Post-drug escape threshold was determined 20 min after the last drug or vehicle injection.

Expt 6: BDNF effect on MAP

Previously naive chemitrode-implanted animals were submitted to electrical stimulation procedure to determine escape threshold 5–7 d after surgery, as described above. Twenty-four hours later, rats were anaesthetized with urethane (1.25 g/kg i.p.) and a catheter (a 4 cm PE-10 segment heat-bound to a 8 cm PE-50 segment; Clay Adams, USA) was inserted into the abdominal aorta through the femoral artery for blood pressure recording. One hour after anaesthesia, MAP was recorded using an HP-7754A amplifier (Hewlett Packard, USA) connected to a signal acquisition board (MP-100A; Biopac, USA) and computer processed. Blood pressure responses were calculated based on the average mean blood pressure at the response’s plateau. Thereafter, electrical stimuli were applied to the DPAG, starting with a current intensity 20 μA below the animal’s pre-determined escape threshold and then increased until a 20-mmHg rise in MAP was recorded. Animals were randomly allocated in four groups: vehicle/vehicle (n = 5), BDNF/vehicle (n = 5), vehicle/bicuculline (n = 5) and BDNF/bicuculline (n = 5). The drug injection protocol was performed as described in expt 5. The variation in MAP threshold (Δ) was then calculated and refers to the difference between MAP increase threshold values (μA) obtained post- and pre-DPAG injection of drugs.

**Histology**

After the experiments, animals were sacrificed under deep anaesthesia with chloral hydrate. The brain was perfused through the heart with saline solution followed by 10% formalin solution, before being removed and fixed in 10% formalin. Frozen, 55-μm sections were cut using a microtome to localize the
positions of the electrode tips according to the atlas of Paxinos & Watson (2007). Only data from rats having electrode tips inside the DPAG (dorsomedial and dorsolateral columns) were included in the statistical analysis.

**Statistical analysis**

The effects of intra-DPAG drug injections were analysed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test, when appropriate. Data are expressed as mean ± S.E.M.

**Results**

Figure 1 shows the sites of electrical stimulation/drug injection in DPAG of animals tested in this study.

**Expt 1: BDNF effect on the escape threshold**

As shown in Fig. 2a, intra-DPAG administration of BDNF dose-dependently raised the escape threshold $[F(3, 17) = 4.06, p < 0.05]$.  

**Expt 2: K252a microinjection and BDNF effect**

As seen in Fig. 2b, previous intra-DPAG administration of K252a blocked the effect of BDNF in raising the escape threshold $[F(3, 17) = 13.22, p < 0.01]$. K252a alone had no effect on this index.

**Expt 3: ketanserin microinjection and BDNF effect**

One-way ANOVA revealed that the groups tested differed in their effects on the escape threshold $[F(3, 17) = 17.58, p < 0.01]$. Fig. 3a shows that ketanserin,
at the dose used, did not interfere with the effect of BDNF on the escape threshold. Ketanserin alone was ineffective. However, as illustrated in Fig. 3b, the same dose of this antagonist counteracted the effect of intra-DPAG injection of 5-HT in raising the escape threshold \([F(2, 12) = 33.18, p < 0.01]\).

**Expt 4: WAY-100635 microinjection and BDNF effect**

As in expt 3, one-way ANOVA revealed a significant difference among groups tested \([F(3, 24) = 12.34, p < 0.01]\). Figure 4a shows that WAY-100635, at a dose of 0.37 nmol, did not change BDNF-induced increase in the escape threshold. However, as shown in Fig. 4b, WAY-100635, at the same dose, was able to counteract the effect of 5-HT on the escape threshold \([F(2, 12) = 65.08, p < 0.01]\).

**Fig. 4.** (a) Lack of effect of WAY-100635 (0.37 nmol) on brain-derived neurotrophin factor (BDNF) (0.2 ng)-induced increase in \(\Delta\) escape threshold and (b) blocking effects of WAY-100635 (0.37 nmol) on 5-HT (10 nmol)-induced increase in escape threshold (*\(p < 0.05\) from control group).

**Fig. 5.** (a) Effect of bicuculline (Bic) microinjected in the dorsal periaqueductal grey matter on the variation (\(\Delta\)) of escape threshold and (b) blocking effect of bicuculline (5 pmol) on brain-derived neurotrophin factor (BDNF) (0.2 ng)-induced increase in \(\Delta\) escape threshold (*\(p < 0.05\) from control group).

**Expt 5: bicuculline microinjection and BDNF effect**

Figure 5a shows that bicuculline microinjection in DPAG caused a dose-related decrease in the escape threshold \([F(3, 19) = 9.26, p < 0.05]\). As illustrated in Fig. 5b, the effect of BDNF in raising the escape threshold was antagonized by intra-DPAG injection of 5 pmol bicuculline \([F(3, 16) = 22.58, p < 0.01]\). This dose of the GABAa antagonist alone did not interfere with the escape threshold.

**Expt 6: bicuculline microinjection and BDNF effect on MAP**

DPAG electrical stimulation was able to evoke escape performances, as in previous experiments. The mean basal escape threshold (mean ± S.E.M.) was 77 ± 8.9 μA and the mean threshold to induce MAP increase was
increase in MAP induced by stimulation of DPAG, another well-known component of the escape reaction (Schenberg et al. 1993). There is plenty of evidence suggesting that neurotrophins can act within a short timescale, as in the present analysis (Bariohay et al. 2008; Deltheil et al. 2009). The so-called BDNF fast actions have been mainly associated with the peptide’s facilitatory effect on synaptic transmission which has been attributed to different mechanisms such as increase in calcium influx (Sakai et al. 1997), enhancement of synapsin I phosphorylation (Jovanovic et al. 2000) and/or activation of specific sodium channels (Hilborn et al. 1998).

Of importance to the present work, in the PAG/DRN, BDNF has been reported to facilitate 5-HT neurotransmission (Siuciak et al. 1994, 1998). A wealth of studies shows that in DPAG, 5-HT, via activation of 5-HT₁₅ or 5-HT₂A/2C receptors, inhibits escape performance, as observed with BDNF in expt 1 (Nogueira & Graeff, 1995; Zanoveli et al. 2007). However, this mechanism does not seem to account for the present BDNF panicolytic-like effect in view of the fact that intra-DPAG injection of WAY-100635 and ketanserin, 5-HT₁₅ and 5-HT₂A/2C receptor antagonists, respectively, did not interfere with BDNF-induced increase in escape threshold. The observation that the same doses of these antagonists counteracted the panicolytic-like effect caused by 5-HT injection in DPAG supports the idea that BDNF does not operate through modulation of 5-HT neurotransmission.

It is important to highlight that Siuciak & co-workers (1998) reported that injection of the neurotrophin in the PAG/DRN area causes a local increase in tryptophan hydroxylase mRNA levels that is accompanied by a facilitation of 5-HT turnover. Given the heterogeneity of the tissue analysed, it is possible that BDNF-induced changes on the 5-HT system as reported by the authors are restricted to areas not investigated in this study such as the DRN. In support of this idea, tryptophan hydroxylase is found in cells that synthesize 5-HT which differently from DRN are only rarely found in DPAG (Coimbra et al. 2006). Moreover, as Siuciak’s neurochemical assays were performed 24 h after BDNF infusion, it is also conceivable that a longer period of time than that employed by us (30 min) is required to reveal a facilitatory effect of BDNF on 5-HT neurotransmission.

In contrast to 5-HT antagonists, intra-DPAG injection of a subeffective dose of the GABAₐ receptor antagonist bicuculline abolished BDNF-induced increase in the escape threshold. Furthermore this antagonist also blocked the inhibitory effect of BDNF on the MAP increase evoked by the electrical stimulation of DPAG.
These data fully support our idea that this neurotrophin panicolytic-like effect occurs via GABA\(_A\)-dependent mechanisms. In line with this idea, injection of GABA-mimetic drugs in DPAG inhibits the expression of escape behaviour in the present experimental model (de Bortoli et al. 2008) or in the elevated T-maze (Bueno et al. 2005). Evidence obtained by in-vitro and in-vivo studies lends further support to this hypothesis. For instance, a recently published study (Vaz et al. 2008), reveals that BDNF, through TrkB receptor activation, inhibits carrier-mediated GABA uptake by isolated nerve terminals. This inhibitory effect on GABA transport is very fast, being already seen after 1 min incubation with BDNF. There is also evidence that the neurotrophin can regulate the functioning of GABA\(_A\) receptors by regulating their phosphorylation state. In cultured hippocampal neurons, BDNF-induced GABA\(_A\) receptor phosphorylation causes a transient enhancement of the receptor functioning as evidenced by increases in miniature IPSC amplitudes (Jovanovic et al. 2004). Hypothetically, the blockade of GABA\(_A\) receptors as reported in the current study may interfere with these modulatory effects of BDNF on GABA signalling.

The interaction between BDNF/TrkB and GABA has also been described in intact animals. Thus, microinjection of BDNF in the rat dorsal vagal complex (Bariohay et al. 2008), similarly to what has been observed with GABA\(_A\)-enhancing drugs (Wang & Bieger, 1991), dose-dependently inhibits rhythmic swallowing, a motor component of feeding behaviour. Over the same time-frame adopted herein, local injection of a subactive dose of bicuculline counteracted this effect. Conversely, co-injection of BDNF and GABA at subthreshold doses causes a significant inhibition of swallowing, indicating a synergistic interaction.

Overall, the present results add to previous preclinical evidence showing the involvement of BDNF in the regulation of anxiety-like behaviours (Deltheil et al. 2009; Maron et al. 2009; Pandey et al. 2006). They broadened the existing knowledge in the field by implicating this neurotrophin in the regulation of a defensive behaviour that has been linked to a specific anxiety pathology: panic disorder.

Certainly, more studies are required to confirm the involvement of BDNF in panic. For instance, preliminary clinical studies have failed to find a difference of plasma BDNF levels between healthy controls and panic disorder patients (Kobayashi et al. 2005). Moreover, no significant association was found between plasma BDNF levels and the occurrence or severity of CCK-4-induced panic attacks in healthy volunteers (Maron et al. 2009). However, as discussed by the latter authors, caution is advised in interpreting these results since it is not clear whether plasma BDNF, which mostly represents released BDNF from blood platelets can appropriately reflect the central functions of this neurotrophin.

The present results prompted us to explore whether BDNF/TrkB signalling in DPAG is implicated in the beneficial effects of antidepressants in panic disorder.

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Statement of Interest

None.

References


