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Acute Effects of *Sceletium tortuosum* (Zembrin), a Dual 5-HT Reuptake and PDE4 Inhibitor, in the Human Amygdala and its Connection to the Hypothalamus

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The South African endemic plant *Sceletium tortuosum* has a long history of traditional use as a masticatory and medicine by San and Khoikhoi people and subsequently by European colonial farmers as a psychotropic in tincture form. Over the past decade, the plant has attracted increasing attention for its possible applications in promoting a sense of wellbeing and relieving stress in healthy individuals and for treating clinical anxiety and depression. The pharmacological actions of a standardized extract of the plant (Zembrin) have been reported to be dual PDE4 inhibition and 5-HT reuptake inhibition, a combination that has been argued to offer potential therapeutic advantages. Here we tested the acute effects of Zembrin administration in a pharmaco-fMRI study focused on anxiety-related activity in the amygdala and its connected neurocircuitry. In a double-blind, placebo-controlled, cross-over design, 16 healthy participants were scanned during performance in a perceptual-load and an emotion-matching task. Amygdala reactivity to fearful faces under low perceptual load conditions was attenuated after a single 25 mg dose of Zembrin. Follow-up connectivity analysis on the emotion-matching task showed that amygdala–hypothalamus coupling was also reduced. These results demonstrate, for the first time, the attenuating effects of *S. tortuosum* on the threat circuitry of the human brain and provide supporting evidence that the dual 5-HT reuptake inhibition and PDE4 inhibition of this extract might have anxiolytic potential by attenuating subcortical threat responsivity.
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INTRODUCTION

The South African endemic plant *Sceletium tortuosum* (L.) N.E. Br. (synonym *Mesembryanthemum tortuosum* L.), of the succulent family Mesembryathemaceae, has a long history of traditional use by San and Khoikhoi people as a masticatory and medicine (Smith *et al*, 1996) and later by colonial farmers as a psychotropic in tincture form (Pappe, 1868). Over the past 15 years, the plant has attracted increasing attention for its hypothesized applications in promoting a sense of wellbeing and relieving stress in healthy individuals and for treating anxiety and depression in clinically anxious and depressed patients (Gericke and Viljoen, 2008). A recent *in vivo* study in rats demonstrated a positive effect of an extract of *S. tortuosum* on restraint-induced anxiety (Smith, 2011), and a small series of case reports described preliminary evidence for antidepressant and anxiolytic activity in patients suffering from major depression who were treated with tablets of milled *S. tortuosum* raw material (Gericke, 2001).

The mechanisms of action on the central nervous system (CNS) of a standardized extract of *S. tortuosum* (Zembrin) were recently identified as comprising blockade of the serotonin (5-HT) transporter and selective inhibition of the phosphodiesterase-4 (PDE4) enzyme (Harvey *et al*, 2011). 5-HT reuptake inhibitors (SSRIs) are widely used for the treatment of anxiety disorders and depression (Pringle *et al*, 2011). However, a combination of SSRIs with a PDE4 inhibitor has been argued to have synergistic therapeutic potential in CNS disorders by providing greater symptomatic efficacy and broader therapeutic utility than either as a drug on its own. In particular, repeated treatment with SSRIs can upregulate PDE4 (Ye *et al*, 2000), which in turn reduces the sensitivity to SSRIs in response to long-term treatment, suggesting that dual treatment with SSRIs and PDE4 inhibitors may be a promising approach (Cashman *et al*, 2009).

Cyclic nucleotide PDEs comprise a diverse group of enzymes that are important regulators of signal transduction.

PDEs are classified into 11 families based on sequence homology, substrates and regulation by modulators. Enzymes in the PDE4 family catalyze the hydrolysis of cyclic AMP (cAMP) and have a critical role in controlling the intracellular concentration of cAMP and increasing phosphorylation of cAMP-response element-binding protein (Li *et al*, 2009). PDE4s are found throughout the brain (Perez-Torres *et al*, 2000), but their levels are decreased in unmedicated depressed individuals (Fujita *et al*, 2012). This reflects a downregulation of the cAMP cascade that can potentially be restored using PDE4 inhibitors (Duman *et al*, 1999), an idea which is supported by an increasing number of animal studies that indicate the potential of PDE4 inhibitors for novel treatments of anxiety and depression (Halene and Siegel, 2007; Li *et al*, 2009). Indeed, the prototypical PDE4 inhibitor rolipram has been shown in both animal (Saccomano *et al*, 1991) and clinical (Fleischhacker *et al*, 1992) studies to have antidepressant activity.

A consistent challenge in translating these pre-clinical studies of PDE4s into clinical practice has been the side effects (Duman *et al*, 1999); in particular nausea and vomiting (Rock *et al*, 2009). The combination of SSRIs and PDE4 inhibitors might therefore not only have synergistic therapeutic potential but may allow for lower PDE4 doses that are better tolerated. Zembrin has been found to be safe and well-tolerated at doses of 8 and 25 mg taken orally once a day in a randomized, double-blind, parallel-group, placebo-controlled clinical trial, supporting the ethnobotanical record of safe use of *S. tortuosum* (Nell *et al*, 2013). Here, we report a functional magnetic resonance imaging (fMRI) study that is the first to test the activity of *S. tortuosum* (Zembrin) in the human brain and is thereby also the first study on the effects of a dual PDE4 and 5-HT reuptake inhibitor in humans. The present study was designed to test the effects of a single administration of Zembrin (25 mg) on anxiety-related activity in the human brain. A reliable and often used index of fear responsivity and anxiety in the human brain is the blood oxygenation level-dependent (BOLD) response to facial threat in the bilateral amygdala as measured with fMRI (Freitas-Ferrari *et al*, 2010). The amygdala can be considered a hub in the brain's threat system involved in threat detection and subsequent promotion and regulation of defensive reactions in the subcortical threat circuit, particularly the hypothalamus, midbrain, and brainstem (Davis and Whalen, 2001; Walker *et al*, 2003). Moreover, the amygdala relays threatening information to the cortex and orchestrates, together with cortical structures, the regulation of fear and anxiety (Kim *et al*, 2011; Terburg *et al*, 2012). Given this diverse role in threat processing, we used two fMRI experiments to independently assess anxiety-related amygdala activation (Bishop *et al*, 2007) and functional connectivity of the amygdala with the threat circuit of the brain (Fisher *et al*, 2011; Hariri *et al*, 2002; Kirsch *et al*, 2005; van Wingen *et al*, 2010; van Wingen *et al*, 2008). As the amygdala is involved in the regulation of fear responsivity in the subcortical threat circuit (hypothalamus and midbrain), as well as with controlling mechanisms in the prefrontal cortex (PFC), we specifically focused the latter analysis on these areas. We hypothesized that administration of Zembrin would reduce anxiety-related amygdala activity as well as functional connectivity of the amygdala with the cortical and/or subcortical threat circuit.

MATERIALS AND METHODS

Investigational Product

The active ingredient of Zembrin is a standardized and characterized aqueous ethanolic (purified water 30% V/V and ethanol 70% V/V, spray-dried onto a maltodextrin carrier) extract of the above ground material of a cultivated traditionally used selection of the South African plant *S. tortuosum*. Figure 1 shows the chemical structures of the four active alkaloids (mesembrenone, mesembrenol, mesembranol, and mesembrine), which were quantified by high pressure liquid chromatography (HPLC) analysis against validated analytical reference standards (Harvey *et al*, 2011). See Supplementary Information for a detailed description.

Each active opaque white size-1 hardshell gelatin capsule, lot number NG022, contained 25 mg of Zembrin together with inert excipients, equivalent to 50 mg of dry raw above-ground *S. tortuosum* plant material. Placebo capsules, lot number NG009, contained only inert excipients. Both placebo and active capsules were identical in size, color, opacity, and odor and were manufactured to GMP by Archon Vitamin Corporation, New Jersey, USA. This study was approved by the Clinical Trials Committee of the Medicines Control Council of South Africa.

Participants and Procedure

Sixteen (eight female) right-handed, healthy undergraduate students (aged 18–21 years, mean = 19.2 years) from the University of Cape Town participated in this study. All participants were right-handed, medication-free, had no history of neurological disease or psychopathology (screened using Mini-International Neuropsychiatric Interviews (Sheehan *et al*, 1998)), had normal or corrected-to-normal vision, and received transportation and remuneration for research time (research credit or ZAR200).

Each participant was scanned once after Zembrin and once after placebo administration, separated by 5–9 days (mean = 6.8 days), and counterbalanced across participants. Researchers remained blind to administration conditions. Each scan session took place promptly at 2 h post administration. Participants were monitored for the entire duration between administration and scanning and were asked to report any mood changes or side effects (see Supplementary Table S1). After the final session,
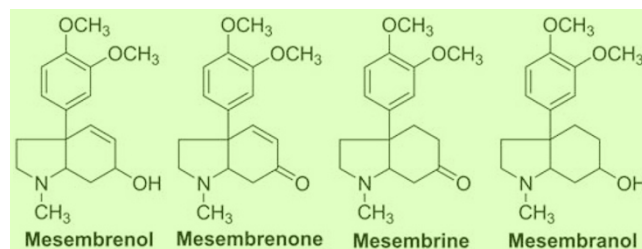

Figure 1 Structures of the four main mesembrine alkaloids that are quantified by HPLC to define the alkaloid content and composition of the extract of *Sceletium tortuosum* (Zembrin).

participants were asked to identify the session that involved administration of the investigational product, which was correctly identified by nine participants, which is not significantly (one-tailed binomial test; $P = 0.30$) above chance level. Response to this question, as well as gender, were entered as covariates in all fMRI analyses but did not change the results.

Perceptual-Load Task (PLT)

The PLT was adapted from Bishop *et al* (2007). In each trial, participants saw a string of six letters superimposed on a task-irrelevant unfamiliar face for 200 ms. Participants were required to decide whether the letter string contained an 'X' or 'N'. The letter string comprised six Xs or Ns in the low-load condition, and a single target letter and five non-target letters (HKMWZ) arranged in a semi-random order in the high-load condition. Target letters appeared in each position equally often. Participants responded by a single key press using button boxes held in each hand. 'N' and 'X' responses were counterbalanced for hand across participants. The faces were of eight different individuals with fearful and neutral expressions (Ekman and Friesen, 1976). Data were acquired for a total of 12 blocks in the high-load and 12 blocks in the low-load condition wherein each block consisted of 4 trials. Load stayed constant across all trials within a block, but emotion varied on a trial-by-trial basis such that each block contained two fearful and two neutral faces presented in a random order. Within blocks, the interstimulus interval was randomly jittered with a mean of 4.5 s and a minimum of 3 s.

Previous research has shown that the PLT can assess state-anxiety-related amygdala activity. Fearful compared with neutral faces induce threat responses, which under high-load conditions are inhibited in favor of task performance (Bishop, 2008; Lavie, 1995). In low-load conditions, however, inhibition of the threat response is not necessary for correct performance, and amygdala reactivity to the fearful compared with neutral faces correlates with state anxiety (Bishop *et al*, 2007).

Emotion-Matching Task (EMT)

The EMT was adapted from Hariri *et al* (2002). Four blocks of an emotion-matching task were interleaved with five blocks of a sensorimotor-control task involving shape matching. During each 5-s trial in the emotion condition, participants saw a target face on top and two probe faces at the bottom. Participants' task was to match the emotion of the face on top with one of the other faces using a left or a right key press (corresponding to the position of the face). During the sensorimotor-control shape-matching trials, participants saw oval shapes in a configuration analogous to the faces and were instructed to match the orientation of the oval (vertical or horizontal ellipses) on top to the ovals on the bottom. The emotional faces expressed anger or fear (Tottenham *et al*, 2009), and luminosity of the oval shapes was matched to that of the faces. Each block consisted of six trials, and blocks were interspersed by a 2-s presentation of instructions for the next block (ie 'match emotion' or 'match shape').

The EMT contrasts emotion with shape-matching blocks and is thereby a reliable amygdala activator (Hariri *et al*, 2002), which is stable over multiple sessions (Manuck *et al*, 2007). Recent evidence also indicates that the EMT provides a valid measure of pharmacological and state-related changes in connectivity patterns of the amygdala with cortical (van Wingen *et al*, 2010; van Wingen *et al*, 2008) as well as subcortical structures (Kirsch *et al*, 2005).

MRI Acquisition

Participants were scanned in a 3-T Allegra Siemens head scanner (VA25 platform, Siemens Medical Systems GmBH, Erlangen, Germany), using a four-channel receiver. A high-resolution T1-weighted anatomical volume (magnetization-prepared rapid gradient echo) was collected in between the two functional runs using the following parameters: TR/TE = 2.53 s/6.6 ms, FA = 7°, FOV = 256 × 256 × 171, and voxel-size = 1.0 × 1.0 × 1.3 mm. Functional volumes (echo-planar imaging) were obtained with 36 interleaved-ascending axial slices, EPI factor = 64, TR/TE = 2 s/27 ms, flip angle = 70°, FOV = 224 × 224 × 145 mm, and voxel-size = 3.5 mm isotropic. Before the start of each task, five volumes were acquired and discarded to allow for stabilization of the MR signal, and 255 (PLT) and 150 (EMT) functional volumes were acquired for data-analysis. Task-stimuli were back projected onto a screen visible via an angled mirror attached to the helmet coil. Each task was explained to the participants in detail 15 min before the scan, and they completed practice rounds in the scanner before the start of each task.

MRI Analysis

All MR images were processed and analyzed using SPM8 (www.fil.ion.ucl.ac.uk/spm; Wellcome Department of Imaging Neuroscience, London, UK). Functional volumes were realigned to the first image in the series. The anatomical volume was coregistered to the mean realigned image and transformed to standard space (Montreal Neurological Institute; MNI) using the unified model as implemented in SPM8. As the PLT is an event-related design, these images were slice-time corrected with the 19th slice as reference. Functional volumes were transformed to MNI space using the parameters from the normalization of the anatomical volume, resampled to 2 mm isotropic, and smoothed with an 8-mm full-width half-maximum Gaussian smoothing kernel.

For both tasks, we applied a general linear model (GLM) to the single-subject data using a canonical hemodynamic response function, with the realignment parameters and a vector with the timing of the button presses convolved with a hemodynamic function as regressors of no interest, and a high-pass filter cutoff of 128 s. For both tasks, mean BOLD signal change from the bilateral amygdala were subsequently extracted for all individual contrast maps using the MarsBar toolbox (http://marsbar.sourceforge.net) and tested in repeated measures ANOVAs. In the GLM for the PLT, each trial was modeled with a box-car function for the duration of the stimulus and separate contrast maps were computed for each condition: LOAD (high, low), and EMOTION (fear, neutral). Contrast maps were entered in a full-factorial design and tested for effects of LOAD

(high > low), EMOTION (fear > neutral), DRUG (Zembrin *versus* placebo), and their interactions (whole-brain family wise error (FWE) corrected $P < 0.05$, and extend-threshold 20 voxels).

In the GLM for the EMT, each block was modeled with a 30-s box-car function and separate contrast maps were computed for emotion and shape matching. A full-factorial design was used to test for main effects of CONDITION (emotion > shape), DRUG (Zembrin *versus* placebo) and their interaction (whole-brain FWE-corrected $P < 0.05$, extend-threshold 20 voxels). Furthermore, we performed a region of interest (ROI) analysis to assess activity in the threat circuit (ventro-medial PFC/orbitofrontal cortex (vmPFC/OFC), amygdala, hypothalamus, and midbrain) with a ROI volume FWE-corrected $P < 0.05$. ROIs were based on the Wake Forest University pickatlas (http://www.fmri.w-fubmc.edu/download.htm), except for the hypothalamus, which was constructed by drawing an 8-mm sphere around the central coordinate (MNI: 0–2–13) of hypothalamus subregions recently described by Baroncini *et al* (2012).

The peak voxels of the contrast of interest indexing anxiety-related amygdala reactivity in the PLT were used for connectivity analysis in the EMT targeting the threat circuit. For each participant, time course (first eigenvariate) was extracted from the spheres of 6 mm around these peak voxels, and mean corrected for general task effects. These were used to assess connectivity for the whole task and both conditions separately by entering them in the original GLM, as well as for psycho-physiological interaction (PPI) analysis as implemented in SPM8 to assess differences between conditions. The resulting contrast maps were entered in the separate full-factorial models to test for DRUG effects in functional connectivity of the bilateral amygdala with the threat circuit (ROI-corrected).

RESULTS

PLT

One participant performed below chance level (47% correct), which was > 3 SDs below average (85%, SD = 4), and was therefore excluded from analysis. A $2 \times 2 \times 2$ repeated-measures ANOVA on the error rates with DRUG, LOAD, and EMOTION as within-subject factors revealed a significant effect of LOAD ($F(1,14) = 194.2$, $P < 0.00001$) but with no other main effects or interactions (all $Ps > 0.27$). Reaction times revealed the same pattern: a significant effect of LOAD ($F(1,14) = 57.4$, $P < 0.00001$) but with no other main effects or interactions (all $Ps > 0.28$; see Figure 2a).

In line with earlier studies with the PLT, whole-brain FWE-corrected positive main effects of LOAD (high > low) were observed bilaterally in the parietal and occipital cortices, anterior insula, precentral, and midfrontal PFC, and dorsal anterior cingulate cortex (see Table 1 and Figure 2b). No other main effects or interactions were observed using this statistical threshold.

A $2 \times 2 \times 2$ repeated-measures ANOVA on the extracted mean signal in the bilateral amygdala, with DRUG, LOAD, and EMOTION as within-subject factors, showed a significant three-way interaction ($F(1,14) = 6.81$, $P = 0.028$, partial $\eta^2 = 0.30$), as well as a main effect of LOAD ($F(1,14) = 13.02$, $P = 0.003$, partial-$\eta^2 = 0.48$). As hypothesized,
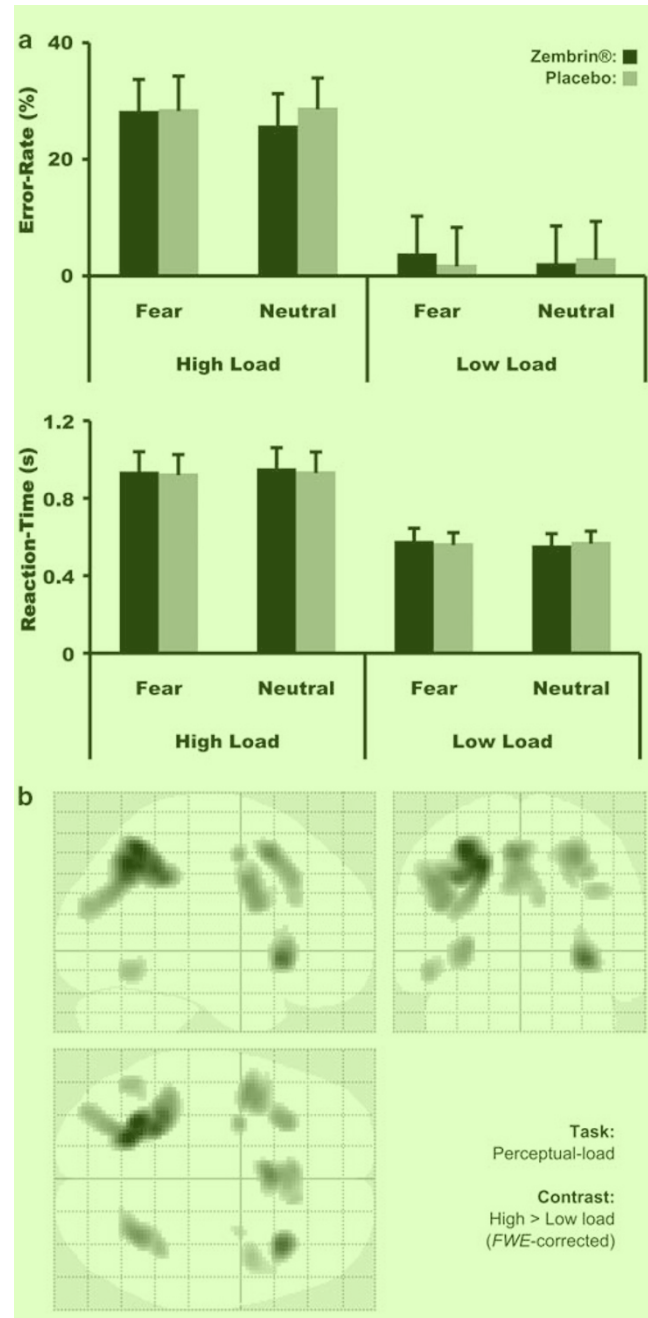

Figure 2 (a) Mean error rates and reaction times on the PLT (error bars represent SEM). (b) Increased BOLD signal in the high-load compared with low-load condition of the PLT plotted in a glass brain (whole-brain FWE-corrected, $P < 0.05$).

the three-way interaction was due to a significant attenuation of amygdala activity after Zembrin administration on the contrast fear > neutral in the low-load condition ($F(1,14) = 6.90$, $P = 0.020$, partial $\eta^2 = 0.33$, see Figure 3a for the associated contrast estimates), which was non-significant in the high-load condition ($F(1,14) = 0.003$, $P = 0.956$).

Finally, to determine the peak voxels of this effect for further connectivity analyses we performed an ROI analysis on this particular contrast, which confirmed the significantly reduced amygdala reactivity for the left (58 voxels, $P = 0.008$, peak voxel − 26 0, − 18, $P = 0.004$) and right (13 voxels, $P = 0.025$, peak voxel 24 4, –20, $P = 0.010$) amygdala (see Table 1 and Figure 3b).

Table I Significant Clusters from the fMRI Analyses [1]

	Hemisphere	Cluster size	Cluster P-value	Peak T-value	Coordinate		[2]
					X	Y	Z
Perceptual load task:							
High > low load (see also Figure 2b)							
Parietal/occipital cortex	Left	1572	<0.001	9.41	−28	−56	52
	Right	543	<0.001	7.42	30	−54	50
Anterior insula	Left	246	<0.001	6.90	−30	24	0
	Right	326	<0.001	8.34	34	22	−4
Precentral PFC	Left	575	<0.001	7.28	−44	4	36
	Right	128	<0.001	6.15	40	6	32
Midfrontal PFC	Left	50	<0.001	6.63	−28	0	50
	Right	22	<0.01	5.48	28	0	50
Temporal/fusiform cortex	Left	136	<0.001	6.31	−48	−60	−10
Dorsal ACC/SMC	Left/right	620	<0.001	7.71	−2	16	50
Placebo > Zembrin (low load: fear > neutral) (see also Figure 3b)							
Amygdala	Left	58	<0.01[a]	3.81	−26	0	−18
Amygdala	Right	13	<0.05[a]	3.56	24	4	−20
Dorsal striatum	Left	390	<0.05[b]	4.51	−10	0	16
Dorsal Striatum	Right	629	<0.01[b]	5.36	26	4	18
Precuneus	Left/right	230	<0.05[b]	4.03	−12	−30	42
Emotion matching task:							
Emotion > shape matching (see also Figure 4a)							
Fusiform cortex/cerebellum	Left	337	<0.001	8.56	−38	−46	−20
	Left	82	<0.001	6.77	−36	−70	−20
	Right	2546	<0.001	11.72	40	−42	−22
Frontal-inferior PFC	Left	263	<0.001	6.50	−42	12	26
	Right	718	<0.001	8.33	42	16	24
Temporal cortex	Left	190	<0.001	6.02	−26	16	−30
	Right	88	<0.001	6.86	48	−54	8
	Right	104	<0.001	6.36	56	−38	10
Amygdala	Left	107	<0.001	6.28	−24	0	−20
	Right	110	<0.001	6.59	22	−4	−16
Hippocampus	Left	90	<0.001	6.44	−22	−24	−4
	Right	36	<0.01	6.09	22	−26	−4
Brainstem	Right	21	<0.01	5.70	18	−32	−48
Precentral PFC	Right	43	<0.01	5.63	48	0	52
Ventro-medial PFC/OFC	Left	220	<0.001	5.17	−24	16	−26
		85	<0.01	4.52	−32	28	−4
Ventro-medial PFC/OFC	Right	458	<0.001	5.22	20	12	−24
Midbrain	Left/Right	427	<0.001[a]	6.03	−20	−28	−4
Hypothalamus	Left/right	53	<0.05[a]	4.65	2	2	−18
Functional connectivity with amygdala: placebo > Zembrin							
Main effect (see also Figure 4b)							
Hypothalamus	Left/right	8	<0.05[a]	3.64	0	−8	−8
Emotion > shape matching							
Hypothalamus	Left/right	13	<0.10[a]	2.76	0	−2	−4
Emotion matching							
Hypothalamus	Left/right	47	<0.05[a]	3.73	−2	0	−6

Abbreviations: ACC, anterior cingulate cortex; OFC, orbitofrontal cortex; PFC, prefrontal cortex; SMC, supplementary motor cortex. [3]
All effects are whole-brain FWE-corrected with an extend threshold of 20 voxels unless stated otherwise. Coordinates refer to MNI space.
[a]FWE-corrected for ROI volume.
[b]FWE-corrected cluster-threshold with uncorrected cluster-defining threshold $P < 0.001$.
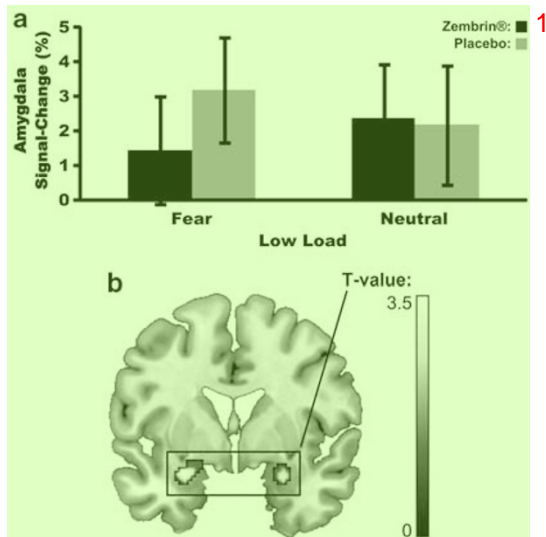
Figure 3 (a) Mean extracted BOLD signal change from the bilateral amygdala on low-load fearful and neutral trials during the PLT (error bars represent SEM). (b) Increased BOLD signal for placebo compared with Zembrin on low-load fearful compared with neutral trials during the PLT in the bilateral amygdala plotted on a template brain ($P < 0.01$, uncorrected for visualization).

Finally, a more liberal whole-brain analysis (FWE-corrected cluster threshold $P < 0.05$, cluster-defining threshold $P < 0.001$ uncorrected) showed a three-way interaction within the dorsal striatum, precuneus, and bilateral postcentral gyrus. Follow-up contrasts established that the effects in the dorsal striatum and precuneus emerged from the same contrast as the effect in the amygdala (placebo > Zembrin (low load: fear > neutral), see Table 1), but the effect in the bilateral postcentral gyrus was not significant in any of the simpler contrasts.

EMT

The participant excluded from the PLT also scored near to chance level (56% correct) on the EMT, thus was also excluded here. No differences were found in performance for CONDITION or DRUG (emotion matching: 92% correct, SD = 9, shape matching: 93%, SD = 13).

Whole-brain FWE-corrected positive main effects of CONDITION (emotion > shape) were observed bilaterally in the fusiform and temporal cortices, cerebellum, frontal inferior PFC, amygdala, and hippocampus (see Table 1 and Figure 4a). No main effect of DRUG or interaction was observed using this or a more liberal (FWE-corrected cluster threshold $P < 0.05$, cluster-defining threshold $P < 0.001$ uncorrected) statistical threshold. ROI analysis on the threat circuit shows reliable activation in the midbrain, hypothalamus, amygdala, and vmPFC/OFC on the emotion > shape-matching contrast (see Table 1), again with no main or interaction effects of DRUG. A 2×2 repeated-measures ANOVA on the extracted mean signal in the bilateral amygdala, with DRUG and CONDITION as within-subject factors, revealed a marginally significant interaction effect ($F(1,14) = 3.13$, $P = 0.099$, partial $\eta^2 = 0.18$) indicating marginally stronger amygdala activity on the emotion > shape-matching contrast in the Zembrin
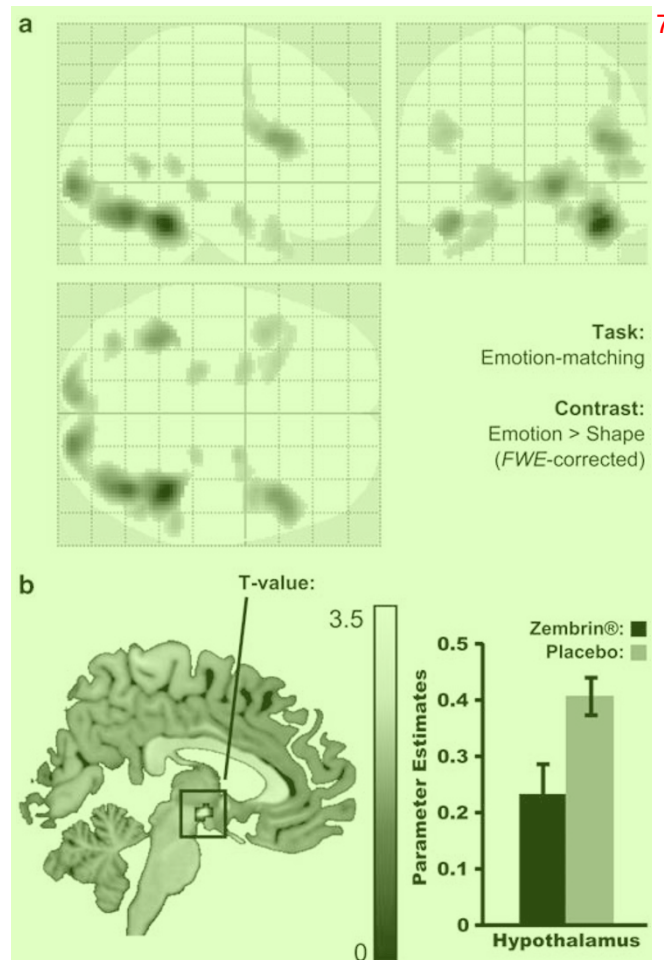

Figure 4 (a) Increased BOLD signal in the emotion-matching compared with shape-matching blocks during the EMT plotted in a glass brain (whole-brain FWE-corrected, $P < 0.05$). (b) Stronger functional connectivity of the bilateral amygdala (seed region) with the hypothalamus for placebo compared with Zembrin during the EMT plotted on a template brain ($P < 0.01$, uncorrected for visualization), and parameter estimates of the mean regression coefficient for this cluster, showing that the positive correlation between amygdala and hypothalamus in the placebo condition was reduced after Zembrin administration (error bars represent SEM).

(percentage of signal change; 0.61, SD = 0.53) compared with placebo (0.38, SD = 0.23) condition.

Finally, time course analysis based on the peak voxels in the amygdala as identified in the PLT analysis revealed that the amygdala showed overall decreased functional connectivity with the hypothalamus (8 voxels, $P = 0.034$) after Zembrin compared with placebo administration (see Figure 4b) but no effects in midbrain or vmPFC/OFC. Further specification of this effect revealed that the reduced amygdala–hypothalamus connectivity was marginally larger in the emotion compared with the shape-matching condition (PPI analysis, 13 voxels, $P = 0.068$). This was further confirmed by a significant hypothalamus cluster in the emotion-matching condition (47 voxels, $P = 0.014$), which did not reach significance in the shape-matching condition (peak voxel, uncorrected $P = 0.246$). As ROI correction in larger ROIs such as vmPFC/OFC and midbrain might obscure local effects, we also used a more liberal

uncorrected threshold of $P < 0.001$ for these analyses, but this did not reveal any other significant clusters.

DISCUSSION

In this pharmaco-fMRI study, we tested the effects of a single administration of Zembrin, a *S. tortuosum* extract that can be characterized as a dual PDE4 and 5-HT reuptake inhibitor (Harvey *et al*, 2011). Using two independent fMRI designs, we show that Zembrin compared with placebo administration reduces anxiety-related (Bishop *et al*, 2007) amygdala reactivity and attenuates amygdala–hypothalamus coupling. These results not only have importance as the first evidence on the dampening effects of Zembrin on the brain's threat system but support further work on the clinical applicability of dual PDE4 and 5-HT reuptake inhibitors for the treatment of anxiety disorders and depression.

Anxiety is characterized by hyper-responsivity to mild threats. The task we used to assess amygdala reactivity directly taps into this mechanism as mild threats, ie fearful faces, had to be ignored in favor of a cognitive task, ie letter detection. Although clinically anxious individuals show such threat interference in general (Bar-Haim *et al*, 2007), healthy individuals can successfully ignore task-irrelevant stimuli under high task demands (Lavie, 1995). In line with this, the amygdala is activated by unattended facial fear, particularly under low perceptual load conditions (Silvert *et al*, 2007), which is further increased by state anxiety (Bishop *et al*, 2007). In the present study, this amygdala reactivity to facial fear under low-load conditions was significantly attenuated after Zembrin administration. Although we did not observe general subjective mood effects, the effects noted here arguably reflect a reduction of responsivity of the threat system related to the anxiolytic properties of *S. tortuosum* (Gericke, 2001; Gericke and Viljoen, 2008; Smith *et al*, 1996). It must furthermore be noted that in contrast to the previous study with the PLT, the behavioral data did not show the expected EMOTION × LOAD interaction. Most likely this is due to a ceiling effect in performance in the low-load condition, as the error rate in the current study ($\sim 3\%$) was only half of the error rate in the earlier study ($\sim 6\%$). Crucially, this does not affect our interpretation of the fMRI effects as behavioral performance was previously shown to be independent from state anxiety and its relation to the amygdala's BOLD signal (Bishop *et al*, 2007).

Attenuation of threat responsivity, as measured with the BOLD signal, can result from reduced basal threat responding, as well as increased cognitive control over these threat responses. Our results are in line with the former as during the matching of threatening facial expressions a decrease in functional connectivity of the amygdala and hypothalamus was observed after Zembrin administration. This effect was marginally stronger when contrasted with the shape-matching control condition, whereas cortical areas showed no such main or interaction effects even when using a less stringent statistical threshold. The amygdala and hypothalamus are connected through the bed nucleus of the stria terminalis, which is heavily involved in anxiety-related behaviors (Walker *et al*, 2003), thus

arguably, this effect reflects anxiolytic actions of Zembrin on basal threat responding in this subcortical threat circuit.

Combined, these results provide support for the anxiolytic potential of dual 5-HT reuptake and PDE4 inhibitors (Harvey *et al*, 2011). This combination has previously been argued to have synergistic potential in the treatment of anxiety and depression (Cashman *et al*, 2009), with the added value that lower doses can be used to reduce side effects (Duman *et al*, 1999; Rock *et al*, 2009). Although we cannot separate these pharmacological actions in the present results, the effects seem to compare well with the literature on both the mechanisms (Harmer *et al*, 2009) (Harmer *et al*, 2009). Acute SSRI administration has been shown to increase amygdala reactivity in tasks that required explicit evaluation of threatening facial expression (Bigos *et al*, 2008) but generally reduces amygdala reactivity to unattended facial expressions of threat (Del-Ben *et al*, 2005; Murphy *et al*, 2009). The first effect is marginally supported in the present data and is usually interpreted as an effect of increased agitation and increased sensitivity for social information in general after first use of SSRIs (Anderson *et al*, 2008; Harmer *et al*, 2009), which normally disappears after long-term administration (Pringle *et al*, 2011). The second effect has been interpreted as the underlying mechanism for the anxiolytic properties of SSRIs in long-term administrations, which are often described in terms of functional coupling of prefrontal areas with the amygdala (Fisher *et al*, 2011; McCabe and Mishor, 2011; Pringle *et al*, 2011). In the present data, this is further supported as the reduction of amygdala reactivity was particularly observed in a contrast that has previously been linked to state anxiety (Bishop *et al*, 2007). Finally, although we did not find evidence for the effects on PFC–amygdala coupling in the present study, these effects might be observed in future studies of long-term Zembrin administration.

FMRI studies on PDE4 inhibitors are, to our knowledge, currently not available, but PDE4 inhibitors can be specifically linked to anxiolytic functions in the subcortical threat circuit. Although PDE4s can be found in cortical as well as subcortical areas (Korff *et al*, 2009), especially the sub-types PDE4B and PDE4D (Perez-Torres *et al*, 2000), which are particular the targets of Zembrin (Harvey *et al*, 2011), can be found in the olfactory nucleus, ventral striatum, hypothalamus, and midbrain of mice (Cherry and Davis, 1999). Moreover, PDE4B has been argued to be associated with acute effects on the noradrenergic stress system (Cherry and Davis, 1999), has been linked to panic disorder (Otowa *et al*, 2011), and the prototypical PDE4 inhibitor rolipram has been shown to affect the amygdala (Rutten *et al*, 2009; Werenicz *et al*, 2012), possibly through its enhancing effect on Fos-like immunoreactivity (Bureau *et al*, 2006). Future fMRI research should establish how these effects are reflected in amygdala reactivity to threat, but taken together these data do suggest that PDE4 inhibitors target the subcortical threat circuit, which is further supported by our results.

In sum, we show here that the dual 5-HT reuptake and PDE4 inhibitor Zembrin reduces amygdala reactivity to unattended facial fear and also decouples amygdala–hypothalamus connectivity. These results support the potential anxiolytic actions of Zembrin and provide a foundation for exploring the clinical potential of dual PDE4

and 5-HT reuptake inhibitors for the treatment of anxiety disorders and depression.

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