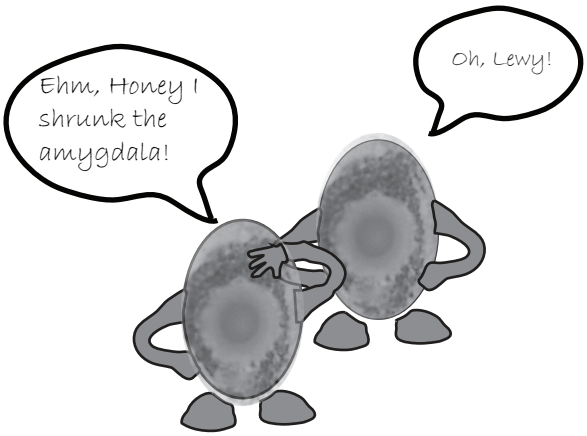

Two

A smaller amygdala is associated with anxiety in Parkinson's disease – a combined FreeSurfer-Voxel-based-morphometry study

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under review

Abstract

Up to 50 percent of all Parkinson's disease (PD) patients develop symptoms of anxiety during the course of their illness, a percentage that is much higher than in the general population. This suggests that pathological alterations associated with PD partly underlie these symptoms. So far only a limited amount of studies has been conducted on the neural correlates of anxiety in PD. The goal of the present cross-sectional study in 110 early stage PD patients (age: 64.6±10.3 years) was to investigate the association between anxiety symptoms and volume of the hippocampus and amygdala, structures that play an important role in the processing of emotional stimuli and have previously been associated with anxiety in the general population. We used both FreeSurfer and voxel-based morphometry (VBM) for the volumetric analyses. Both the FreeSurfer (β = -0.24, P=.001) and VBM (r = -0.23, P=.016) analyses showed a negative correlation between the severity of anxiety symptoms (measured with the Beck Anxiety Inventory) and volume of the left amygdala. This association was independent of the severity of motor symptoms, autonomic dysfunction and medication status and was predominantly driven by the psychological symptoms of anxiety. These results confirm previous studies in the general population showing lower left amygdalar volume in anxious patients. Whether this volume decrease constitutes a premorbid trait, a Parkinson-associated neurobiological susceptibility to anxiety or the consequence of chronic anxiety symptoms remains to be determined by future prospective longitudinal studies. Nonetheless, we tentatively hypothesize that the Parkinson pathology is responsible for the volume loss of the amygdala and the concomitant development of anxiety symptoms.

Introduction

Anxiety symptoms are very common in Parkinson's disease (PD), affecting as many as 50% of all patients (Pontone *et al.* 2009; Leentjens *et al.* 2011b) and yet surprisingly little research has been devoted to its pathophysiology. Anxiety in PD is associated with poorer appreciation of the quality of life (Quelhas and Costa 2009) and has, among others, been linked to more severe motor symptoms (Vazquez *et al.* 1993; Lauterbach *et al.* 2003), autonomic dysfunctions (Berrios *et al.* 1995; Rutten *et al.* 2014), and severity of depressive symptoms (Rutten *et al.* 2014); see (Sagna *et al.* 2014) for a review.

The severity of anxiety symptoms in PD has previously been associated with reduced striatal dopamine transporter (DaT) availability (Weintraub *et al.* 2005; Erro *et al.* 2012), although increased availability has also been reported (Moriyama *et al.* 2011; Ceravolo *et al.* 2013). Lesioning of the substantia nigra pars compacta in rodents leads to anxiety-like behavior that is reversed by dopamine agonist infusion (Carnicella *et al.* 2014; Drui *et al.* 2014), suggesting a causal role for dopamine dysfunction in the development of anxiety symptoms. Nevertheless, studies on whether dopamine replacement therapy can alleviate anxiety symptoms in PD patients have been inconsistent (Vazquez *et al.* 1993; Maricle *et al.* 1995; Richard *et al.* 1996; Lemke *et al.* 2005; Stacy *et al.* 2010) and dysfunction of other neurotransmitters, such as serotonin and noradrenalin, are probably also involved in the development of anxiety in PD.

The amygdala is critical for fear conditioning (Duvarci and Pare 2014), while the hippocampus serves an important role in the declarative memory and the encoding of contextual representations (Maren *et al.* 2013; Eichenbaum and Cohen 2014). Studies in non-PD samples have shown an important role for the hippocampus and amygdala in the pathophysiology of anxiety disorders (Etkin and Wager 2007; Holzschneider and Mulert 2011). Functional imaging studies have consistently shown hyperactivation of the amygdala during symptom provocation in patients with an anxiety disorder (Etkin and Wager 2007). Hippocampal activity is also altered, especially in patients with posttraumatic stress disorders (PTSD), but there are inconsistencies in the direction of the effect (Rauch *et al.* 2006; Thomaes *et al.* 2014). A meta-analysis of structural studies in PTSD showed that PTSD patients have smaller hippocampal and amygdalar volume than healthy controls (Karl *et al.* 2006). Reductions in hippocampal and amygdalar volume have also generally been observed in patients with panic disorder (Massana *et al.* 2003; Hayano *et al.* 2009), social anxiety (Irle *et al.* 2010) and generalized anxiety disorder (Meng *et al.* 2013; Moon *et al.* 2014), although a minority of studies reported volume increases (Schienle *et al.* 2011; Machado-de-Sousa *et al.* 2014). To the best of our knowledge, no morphological studies have so far been performed on anxiety symptoms in PD. In a previous study by our own group, however, we observed a negative correlation between depressive symptoms and bilateral hippocampal and right amygdalar volume (van Mierlo

et al. 2014). Depression and anxiety are frequently co-occuring in PD and may have partly overlapping underlying pathophysiological mechanisms (Dissanayaka *et al.* 2014). Atrophy of the bilateral amygdala was also observed in PD patients with mild depression compared with healthy controls (Surdhar *et al.* 2012). Other studies found mainly cortical differences between depressed and non-depressed PD patients (see Benoit and Robert 2011; Vriend *et al.* 2014c for reviews).

In this study we investigated the volumetric correlates of anxiety symptoms in a large sample of PD patients. Based on previous findings in non-PD patients with anxiety and in PD patients with depression we hypothesized that anxiety symptoms would be associated with reduced volume of the amygdala and hippocampus. Measuring the severity of anxiety symptoms in PD is obscured by the overlap with PD-related somatic symptoms (e.g. motor and autonomic symptoms) (Sagna *et al.* 2014). The clinimetric properties of the existing anxiety rating scales are therefore less suitable to diagnose anxiety in PD (Leentjens *et al.* 2008). To disentangle anxiety symptoms from PD-related motor impairments and autonomic dysfunctions we previously conducted a factor analysis on the Beck Anxiety Inventory (BAI) and showed that the BAI can be partitioned into an affective subscale, reflecting 'psychological' anxiety symptoms, that is unrelated to motor and autonomic failure, and four somatic subscales (Rutten *et al.* 2014). In the current study, we also correlated volume with these subscales and hypothesized that amygdalar and hippocampal volume would show the strongest association with psychological symptoms of anxiety.

Methods

Participants

Patients were consecutive cases diagnosed by a movement disorder specialist according to the UK Parkinson's disease Brain Bank criteria for idiopathic Parkinson's disease (Daniel and Lees 1993) between May 2008 and October 2012. In addition, we used MRI and clinical data from medication-naïve PD patients of a previous independent but parallel fMRI study (Vriend *et al.* 2015). All participants provided written informed consent according to the declaration of Helsinki and the study was approved by the local research ethics committee.

Clinical measures

In all patients, we used the Unified Parkinson's Disease Rating Scale motor section (UPDRS-III) (Fahn *et al.* 1987) and Hoehn and Yahr stage (Hoehn and Yahr 1967) to assess disease severity and disease stage, respectively. Severity of anxiety symptoms were assessed with the BAI (Beck *et al.* 1988) and depressive symptoms with the Beck Depression Inventory (BDI) (Beck *et al.* 1961). We measured severity of autonomic dysfunction with the Scales for Outcomes in Parkinson's disease – Autonomic (SCOPA-AUT) (Visser *et al.* 2004). Patients were

excluded if they missed more than 1/6th (16.7 percent) of the items on the BAI. Otherwise we used mean imputation to fill in the missing items. If possible, mean imputation was also applied to the BDI and SCOPA-AUT items. On these scales, however, >16.7 percent missing items did not lead to exclusion of the patient, only to a missing value on the total score of that particular scale. No imputation was used for the UPDRS-III. Further exclusion criteria were signs of dementia (Mini Mental State Examination [MMSE] ≤ 24) (Folstein *et al.* 1975) and gross brain pathology or movement artefacts visible on the MRI. Patients were also excluded if their imaging and clinical data were not acquired on the same date. Dopamine replacement therapy dosages were converted to levodopa equivalent daily dose (LEDD) as described previously (Olde Dubbelink *et al.* 2013).

BAI subscales

The previously conducted principal component analysis of the BAI resulted in five subscales, corresponding to one affective and four somatic symptom dimensions (thermoregulation, tremble, hyperventilation, and hypotension) (Rutten *et al.* 2014). The affective subscale was unrelated to the severity of motor or autonomic symptoms and we therefore considered this to be a more reliable measure of the psychological anxiety symptoms in PD than the total BAI score. Supplementary Table S2.1 lists the BAI items that belong to each BAI subscale.

Image acquisition

All imaging data were collected on a GE Signa HDxT 3T MRI scanner (General Electric, Milwaukee, U.S.) at the VU University Medical Center (Amsterdam, The Netherlands). We acquired structural images using a 3D T1-weighted sequence with an eight-channel head coil (matrix 256 x 256, field of view=25 cm, TI=450 ms, TE=3.004 ms, voxel size 1 x 0.937 x 0.937 mm, 172 slices).

FreeSurfer preprocessing

We used FreeSurfer 5.3 to automatically segment the left and right amygdala and hippocampus and calculate their volume. Automated segmentation of the amygdala and hippocampus using FreeSurfer has an accuracy comparable to manual tracing (Morey *et al.* 2009). The brain parcellation and segmentation were run using the standard "recon-all" script and all settings were left at default (Fischl *et al.* 2002; Fischl *et al.* 2004). The output was thoroughly inspected for segmentation errors. Quality checking and volume calculation was aided by scripts supplied by ENIGMA (Enhancing Neuro-Imaging Genetics Through Meta-Analysis; http://enigma.ini.usc.edu/). Intracranial volume (ICV) was also calculated to correct for inter-individual differences in total brain size. All volume measures were exported to IBM SPSS 20 (Armonk, NY, USA) for statistical analyses.

Voxel-based morphometry (VBM) preprocessing

To corroborate the results from the FreeSurfer analyses we also conducted

VBM analyses in SPM8 (Wellcome Trust Center for Neuroimaging, London, UK) using the VBM8 toolbox (http://dbm.neuro.uni-jena.de/vbm.html). Images were reoriented to the anterior/posterior commissure axis and segmented into gray matter, white matter and cerebrospinal fluid with a bias-field correction cut-off of 30 mm full width at half-maximum (FWHM). Next a group-specific template was created using high dimensional DARTEL (Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra; default settings) and flow fields were calculated that contain information on the nonlinear deformations between each subject's scan and the DARTEL template. The resulting flow fields were subsequently applied to each subject's gray matter images to warp them to Montreal Neurological Institute (MNI) space using linear affine transformation and nonlinear deformation with the parameters obtained with DARTEL. Gray matter images were modulated to preserve relative regional volumes and correct for individual differences in brain size. Finally, the segmented, modulated and normalized images were smoothed using a 10 mm FWHM Gaussian kernel. Quality checks on the segmented and normalized images, such as segmentation errors and sample homogeneity, were performed with the VBM8 tools.

Data analyses

Correlations between clinical measures, demographics and volume were analyzed with Pearson's r or Spearman's rho (rs) correlation coefficient, depending on the distribution of the variable.

Hierarchical multiple regression analyses were performed to examine the relation between BAI (sub)score and amygdalar or hippocampal volume determined with FreeSurfer. Age, gender and ICV were simultaneously added as nuisance regressors to the first block of all regression models. BAI total score was added to the second block of the model to examine the association between anxiety symptoms and volume of the four regions-of-interest (ROIs), above and beyond the effects of age, gender and ICV. A total of four models with the BAI total score was constructed with left and right amygdalar or hippocampal volume specified as the dependent variables (figure 2.1b).

For multiple regression analyses with the BAI subscales we added an interim step to limit the number of tests that had to be performed (i.e. multiple testing problem). For all four ROIs, age, gender and ICV were simultaneously added as nuisance regressors to the first block. To determine which scales had to be modelled with volume of the four ROIs, we added the score on the BDI and all five BAI subscales to the second block for a backward selection procedure. See figure 2.1 for a schematic representation of this procedure. BDI and BAI subscales were removed step-by-step from the model if their beta (β) had a $P>.05$. The model was subsequently rerun without this (sub)scale and P-values were re-evaluated. This process was repeated until the β's of all remaining scales had a $P<.05$. Because of their known influence on regional volume, age, gender and ICV were always retained in the model irrespective of their significance. Four final models (one for

each ROI) were selected by the procedure and statistically evaluated. The left amygdala regression analysis was modelled with the 'affective' subscale, the right amygdala with the 'hyperventilation' subscale and the left and right hippocampus with the 'hypotension' subscale (figure 2.1). Importantly, the backward selection procedure removed BDI score from all models. We ensured that all models met the assumptions of multiple regression analyses, including normality of the residuals and homoscedasticity. Regressors were considered significant if they fell below an alpha of P<.018. This critical value was established with SISA (http://www.quantitativeskills.com/sisa/calculations/bonhlp.htm), which uses the mean correlation between variables (ROI volumes) that are mutually correlated (mean r = 0.51, eight comparisons) for the alpha correction and allows one to perform a less stringent correction than the classical Bonferroni method for multiple comparisons.

In VBM, statistical analyses were performed in the context of the general linear model. Regression models that showed a significant association between the BAI subscale and FreeSurfer volumes were reproduced in VBM. These ROIs were based on the Automated Anatomical Labeling (AAL) atlas and created with the Wake-Forest University PickAtlas tool 3.0. BAI total score was correlated with gray matter volume of all four ROIs. Correlation coefficients were determined by drawing 3.5 mm spherical ROIs around the peak voxel of significant clusters using MarsBaR, (http://marsbar.sourceforge.net), extracting its mean gray matter estimate to correlate it with their respective BAI (sub)score in SPSS. The ICV, age and gender were added as nuisance regressors to all VBM analyses. Results were considered significant if they fell below an alpha of P<.05, Family-wise error (FWE) corrected for multiple comparisons.

Results

Demographics and clinical data

Data from 111 PD patients remained available for analyses. See the flowchart in figure 2.2 for an overview of the PD patients that met our exclusion criteria. One additional patient was excluded due to a segmentation failure in FreeSurfer. Table 2.1 lists the demographic and clinical data. Thirteen patients were using anxiolytics or mood-stabilizers. The mean BAI total score of the total sample was 12.3±8.3. BAI total (r= 0.25, P=.009), but not the affective subscale (rs= 0.12, P=.22), correlated positively with UPDRS-III. The affective subscale also showed a lower association with autonomic symptoms (rs= 0.19, P=.05) than the BAI total score (BAI total: r= 0.52, P<.001). BAI total score or subscale scores did not correlate with age or LEDD.

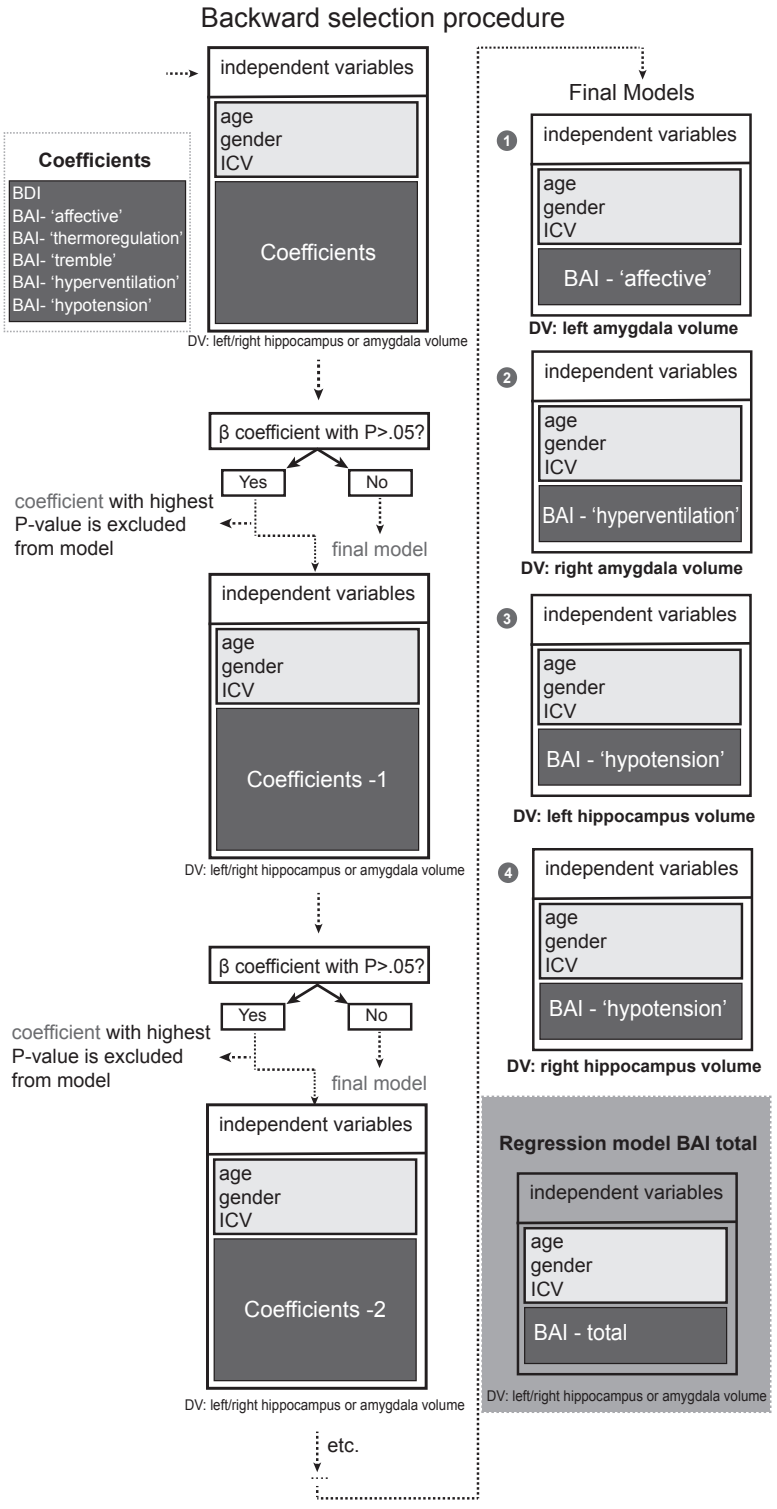
Backward selection procedure
independent variables
age
gender
ICV
Coefficients
Coefficients
BDI
BAI- 'affective'
BAI- 'thermoregulation'
BAI- 'tremble'
BAI- 'hyperventilation'
BAI- 'hypotension'
DV: left/right hippocampus or amygdala volume
β coefficient with P>.05?
Yes
No
coefficient with highest P-value is excluded from model
final model
independent variables
age
gender
ICV
Coefficients -1
DV: left/right hippocampus or amygdala volume
β coefficient with P>.05?
Yes
No
coefficient with highest P-value is excluded from model
final model
independent variables
age
gender
ICV
Coefficients -2
DV: left/right hippocampus or amygdala volume
etc.
Final Models
1
independent variables
age
gender
ICV
BAI - 'affective'
DV: left amygdala volume
2
independent variables
age
gender
ICV
BAI - 'hyperventilation'
DV: right amygdala volume
3
independent variables
age
gender
ICV
BAI - 'hypotension'
DV: left hippocampus volume
4
independent variables
age
gender
ICV
BAI - 'hypotension'
DV: right hippocampus volume
Regression model BAI total
independent variables
age
gender
ICV
BAI - total
DV: left/right hippocampus or amygdala volume

Figure 2.1 (left page) – Schematic overview of the backward stepwise regression selection procedure. Coefficients for the final models were selected by eliminating them one by one from the model according to the significance of their β (P>.05). This procedure continued until all coefficients in the model had a β with P<.05. Age, gender and ICV were always retained in the model irrespective of their significance. The selection procedure was carried out for volume of the left and right amygdala and hippocampus. See the method section for more information. (Inlay) Regression models with the BAI total as coefficient of interest and age, gender and ICV as nuisance covariates. Regression analyses were carried out for volume of the left and right amygdala and hippocampus. Abbreviations: DV = dependent variable, ICV = intracranial volume.

FreeSurfer analyses

The multiple regression analyses showed that there was no relation between BAI total score and volume in all four ROIs beyond the effects age, gender and ICV. The 'affective' subscale showed a negative association with volume of the left amygdala (β=-0.24, P=.001; see figure 2.3a) and the 'hypotension' subscale with volume of the right hippocampus (β=-0.19, P=.012; see Table 2.2). The addition of the 'affective' and 'hypotension' subscales to age, gender and ICV significantly increased the amount of explained variance by the model ('affective': ΔR2= 0.05, P=.001; 'hypotension': ΔR2= 0.04, P=.01). The 'hypotension' subscale was also associated with left hippocampal volume (β=-0.15, P=.05) and the 'hyperventilation' subscale with right amygdalar volume (β=-0.16, P=.025), but these associations fell short of our predefined statistical threshold (P<.018).

As a post-hoc analysis we also added UPDRS-III, SCOPA-AUT, LEDD or lateralization of motor symptoms to the models to exclude the possible influence of additional nuisance regressors on the association. Adding UPDRS-III, SCOPA-AUT, LEDD or lateralization of motor symptoms to the model had no effect on the association between the 'affective' subscale and left amygdalar volume (all Ps<.018). In contrast, after adding UPDRS-III score to the association between the 'hypotension' subscale and left hippocampal volume this association was no longer significant (β=-0.18, P=.03). We also performed the analyses in a subgroup of patients that did not use anxiolytics (benzodiazepines, tricyclic antidepressants, serotonin reuptake inhibitors [SSRIs]) or mood-stabilizers (lithium). Exclusion of these patients did not affect the association between the 'affective' subscale and left amygdalar volume (β=-0.21, P=.007), but did affect the association between the 'hypotension' subscale and right hippocampal volume (β=-0.17, P=.04).

VBM analyses

To confirm the above reported results we conducted the same analyses with VBM. Similar to the FreeSurfer analyses, BAI total score did not correlate with gray matter volume in the left and right hippocampus or the right amygdala. BAI total score did, however, correlate negatively with volume of the left amygdala (ke=149, PFWE =.018, r=-0.27, P=.005; see Table 2.3). In agreement with the FreeSurfer analysis, the 'affective' subscale also correlated with left amygdalar volume (ke=168, PFWE =.012, rs=-0.23, P=.016; see figure 2.3b). No correlation was observed between the 'hypotension' subscale and right hippocampal volume.

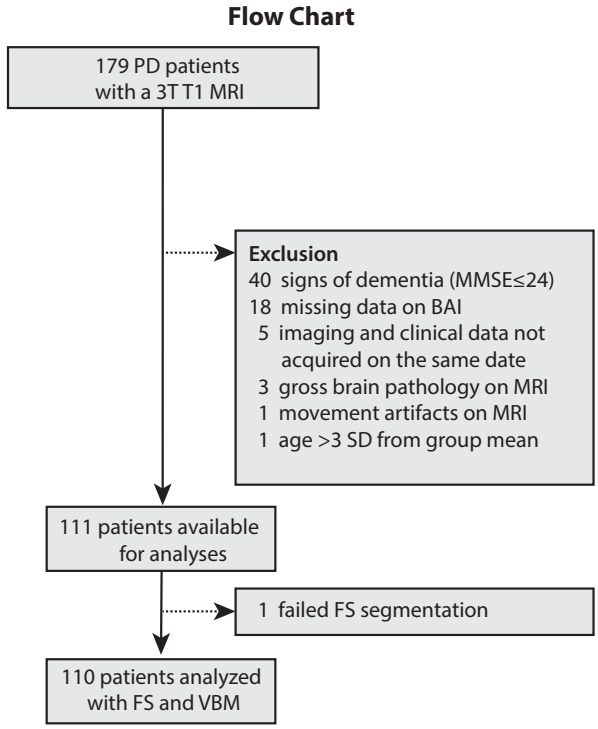

Figure 2.2 – Flow of patients included in this study. Abbreviations: FS = FreeSurfer. VBM = voxel-based morphometry.

Discussion

In this study, we showed that symptoms of anxiety in PD patients, and 'psychological' symptoms of anxiety in particular, show a negative correlation with volume of the left amygdala. This association was not affected by the severity of motor or autonomic symptoms or medication status. Correlations with hippocampal volume did not survive statistical thresholding. Our results are consistent with findings in anxious patients without PD (Massana *et al.* 2003; Karl *et al.* 2006; Hayano *et al.* 2009; Irle *et al.* 2010; Meng *et al.* 2013). The amygdala is critically involved in emotional processing and fear conditioning. Neuroimaging studies in healthy individuals have shown that pharmacologically induced fear (Eser *et al.* 2009), fearful stimuli (Hariri *et al.* 2002), and emotional faces (Fitzgerald *et al.* 2006) all elicit amygdalar activation; see (Shin and Liberzon 2010) for a review. These results are mirrored by findings on fear learning in animal models (Duvarci and Pare 2014). Anxiety disorders, such as panic disorder, generalized anxiety disorder, PTSD, social and specific phobia, all seem to be characterized by an increased reactivity of the amygdala (Etkin and Wager 2007; Shin and Liberzon 2010) and normalization of this activity correlates with symptom improvement

Table 2.1 – Sample characteristics

N patients (% male)	110 (60.0)
Age (years)	64.6 ± 10.3
UPDRS III†	24.9 ± 10.4
H&Y stage (in %)	
0	0.9
1	10.1
1.5	4.6
2	52.3
2.5	20.2
3	11.0
4	0.9
Motor lateralization (L/R/BI)†	48/52/8
Subjective disease duration (years)*	3.3 ± 3.6
MMSE	28.4 ± 1.5
DRT (yes/no)	41/69
LEDD (mg/day)#	436.4 ± 332.7
BAI	12.3 ± 8.3
BDI	10.2 ± 7.1
SCOPA-AUT	37.6 ± 9.1

All variables, except H&Y, motor lateralization and DRT, are listed as mean ± SD. † UPDRS-III scores were missing for two patients. * measured from the first moment patients reported suffering from the classical motor symptoms (rigidity, bradykinesia, tremors, etc.) . # only of patients on dopamine replacement therapy. Abbreviations: UPDRS III = Unified Parkinson's disease rating scale part III (motor section), H&Y= Hoehn & Yahr, L/R/BI = left/right/bilateral, MMSE = Mini Mental State Examination, DRT = dopamine replacement therapy, LEDD = levodopa equivalent daily dose, BAI = Beck Anxiety Inventory, BDI = Beck Depression Inventory, SCOPA-AUT: SCales for Outcomes in PArkinson's disease AUTonomic symptoms.

after treatment (Furmark *et al.* 2005; Felmingham *et al.* 2007; Goossens *et al.* 2007). No functional neuroimaging study has yet been performed on anxiety in PD patients. One study, however, showed that PD patients without anxiety and off medication show a blunted amygdala activation in response to fearful stimuli compared with healthy controls, that was partly corrected by dopamine replacement therapy (Tessitore *et al.* 2002b). Emotional blunting has also been observed in many other studies in PD patients (see Peron *et al.* 2012 for a review). How these deficits in emotional processing relate to the development of anxiety symptoms remains to be determined.

The association between psychological anxiety symptoms and amygdalar volume was lateralized to the left. This result was not due to lateralization of motor symptoms – and the presumed increased pathological load in the

contralateral hemisphere – since adding this variable to the multiple regression model had no effect on the reported result. No differences in amygdalar volume or anxiety symptoms between left, right or bilaterally affected patients occurred (data not shown). Multiple studies have shown that the processing of negative stimuli by the amygdala (Wager *et al.* 2003; Baas *et al.* 2004; Beraha *et al.* 2012) and amygdalar dysfunction in anxiety disorders is left-lateralized (Karl *et al.* 2006; Fisler *et al.* 2013; Ipser *et al.* 2013). Reports on this hemispheric asymmetry may in part be due to differences in methodology, valence of the stimuli (in the case of fMRI studies), or anatomical asymmetry (Beraha *et al.* 2012). Nevertheless, it has been proposed that the left amygdala is more involved in sustained emotional processing, while the right amygdala is more important for rapid and automatic stimulus detection (Glascher and Adolphs 2003). No information is yet available on what the neurobiological mechanism is behind decreases in left amygdalar volume, dysfunction of sustained emotional processing and the development of anxiety. This is partly because most studies on anxiety, including the present, are cross-sectional and we can therefore not draw any firm conclusions on whether decreased amygdalar volume constitutes a neurobiological susceptibility to anxiety or that amygdalar shrinkage is the consequence of chronic anxiety symptoms. The amygdala undergoes severe pathological alterations during the course of PD and may already be affected in the prodromal stages (Harding *et al.* 2002; Braak *et al.* 2003). PD pathology may lead to dysfunction and shrinkage of the amygdala and possibly with that the development of anxiety symptoms. There is some evidence from neuroimaging studies suggesting that amygdala volume is reduced in PD patients compared with matched healthy controls (Bouchard *et al.* 2008; Ibarretxe-Bilbao *et al.* 2009; Morgen *et al.* 2011) and that (surgical) damage to the amygdala is associated with the development of anxiety (Truitt *et al.* 2009; Assefa *et al.* 2012; Knutson *et al.* 2013). On the other hand, anxiety may itself cause of shrinkage of brain structures through overactivation of the hypothalamus-pituitary-adrenal axis (HPA) stress axis (Lupien *et al.* 2009). Particularly the hippocampus seems very vulnerable to the effects of stress. Nevertheless, this mechanism appears less specific for the amygdala, that actually increases in volume due to dendritic hyperthrophy in the basolateral amygdaloid nucleus in response to stress-hormones (Lupien *et al.* 2009; Coplan *et al.* 2014). This increase in volume also correlated with the severity of anxiety symptoms in an animal model (Coplan *et al.* 2014). The fact that the present study and studies in non-PD anxious patients have generally reported decreases in amygdala volume (Massana *et al.* 2003; Karl *et al.* 2006; Hayano *et al.* 2009; Irle *et al.* 2010; Meng *et al.* 2013) suggests that factors other than overactivation of the HPA axis negatively influence amygdala volume in anxious patients. Considering our findings against the background of the above reviewed studies we tentatively hypothesize that the PD pathology is responsible for the observed volume loss of the left amygdala and the concomitant development of anxiety symptoms. The validity of this hypothesis needs to be investigated by future longitudinal

studies on the directionality of PD development, amygdalar volume and anxiety symptoms.

Anxiety and depression frequently co-occur in PD and show overlap in symptoms and pathophysiology (Dissanayaka *et al.* 2014). In a previous VBM study we showed that the severity of depressive symptoms correlated negatively with volume in the bilateral hippocampus and right amygdala (van Mierlo *et al.* 2014). The cluster we observed in the left hippocampus also extended somewhat into the left amygdala. In the present study the BDI was removed from the model during the stepwise selection procedure because it explained less variance in left amygdalar volume than the affective subscale. On the other hand, given the high correlation between anxiety and depressive symptoms we cannot fully exclude the possibility that the correlation is partly confounded by the severity of depressive symptoms. The exclusion of patients with comorbid depressive symptoms could have overcome this possible limitation. Nevertheless, we argue that such a group would not be representative of the majority of PD patients with anxiety symptoms, in whom comorbidity with depression occurs frequently.

Another possible limitation stems from the fact that we used the BAI questionnaire as a measure for anxiety symptoms, rather than a formal diagnosis of an anxiety disorder. A Movement Disorder Society task force classified the BAI and all other available anxiety rating scales as 'suggested' for PD rather than 'recommended', in part because they have limitations in their construct validity (Leentjens *et al.* 2008; Leentjens *et al.* 2011b). We tried to overcome some of the shortcomings of the BAI by using an affective subscale of the BAI that, according to our previous factor analysis, is less susceptible to the severity of PD-related motor and autonomic symptoms (Rutten *et al.* 2014). A recently developed anxiety rating scale for PD, the Parkinson Anxiety Scale (PAS), showed better clinimetric properties than other frequently used anxiety scales in PD (Leentjens *et al.* 2014). This rating scale can be used in future replication studies on the structural brain correlates of anxiety symptoms in PD.

In conclusion, using two different structural brain imaging analysis techniques we demonstrated that symptoms of anxiety in PD are associated with reduced volume of the left amygdala, independent of the severity of motor and autonomic symptoms and medication status. As this was a cross-sectional study, which impedes any clear-cut conclusions on the directionality of this association, we can only speculate that the PD pathology underlies shrinkage of the amygdala and constitutes a risk factor for the development of anxiety.

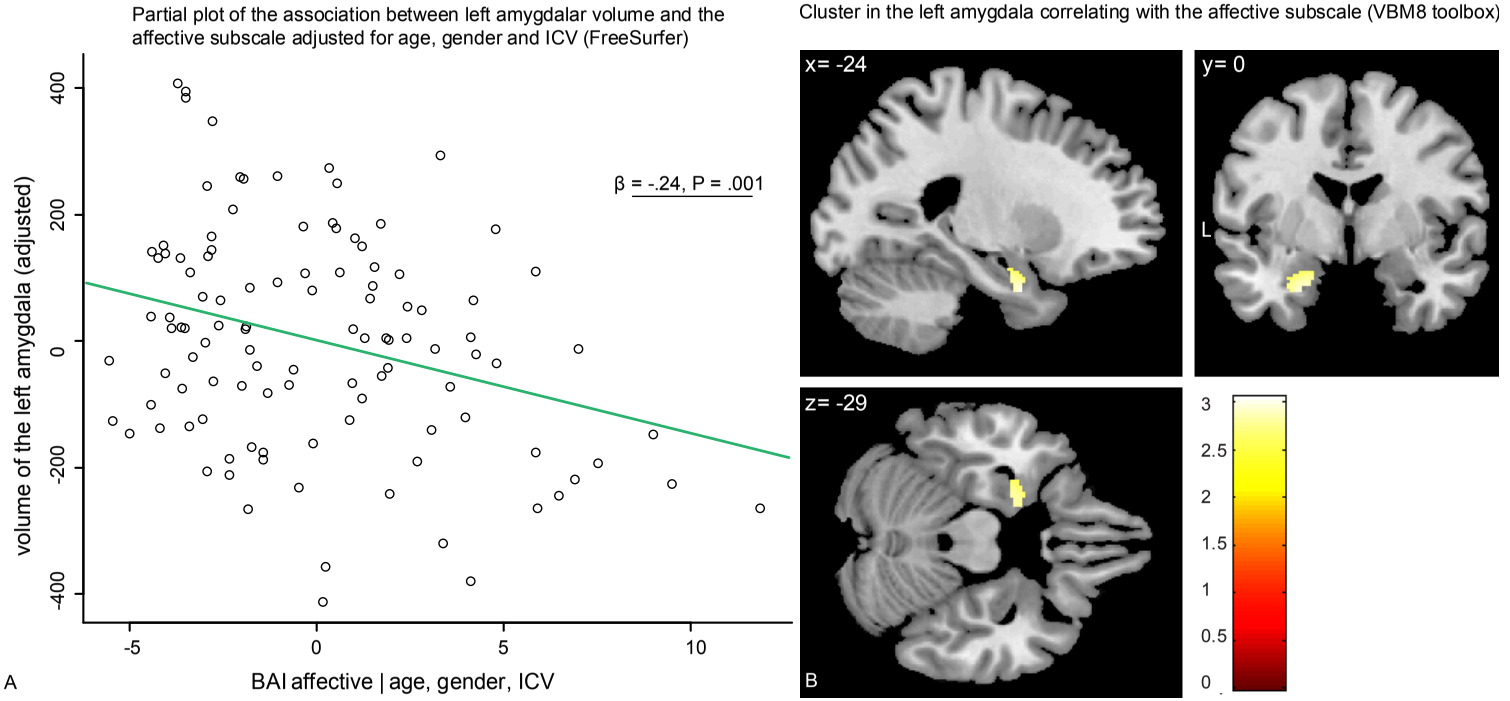

Figure 2.3 – Regression of the 'affective' subscale with volume of the left amygdala determined by FreeSurfer (a) and VBM (b). a) Partial plot of the association between volume of the left amygdala – as determined with FreeSurfer – and the 'affective' subscale while correcting for age, gender and intracranial volume (ICV). b) Cluster of gray-matter volume in the left amygdala – as determined with VBM – correlating negatively with the 'affective' subscale. The VBM analysis was also corrected for age, gender and intracranial volume.

Table 2.3 – Correlation between BAI (sub)scales and VBM volume

						MNI coordinates				
Contrast	**Anatomical region**	**L/R**	**ke**	**P_{FWE}**	**t**	**x**	**y**	**z**	**r**	**P**
-ve BAI total	Amygdala	L	149	.018	2.91	-24	0	-29	-0.27	.005
-ve affective	Amygdala	L	169	.014	3.05	-24	0	-29	-0.23	.016

Abbreviations: -ve = negative correlation, Ke= cluster size

Table 2.2 - Multiple Regression analyses on FreeSurfer volumes

	Model 1						Model 2							
	B	**95% CI**	**SE B**	**β**	**P**	**R2**	**B**	**95% CI**	**SE B**	**β**	**P**	**R2**	**Δ R2**	**Sig.**
Left Amygdala						.44						.50	.05	.001
Constant	949.0	472.6 ; 1425.3	240.3	-	<.001		1102.0	637.2 ; 1566.7	234.4		<.001			
Age	-7.9	-11.2 ; -4.7	1.7	-0.35	<.001		-8.6	-11.8 ; -5.5	1.6	-0.39	<.001			
Gender	66.2	-19.9 ; 152.4	43.5	0.14	0.13		44.1	-39.3 ; 127.6	42.1	0.10	0.30			
ICV	0.001	0.0 ; 0.001	< 0.001	0.41	<.001		0.001	0.0 ; 0.001	0.0	0.39	<.001			
'affective'							-14.7	-23.5 ; -5.9	4.5	-0.24	.001			
Right Amygdala						.49						.51	.02	.03
Constant	1083.7	506.4 ; 1661.0	291.2	-	<.001		1157.6	587.7 ;1727.5	287.4		<.001			
Age	-10.4	-14.4 ; -6.4	2.0	-0.37	<.001		-10.3	-14.2 ; -6.3	2.0	-0.36	<.001			
Gender	112.0	7.6 ; 216.5	52.7	0.19	.04		123.3	-20.4 ; 226.2	51.9	0.21	.02			
ICV	0.001	0.0 ; 0.001	<0.001	0.40	<.001		0.001	0.0 ; 0.001	<0.001	0.37	<.001			
'hyperventilation'							-56.0	-104.8 ; -7.3	24.6	-.16	.025			

	Model 1						Model 2							
	B	**95% CI**	**SE B**	**β**	**P**	**R2**	**B**	**95% CI**	**SE B**	**β**	**P**	**R2**	**Δ R2**	**Sig.**
Left Hippocampus						.38						.41	.02	.05
Constant	4256.5	3207.7 ; 5305.3	529.0		<.001		4447.6	3396.9 ; 5498.3	529.9		<.001			
Age	-23.0	-30.3 ; -15.8	3.6	-0.49	<.001		-23.4	-30.5 ; -16.3	3.6	-0.50	<.001			
Gender	109.5	-80.3 ; 299.2	95.7	0.11	0.26		95.0	-92.6 ; 282.6	94.6	0.10	.32			
ICV	0.001	<0.001 ; 0.001	<0.001	0.22	.025		0.001	0.0 ; 0.001	<0.001	0.21	.03			
'hypotension'							-34.2	-67.6 ; -0.7	16.9	-0.15	.05			
Right Hippocampus						.38						.41	.04	.01
Constant	3854.6	2734.4 ; 4974.8	565.0		<.001		4110.8	3000.8 ; 5220.7	559.7		<.001			
Age	-23.1	-30.9 ; -15.4	3.9	-.47	<.001		-23.6	-31.2 ; -16.1	3.8	-.47	<.001			
Gender	-22.0	-226.3 ; 182.3	103.0	-.02	.83		-39.9	239.5 ; 159.7	100.7	-.04	.69			
ICV	0.001	<0.001 ; 0.002	<0.001	0.33	.001		0.001	0.0 ; 0.002	<0.001	0.33	.001			
'hypotension'							-45.5	-80.9 ; -10.2	17.8	-.19	.012			

Table S2.1 – BAI items per subscale

BAI subscale	BAI item (Q)	Question
Affect	4	Unable to relax
	5	Fear of the worst
	9	Terrified/afraid
	10	Nervous
	14	Fear of losing control
	16	Fear of dying
	17	Scared
Thermoregulation	2	Feeling hot
	20	Flushed face
	21	Hot/cold sweats
Tremble	12	Trembling hands
	13	Shaky/unsteady
Hyperventilation	11	Feeling of choking
	15	Difficulty breathing
Hypotension	1	Numbness/tingling
	6	Dizzy/lightheaded
	8	Unsteady
	19	Faint/lightheaded