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Latent toxoplasmosis and psychiatric symptoms – A role of tryptophan metabolism?

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ABSTRACT

Toxoplasma gondii (TOX) is a common parasite which infects approximately one third of the human population. In recent years, it has been suggested that latent toxoplasmosis may be a risk factor for the development of mental disorders, particularly schizophrenia and anxiety. With regards to depression the results have been varied.

The main objective of this study was to examine subpopulations from the Danish PRISME and GENDEP populations for TOX IgG antibodies. These consisted of: a group with symptoms of anxiety, a group suffering from burnout syndrome, as well as two different subpopulations with depression of differing severity. The secondary objective of this study was to examine whether tryptophan metabolism was altered in TOX-positive subjects within each subpopulation.

Our results show that the anxiety and burnout populations were more likely to be TOX IgG seropositive. Furthermore, we find that the moderate-severe but not mild-moderate depressive subpopulation were associated with TOX seropositivety, suggesting a possible role of symptom severity. Additionally, we found that TOX positive subjects in the anxiety and burnout subpopulations had altered tryptophan metabolism. This relationship did not exist in the mild-moderate depressive subpopulation.

These results suggest that TOX seropositivity may be related to anxiety, burnout and potentially to severity of depression. We furthermore show that the psychiatric symptoms could be associated with an altered tryptophan metabolism.

1. Introduction

Toxoplasma gondii (TOX) is an intracellular protozoan parasite that infects warm-blooded animals including humans. It is believed that approximately one third of the world population is infected, with marked geographical differences (Montoya and Liesenfeld, 2004). The majority of horizontal transmissions to humans are caused by ingestion of tissue cysts in infected undercooked meat and oocysts in food or water contaminated with feline faeces (Tenter et al., 2000). Until recently, latent (or more accurately; 'inapparent') adult-acquired toxoplasmosis was believed to be asymptomatic in immunocompetent individuals. Nevertheless, an increasing number of studies are beginning to provide evidence that latent toxoplasmosis is associated with behavioural changes and mental disorders. Thus, subjects with latent toxoplasmosis show reduced novelty seeking behaviour (Skallova et al., 2005), a higher degree of apprehension (Novotna et al., 2005), and are more likely to be involved in traffic incidents (Flegr et al., 2002). Additionally, many studies have demonstrated that patients suffering from schizophrenia have an increased TOX seropositivity (see Torrey et al. (2007) and Gutierrez-Fernandez et al. (2015) for meta-analyses) and TOX seropositivity has also been associated with anxiety disorders (Groer et al., 2011; Markovitz et al., 2015).

With regards to depression and a potential relationship to TOX infection, the results have been discrepant: One study found TOX titer levels to correlate positively with severity of anxiety and depression in pregnant women (Groer et al., 2011), whereas a recent study

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(Alvarado-Esquivel et al., 2017) did not find any relationship to depression. Pearce and colleagues found an elevated prevalence of TOX seropositivity in patients with a history of bipolar disorder but did not find the same relationship in patients with a history of unipolar depression (Pearce et al., 2012). A recent meta-analysis did not find an association between TOX seropositivety and depression (Sutterland et al., 2015). Nevertheless, TOX positive women have shown an increased risk of self-directed violence (Pedersen et al., 2012), and several studies have also found a strong relationship between TOX infection and suicidality and suicide (Ling et al., 2011; Okusaga et al., 2011; Yagmur et al., 2010; Zhang et al., 2012).

TOX initially multiplies in almost every tissue of the body (acute phase) and then forms long-lived cysts in muscle- and liver tissue as well as nerve cells during the latent/chronic stage of the infection (Lyons et al., 2002). Infected neurons show functional abnormalities and increased cell death. Furthermore, the infection leads to subclinical neuroinflammation as seen by increased gliosis and cytokine production (Dellacasa-Lindberg et al., 2011; Schluter et al., 1997). Additionally, alterations of the kynurenine pathway have also been shown with an increased kynurenine/tryptophan (KYN/TRP) ratio and elevated kynurenic acid (KYNA) levels (Engin et al., 2012; Guidetti et al., 2006). It is likely that such alterations of the kynurenine pathway are mediated by cytokine-induced hyper-activation of indoleamine 2,3-dioxygenase (IDO) and it is well known that such hyper-activation of IDO and subsequent alterations of the KYN-pathway are likely to drive the behavioural changes (see e.g. O'Connor et al. (2009)).

As described above, the results related to TOX and a potential association with affective disorders have been varied. To help resolve this, we here investigated several subpopulations with affective symptoms of varying degree of severity or symptoms of anxiety. The aim was twofold: i) to further explore the putative association between latent toxoplasmosis and mental disorders, and ii) to examine tryptophan (TRP) metabolites in three out of four subpopulations.

2. Materials and methods

2.1. Study population

Subjects from the Danish 2007 PRISME study were included in the study of anxiety, burnout and depression. These subjects were either municipal or hospital employees within large public service workplaces in the county 'Central Denmark Region'. The participants went through a semi-structured diagnostic interview (SCAN interview (Wing et al., 1998)) followed by blood sampling. The population initially included 595 individuals. From this population, a control group (n = 156) with no affective, anxiety symptoms or alcohol addictions was extracted, as well as subpopulations consisting of subjects showing symptoms of anxiety (SCAN interview (4.001), n = 106), burnout syndrome (defined as a score of 4 or more on the Copenhagen Burnout Inventory (CBI) (Kristensen et al., 2005), n = 53) or depression (ICD-10 diagnostic criteria, obtained from the SCAN interview, n = 88). Of these, 36 suffered from mild depression (F32.0), 37 from moderate depression (F32.1) and 15 from severe depression (F32.2)). As a measure of depressive symptomatology, the symptom checklist for depression (SCL-DEP6) from the common mental disorder questionnaire (CMDQ) was extracted. This had been filled in by the subjects a few months before the clinical interview. Here, values below 3 indicate a less than 40% risk of having a clinical depression of any severity (Christensen et al., 2005). This subpopulation is referred to as Depression1. A detailed description of the PRISME samples are described in Kolstad et al. (2011). Furthermore, serum from 56 individuals from the Danish contribution to the GENDEP trial were included in the study of depression. These subjects were diagnosed with ICD-10/DSM-IV unipolar major depression of at least moderate severity (8 suffered from moderate depression, 17 from severe depression, and 31 from very severe depression according to the 17-item Hamilton Depression Rating Scale (HDRS)). Severity of depression was also evaluated using the Montgomery-Åsberg Depression Rating Scale (MADRS) (Hamilton, 1960; Montgomery and Asberg, 1979) and the Beck Depression Inventory (BDI) (Beck et al., 1961). This subpopulation is referred to as Depression2. A detailed description of the GENDEP sample is described in Uher et al. (2009). Informed consent of the participants was obtained after the nature of the procedures had been explained. The study was approved by the ethics boards in Denmark and was carried out in accordance with the Declaration of Helsinki.

2.2. Blood samples

Blood for serum samples was collected in anticoagulant-free tubes between 9 a.m. and 3 p.m., centrifuged (1550 g, 10 min, 4 °C) and stored at −80 °C until further analysis.

2.3. Toxoplasma gondii IgG ELISA

Serum samples were tested for IgG antibodies to TOX using Enzyme-Linked Immunosorbent Assay (ELISA) kits from Fitzgerald Industries International (Acton, MA, USA) following manufacturer's instructions. Briefly, 10 µl of sample serum was diluted 1:21 in sample diluent. Calibrators, positive and negative controls were included and samples were read spectrophotometrically at 450 nm. All samples were run in duplicates. A threshold optical density reading was used to determine seropositivity. An antibody index of < 0.9 was reported as negative and an antibody index of > 1.1 was considered positive.

2.4. Tryptophan metabolites

Measurements of the TRP metabolites 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxykynurenine (3-HKYN), anthralinic acid (ANA), xanthurenic acid (XT), 3-hydroxyanthranilic acid (3-HANA), quinolinic acid (QUIN), picolinic acid (PA) and nicotinamide (NTA), were performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using isotopologues as internal standards. Briefly, plasma samples were diluted by a factor 95 (v/v) in 0.2% acetic acid in aqueous solution containing internal standards. Diluted samples were then filtrated through 3 kDa Amicon Ultra filter (EMD Millipore, Billerica, MA, USA) by centrifugation (13,500 g, 60 min, 4 °C). Resultant filtrates were hereafter directly injected (20 µL) into a Waters Acquity HPLC system (Waters Corporation, Milford, MA, USA) equipped with an YMCTM ODS-AQTM 2 9100 mm, 3 µm particle column using a gradient elution (A: 0.5% formic acid in aqueous solution/B: 1% formic acid in acetonitrile). Separated metabolites were detected by a Waters Quattro Premier XE triple quadrupole mass spectrometer, Waters Corporation, Milford, MA, USA), operating in the MS/MS mode as previously described (Mazarei et al., 2013). Average concentrations were based on triplicate measures. All solvents and reagents were of HPLC grade or better with purities > 99%.

2.5. Statistical analyses

Statistical analysis was performed using SPSS 21 software. Chi-square tests were used for categorical comparisons (gender and TOX status) and independent t-tests for continuous variables (age, BMI, and psychiatric ratings). Logistic regression models were used to examine TOX status and potential confounders (gender, age, and BMI) in controls versus case groups. To examine tryptophan metabolites and potential confounding factors, regression models were applied to the anxiety, burnout and depression1 subpopulations. Within each of these subpopulations TOX positive subjects were compared with TOX status as the independent variable and tryptophan metabolites as well as age, gender and BMI as dependent variables. Variables were omitted based

Table 1
Characteristics of the populations.

Variable	Control	Anxiety	Burnout	Depression1	Depression2
	(n = 156)	(n = 106)	(n = 53)	(n = 88)	(n = 56)
Gender, n females (%)	121 (77.6)	93 (88.0)*	47 (88.7)**	73 (83.0)	41 (73.2%)
Age, mean (± SD)	45.7 (± 10.8)	45.9 (± 9.4)	45.9 (± 9.9)	45.9 (± 9.1)	40.0 (± 10.2)***
BMI, mean (± SD)	24.1 (± 4.1)	24.6 (± 5.1)	25.1 (± 5.0)	24.0 (± 6.0)	26.5 (± 5.7)**
Civil status, n single[$] (%)	38 (24.4)	32 (30.2)	18 (34.0)	29 (33.0)	16 (28.6)
Education, n long[§] (%)	113 (72.4)	81 (77.1)	36 (67.9)	68 (77.3)	n/a
SCL-DEP6, mean (± SD)	0.1 (± 0.4)	2.9 (± 1.8)***	2.7 (± 1.8)***	3.6 (± 1.6)***	n/a
MADRS, mean (± SD)	n/a	n/a	n/a	n/a	28.4 (± 4.2)
HDRS, mean (± SD)	n/a	n/a	n/a	n/a	22.7 (± 3.4)
BDI, mean (± SD)	n/a	n/a	n/a	n/a	30.2 (± 9.0)
TOX IgG status, n positive (%)	34 (21.8)	35 (33.0)*	22 (41.5)**	25 (28.4)	17 (30.4%)

Chi-square test for categorical variables; independent *t*-test for continuous variables. [$] Refers to single, divorced and widowed subjects. [§] Refers to medium-long and long higher education (> 3 years) e.g. school teacher, nurse, engineer, medical doctor, lawyer. *p < 0.05, **p < 0.01, ***p < 0.001 compared to controls. Abbreviation: n/a – not applicable.

on their p-values using step-wise exclusion until all p-values were < 0.1 or until the change in β value from the original analysis exceeded 20%. Non-normally distributed data were ln transformed. Alpha level of significance was set at p < 0.05.

3. Results

3.1. Anxiety symptoms

Table 1 shows sample characteristics for controls and clinical subpopulations. We observed significant differences between the control and anxiety group for gender and TOX status. No difference in civil status or education level was found. More females were found in the anxiety group and more anxiety subjects were TOX positive. In the current population, a significant difference in the SLC-DEP6 score was found between anxiety subjects and controls, with anxiety subjects having a significantly higher depression rating than controls.

In the logistic regression model including TOX status as well as gender, age, and BMI, we also found significant effects of TOX status and gender. Again, the results show that anxiety subjects are more likely to be TOX positive than control subjects even when correcting for potential confounding factors (Table 2a).

3.2. Burnout

When comparing the burnout subpopulation with control subjects, we observed significant differences for gender and TOX status (Table 1). Also, more females and more TOX positive subjects are found in the case group than in the control group. . No difference in civil status or education level was found. A significant difference in SCL-DEP6 is also found in this subpopulation, but according to the definition the population has less than 40% chance of suffering from clinical depression.

In the logistic regression model including TOX status as well as gender, age, and BMI as potential confounding factors we observed the same pattern; a significant difference between controls and cases is found for TOX status as well as gender. Subjects suffering from burnout are more likely to be TOX-positive (Table 2b).

Table 2a
Adjusted Odds Ratios from logistic regression analysis comparing control subjects to anxious subjects on TOX status and the potential confounders gender, age and BMI.

Variable	Adjusted Odds Ratio	95% CI	p-value
Tox status, neg vs pos	2.054	1.141–3.699	0.016
Gender, female vs male	0.360	0.171–0.757	0.007
Age	1.004	0.979–1.031	0.738
BMI	1.032	0.975–1.093	0.280

Table 2b
Adjusted Odds Ratios from logistic regression analysis comparing control subjects with burnout on TOX status and the potential confounders gender, age and BMI.

Variable	Adjusted Odds Ratio	95% CI	p-value
Tox status, neg vs pos	3.427	1.666–7.048	< 0.001
Gender, male vs female	2.843	1.059–7.629	0.038
Age	0.991	0.959–1.025	0.605
BMI	1.011	0.946–1.081	0.747

3.3. Depression1

No relationship between TOX infection and depression was found for this subpopulation (Table 1). Correcting for potential confounding factors in the logistic regression model supports this finding, i.e. that no relationship between TOX seropositivety and depression exists in this subpopulation (Table 2c).

3.4. Depression2

Sample characteristics for the moderate to severely depressed individuals (measured with MADRS, HDRS and BDI, see Table 1) compared to healthy controls are presented in Table 2d and show a marked age and BMI difference with depressed patients being younger and having a higher BMI. Without adjustment for confounding factors, no significant difference in TOX status is found.

When a regression model is created including the potential confounders, it is evident that TOX-status differ between groups; as can be seen in Table 2d moderate to severely depressed individuals are more likely to be TOX-positive than healthy controls.

Table 2c
Adjusted Odds Ratios from logistic regression analysis comparing control subjects with depression on TOX status and the potential confounders gender, age and BMI.

Variable	Adjusted Odds Ratio	95% CI	p-value
Tox status, neg vs pos	1.540	0.822–2.885	0.177
Gender, male vs female	1.536	0.766–3.081	0.227
Age	1.003	0.976–1.030	0.844
BMI	1.008	0.953–1.065	0.787

Table 2d
Adjusted Odds Ratios from logistic regression analysis comparing control subjects to moderate to severely depressed subjects on TOX status, adjusted for gender, age and BMI.

Variable	Adjusted Odds Ratio	95% CI	p-value
Tox status, neg vs pos	3.160	1.333–7.490	0.009
Gender, female vs male	1.356	0.570–3.222	0.491
Age	0.913	0.879–0.948	< 0.001
BMI	1.148	1.052–1.252	0.002

Table 3a
Stepwise exclusion regression model including tryptophan metabolites and potential confounding factors for the anxiety population comparing TOX positive to TOX negative subjects.

Variable	Adjusted Odds Ratio	95% CI	p-value
NTA	0.983	0.967–0.9999	0.049
KYN	1.004	1.00001–1.009	0.049
TRP	0.9999	0.999876–1.0	0.051
AA	0.922	0.843–1.009	0.076
Gender	2.140	1.073–4.269	0.031

3.5. Tryptophan metabolism

Tryptophan metabolites were examined for the anxiety, burnout and Depression1 subpopulations. The results after stepwise exclusion for the anxiety population comparing TOX positive vs TOX negative subjects are found in Table 3a. As can be seen, a tendency for KYN to be elevated, while NTA and TRP appears decreased (borderline significance) in the TOX positive subjects compared to TOX negative subjects.

For the burnout population, a similar tendency is found for TRP, with decreased values in TOX positive subjects, whereas 5-HTP is increased (see Table 3b).

For Depression1 no significant alterations in TRP metabolites were found (Table 3c).

4. Discussion

These current results are in agreement with the previously shown association between TOX and anxiety. Additionally, we show an association between TOX and burnout symptoms and further suggest that the relationship between TOX and depression is dependent on symptom severity. Furthermore, we suggest that these symptoms are related to alterations of TRP metabolism. We did not find any associations between quantitative levels of TOX antibodies and symptom severity

Table 3b
Stepwise exclusion regression model including kynurenine metabolites and the potential confounding factors age, gender and BMI for the burnout population comparing TOX positive to TOX negative subjects.

Variable	Adjusted Odds Ratio	95% CI	p-value
PA	0.938	0.783–1.124	0.491
QA	1.013	0.995–1.032	0.160
3-HK	0.952	0.766–1.184	0.658
5-HT	1.007	0.990–1.024	0.436
5-HTP	2.222	1.023–4.829	0.044
KYN	1.007	0.983–1.031	0.578
3-HAA	0.855	0.484–1.511	0.590
TRP	0.9997	0.9994–0.9999	0.044
XT	0.691	0.474–1.009	0.056
KYNA	1.393	0.947–2.049	0.092
5-HIAA	0.997	0.994–1.001	0.169
AA	0.944	0.775–1.150	0.567
Age	1.027	0.965–1.093	0.403
Gender	2.832	0.748–10.721	0.125

Table 3c
Stepwise exclusion regression model including kynurenine metabolites and potential confounding factors for the Depression1 population comparing TOX positive to TOX negative subjects.

Variable	Adjusted Odds Ratio	95% CI	p-value
5-HTP	1.014	0.758–1.354	0.928
KYN	1.002	0.998–1.006	0.378
5-HIAA	0.999	0.997–1.001	0.230
Age	1.054	1.021–1.089	0.001

wherefore we concentrate the discussion on examinations of TOX seropositivity.

We find that the anxiety population show an increased risk of being TOX positive compared to healthy controls. This was found in both uncorrected analyses, but also when correcting for potential confounding factors such as age and gender (Tables 1 and 2a). This corresponds well with previously published results (Groer et al., 2011; Markovitz et al., 2015).

One previous study examining the relationship between TOX seropositivity and psychiatric disease found no relationship between TOX and self-reported burnout syndrome (Flegr and Horáček, 2018). We have, to our knowledge for the first time, examined whether subjects suffering from clinically diagnosed burnout syndrome are more likely to be TOX positive than healthy controls. Our findings in both uncorrected and corrected models show that burnout subjects are indeed more likely to be TOX positive. Burnout is a condition which is associated with high levels of stress (Freudenberger, 1986) and it has previously been suggested that TOX infection leads to long-term subliminal stress (Lindova et al., 2010). In the current study we have exclusively included subjects suffering from clinically diagnosed burnout (defined as scoring 4 or more on CBI). It is likely that self-reported burnout syndrome is less severe than clinically diagnosed burnout. The findings of Flegr and Horáček (2018) could therefore be attributed to the lack of a firm clinical diagnosis explaining the discrepancy between results. One explanation for our finding could therefore be that TOX infected subjects already are exposed to subliminal stress levels and that further stressful events and factors will have an additive effect in genetically or otherwise vulnerable subjects so that these subjects are less resilient and develop clinically defined burnout syndrome.

Regarding the potential relationship between TOX and depression, previous studies have found varying results. Suvisaari et al. (2017) found that TOX IgG titer levels correlated positively with depression scores, Flegr and Escudero (2016) found that TOX positivity was associated with depression in male but not female subjects whereas Pearce et al. (2012) did not find any association between unipolar depression and TOX infection. Moreover, a recent meta-analysis concluded that no relationship between TOX and depression existed (Sutterland et al., 2015). One study found that TOX infection affected susceptibility to depression and severity of depressive symptoms in pregnant women (Nourollahpour Shiadeh et al., 2016). Another study showed that male subjects infected with *Bartonella henselae* displayed more severe depressive symptoms when co-infected with TOX (Flegr et al., 2018). Our finding could be similar; the Depression1 subpopulation consists primarily of subjects suffering from mild-moderate depression -subjects who are still able to keep a job and keep a normal life are not more likely to be TOX positive. On the other hand, psychiatric patients with primarily severe or very severe depression are indeed more likely to be infected with TOX. We suggest that genetically or otherwise vulnerable subjects, prone to develop depressive symptoms, will display more severe symptoms when TOX infected. More resilient subjects will not be affected by a TOX infection. This could explain why previous findings on this topic have been very varied and is similar to the reported potential difference between a relationship linking TOX and self-rated vs. clinically defined burnout syndrome.

Tryptophan metabolites were examined in TOX positive versus TOX

negative subjects in the anxiety, burnout, and Depression1 subpopulations. In the anxiety subpopulation KYN is elevated while NTA and TRP (borderline significance) are decreased (see Table 3a) in the TOX positive subjects. This suggests alterations in the metabolism of tryptophan. That TRP is decreased suggest an overall increase in degradation of tryptophan. The fact that KYN is elevated suggest that it is the kynurenine pathway which here is overactivated rather than an increased synthesis of 5-HT. The decreased NTA levels further suggest alterations in the metabolism, possibly with a decrease in the QUIN branch.

In the burnout population, we also see a tendency for a decrease in TRP, again suggesting that TRP metabolism is increased. Here, we furthermore find an increase in 5-HTP suggesting that in this case also the serotonin pathway is affected with a possible upregulation in this pathway. Despite being a precursor for 5-HT, early studies administering 5-HTP to psychiatric patients, did not show any effect of such treatment (Nolen, 1989; Nolen et al., 1985, 1988). Potentially in line with this, we do not show elevated levels of 5-HT, despite the increased 5-HTP levels. 5-HTP has also been shown to increase levels of cortisol, which could be related to a stress response (den Boer and Westenberg, 1990; Dinan, 1996).

In the Depression1 where no association between TOX seropositivity and symptoms were found, we did not find any differences in TRP metabolism either. This population, which is not affected by TOX and furthermore, TOX does not appear to affect TRP metabolism supports the notion that altered TRP metabolism could be related to the development of symptoms.

The conversion from TRP to KYN is mediated by the enzymes IDO and TDO. TDO is induced by corticosteroids and glucagon, whereas IDO is induced by proinflammatory cytokines (Lestage et al., 2002). It is well known that chronic TOX infection leads to microgliosis and increased production of proinflammatory cytokines. Specifically, IFN-γ, one of the main inducers of IDO, has been shown to be highly upregulated (Dellacasa-Lindberg et al., 2011; Fischer et al., 1997; Schluter et al., 1997). Interestingly, Cattaneo et al. (2013) examined cytokine gene expression in one part of the GENDEP population and show an increased expression of proinflammatory cytokines in patients compared to controls. One could therefore speculate that the same was true for this part of the GENDEP population, as TOX is known to promote the expression of proinflammatory cytokines (Henriquez et al., 2009; Schluter et al., 1997), and several studies have suggested a link between increased KYN pathway activation and psychiatric disorders (Cervenka et al., 2017; Dantzer, 2017). Additionally, Groer et al. (2011) finds a positive relationship between TOX and prenatal depression and furthermore finds elevated plasma TNF-α in TOX positive women. Together, these results point towards TOX infection leading to a low grade neuroinflammation, which in turn activates IDO and this subsequently leads to the altered TRP metabolism we find in this study. It should be noted that diet and certain medications can potentially affect levels of the kynurenine pathway.

A limitation of this study is that we did not have the opportunity to examine trypthophan metabolites in the severely depressed individuals. Furthermore, it should be noted that the control population has a slightly lower prevalence of TOX seropositivity than expected and this could potentially influence the results. We do not find any indication that socioeconomic status differs between the groups, but place of upbringing would have been interesting to include as exposure to TOX can vary based on socio-demographic factors.

To summarise, we find that TOX seropositivety is related to symptoms of anxiety, burnout, and potentially to severity of depression. This information opens up for the possibility of using TOX seropositivity as a potential biomarker for vulnerability to psychiatric disease. Moreover, we stress the importance of stratifying depression diagnoses according to severity. Lastly, we show that the behavioural alterations are associated with an altered TRP metabolism. This finding should be explored further.

Conflicts of interest

David Budac was employed at Lundbeck at the time of the experiment but now works for Psychogenics Inc. Gregers Wegener reported having received lecture/consultancy fees from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, Pfizer Inc, J&J Inc., Shire A/S, HB Pharma A/S, Arla Foods A.m.b.A., Alkermes Inc, and Mundipharma International Ltd. These companies had no role in the design and conduct of the study; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication. No other authors report potential conflicts of interest.

Role of the funding sources

The funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication.

Contributors

CBR contributed substantially to conception and design, acquisition of data, data analysis and interpretation of data. She furthermore drafted the article and gave final approval for publication. HNB contributed substantially to interpretation of data, revised the article critically and gave final approval for publication. OM contributed substantially to conception and design, revised the article critically and gave final approval for publication. AE contributed substantially to data analysis, revised the article critically and gave final approval for publication. DB contributed substantially to acquisition of data, revised the article critically and gave final approval for publication. LK contributed substantially to conception and design, revised the article critically and gave final approval for publication. GW contributed substantially to conception and design, revised the article critically and gave final approval for publication.

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