

Original Article

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In major depression, increased kappa and mu opioid receptor levels are associated with immune activation

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Abstract

Objective: This study was carried out to delineate differences between major depressive disorder (MDD) and healthy controls in dynorphin and kappa opioid receptor (KOR) levels in association with changes in the β -endorphin – mu opioid receptor (MOR) and immune-inflammatory system. **Methods:** The present study examines dynorphin, KOR, β -endorphin, MOR, interleukin (IL)-6 and IL-10 in 60 drug-free male participants with MDD and 30 age-matched healthy males. **Results:** Serum dynorphin, KOR, β -endorphin and MOR are significantly higher in MDD as compared to controls. The increases in the dynorphin/KOR system and β -endorphin/MOR system are significantly intercorrelated and are both strongly associated with increased IL-6 and IL-10 levels. Dynorphin, β -endorphin, KOR and both cytokines showed a good diagnostic performance for MDD versus controls with a bootstrapped ($n = 2000$) area under the receiver operating curve of 0.972. The dynorphin/KOR system is significantly decreased in depression with comorbid nicotine dependence. **Conclusion:** Our findings suggest that, in MDD, immune activation is associated with a simultaneous activation of dynorphin/KOR and β -endorphin/MOR signaling and that these opioid systems may participate in the pathophysiology of depression by (a) exerting immune-regulatory activities attenuating the primary immune response and (b) modulating reward responses and mood as well as emotional and behavioural responses to stress.

Significant outcomes

- Serum dynorphin and kappa opioid receptor (KOR) levels are significantly increased in depression (MDD) suggesting that dynorphin/KOR signaling is increased.
- In MDD, dynorphin/KOR and β -endorphin/mu opioid (MOR) signaling are significantly intercorrelated and associated with immune activation.
- It is hypothesised that both KOR and MOR systems participate in the pathophysiology of depression by exerting immune regulatory as well as emotional and behavioural effects.

Limitations

- It would have been more interesting if we had measured more cytokines including those of M1 macrophage, T helper (Th)-1, Th-2, Th-17 and T regulatory phenotypes.
- This study examined male subjects, and therefore our study deserves replication in females.

Introduction

Major depressive disorder (MDD) is a chronic relapsing disorder characterised by the recurrence of major depressive episodes. A recent meta-analysis reported that, from 1994 to 2014, the lifetime prevalence of depression was 10.8% (Lim *et al.*, 2018). There is now evidence that MDD is associated with changes in immune functioning and activation of immune-inflammatory pathways comprising increased production of pro-inflammatory [e.g. interleukin-6 (IL-6)] as well as immune-regulatory (e.g. IL-10) cytokines (Maes *et al.*, 1990; Al-Hakeim *et al.*, 2008; Catena-Dell'osso *et al.*, 2013; Young *et al.*, 2014; Köhler *et al.*, 2017; Al-Hakeim *et al.*, 2018a; Maes & Carvalho, 2018). The latter are part of a regulatory system that down-regulates the



primary immune-inflammatory response via multiple negative feedback systems, collectively named the 'compensatory immune regulatory system' (CIRS) (Al-Hakeim *et al.*, 2018b; Maes & Carvalho, 2018).

Aberrations in endogenous opioid peptides and their receptors may occur in MDD. The opioid system comprises different neuropeptide families including dynorphins, endorphins and enkephalins, and three receptor subtypes, namely kappa opioid receptor (KOR), mu opioid receptor (MOR) and delta opioid receptor (DOR) receptors (Snyder, 2004). β -Endorphin and enkephalins bind to MORs and DORs, while dynorphin binds predominately to KORs (Ninković, 2013). Both opioid receptors and opioid peptides are expressed throughout peripheral and central nervous systems (Le Merrer *et al.*, 2009). A growing body of research indicates that those endogenous opioids and their receptors are involved in emotional and behavioural responses to stress, regulation of mood and the pathophysiology of MDD (Al-Fadhel *et al.*, 2019; Peciña *et al.*, 2019). MDD is often accompanied by increased baseline and post-dexamethasone β -endorphin concentrations, although there are some reports that β -endorphin levels may be lowered (Facchinetti *et al.*, 1986; Maes *et al.*, 1990; Darko *et al.*, 1992; Lutz & Kieffer, 2013). In animal models, increased β -endorphin secretion is associated with depressive-like behaviours (Lutz & Kieffer, 2013). MOR is a key component of reward processing, while acute activation of MOR attenuates depressive-like behaviours in some but not all studies (Lutz & Kieffer, 2013). Recently, we reported that MOR levels were significantly higher in MDD patients than controls (Al-Fadhel *et al.*, 2019), while post-mortem studies showed increased MOR binding in suicide victims (Escriba *et al.*, 2004). Positron emission tomography, on the other hand, showed contradictory results with increased binding potential for [11 C]-carfentanil during depression but decreased binding potential in women with MDD (Pecina & Zubieta, 2018). Subjects with a lower expression of MORs and a reduced capacity to release endogenous opioids may show increased vulnerability to develop depressive symptoms and show a poor treatment response to selective serotonin reuptake inhibitors (SSRIs) (Kennedy *et al.*, 2006; Pecina *et al.*, 2015a). In addition, reduced baseline MOR availability in the nucleus accumbens predicts a lack of response to SSRIs (Pecina *et al.*, 2015b). A variety of stressors may increase KOR signaling, which is involved in stress-induced alterations in brain reward systems and associated despair- and depression-like responses and depressive disorders (Carlezon & Thomas, 2009; Bruchas *et al.*, 2010; Knoll & Carlezon, 2010; Lutz & Kieffer, 2013; Bailey & Husbands, 2018). Consequently, KOR signaling is sometimes regarded as an 'anti-reward dysphoric system', which once activated may increase risk towards MDD (Lalanne *et al.*, 2014; Taylor & Manzella, 2016). The overall conclusion from animal models supports the idea that the KOR system may underpin aspects of sadness and dysphoria but that the cause of the opioid alterations in MDD remains inconclusive (Nummenmaa & Tuominen, 2018). Nevertheless, in a pilot study, no differences in KOR binding (using [11 C]GR103545) could be found between MDD patients and controls (Miller *et al.*, 2018), while prodynorphin mRNA was significantly lower in MDD and bipolar depression as compared with controls (Hurd, 2002). However, there is a lack of KOR measurements in the literature, which in part may be explained by limitations in techniques to measure KOR and inadequate knowledge about the exact source of KOR in plasma (Suzuki *et al.*, 2001; Rittner *et al.*, 2008). There is now evidence that the immune and opioid systems show functional relationships. Monocytes, macrophages, granulocytes,

and T and B-lymphocytes express opioid peptides, including β -endorphin and pro-opiomelanocortin (POMC) and all POMC processing enzymes (Mousa *et al.*, 2004; Pomorska *et al.*, 2014). Circulating leukocytes may produce opioid peptides and migrate into inflamed tissues where they exert immune stimulatory and regulatory activities (Rittner *et al.*, 2005; Pomorska *et al.*, 2014). Lymphocyte functions involved in the host defense are modulated by β -endorphin and dynorphin (Bidlack *et al.*, 2006). Associations between both systems are also detected in patients with MDD. For example, lowered cell-mediated hypersensitivity and natural killer activity are significantly associated with increased β -endorphin levels in depression (Maes *et al.*, 1991b; Pomorska *et al.*, 2014). Castilla *et al.* (1992) reported an increased opioid tone in depression and a concomitant suppression of monocytic functions. Recently, we reported significant associations between MOR levels and IL-10 in patients with major depression (Al-Fadhel *et al.*, 2019). Nevertheless, the associations between immune activation and the KOR/dynorphin system were not studied in patients with MDD.

There is also a strong comorbidity between MDD and nicotine dependence (ND) with increased inflammatory potential and affiliated nitro-oxidative pathways in comorbid MDD and ND (Nunes *et al.*, 2013; Nunes *et al.*, 2015a,b). Nicotine has a biphasic effect on the opioid system with anti-opioid effects at lower doses, while nicotine administration may entrain opioid activity 'during the acquisition and re-acquisition of nicotine self-administration' (Pomerleau, 1998). Nevertheless, the effects of comorbid MDD and ND on the KOR system have not been examined.

Hence, the present study was carried out to delineate (a) KOR/dynorphin system in association with MOR/endorphin system and serum IL-6 and IL-10 levels in MDD patients as compared to healthy controls and (b) examine the effects of comorbid MDD and ND on these associations.

Subjects and methods

Participants

The present study examined 60 drug-free MDD males and 30 age-matched healthy males. Participants were recruited at 'The Psychiatry Unit', Al-Hakeem General Hospital and at a private psychiatric clinic, Najaf Governorate, Iraq during the period of January–July 2017. This study is a first report on KOR and dynorphin values in this sample. These biomarkers were measured in the same study group on which we published MOR, β -endorphin, IL-6 and IL-10 data (Al-Fadhel *et al.*, 2019), except that one patient and one control were substituted by two new cases with complete dynorphin/KOR measurements. The 4th edition of Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2000) was used to make the diagnosis of MDD. The 24-item Hamilton Depression Rating Scale (HDRS) was employed 1 or 2 days before blood was drawn to measure severity of depression. We included only patients who showed a HDRS score of more than 21. Patients were divided into those with and without ND, and all ND patients showed heavy nicotine use of more than 20 cigarettes/day.

We completed a full medical history using a semi-structured interview. Exclusion criteria for patients and controls were immune disorders including diabetes mellitus, autoimmune disorders, inflammatory bowel disorder, psoriasis, chronic obstructive pulmonary diseases (COPD) and chronic kidney disease. Excluded were also subjects with neurodegenerative/neuroinflammatory diseases including stroke, multiple sclerosis, and Alzheimer's and

Parkinson's diseases. Only drug-free patients were included in this study. MDD patients were excluded when they showed other-axis I diagnosis including bipolar disorder, substance abuse, psycho-organic disorders and schizophrenia. Serum C-reactive protein (CRP) levels were lower than 6 mg/l, thereby excluding subjects with overt inflammation. Written informed consent was obtained from all participants, according to the guidelines laid down in the current version of the Declaration of Helsinki, after approval from the ethics committee (IRB) of the College of Science, University of Kufa, Iraq (229-1/2017).

Methods

Five milliliters of venous blood samples was drawn, utilising disposable needle and plastic syringes, from patients and controls. The samples were transferred into a clean plain tube. Blood was left at room temperature for 15 min for clotting, centrifuged 3000 rpm for 10 min and then serum was separated and transported into two Eppendorf tubes to be stored at -80°C until thawed for assay. Serum CRP was measured using a kit supplied by Spinreact[®], Girona, Spain. This test is based on the principle of the latex agglutination. Commercial ELISA kits were used to measure KOR (Human Kappa-Type Opioid ELISA Kit) and MOR (Human Mu-Type Opioid Receptor ELISA Kit) (MyBioSource, San Diego CA, USA). The sensitivity of the KOR and MOR assays were 0.019 and 0.029 ng/ml, respectively, and the detection ranges were 0.05–15 ng/ml and 0.05–30 ng/ml, respectively. MyBioSource specifies that 'no significant cross-reactivity or interference between human OPRM1 and analogues was observed'. Dynorphin was assayed using the Human Dynorphin ELISA Kit supplied by (Cusabio Co, Wuhan, China) and the β -Endorphin was assayed by Human β -Endorphin ELISA Kit supplied by (Melsin Medical Co., Ltd Changchun, China) which show a sensitivity of 7.8 and 0.1 pg/ml, respectively. IL-6 and IL-10 were assayed using the Human Interleukin-6 and Interleukin-10 ELISA Kits (Cusabio Co) which have a sensitivity of 2.4 and 3.1 pg/ml, respectively. We followed all procedures according to the manufacturer's instructions. The intra-assay coefficients of variation (precision within-assay) were <8.0% for all analytes.

Statistical analysis

Differences in scale variables between diagnostic groups were examined using the analysis of variance. Associations between nominal variables were assessed using the analysis of contingency tables (χ^2 test). We used Pearson's product moment correlation coefficients to check associations between scale variables. Multivariate general linear model (GLM) analysis was used to assess the effects of diagnosis (MDD with and without ND versus controls) as primary explanatory variable, while adjusting for extraneous variables (age and BMI). Tests for between-subject effects were employed to assess the effects of significant explanatory variables on biomarkers. Multiple post-hoc comparisons among treatment means were assessed using protected least significant difference (LSD) values. Differences in the biomarkers among classes are displayed as mean (SE) values computed on their z scores. Binary logistic regression analysis was employed to delineate the most important predictors of MDD versus controls, with computation of odds ratios and 95% confidence intervals. We computed the area under the curve (AUC) to determine the diagnostic accuracy of the biomarkers as well as the bootstrapped (2000 bootstraps) AUC values. An optimal diagnostic tool will have an

AUC of 1, which indicates 100% sensitivity and specificity (Bewick *et al.*, 2004). Tests were two-tailed and a *p*-value of 0.05 was used for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25, 2017.

Based on the measurements of IL-6, IL-10, β -endorphin, dynorphin, KOR and MOR, we computed z unit weighted composite scores as follows:

DYN-KOR: index of KOR signaling computed as z transformation of dynorphin (zDYN) + zKOR.

DYN-END: integrated index of circulating opioid peptides, computed as zDYN + z β -endorphin.

KOR-MOR: index of opioid receptor status computed as zKOR + zMOR.

IL6-IL10: index of immune activation computed as zIL-6 + zIL-10.

Results

Descriptive statistics

Table 1 shows the socio-demographic data as well as the raw values of the biomarkers used in this study. Patients were divided into those with (MDD + ND) and without ND (MDD). There were no significant differences in age, BMI and urban/rural ratio between the three study groups. There were somewhat more married subjects in MDD + ND than in controls, while there were more unemployed people in both depressed subgroups than in controls. This table also shows the raw measurements (not adjusted for extraneous variables) of the different biomarkers. Figure 1 shows the differences in biomarker profile between the three study groups. Shown are the group mean values (\pm SE) after z transformations were made. The Beck depression inventory (BDI-II) score was not significantly different between both MDD subgroups. In the MDD group, there was no significant correlation between the BDI score and any of the biomarkers.

Table 2 shows the intercorrelation matrix (obtained using partial correlation coefficients after adjusting for age, BMI and ND) between different biomarkers in the 90 subjects included in this study. Dynorphin (DYN) was significantly correlated with β -endorphin (END), MOR, KOR-MOR and IL6-IL10. KOR was significantly associated with END, MOR, DYN-END and IL6-IL10. DYN-KOR was significantly correlated with END, MOR and IL6-IL10. END levels were significantly correlated with MOR, KOR-MOR and IL6-IL10. DYN-END was significantly associated with KOR-MOR and IL6-IL10, while KOR-MOR was positively associated with IL6-IL10. Figure 2 shows the association between KOR and IL6-IL10, while Fig. 3 shows the correlation between DYN-END and IL6-IL10. Figure 4 shows the association between KOR-MOR and IL6-IL10. These correlations in the figures were not adjusted for covariates.

Biomarker differences between controls and patients

In order to examine differences in the 10 biomarkers between the diagnostic groups, we have carried out multivariate GLM analysis while adjusting for age and BMI. Table 3 shows the outcome of the multivariate as well as univariate (tests for between-subject effects) GLM analyses. The biomarkers entered in this analysis were DYN, END, KOR, MOR, IL-6 and IL-10 levels as well as z unit weighted composite scores, namely DYN-KOR, DYN-END, KOR-MOR and IL6-IL10. We found a highly significant effect of diagnosis with an effect size of 0.483, while age and BMI were not significant. There were highly significant associations between all 10

Table 1. Socio-demographic, clinical and biomarker data in major depressed patients with nicotine dependence (MDD + ND), patients without ND (MDD) and healthy controls (HC)

Variables	HC ^A	MDD ^B	MDD + ND ^C	F/ χ^2	df	p
	n = 30	n = 35	n = 25			
Age (years)	30.3 (8.8)	31.1 (11.8)	33.8 (9.9)	0.82	2/87	0.443
BMI (kg/m ²)	26.2 (2.8)	24.2 (3.6)	25.3 (3.8)	2.60	2/87	0.080
Single/Married	16/14 ^C	14/21	5/20 ^A	6.41	2	0.041
Urban/Rural	5/25	7/28	8/17	2.02	2	0.364
Employment (N/Y)	4/26 ^{BC}	16/19 ^A	11/14 ^A	8.90	2	0.012
Dynorphin (pg/ml)	17.04 (2.55) ^B	18.83 (1.86) ^{AC}	17.50 (1.79) ^B	6.35	2/87	0.003
KOR (ng/ml)	8.32 (1.71) ^{BC}	11.99 (3.99) ^{AC}	10.02 (1.81) ^{AB}	13.46	2/87	<0.001
END (pg/ml)	24.90 (5.27) ^{BC}	34.95 (4.02) ^A	33.02 (2.75) ^A	49.94	2/87	<0.001
MOR (ng/ml)	3.01 (0.83) ^{BC}	4.43 (1.49) ^A	4.40 (1.24) ^A	12.97	2/87	<0.001
IL-6 (pg/ml)	11.4 (2.8) ^{BC}	16.2 (3.7) ^A	16.7 (4.3) ^A	18.78	2/87	<0.001
IL-10 (pg/ml)	6.0 (1.7) ^{BC}	8.9 (2.2) ^A	8.9 (2.0) ^A	21.78	2/87	<0.001
BDI-II	–	48.3 (11.0)	50.9 (8.5)	0.96	1/58	0.336

BMI, body mass index; KOR, kappa opioid receptor; END, β -endorphins; MOR, mu opioid receptor; IL, interleukin; BDI-II, Beck depression inventory.

^{A,B,C}Results of pairwise comparisons between group means.

Results are shown as mean (\pm SD).

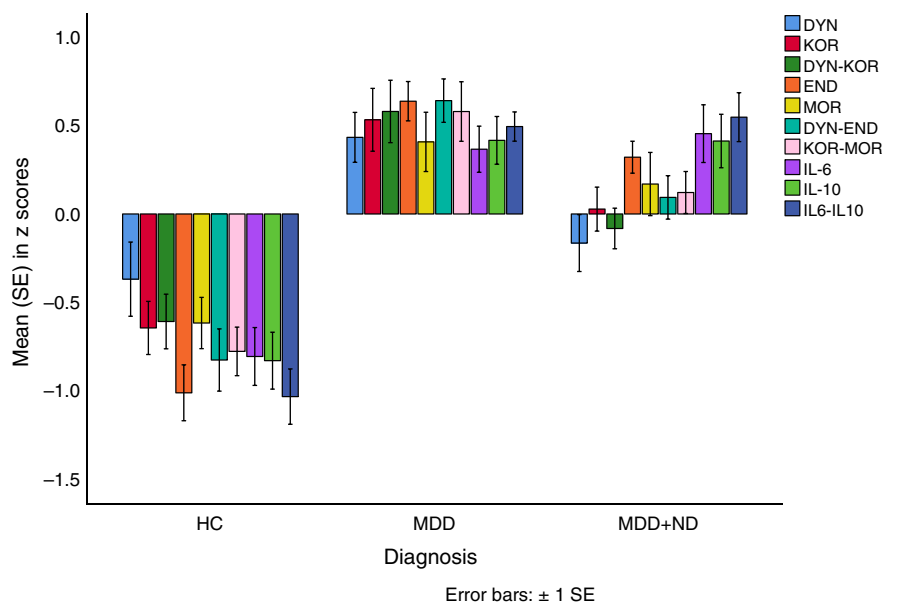


Fig. 1. Differences in biomarker profile between controls (HC) and major depressed patients with (MDD + ND) and without (MDD) nicotine dependence. Shown are the group mean values (\pm SE) after z transformations were made. DYN, dynorphin; KOR, kappa opioid receptor; DYN-KOR, index of KOR signaling computed as z transformation of dynorphin (z DYN) + z KOR; END, β -endorphin; MOR, mu opioid receptor; DYN-END, integrated index of circulating opioid peptides, computed as z DYN + z END; KOR-MOR, index of opioid receptor status computed as z KOR + z MOR; IL, interleukin; IL6-IL10, index of immune activation computed as z interleukin-6 (z IL6) + z IL10.

biomarkers and diagnosis, with the strongest associations between diagnosis and IL6-IL10, END, DYN-END, IL-10 and KOR-MOR (with effect sizes > 0.300). Table 4 shows the model-generated estimated marginal mean values (in z scores) obtained by the GLM analyses shown in Table 3. As such, the z transformed biomarker data were adjusted for age and BMI and the differences observed between the study groups may be expressed in standard deviations. Pairwise multiple post-hoc analyses showed that DYN was significantly higher in MDD as compared with controls and MDD + ND. KOR, DYN-KOR and DYN-END were significantly different between the three study groups and increased from controls \rightarrow MDD + ND \rightarrow MDD. END, MOR, KOR-MOR, IL-6, IL-10 and IL6-IL10 were significantly higher in both MDD groups than in controls.

In order to delineate the most important biomarkers predicting MDD as well their odds ratios, we performed binary logistic regression analysis. Table 5 shows the outcome of a stepwise binary logistic regression analyses with MDD (versus controls) as dependent variable. IL6-IL10 and DYN-END were the best predictors of MDD ($\chi^2 = 77.16$, $df = 2$, $p < 0.001$) with an effect size of 0.800 (Nagelkerke value).

Table 6 shows the results of receiver operating characteristic (ROC) analyses discriminating MDD from controls. The AUC ROC curves for DYN, KOR, DYN-KOR and DYN-END were computed on non-smoking MDD patients versus controls (because ND has an effect on these biomarkers). The other biomarkers were examined in all MDD patients versus controls. In the same table, we also show the bootstrapped AUC values after

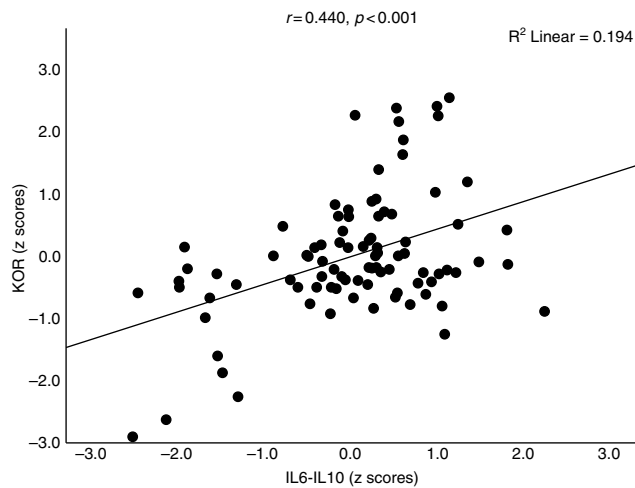
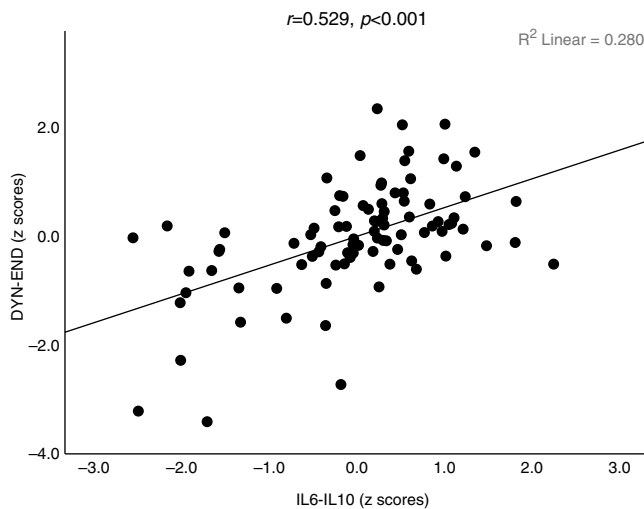
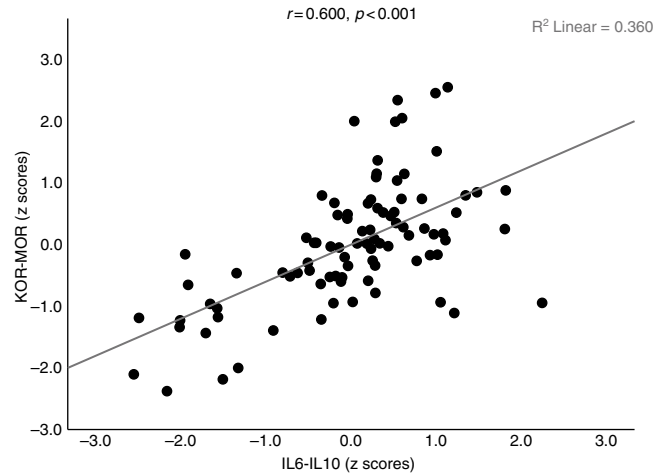
Table 2. Inter-correlation matrix among biomarkers data

Variables	DYN	KOR	DYN-KOR	END	MOR	DYN-END	KOR-MOR
KOR	0.390						
DYN-KOR	0.833	0.834					
END	0.433	0.436	0.522				
MOR	0.405	0.294	0.419	0.365*			
DYN-END	0.850	0.488	0.802	0.843	0.455		
KOR-MOR	0.494	0.819	0.788	0.499	0.789	0.587	
IL6-IL10	0.382	0.460	0.505	0.539	0.550	0.543	0.626

KOR, kappa opioid receptor; DYN-KOR, index of KOR signaling computed as z transformation of dynorphin (zDYN) + zKOR; END, β -endorphin; MOR, mu opioid receptor; DYN-END, integrated index of circulating opioid peptides, computed as zDYN + zEND; KOR-MOR, index of opioid receptor status computed as zKOR + zMOR; IL6-IL10, index of immune activation computed as z interleukin-6 (zIL6) + zIL10.

All $p < 0.001$, except *: $p < 0.01$ ($n = 90$).

Shown are the partial correlation coefficients after adjusting for age, body mass index and nicotine dependence.

**Fig. 2.** Association between kappa opioid receptor (KOR) levels and immune activation as indicated by a z unit weighted composite (IL6-IL10) score based on interleukin (IL)-6 and IL-10 assays.**Fig. 3.** Association between opioid peptides as indicated by a z unit weighted composite score based on dynorphin (DYN) and β -endorphin (END) assays (KOR-MOR) and immune activation as indicated by a z unit weighted composite score based on interleukin (IL)-6 and IL-10 assays (IL6-IL10).**Fig. 4.** Association between opioid receptor levels as indicated by a z unit weighted composite score based on kappa (KOR) and mu (MOR) opioid receptor levels (KOR-MOR) and immune activation as indicated by a z unit weighted composite score based on interleukin (IL)-6 and IL-10 assays (IL6-IL10).

2000 bootstraps. We found that DYN-END, END, KOR-MOR and IL6-IL10 yielded very high (bootstrapped) AUC (all > 0.849) separating MDD from controls. The best bootstrapped AUC was delineated for the combination of IL6-IL10 and DYN-END (0.972).

Discussion

The first major finding of this study is that serum dynorphin and KOR were significantly increased in MDD as compared to controls and that increases in those opioid peptides and their receptors are interrelated, suggesting that dynorphin/KOR signaling is significantly increased in MDD. However, two previous clinical studies were unable to observe signs of increased dynorphin/KOR signaling in depression (see Introduction: Hurd, 2002; Miller *et al.*, 2018). As described in the Introduction, increased KOR signaling results in anti-reward and dysphoric effects, thereby contributing to depressive behaviours (Carlezon & Thomas, 2009; Bruchas *et al.*, 2010; Knoll & Carlezon, 2010; Lutz & Kieffer, 2013; Lalanne *et al.*, 2014; Taylor & Manzella, 2016; Bailey & Husbands, 2018). Increased KOR signaling in limbic brain regions as a consequence of acute stress may induce many behavioural and pathophysiological alterations while release of dynorphin may occur secondary to stress or may cause stress hormone release (Bilkei-Gorzo *et al.*, 2008; Land *et al.*, 2008). Dynorphin-mediated KOR activation may mediate the acute effects of stress while KOR activation may decrease neuronal activity (Knoll & Carlezon, 2010). Excitatory inputs are negatively regulated by dendritic dynorphin release in the hypothalamus and hippocampus via activation of presynaptic KORs (Iremonger & Bains, 2009) and this mechanism may generalise to neuronal circuits that are involved in mood and motivation (Reyes *et al.*, 2009). Moreover, KORs may also regulate serotonergic (Bilkei-Gorzo *et al.*, 2008) and noradrenergic (Reyes *et al.*, 2007) systems, which may play a role in MDD. In animal models of the forced swim test, blockade of KORs decreases the immobility time (Mague *et al.*, 2003). In stress conditions, KOR signaling elevates physical capacity (through analgesia) and the motivation to escape threat (through increasing aversion) thereby facilitating adaptive responses (Knoll & Carlezon, 2010).

The results of the current study on dynorphin and KOR are in agreement with findings that β -endorphin and MOR are

Table 3. Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variable while adjusting for extraneous variables

Tests	Dependent variables	Explanatory variables	F	df	P	Partial η^2
Multivariate	10 Biomarkers	Diagnosis	12.37	12/160	<0.001	0.481
		Age	2.01	6/80	0.074	0.131
		BMI	1.51	6/80	0.185	0.102
Between-subject effects	DYN	Diagnosis	7.46	2/85	0.001	0.149
	KOR	Diagnosis	15.13	2/85	<0.001	0.263
	DYN-KOR	Diagnosis	16.99	2/85	<0.001	0.286
	END	Diagnosis	50.50	2/85	<0.001	0.543
	MOR	Diagnosis	11.28	2/85	<0.001	0.210
	DYN-END	Diagnosis	31.10	2/85	<0.001	0.423
	KOR-MOR	Diagnosis	23.71	2/85	<0.001	0.358
	IL6	Diagnosis	20.98	2/85	<0.001	0.330
	IL10	Diagnosis	23.32	2/85	<0.001	0.354
	IL6-IL10	Diagnosis	51.30	2/85	<0.001	0.547

Diagnosis, healthy controls versus depression with and without nicotine dependence; DYN, dynorphin; KOR, kappa opioid receptor; DYN-KOR, index of KOR signaling computed as z transformation of dynorphin (zDYN) + zKOR; END, β -endorphin; MOR, mu opioid receptor; DYN-END, integrated index of circulating opioid peptides, computed as zDYN + zEND; KOR-MOR, index of opioid receptor status computed as zKOR + zMOR; IL6-IL10, index of immune activation computed as z interleukin-6 (zIL6) + zIL10.

Table 4. Model-generated estimated marginal means of the 10 biomarkers obtained by GLM analysis shown in Table 2 in controls (HC) and major depressed (MDD) patients, divided into those with (MDD + ND) and without (MDD) nicotine dependence

Variables	HC ^A	MDD ^B	MDD + ND ^C
	n = 30	n = 35	n = 25
DYN	-0.438(0.175) ^B	0.472(0.161) ^{A,C}	-0.134(0.189) ^B
KOR	-0.689(0.163) ^{B,C}	0.548(0.150) ^{A,C}	0.059(0.176) ^{A,B}
DYN-KOR	-0.677(0.161) ^{B,C}	0.612(0.147) ^{A,C}	-0.045(0.173) ^{A,B}
END	-1.052(0.128) ^{B,C}	0.653(0.118) ^A	0.349(0.138) ^A
MOR	-0.622(0.160) ^{B,C}	0.369(0.146) ^A	0.231(0.172) ^A
DYN-END	-0.892(0.145) ^{B,C}	0.673(0.133) ^{A,C}	0.129(0.156) ^{A,C}
KOR-MOR	-0.808(0.147) ^{B,C}	0.565(0.135) ^A	0.178(0.158) ^A
IL6	-0.834(0.156) ^{B,C}	0.388(0.143) ^A	0.457(0.168) ^A
IL10	-0.866(0.153) ^{B,C}	0.435(0.141) ^A	0.430(0.165) ^A
IL6-IL10	-1.074(0.128) ^{B,C}	0.520(0.118) ^A	0.560(0.138) ^A

DYN, dynorphin; KOR, kappa opioid receptor; DYN-KOR, index of KOR signaling computed as z transformation of dynorphin (zDYN) + zKOR; END, β -endorphin; MOR, mu opioid receptor; DYN-END, integrated index of circulating opioid peptides, computed as zDYN + zEND; KOR-MOR, index of opioid receptor status computed as zKOR + zMOR; IL6-IL10, index of immune activation computed as z interleukin-6 (zIL6) + zIL10.

^{A,B,C}Results of pairwise comparisons between group means.

All results are shown as mean (SE) and as z scores.

upregulated in MDD and that the increases in β -endorphin and MOR are significantly intercorrelated, indicating increased β -endorphin/MOR signaling in MDD (Al-Fadhel et al., 2019). Previously, one paper showed increased expression of MOR in suicide victims, whereas positron emission tomography (PET) scan studies showed controversial results on MOR binding profiles (see Introduction). As reviewed in the Introduction, there are also some studies reporting lowered β -endorphin levels and suggesting lowered MOR signaling in MDD. These contradictory results may

Table 5. Results of binary logistic regression analysis with major depression (controls as reference group) as dependent variable

Variable	B	SE	W	df	p	OR	95% CI
IL-6-IL-10	3.784	1.195	10.02	1	0.002	44.01	4.23–458.17
DYN-END	2.000	0.916	4.77	1	0.029	7.39	1.23–44.49

OR, Odds ratio with 95% confidence intervals; IL6-IL10, computed as z (z interleukin-6 (zIL6) + zIL10); DYN-END, computed as z (z dynorphin (zDYN) + z β -endorphin (zEND)).

be explained by differences in the source (type of tissue or cells) used to measure these receptors (Stein et al., 2003; Rittner et al., 2008). Interestingly, MOR activation may elevate mood and therefore may improve depressive states (Shippenberg et al., 2008). All in all, the results of different studies indicate that the opioid system as a whole is dysregulated in MDD with an upregulated dynorphin/KOR system and both up- and downregulation of the β -endorphin/MOR system. A plausible reason for the disparity of reported changes in β -endorphin levels in blood is the use of different inclusion criteria for patients and the heterogeneity of MDD.

The second major finding of this study is that the indices of dynorphin/KOR and β -endorphin and MOR levels are significantly intercorrelated and are strongly associated with immune inflammatory response system (IRS)/CIRS activation. Heightened IRS and CIRS responses are commonly observed in patients with MDD, with increased levels of immune-inflammatory biomarkers and immune-regulatory compounds (Krishnadas & Cavanagh, 2012; Al-Hakeim et al., 2015; Haapakoski et al., 2015; Köhler et al., 2017), including serum IL-6 and IL-10 (Köhler et al., 2017; Maes & Carvalho, 2018; Al-Fadhel et al., 2019). Moreover, we observed that these opioid and IRS/CIRS responses yielded a good diagnostic performance for MDD with a bootstrapped ($n = 2000$) AUC of 0.972 (both opioid peptides combined with both cytokines). In this respect, it is interesting to note that classical neurotransmitters transduce information between neurons while cytokines and neuropeptides coordinate neuronal circuits or networks (Ludwig & Leng, 2006;

Table 6. Results of ROC

Variables	Area ROC	SE	<i>p</i>	95% CI	Bootstrapped AUC	
					Boot-AUC	95%CI
DYN*	0.651	0.069	0.036	0.517–0.786	0.664	0.523–0.785
KOR*	0.805	0.053	<0.001	0.702–0.909	0.786	0.670–0.882
DYN-KOR*	0.787	0.056	<0.001	0.677–0.896	0.786	0.677–0.877
DYN-END*	0.936	0.936	<0.001	0.883–0.990	0.922	0.852–0.971
END	0.952	0.020	<0.001	0.913–0.990	0.944	0.900–0.978
MOR	0.755	0.053	<0.001	0.652–0.858	0.742	0.633–0.897
KOR-MOR	0.851	0.039	<0.001	0.774–0.928	0.850	0.759–0.911
IL6-IL10	0.947	0.023	<0.001	0.902–0.993	0.931	0.877–0.978
IL6-IL10 and DYN-END	0.972	0.014	<0.001	0.944–1.000	0.972	0.932–0.994

ROC, receiver operating characteristic; DYN, dynorphin; KOR, kappa opioid receptor; DYN-KOR, index of KOR signaling computed as z transformation of dynorphin (zDYN) + zKOR; END, β -endorphin; MOR, μ opioid receptor; DYN-END, integrated index of circulating opioid peptides, computed as zDYN + zEND; KOR-MOR, index of opioid receptor status computed as zKOR + zMOR; IL6-IL10, index of immune activation computed as z interleukin-6 (zIL6) + zIL10; IL6-IL10 and DYN-END, as obtained using binary logistic regression (shown in Table 5).

Analysis discriminating major depression from normal controls.

*Performed in non-smokers only (all other analyses in all participants).

Leonard & Maes, 2012). This may explain that combinations of immune and opioid biomarkers have a higher sensitivity, specificity and diagnostic performance for MDD versus controls. Nevertheless, our data are insufficient to conclude that these measurements may be used as a biomarker for MDD because this conclusion entails that a biomarker set should be highly sensitive and specific for MDD versus other medical and neuropsychiatric disorders which are often characterised by aberrations in KOR/MOR and IRS/CIRS systems.

As described in the Introduction, there are many reciprocal relationships between the opioid and immune systems. Prossin *et al.* (2011) reported that, in MDD, increased IL-18 (another pro-inflammatory protein) was positively associated with baseline MOR nondisplaceable binding potential and with sadness-associated MOR activation in amygdala, subgenual anterior cingulate and ventral basal ganglia. During inflammation, pro-inflammatory cytokines including IL-1 β and IL-6 may stimulate hypothalamic corticotrophin releasing hormone (CRH) release (Maes *et al.*, 1990; Turnbull & Rivier, 1999). In response to CRH and cytokines, peripheral blood mononuclear cells may secrete opioids (Rittner *et al.*, 2005). Nevertheless, the major sources of local endogenous opioid ligands including β -endorphin and dynorphin are leukocytes (Machelska *et al.*, 2003). For example, secretory granules with β -endorphin are present at the periphery of granulocytes, monocytes, macrophages and lymphocytes, and may undergo exocytosis (Ninković, 2013). During early inflammation, neutrophils contain most opioids while during later stages macrophages and monocytes and activated T and B cells are more important sources (Stein *et al.*, 2003; Rittner *et al.*, 2008). This may explain that leukocytes have analgesic effects by releasing opioids that consequently activate opioid receptors of nociceptors in the periphery (Ninković, 2013).

Dynorphins produced during inflammatory conditions display anti-inflammatory effects by attenuating translocation of nuclear factor-KB and consequently production of tumour necrosis factor- α and IL-1 β (Rahiman *et al.*, 2017). Inhibition of nuclear-factor KB is likely a major mechanism explaining the anti-inflammatory

effects of opioids (Rahiman *et al.*, 2017). Also, activation of KOR by dynorphins negatively regulates many immune cell functions including cytokine production by macrophages (Alicea *et al.*, 1996), T cell proliferation (Guan *et al.*, 1997), phagocytoses (Szabo *et al.*, 1993) and antibody production (Morgan, 1996). Moreover, dynorphins may exert some of their activities by binding to other receptors, including N-methyl-D-aspartate (NMDA) receptors, activating the hypothalamic–pituitary–adrenal (HPA) axis, inhibiting dopaminergic neurons and mediating changes in calcium channels leading to increased intracellular calcium levels (Jin *et al.*, 1997; Schwarzer, 2009).

Immune cells may upregulate POMC mRNA and contain all enzymes to process POMC into β -endorphin and adrenocorticotropic hormone (ACTH) (Machelska *et al.*, 2003). These neuropeptides are secreted by the pituitary and arcuate nucleus (Stengaard-Pedersen & Larsson, 1981) leading to a concomitant increase in ACTH, β -endorphin and cortisol in MDD (Maes *et al.*, 1990; Maes *et al.*, 1991a; Maes *et al.*, 1992; Al-Hakeim, 2008; Al-Fadhel *et al.*, 2019). During inflammatory conditions, ‘CRH stimulates peripheral release of β -endorphin from immune cells’ (Berczi & Quintanar-Stephano, 2016) and, therefore, activation of immune-inflammatory pathways likely contributes to circulating levels of β -endorphin. Animal data show that β -endorphins have immune-regulatory effects (Silberstein *et al.*, 2009), which may explain the protective effects of CRH-stimulated elevations of β -endorphin during immune activation (Khairova *et al.*, 2009). Inflammation also induces MOR transcription and increased MOR potency as observed in intestinal inflammation (Pol *et al.*, 2001). MORs, in turn, have anti-inflammatory effects and MOR agonists may be used to dampen immune-inflammatory responses in, for example, inflammatory bowel disease (Philippe *et al.*, 2003). All in all, our findings suggest that, in MDD, immune activation is associated with a simultaneous upregulation of dynorphin/KOR and β -endorphin/MOR signaling, which both may have immune-regulatory properties and, therefore, it may be hypothesised that increased signaling of both opioid systems may contribute to CIRS activities in MDD. Moreover, it may be hypothesised that increased dynorphin/KOR and β -endorphin/MOR signaling may play a role in depressive symptomatology whereby KOR has detrimental effects and MOR more protective effects.

The third major finding of this study is that KOR and the dynorphin/KOR signaling index are both significantly decreased in MDD subjects with comorbid ND, while there are no significant effects on β -endorphin or MOR levels (although there is a trend towards a decrease). Smoking is highly prevalent among people with MDD (Nunes *et al.*, 2012) and is regarded as a means to cope with depressed mood and counteract fatigue and inactivity (Piper *et al.*, 2004). As explained in the Introduction, smoking is also a risk factor for depression through activated immune-inflammatory and oxidative pathways, while comorbid MDD and ND are characterised by lowered antioxidant and increased neuro-immune, neuro-oxidative and degenerative biomarkers (Rittner *et al.*, 2005; Bidlack *et al.*, 2006; Nunes *et al.*, 2012). Isola *et al.*, (2009) proposed that nicotine administration may influence dynorphin through alterations in dopamine and glutamate metabolism. Nicotine exposure increases dopamine release in mesolimbic terminal fields (Di Chiara, 2000) and, therefore, lowered dynorphin/KOR levels in comorbid MDD and nicotine use may be due to nicotine-induced dopamine release. Moreover, KOR activation may impact the reinforcing effects of nicotine (Ismayilova & Shoaib, 2010), while KOR agonists may lower locomotor activity induced by nicotine (Hahn *et al.*, 2000). Mice without the prodynorphin

gene show an increased intake of nicotine as compared to wild-type mice, indicating that dynorphin/KOR signaling may attenuate the reinforcing effects of nicotine (Galeote et al., 2009). Therefore, it is also possible that MDD patients with relatively lower dynorphin/KOR signaling display increased reinforcement effects of nicotine and thus more ND behaviours. Furthermore, due to lowered dynorphin/KOR signaling, heavy smokers may show lowered anti-inflammatory protection and, therefore, may be more vulnerable to the detrimental effects of smoking-induced IL-6 and TNF- α signaling (Lesperance et al., 2004; Nunes et al., 2012; Gaspersz et al., 2017).

Limitations of the study

The results of this study should be discussed with respect to its limitations. One limitation is that it would have been more interesting if we had measured DOR and many more cytokines including those produced by M1 macrophage, T helper (Th)-1, Th-2, Th-17 and T regulatory phenotypes in order to examine the associations of opioids with the full spectrum of IRS and CIRS cytokines and their ratios. Secondly, this study examined male subjects only and therefore, our study should be replicated in females. Thirdly, while the manufacturer of the kit used to assay MOR stated that there is no significant cross-reactivity between human MOR and analogues no such statement was reported for the KOR kit and therefore, cross-reactivity is not ruled out completely. Fourthly, it would be important to examine the effects of dynorphin and β -endorphin on the production of IRS/CIRS cytokines and the effects of immune activation (by mitogen or lipopolysaccharides (LPS) stimulation) on KOR-MOR protein production by stimulated and unstimulated whole blood cultures of patients and controls.

Conclusions

Serum dynorphin and KOR and β -endorphin and MOR are significantly increased in MDD as compared with controls. The increases in dynorphin/KOR and β -endorphin/MOR signaling are significantly intercorrelated with signs of IRS/CIRS activation. KOR and the dynorphin/KOR signaling index are both significantly decreased in depressed subjects with comorbid nicotine use. The findings of the present study suggest that the immune activation in MDD is associated with a simultaneous activation of dynorphin/KOR and β -endorphin/MOR signaling and that these opioid systems may participate in the pathophysiology of depression by exerting immune-regulatory activities as well as emotional and behavioural effects.

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