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In major depression, increased serum dynorphin and kappa opioid receptor levels are positively associated with mu opioid receptor levels and immune activation and are attenuated by nicotine dependence.

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Abstract

Background: There is now evidence that immune and opioid systems show functional reciprocal relationships and that both systems may participate in the pathophysiology of major depression (MDD).

Objective: The present study was carried out to delineate differences between MDD patients and healthy controls in dynorphin and kappa opioid receptor (KORs) in association with levels of β-endorphins and mu opioid receptors (MORs), interleukin-6 (IL-6) and IL-10.

Method: The present study recruited 60 drug-free male participants with MDD aged 24-70 year and 30 age-matched healthy males as control group and measured serum levels of dynorphin, KOR, β-endorphin, MOR, IL-6 and IL-10.

Results: Serum dynorphin, KOR, β-endorphin and MOR are significantly increased in MDD as compared with controls. The increases in the dynorphin/KOR system and β-endorhin/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, whereby both opioid peptides and cytokines show a bootstrapped (n=2000) area under the receiver operating curve of 0.972. KOR and the dynorphin/KOR system are both significantly decreased in depressed subjects with comorbid nicotine dependence.

Conclusion: Our findings suggest that in MDD, immune activation is associated with a simultaneous activation of dynorphin/KOR and β-endorhin/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 behavioral responses to stress.

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Keywords: Depression, cytokines, inflammation, endogenous opioid, opioid receptor.

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-endorhin/mu opioid (MOR) signaling are significantly intercorrelated and associated with immune activation.
- Both KOR and MOR systems may participate in the pathophysiology of depression by exerting immune regulatory as well as emotional and behavioral 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 should be replicated in females.

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Introduction

Major depressive disorder (MDD) is a chronic relapsing disorder characterized 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% (1). 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) mediators (2, 3, 4, 5, 6, 7, 8). 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) (8, 9).

Aberrations in endogenous opioid peptides and their receptors are other characteristics of MDD. The opioid system comprises three families of neuropeptides, namely endorphins, enkephalins, and dynorphins, and three cognate receptor subtypes, namely μ (MOR), δ (DOR), and κ (KOR) receptors (10). β-Endorphin and enkephalins bind to MORs and DORs, while dynorphin bind predominately to KORs (11). Both opioid receptors and opioid peptides are expressed throughout peripheral and central nervous systems (12). A growing body of research indicates that those endogenous opioids and their receptors are involved in emotional and behavioral responses to stress, regulation of mood and the pathophysiology of MDD (13, 14). MOR is a key component of reward processing, while acute activation of MOR attenuates depressive-like behaviors in some but not all studies (15). MDD is accompanied by increased baseline and post-dexamethasone β-endorphin concentrations (2, 16, 17, 18). In animal models, increased β-endorphin secretion is associated with depressive-like behaviors suggesting that the opioid system could be a new drug target to treat depression (19). Recently, we reported that

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MOR levels were significantly higher in MDD patients than controls (13), while post-mortem studies showed increased MOR binding in suicide victims (20). Positron emission tomography (PET), on the other hand, showed contradictory results with increased binding potential for [^{11}C]-carfentanil during depression, but decreased biding potential in women with MDD (21).

A variety of stressors may increase KOR signaling, which is implicated in stress-induced changes in brain reward systems, which, in turn, may contribute to despair- and depression-like responses and depressive disorders (15, 22, 23, 24, 25). As such, some authors regard KOR signaling as an anti-reward dysphoric system increasing risk towards depression (26, 27). 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 (28). Nevertheless, in a pilot study, no differences in KOR binding (using [11C]GR103545) could be found between MDD patients and controls (29), while prodynorphin mRNA was significantly lower in MDD and bipolar depression as compared with controls (30).

There is now evidence that immune and opioid systems show functional relationships. T and B-lymphocytes, granulocytes, and monocytes/macrophages express opioid peptides, including β-endorphin and pro-opiomelanocortin (POMC) and all POMC processing enzymes (31, 32). Opioid peptides are synthesized in circulating leukocytes that, directed by chemokines and adhesion molecules, may migrate to inflamed tissues where they may exert immune stimulatory as well as immune inhibitory activities (32,33). β-endorphin and dynorphin peptides modulate functions of lymphocytes and other cells involved in host defense and immunity (34). Associations between both systems are also detected in patients with MDD. For example, lowered cell-mediated hypersensitivity and natural killer (NK) activity are significantly associated with increased β-endorphin levels in depression (32, 35). Castilla et al. (1992)

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reported an increased opioid tone in depression and a concomitant suppression of monocytic functions (36). Recently, we reported significant associations between MOR levels and IL-10 in patients with major depression (13). 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 tobacco use (NU) or nicotine dependence (ND) with increased inflammatory potential and affiliated nitro-oxidative pathways in comorbid MDD and NU/ND (37, 38, 39). 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" (40). Nevertheless, the effects of comorbid MDD and ND on the KOR system has not been examined.

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

Subjects and Methods

Participants

The present study recruited 60 drug-free male participants with MDD aged 24-70 year and 30 age-matched healthy males as a control group. 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 to July 2017. This study reports KOR and dynorphin values in the same study group on which we published MOR, β-endorphin, IL-6 and IL-10 data (13), except that one patient and one control were substituted by two new cases with

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dynorphin/KOR measurements. The diagnosis was made using criteria of the 4th edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [American Psychiatric Association 2000]. Severity of depressive symptoms was assessed using the 24-item Hamilton Depression Rating Scale (HDRS) one or two days before blood was drawn. Only patients with a total HDRS score >21 were enrolled in the present study. Patients were divided in those with and without ND and all ND patients showed heavy nicotine use of more than 20 cigarettes/day. Patients were evaluated using a full medical history. We excluded subjects with systemic disease that may affect immune parameters, including diabetes mellitus, autoimmune disorders, neuro-inflammatory disorders, inflammatory bowel disorder, and chronic kidney disease. We also excluded subjects with neurodegenerative/neuroinflammatory disease including stroke, multiple sclerosis, and Alzheimer and Parkinson's disease. We also excluded MDD patients who were medicated, and subjects with other-axis I diagnosis including schizophrenia, psycho-organic disorders and substance abuse. To eliminate any effects of overt inflammation from other disorders, serum C-reactive protein (CRP) was evaluated in all samples and we excluded subjects with CRP values >6 mg/L. 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 were drawn, utilizing 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

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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®, Spain. This test is based on the principle of the latex agglutination. Commercial ELISA sandwich kits were used to measure KOR, MOR, β-endorphin, dynorphin, IL-6 and IL-10 (MyBioSource, Inc, CA, USA; and CUSABIO Co, China). We followed exactly all procedures according to the manufacturer's instructions. The intra-assay coefficients of variation (CV) (precision within-assay) were < 7.0% for all analytes.

Statistical analysis

Differences in scale variables between diagnostic groups were examined using analysis of variance (ANOVA). Associations between nominal variables were assessed using 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-subjects effects were employed to assess the effects of significant explanatory variables on biomarkers. Multiple post-hoc comparisons among treatment means were assessed using protected 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 Odd's 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

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specificity (41). Tests were 2-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. Statistical analyses were conducted in accordance with the International Conference on Harmonisation E9 statistical principles (November 2005).

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 nicotine dependence (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 (±SE) after z transformations were made. The BDI-II score was not significantly different between both MDD subgroups.

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Table 2 shows the intercorrelation matrix 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 **Figure 3** shows the correlation between DYN-END and IL6-IL10. **Figure 4** shows the association between KOR-MOR and IL6-IL10.

Biomarker differences between controls and patients groups

Table 3 shows the outcome of a multivariate GLM analysis with the 10 biomarkers as dependent variables while adjusting for age and BMI. The dependent variables were DYN, END, KOR, MOR, IL-6, and IL-10 levels as well as z unit weighed 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 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. Pair-wise 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

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increased from controls → MDD+ND → MDD. END, MOR, KOR-MOR, IL-6, IL-10 and IL6-IL10 were significantly higher in both MDD groups than in controls.

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 (X^2=77.16, df=2, p<0.001) with an effect size of 0.800 (Nagelkerke value).

Table 6 shows the results of 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 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 with 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: 29, 30). As described in the Introduction, increased KOR signaling results in anti-reward and dysphoric effects thereby contributing to depressive behaviors (15, 22, 23, 24, 25, 26, 27). Acute stress induces numerous physiologic and behavioral effects that are mediated by KOR signaling in limbic brain regions,

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while dynorphin release can be both a cause and consequence of stress hormone release, or may occur as a direct result of stress-induced increases in neuronal activity (42, 43). Evidence suggests that the acute effects of stress are caused, at least in part, by dynorphin-mediated KOR activation. The predominant effect of KOR stimulation is decreased neuronal activity in cell populations that express KORs (24). Dendritic dynorphin release in the hippocampus and hypothalamus negatively regulates excitatory inputs via retrograde activation of presynaptic KORs (44). This inhibitory mechanism may generalize to other neuronal populations often implicated in the regulation of mood and motivation, such as the amygdala and striatum, which express dendritic dynorphin (45). KORs are also involved in the regulation of serotonergic (42) and noradrenergic (46) systems, which may play a role in MDD. Blockade of KORs decrease immobility time in animal models of the forced swim test, suggesting these receptors may show antidepressant-like effects (47). On the other hand, during acute stress, KOR signaling may increase physical ability (by producing analgesia) and motivation to escape threat (by producing aversion) thereby facilitating adaptive responses **(**24).

Our results on dynorphin and KOR are in agreement with our findings that also β-endorphin and MOR are upregulated in MDD and that the increases in β-endorphin and MOR are significantly intercorrelated, indicating increased β-endorphin/MOR signaling in MDD (13). Previously, one paper showed increased expression of MOR in suicide victims, whereas PET scan studies showed controversial results on MOR binding profiles (see Introduction). Interstingly, MOR (and DOR) activation may elevate mood and therefore may improve depressive states (48).

The second major finding of this study is that dynorphin/KOR and β-endorhin/MOR signaling indices are significantly intercorrelated and are strongly associated with IRS/CIRS

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activation. Heightened IRS and CIRS responses are commonly observed in patients with MDD, with increased levels of immune-inflammatory biomarkers and immune-regulatory compounds, (5, 49, 50, 51), including serum IL-6 and IL-10 concentrations (5, 8, 13). Moreover, we observed that these opioid and IRS/CIRS responses yielded a good diagnostic performance for MDD with a bootstrapped (n=2000) area under the curve of 0.972 (for both opioid peptides combined with both cytokines). In this respect it is intersting to note that recent theories suggest that classical neurotransmitters convey information between pairs of neurons, whereas neuropeptides and cytokines convey information and coordinate activities across broader networks of neurons (52, 53). This may explain that combinations of immune and opioid biomarkers have a high sensitivity, specificity and diagnostic performance for MDD. All in all, our results show that increased dynorphin/KOR and β-endorhin/MOR signaling in MDD are strongly associated with the peripheral IRS/CIRS response in that illness.

As described in the Introduction, there are many reciprocal relationships between the opioid and immune systems. During inflammation, pro-inflammatory cytokines including IL-1β and IL-6 may stimulate hypothalamic corticotrophin releasing hormone (CRH) release (2, 54). In response to CRH and cytokines, peripheral blood mononuclear cells may secrete opioids (33). Nevertheless, the major sources of local endogenous opioid ligands including β-endorphin and dynorphin are leukocytes (55). For example, in macrophages, monocytes, granulocytes and lymphocytes, β-endorphin is present in secretory granules arranged at the cell periphery ready for exocytosis (11). Quantitative analysis revealed that in early inflammation, granulocytes (especially neutrophils) are the major opioid-containing leukocytes, whereas in later stages of inflammation, monocytes or macrophages and lymphocytes (especially activated T and B cells)

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predominate (56, 57). Therefore, leukocytes are able to exert analgesic effects by releasing opioid peptides which bind to opioid receptors of the nociceptors in the periphery (11).

Dynorphins produced during inflammatory conditions display anti-inflammatory effects by attenuating translocation of nuclear factor-KB and consequently production of tumor necrosis factor-α and IL-1β (58). Inhibition of nuclear-factor KB is likely a major mechanism explaining the anti-inflammatory effects of opioids (58). Also, activation of KOR by dynorphins negatively regulates many immune cell functions including cytokine production by macrophages (59), T cell proliferation (60), phagocytoses (61) and antibody production (62). Moreover, dynorphins may exert some of their activities by binding to other receptors, including NMDA receptors, activating the HPA-axis, inhibiting dopaminergic neurons, and mediating changes in calcium channels leading to increased intracellular calcium levels (63, 64).

Immune cells contain and upregulate signal-sequence encoding mRNA of the β-endorphin precursor POMC and the entire enzymatic machinery necessary for its processing into the functionally active peptide (55). POMC neuropeptides such as β-endorphin and ACTH are produced in both the anterior and intermediate lobes of the pituitary, as well as in a cluster of neurons in the hypothalamic arcuate nucleus (65) leading to a concomitant increase in ACTH, β-endorphin and cortisol in MDD (2, 6, 13, 66, 67). During inflammatory conditions, "CRH stimulates peripheral release of β-endorphin from immune cells" (68) and, therefore, activation of immune-inflammatory pathways likely plays an important role in contributing to circulating β-endorphin levels. Animal data show that β-endorphin have anti-inflammatory and immunosuppressive properties (69) explaining the positive benefits of CRH-mediated increases in β-endorphin levels under inflammatory conditions (70). Inflammation also induces MOR transcription and increased MOR potency as observed in intestinal inflammation (71). MORs, in

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turn, have anti-infammatory effects and MOR agonists may be used to dampen immune-inflammatory responses during for example inflammatory bowel disease (72). All in all, our findings suggest that in MDD a) immune activation is associated with a simultaneous activation of dynorphin/KOR and β-endorhin/MOR signaling, which both may have CIRS activities attenuating the primary immune response and b) both increased dynorphin/KOR and β-endorhin/MOR signaling may play a role in depressive symptomatology albeit with divergent effects, namely detrimental (KOR) versus more protective (MOR) effects.

The third major finding of this study is that KOR and the index dynorphin/KOR signaling are both significantly decreased in MDD subjects with comorbid nicotine dependence, 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 (73) and is commonly cited as a means to cope with depressed mood and counteract fatigue and inactivity (74). As explained in the introduction, smoking is also a risk factor for depression through activated immune-inflammatory and oxidative pathways, while comorbid MDD and nicotine dependence are characterized by lowered antioxidant and increased neuro-immune, neuro-oxidative and degenerative biomarkers (38, 39, 73). Isola et al. (2009) proposed that nicotine administration may influence dynorphin primarily through dopamine release and that glutamate plays a modulatory role (75). Nicotine exposure increases dopamine release in mesolimbic terminal fields (76) and, therefore, one possibility is that the lowered dynorphin/KOR levels in comorbid MDD and nicotine use may be due to dopamine release in response to nicotine. Moreover, KOR activation may attenuate the reinforcing effects of nicotine (77), while KOR agonists attenuate nicotine-induced locomotor activity (78). Additionally, mice lacking the prodynorphin gene compared to wild-type mice self-administered nicotine at lower doses, suggesting that the

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dynorphin/KOR system plays an inhibitory role in the reinforcing effects of nicotine (79). Therefore, it is aslo possible that MDD patients with releatively lower dynorphin/KOR signaling display increased reinforcement effects of nicotine and thus more nicotine dependence behaviors. 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 (73, 80, 81).

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 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 (8). Secondly, this study examined male subjects only and therefore our study should be replicated in females.

Conclusion

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-endorhin/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. Our findings suggest that in MDD immune activation is associated with a simultaneous activation of dynorphin/KOR and β-endorhin/MOR signaling and that these opioid

systems may participate in the pathophysiology of depression by exerting CIRS activities attenuating the primary immune response as well as emotional and behavioral effects.

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Declaration of interest

The authors have no financial conflict of interests.

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Authorships.

All authors contributed significantly to the paper and approved the final version.

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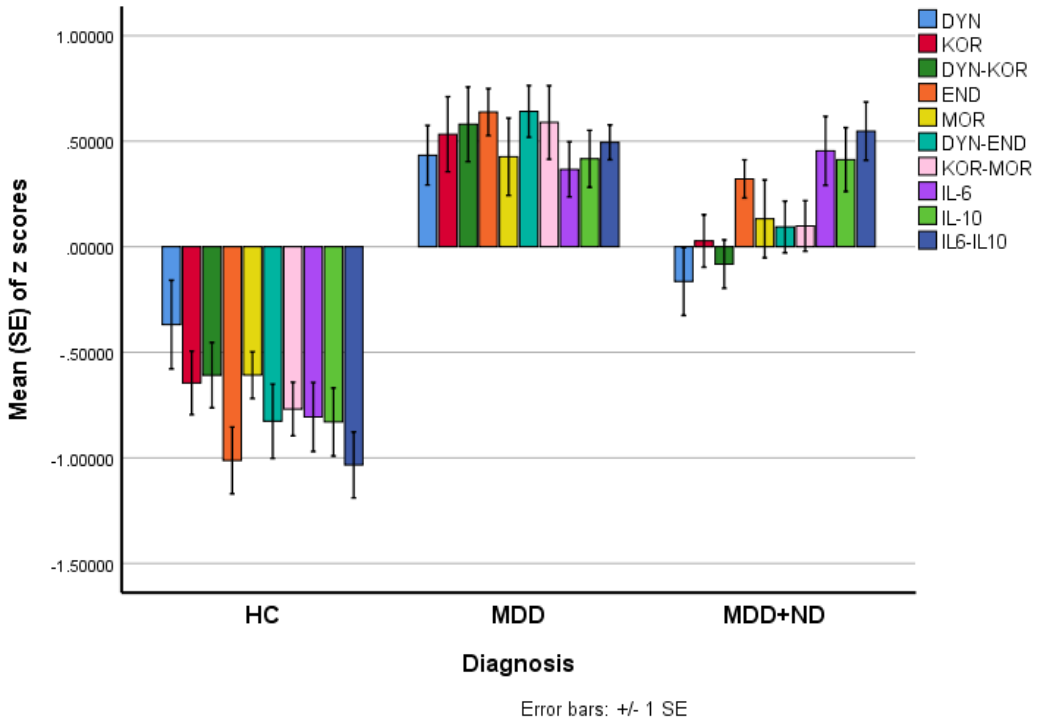

Figure 1 Differences in biomarker profile between controls (HC) and major depressed patients with (MDD+ND) and without (MDD) nicotine dependence.

Shown are the group mean values (±SE) after z transformations were made.

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.
IL: interleukin
IL6-IL10: index of immune activation computed as z interleukin-6 (zIL6) + zIL10

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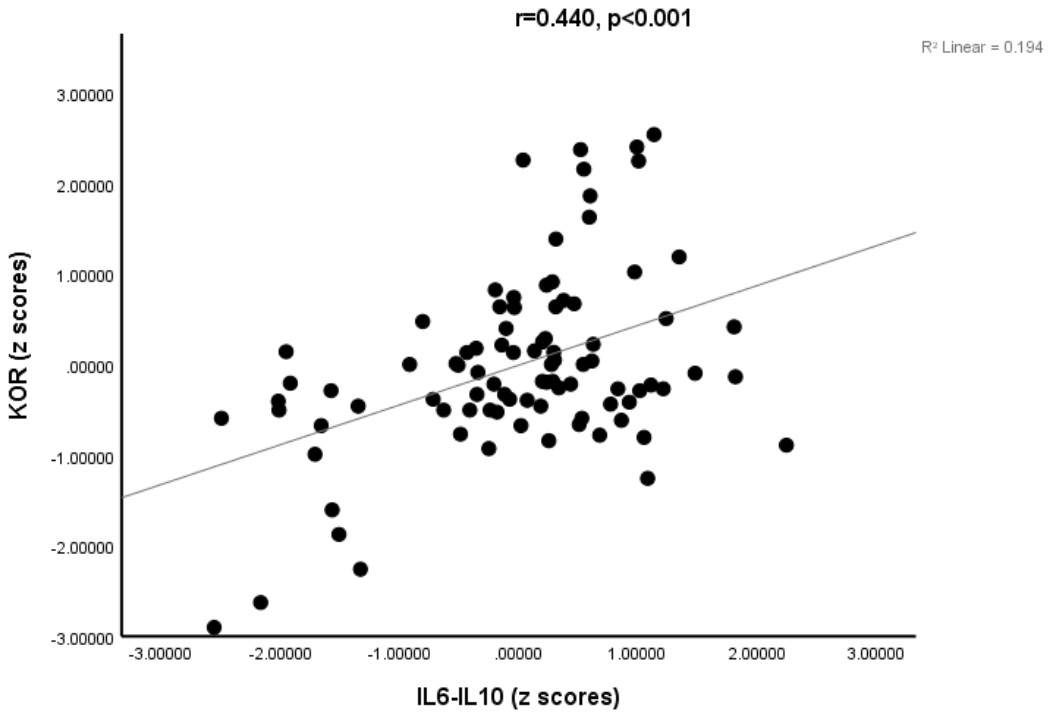

Figure 2 Association between kappa opioid receptor (KOR) levels and immune activation as indicated by a z unit weighted composite (IL6-IL10) score based on interleukin (IL)-6 and IL-10 assays.

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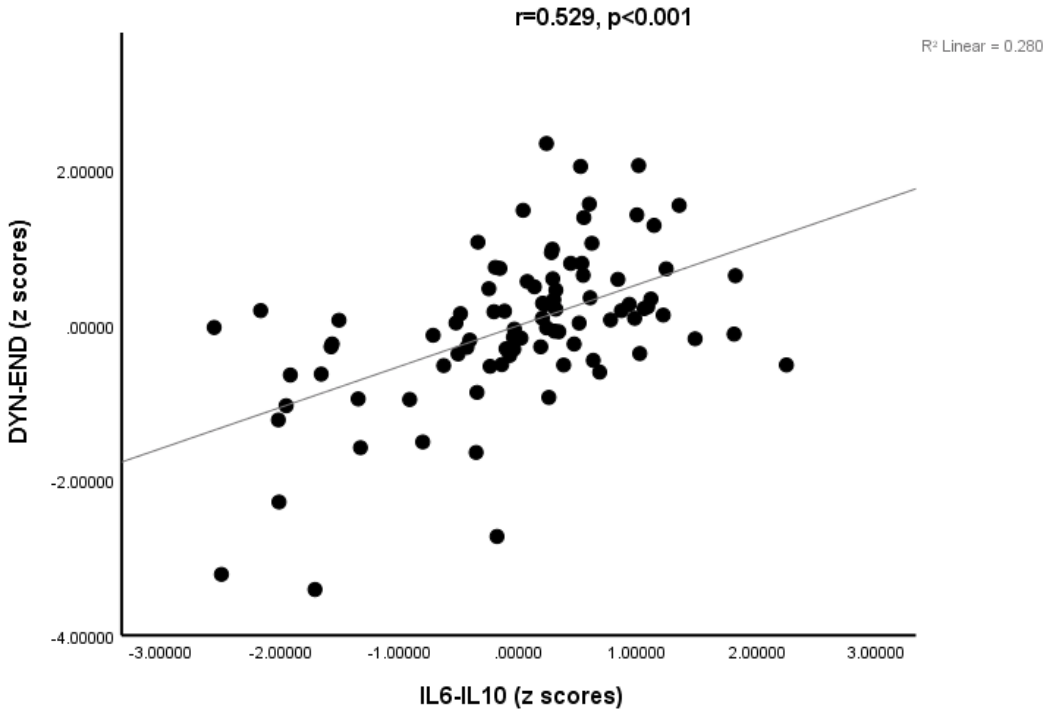

Figure 3 Association between opioid peptides as indicated by a z unit weighted composite score based on dynorphin (DYN) and β-endorphin (END) assays (KOR-MOR) and immune activation as indicated by a z unit weighted composite score based on interleukin (IL)-6 and IL-10 assays (IL6-IL10).

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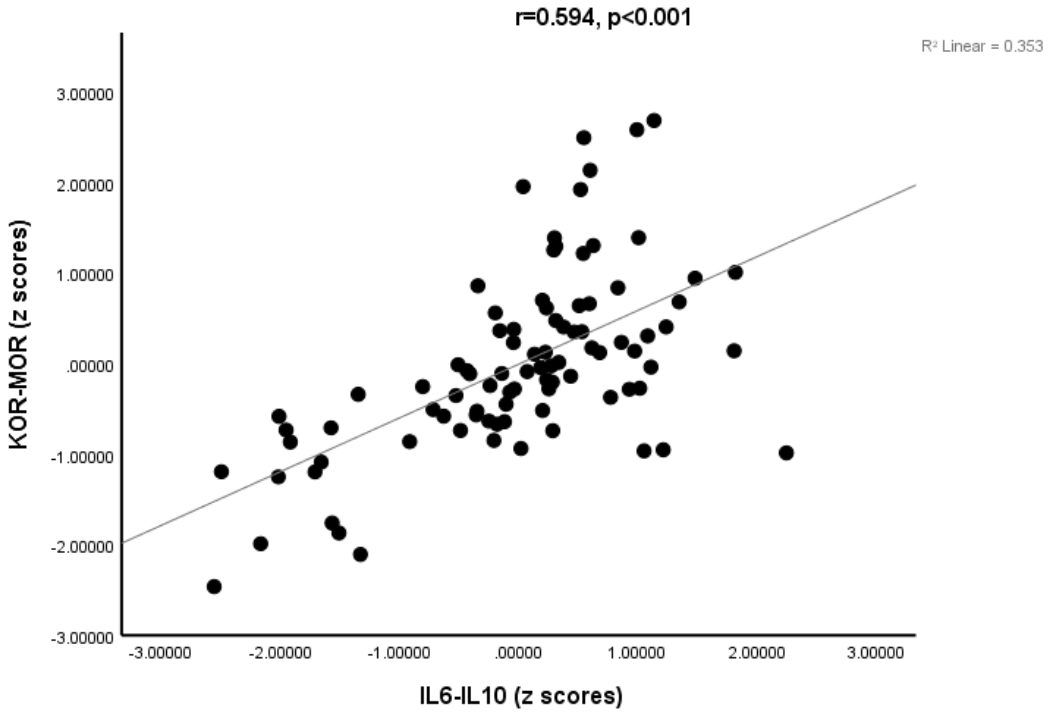

Figure 4 Association between opioid receptor levels as indicated by a z unit weighted composite score based on kappa (KOR) and mu (MOR) opioid receptor levels (KOR-MOR) and immune activation as indicated by a z unit weighted composite score based on interleukin (IL)-6 and IL-10 assays (IL6-IL10).

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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] n=30	MDD [B] n=35	MDD+ND [c] n=25	F/χ^2	df	p
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 [B,C]	16/19 [A]	11/14 [A]	8.90	2	0.012
Dynorphin (pg/ml)	17.04(2.55) [B]	18.83(1.86) [A,C]	17.50(1.79) [B]	6.35	2/87	0.003
KOR (pg/ml)	8.32(1.71) [B,C]	11.99(3.99) [A,C]	10.02(1.81) [A,B]	13.46	2/87	<0.001
END (pg/ml)	24.90(5.27) [B,C]	34.95(4.02) [A]	33.02(2.75) [A]	49.94	2/87	<0.001
MOR (pg/ml)	3.01(0.83) [B,C]	4.43(1.49) [A]	4.03(1.27) [A]	10.94	2/87	<0.001
IL-6 (pg/ml)	11.4(2.8) [B,C]	16.2(3.7) [A]	16.7(4.3) [A]	18.78	2/87	<0.001
IL-10 (pg/ml)	6.0(1.7) [B,C]	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

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Table 2: Inter-correlation matrix among biomarkers data.

Variables	DYN	KOR	DYN-KOR	END	MOR	DYN-END	KOR-MOR
KOR	0.388**						
DYN-KOR	0.833**	0.833**					
END	0.396**	0.440**	0.502**				
MOR	0.339**	0.328*	0.400**	0.358**			
DYN-END	0.835**	0.495**	0.799**	0.835**	0.417**		
KOR-MOR	0.209*	0.738**	0.568**	0.477**	0.815**	0.410**	
IL6-IL10	0.316**	0.440**	0.454**	0.568**	0.529**	0.529**	0.594**

*: $p<0.05$ **: $p<0.001$ (n=90)

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

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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	12.46	12/160	<0.001	0.483
		Age	1.61	6/80	0.155	0.108
		BMI	1.43	6/80	0.215	0.097
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	10.52	2/85	<0.001	0.198
	DYN-END	Diagnosis	31.10	2/85	<0.001	0.423
	KOR-MOR	Diagnosis	22.71	2/85	<0.001	0.348
	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

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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] n=30	MDD [B] n=35	MDD+ND [C] 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(0.1.38) [A]
MOR	-0.607(0.163) [B,C]	0.389(0.149) [A]	0.149(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	-0.795(0.149) [B,C]	0.575(0.137) [A]	0.149(0.160) [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]

All results are shows as mean (SE) and as z scores

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

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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: Odd's 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))

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Table 6: Results of receiver operating characteristic (ROC) analysis discriminating major depression from normal controls.

Variables	Area ROC	SE	p	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	<0001	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	0.754	0.053	<0.001	0.651-0.858	0.742	0.633-0.897
KOR-MOR	0.849	0.039	<0.001	0.772-0.925	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

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

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

IL6-IL10 and DYN-END: as obtained using binary logistic regression (shown in Table 5)