Journal of Affective Disorders

Full-Length Research Paper.

Revised Ms JAD_2020_141: **May 24, 2020**

Regulation of cannabinoid CB_1 and CB_2 receptors, neuroprotective mTOR and pro-apoptotic JNK1/2 kinases in postmortem prefrontal cortex of subjects with major depressive disorder

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ABSTRACT

***Background*:** Dysregulations of endocannabinoids and/or cannabinoid (CB) receptors have been implicated in the pathophysiology and treatment of major depressive disorder (MDD).

***Methods*:** CB_1 and CB_2 receptors, neuroprotective mTOR (mechanistic target of rapamycin) and pro-apoptotic JNK1/2 (c-Jun-N-terminal kinases) were quantified by immunoblotting in postmortem prefrontal cortex of MDD and controls, and further compared in antidepressant (AD)-free and AD-treated subjects. Neuroplastic proteins (PSD-95, Arc, spinophilin) were quantified in MDD brains.

***Results*:** Total cortical CB_1 glycosylated (~54/64 kDa) receptor was increased in MDD (+20%, n=23, p=0.02) when compared with controls (100%, n=19). This CB_1 receptor upregulation was quantified in AD-treated (+23%, n=14, p=0.02) but not in AD-free (+14%, n=9, p=0.34) MDD subjects. In the same MDD cortical samples, CB_2 glycosylated (~45 kDa) receptor was unaltered (all MDD: +11%, n=23, p=0.10; AD-free: +12%, n=9, p=0.31; AD-treated: +10%, n=14, p=0.23). In MDD, mTOR activity (p-Ser2448 TOR/t-TOR) was increased (all MDD: +29%, n=18, p=0.002; AD-free: +33%, n=8, p=0.03; AD-treated: +25%, n=10, p=0.04). In contrast, JNK1/2 activity (p-Thr183/Tyr185/t-JNK) was unaltered in MDD subjects. Cortical PSD-95, Arc, and spinophilin contents were unchanged in MDD.

***Limitations*:** A relative limited sample size. Some MDD subjects had been treated with a variety of ADs. The results must be understood in the context of suicide victims with MDD.

***Conclusions*:** The upregulation of CB_1 receptor density, but not that of CB_2 receptor, as well as the increased mTOR activity in PFC/BA9 of subjects with MDD (AD-free/treated) support their contributions in the complex pathophysiology of MDD and in the molecular mechanisms of antidepressant drugs.

Keywords:

Major depressive disorder

Antidepressant drugs

Postmortem human brain (dorsolateral PFC)

Cannabinoid CB_1 and CB_2 receptors

Mechanistic target of rapamycin (mTOR)

c-Jun-N-terminal kinase (JNK1/2)

1. Introduction

The cannabinoid (CB) CB_1 and CB_2 receptors (Matsuda et al., 1990; Munro et al., 1993) are Gαi protein-coupled receptor entities (primarily signaling through inhibition of adenylyl cyclase, AC) that mediate the actions of endoCBs (e.g. anandamide) through various cellular pathways (Mechoulam and Parker, 2013; Cristino et al., 2020). The differential expression of brain CB_1 and CB_2 receptors (neuron and/or astrocytes and/or microglia) has been extensively documented (see Álvaro-Bartolomé et al., 2010; Salort et al., 2017a and other references therein). Besides the classic CB_1 receptor (Mechoulam and Parker, 2013), the relatively less investigated CB_2 receptor has also been shown to play relevant roles in synaptic functions (Fernández-Ruiz et al., 2007; Morgan et al., 2009; Li and Kim, 2016; Li et al., 2019) and various brain disorders (Ishiguro et al., 2010; García-Gutiérrez et al., 2010; García et al., 2015; Aymerich et al., 2018; Espejo-Porras et al., 2019).

Over the past decades, numerous studies have revealed the involvement of endoCBs and CB receptors in the pathophysiology and/or treatment of major psychiatric syndromes such as schizophrenia (reviewed in Urigüen et al., 2013; Jacobson et al., 2019; Navarrete et al., 2020) and major depressive disorder (MDD) (reviewed in Hillard and Liu, 2014; Poleszak et al., 2018; Navarrete et al., 2020). In MDD, several postmortem brain studies have reported an increased expression and/or activity of CB_1 receptors in the dorsolateral prefrontal cortex (PFC) of depressed suicides, most of them under antidepressant (AD) treatments (Mato et al., 2001; Hungund et al., 2004; Choi et al., 2012; Urigüen et al., 2013). In contrast to the upregulation of CB_1 receptor in MDD brains, most preclinical and some clinical studies (reviewed in Hillard and Liu, 2014) have suggested that depressive syndromes are associated with deficient CB_1 receptor-mediated signaling (e.g., induction of depressed mood and suicide by the CB_1 antagonist/inverse agonist rimonabant; Christensen et al., 2007) and that conventional AD drugs (e.g. norepinephrine and serotonin reuptake inhibitors; Hill and

Gorzalka, 2005; Cryan et al., 2005) act through an enhancement of brain CB_1 receptor signaling (see McLaughlin et al., 2007). Remarkably, and in contrast to the human CB_1 receptor, the status of brain CB_2 receptors in subjects with MDD has not been assessed yet.

Several signaling pathways (in addition of AC inhibition) are associated with the activation of CB_1 and CB_2 receptors (Mechoulam and Parker, 2013; Basavarajappa et al., 2017), and among them the regulation of neuroprotective mTOR kinase (Saxton et al., 2017) and pro-apoptotic JNK1/2 kinases (Win et al., 2018) by CB receptors (Álvaro-Bartolomé et al., 2010; Salort et al., 2017a) could have relevant roles in the pathophysiology and/or treatment of MDD (Jernigan et al., 2011; Ignácio et al., 2015; Jovicic et al., 2015).

Against this background, the present study quantified the immunocontents of CB_1 and CB_2 receptors and the basal activations of mTOR (p-TOR/t-TOR) and JNK1/2 (p-JNK/t-JNK) kinases in postmortem PFC of unmedicated and AD-treated MDD subjects and healthy matched controls. Markers involved in brain neuroplasticity (Sarrouilhe et al., 2006; Chen et al., 2011) such as PSD-95 (postsynaptic density protein of 95 kDa), Arc (activity-regulated cytoskeleton-associated protein), and spinophilin were also quantified in the same brain samples (MDD and controls). The major findings reveal upregulation of brain CB_1 (but not CB_2) receptor density and increased activity of mTOR in the PFC of subjects with MDD. A preliminary account of this work was given at the 30th Congress of the European College of Neuropsychopharmacology (ECNP), Paris, France (Salort et al., 2017b).

2. Methods

2.1. Postmortem human brains and compliance with ethical issues

The human corpses (men and women) were refrigerated within 2-5 h of death and stored at 4ºC until autopsy. Samples of the dorsolateral prefrontal cortex (PFC) from caucasian healthy controls and caucasian subjects with diagnosis of MDD (Otte et al., 2016) were obtained at

autopsy from the Institute of Forensic Medicine, University of Geneva, Switzerland, and the Basque Institute of Legal Medicine, Bilbao, Spain. Clinical diagnoses and drug treatments for MDD subjects, and eventually for healthy controls (Table 1), were taken from the medical examiner and/or records of general and psychiatric hospitals (DSM-IV or ICD-10 criteria; Urigüen et al., 2009; Rivero et al., 2014). Toxicological screenings (quantitative assays for ADs, antipsychotics, other psychotropic drugs, including cannabis, and ethanol) were performed on blood/urine samples at the Toxicology Unit, Institute of Forensic Medicine, Geneva, and at the National Institute of Toxicology, Madrid, Spain (Urigüen et al., 2009; García-Fuster et al., 2014). The study was in compliance with the respective national policies of research and ethical review boards for human postmortem brain studies, and the neurochemical experiments were approved by the local Ethics Committee of Clinical Investigation (CEIC-CAIB) and followed the guidelines of the University of the Balearic Islands (UIB).

2.2. Brain area, subjects selection and brain markers of tissue quality

Specimens of right PFC (Hecht, 2010; Velichkowsky et al., 2018), Brodmann's area 9 (BA9), were cut at autopsy extending dissection from the pial surface to white matter (Rajkowska and Goldman-Rakic, 1995), and special care was taken of including only gray mater (samples of about 2 g). The PFC/BA9 was selected for examination because it is one of the most consistently implicated areas in the pathophysiology of MDD (Rajkowska et al., 1999; Hecht, 2010; Goodwin, 2016; Millan et al., 2016). Samples were immediately stored at -80ºC until homogenization and membrane preparation; the sample storage period (up to 36 months) was similar for control and MDD subjects.

Control healthy subjects for the present study were chosen on the basis of the following cumulative criteria: negative medical information on the presence of neuropsychiatric

disorders or drug abuse, accidental cause of death, and negative results in toxicological screening for psychotropic drugs except ethanol (19 healthy controls, 11 women and 8 men, met these criteria, Table 1). The selected healthy controls and MDD subjects (Table 1) had been used in previous studies which dealt with the regulation of brain markers associated with depression (Rivero et al., 2013, 2014; Keller and García-Sevilla, 2017), including those markers related to apoptotic cell death in depressed/suicide brains (García-Fuster et al., 2014) and in rat brain cortex (García-Fuster and García-Sevilla, 2016). Control subjects (Table 1) mainly died of accidental or violent causes (heart attack, traffic trauma, drowning, homicide), about the same period of time of MDD subjects, and had a negative toxicology in blood and/or urine samples for psychotropic drugs (including cannabis), except for benzodiazepines (oxazepam, midazolam) in two subjects. Suicide (hanging, jumping, drug overdose) was the main cause of death in MDD subjects (Table 1). After quantitative toxicology (Table 1; ethanol was also detected in some controls and depressed; blood concentration, range: 0.05-1.13 g/l), subjects with MDD (AD-free and AD-treated) were matched to healthy controls for sex, age, postmortem interval (PMI) and brain pH (Table 1) as closely as possible (McCullumsmith and Meador-Woodruff, 2011; Bao and Swaab, 2018). In a few cases and due to a lesser availability of healthy controls (n=19), compared with MDD (n=23), the same control subject was matched with two MDD (AD-free and AD-treated; same sex and very similar age, PMI, brain pH, and NSE-2 content) and the three samples were run together in the same experiment (e.g. Fig 1A, immunoblot for F3, T4, C6; see figure legends). Therefore, the different number of total control (n=19) and total MDD (n=23) subjects precluded a perfect one to one matching experimental design (see 4.1 Limitations).

Control and MDD subjects had minimal agonal states (Hardy et al., 1985; Bao and Swaab, 2018), as reflected by the normal and similar brain pH values (Table 1); i.e. absence of acidosis related to increased lactic acid in the PFC/BA9. Brain pH is a better indicator of

postmortem human brain tissue quality (e.g. for protein degradation) than the assessment of RNA integrity numbers (RIN) or the quantification of mRNA expression itself (Preece and Cairns, 2003; Urigüen et al., 2009; Sonntag et al., 2016). In addition, the content of neuron-specific enolase (NSE-2), a marker of neuronal damage (Nogami et al., 1998; Keller and García-Sevilla, 2017), was very similar in the PFC/BA9 of controls and MDD subjects (Table 1), also indicating good tissue quality. Besides these brain markers, the PMI is the most important confounding variable (protein degradation with increased PMI) in this type of human brain studies (Bao and Swaab, 2018). In a previous postmortem study, the contents of CB_1 and CB_2 receptors, but not NSE-2, were shown to decline with the length of PMI (5-79 h) in the PFC/BA9 of 12 healthy subjects, with similar rates of protein degradation for both CB receptors (Álvaro-Bartolomé and García-Sevilla, 2013). In the present study, the effect of PMI on the contents of pro-survival mTOR (p-Ser2448 mTOR and total mTOR) and pro-apoptotic JNK1/2 (p-Thr183/Tyr185 JNK and total JNK) in the PFC/BA9 were also assessed (see Results 3.3; Fig. 3B) in the same cohort of healthy subjects (Álvaro-Bartolomé and García-Sevilla, 2013).

2.3. Sample preparation, electrophoresis/Western blot analysis, and quantification of targets

Brain samples (PFC/BA9 ~100 mg) were prepared for the immunodetection of CB_1 and CB_2 receptor proteins (glycosylated forms) as well as for mTOR and JNK1/2 kinases (phosphorylated and total forms) as described previously with minor modifications (e.g. García-Fuster et al., 2014; Keller and García-Sevilla, 2017). Sample preparations (total homogenate) contained a mixture of six protease inhibitors (AEBSF, aprotinin, leupetin, bestatin, pepstain A, E-64; Protease Inhibitor Cocktail, P-8340, Sigma-Aldrich, St Louis, MO) and various phosphatase inhibitors (Phosphatase Inhibitor Cocktail 3, P-0044, Sigma-Aldrich), and protein concentration was determined by the biuret reaction using bicinchoninic

acid for the colorimetric detection of cuprous cation (BCA ProteinAssay Reagent; Pierce Chemical Co., USA) with bovine serum albumin as the standard. Aliquots of PFC/BA9 samples were combined with equal volumes of electrophoresis loading buffer (100 mM Tris HCl, pH 6.8, 3% SDS, 20% glycerol, 5% β-mercaptoethanol, and ~0.1% bromophenol blue), denaturated (95ºC for 4 min), and stored at –80ºC until use.

In routine experiments, brain proteins (PFC/BA9 homogenate: 40 μg) were resolved by electrophoresis on 7.5-10% SDS-PAGE minigels (6x8 cm, 1 mm thickness, 15 wells) (Bio-Rad Laboratories, USA), transferred (2–3 h, 4ºC) to nitrocellulose membranes (Western blotting), and blocked at room temperature for 1 h in phosphate-buffered saline (PBS for non-phosphorylated proteins: 137 mM NaCl, 2.7 mM KCl, 12 mM Na_4HPO_4, 1.38 mM KH_2PO_4; pH 7.2) or in Tris-buffered saline (TBS for phosphorylated proteins: 137 mM NaCl, 20 mM Tris-HCl, pH 7.6), containing 5-10% non-fat dry milk and 0.1-0.2% Tween 20 to block nonspecific protein binding sites (blocking solution). The nitrocellulose membranes were incubated overnight at 4ºC with the appropriate primary epitope-affinity purified polyclonal or monoclonal antibody (dilution range: 1/500 to 1/10,000) as shown in Table 2. Horseradish peroxidase-conjugated secondary antibodies and ECL detection system (Amersham, Buckinghamshire, UK) were used to visualize immunoreactivity of target proteins on autoradiographic films (Amersham ECL Hyperfilm).

The immunoreactive bands were quantified (integrated optical density, IOD) by densitometric scanning (GS-800 densitometer, Bio-Rad). The amount of a target protein in PFC/BA9 samples from MDD subjects was compared with that of matched controls (100%) in the same gel, and for CB_1 and CB_2 receptors and neuroplastic targets (PSD-95, Arc and spinophilin) the data were normalized to the contents of β-actin. The activations of mTOR and JNK1/2 kinases (see Salort et al., 2017a) were expressed as the ratio of phosphorylated kinase (p-form) to total enzyme (t-TOR or t-JNK) and reported as a percentage of that in the

corresponding control group (100%). Each paired of matched subjects (MDD and healthy control) was quantified in 2-4 gels and the mean value was used as a final estimate.

2.4. Specificity of CB_1 and CB_2 receptor antibodies

The specificities of anti-CB_1 receptor antibody (recognizing the mature ≈54-64 kDa glycosylated receptor forms; Song and Howlett, 1995; Porcella et al., 2002; López de Jesús et al., 2006; Álvaro-Bartolomé and García-Sevilla, 2013; Ruehle et al., 2017) and anti-CB_2 receptor antibodies (recognizing the ≈45 kDa glycosylated receptor; Nowell et al., 1998; Olson et al., 2003; Vandeputte et al., 2011; Álvaro-Bartolomé and García-Sevilla, 2013) had been previously assessed in human, rat and mouse brain tissues, as well as in brain cortices of WT and CB_1 KO or CB_2 KO mice (Álvaro-Bartolomé and García-Sevilla 2013), or in mice overexpressing CB_2 receptors (CB2xP) (Salort et al., 2017a).

2.5. Specificity of phosphorylated mTOR and JNK kinase antibodies

The specificity of p-Ser2448 mTOR and p-Thr183/Tyr185 JNK1/2 antibodies for phosphorylated epitopes was tested on Western blots of human brain as described previously (Ferrer-Alcón et al. 2000). Briefly, total homogenate of human PFC/BA9 (sex: female, age: 30 year-old, PMI: 15 h) was incubated in the absence (control, C) or presence of calf intestinal mucosa alkaline phosphatase (AP, 95 units, Product 79390, Sigma-Aldrich, Germany), as well as other AP samples that were incubated with 100 mM sodium pyrophosphate (inhibited control, IC). The reaction was terminated with the addition of sodium pyrophosphate (100 mM) to control and AP samples (Ferrer-Alcón et al., 2000) (see Results 3.3; Fig. 3A). To immunodetect the total content of mTOR and JNK1/2 kinases, independently of the protein phosphorylation state, the p-kinase blots were stripped and then

reprobed with the corresponding antibody for total enzyme protein, which also served as control for sample loading and protein transfer (see details in Salort et al., 2017a).

2.6. Neurochemical data and experimental design/statistical analysis

The results were analyzed using the program GraphPad PrismTM, version 6.0 (GraphPad Software, La Jolla, CA, USA). All data sets were expressed as mean values ± standard error of the mean (SEM) for a given group of subjects. The number of cases investigated (Table 1) in MDD subgroups (C, AD-free, AD-treated) is indicated in the Figure legends (different numbers indicate that some brain samples were exhausted). The experimental design of this study incorporated the pairing of subjects (MDD and healthy controls) matched for different variables such as gender, age, PMI, and brain pH (Table 1), and a statistical test that takes pairing into account was used for the main evaluations: i.e. the two-tailed one-sample *t*-test (similarly to paired data sets with N-1 degrees of freedom) compares the experimental mean (percent of control) with a hypothetical mean of 100 (Motulsky, 1995). The two subgroups of MDD subjects (AD-free and AD-treated) were similarly compared with the corresponding matched controls (one-sample *t*-test). Differences for target protein contents between AD-free and AD-treated subjects were assessed by unpaired Student's *t*-test. Analyses of covariance (ANCOVA) indicated that age and PMI as well as subject gender (unpaired *t*-test) or male/female ratio did not influence the reported results (data not shown; see Keller and García-Sevilla, 2017). To note that recent positron emission tomography (PET) studies with subtype-selective radioligands have reported opposite gender differences in brain CB_1 receptor availability, with higher (Van Laere et al., 2008; Laurikainen et al., 2019) or lower (Normandin et al., 2015) receptor binding in men. In the current study, no significant sex differences were observed for the immunoreactive content of CB_1 or CB_2 receptor in the PFC/BA9 of control and MDD subjects (data not shown). Pearson's correlation coefficient (*r*)

was calculated to assess possible interdependences between variables. The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Immunodensity of CB_1 glycosylated receptor in the PFC/BA9 of subjects with MDD, and in subgroups of antidepressant (AD)-free and antidepressant (AD)-treated subjects

In subjects with MDD and regardless of AD drug treatment (n=23), the immunodensity of total CB_1 glycosylated (~54/64 kDa) receptor forms in the PFC/BA9 was significantly increased (+20±8%, p=0.02) when compared with the content (100%) in matched controls (Fig. 1A). In these MDD subjects (n=23), the cortical density of 54 kDa CB_1 receptor was also increased (+21±6%, p=0.004) and that of 64 kDa receptor form was marginally increased (+30±15%, p=0.06) in the same PFC/BA9 samples (Fig. 1A). In the subgroup of AD-free subjects (n=9), the total density of CB_1 glycosylated (~54/64 kDa) receptor (+14±14%, p=0.34), and that of individual 54 kDa receptor form (+17±9%, p=0.08) and 64 kDa receptor form (+20±27%, p=0.47) were not significantly augmented in the PFC/BA9 of MDD subjects (Fig. 1A). In contrast, the subgroup of AD-treated MDD subjects (n=14) showed increases in the cortical content of total CB_1 glycosylated (~54/64 kDa) receptor (+23±9%, p=0.02), that of 54 kDa receptor form (+24±9%, p=0.02), and that of 64 kDa receptor form (+36±18%, p=0.07) (Fig. 1A).

Notably, a statistically significant increase in the immunodensity of total CB_1 glycosylated (~54/64 kDa) receptor forms was also observed when the individual pairs of MDD subjects and matched controls were analyzed (Fig. 1B; pairwise comparison) and then expressed as mean percentages of the pool of controls (Fig. 1B; healthy controls: 101±4%, n=19; MDD subjects: 115±6%, n=23, p=0.03). This pairwise comparison better visualized the upregulation of CB_1 glycosylated receptor in the PFC/BA9 of MDD subjects (Fig. 1B).

3.2. Immunodensity of CB_2 glycosylated receptor in the PFC/BA9 of subjects with MDD, and in subgroups of antidepressant (AD)-free and antidepressant (AD)-treated subjects

In the same subjects with MDD and regardless of AD drug treatment, the immunodensity of CB_2 glycosylated receptor form (~45 kDa) in the PFC/BA9 (same brain samples) was found unchanged (+11±6%, n=23, p=0.10) when compared with the content (100%) in matched controls (Fig. 2A). Similar negative results for the contents of cortical CB_2 receptor were observed in the subgroups of AD-free (+12±11%, n=9, p=0.31) and AD-treated (+10±8%, n=14, p=0.23) MDD subjects (Fig. 2A).

This lack of CB_2 receptor regulation in the PFC/BA9 of MDD subjects, which was at variance with that of CB_1 receptor, was confirmed when the individual pairs of MDD subjects and matched controls were further analyzed (Fig. 2B; pairwise comparison) and then expressed as mean percentages of the pool of controls (Fig. 2B; healthy controls: 110±9%, n=19; MDD subjects: 114±7%, n=23, p=0.63).

3.3. Dephosphorylation of p-Ser2448 mTOR and p-Thr183/Tyr185 JNK1/2, and effects of postmortem interval (PMI) on phosphorylated and total kinases in the PFC/BA9 of healthy subjects

The specificity of anti-p-mTOR and anti-p-JNK1/2 antibodies for phosphorylated epitopes (Ser2448 and Thr183/Tyr185, respectively) was tested in dephosphorylation experiments on Western blots of PFC/BA9 (female, 30 year-old, PMI 15 h) using calf intestinal mucosa alkaline phosphatase (AP) (Fig. 3A; see other details in Methods, Section 2.5). The characterization of these p-antibodies revealed the identification p-Ser2448 mTOR kinase (major p-band of ~250-280 kDa; and a minor p-form of lower MW ~140-150 kDa), as well as p-Thr183/Tyr185 JNK1/2 kinase forms (~52-54 kDa kDa) in the human prefrontal cortex (see Fig. 3A and legend).

On the other hand, the length of PMI (ranging from 5 to 79 h) progressively reduced the contents of p-Ser2448 mTOR (r=−0.61, *p*=0.048, n=11) and total mTOR (r=−0.37, *p*=0.23, n=12) in the PFC/BA9 following the expected linear decay models (Fig. 3B, left panels). Similarly, the PMI (5-79 h) also decreased the contents of p-Thr183/Tyr185 JNK (r=−0.67, *p*=0.024, n=11) and total JNK (r=−0.83, *p*=0.0009, n=12) in the PFC/BA of healthy subjects (Fig. 3B, right panels).

3.4. Activation of neuroprotective mTOR and pro-apoptotic JNK1/2 kinases in the PFC/BA9 of subjects with MDD, and in subgroups of antidepressant (AD)-free and antidepressant (AD)-treated subjects

In subjects with MDD and regardless of AD drug treatment, the basal activation of mTOR kinase (p-Ser2448 TOR/t-TOR) was increased (+29±8%, n=18, *p*=0.002) in the same PFC/BA9 when compared with the activity (100%) in matched controls (Fig. 4A, left panel). The activation of cortical mTOR was also observed in the subgroups of AD-free (+33±13%, n=8, *p*=0.03) and AD-treated (+25±10%, n=10, *p*=0.04) MDD subjects (Fig. 4A, left panel). The upregulation of mTOR activity in AD-free MDD was due to an increased cortical immunocontent of p-mTOR (+34%) without changes in total (t) mTOR enzyme density (−6%) (Fig. 4A, right panel), which resulted in a significant mTOR basal activation (shown in Fig. 4A, left panel). In AD-treated MDD subjects, less clear-cut results were observed for the cortical immunodensities of mTOR forms (Fig. 4A, right panel), although the kinase activation was found significantly increased (Fig. 4A, left panel).

In the same MDD subjects and regardless of AD drug treatment, the basal activation of JNK1/2 kinases (p-Thr183/Tyr185/t-JNK) was found unchanged (+3±14%, n=10, *p*=0.2) in the PFC/BA9 when compared with that (100%) in matched controls (Fig. 4B). Similarly, no

activation of pro-apoptotic JNK1/2 kinases was observed in the subgroups of AD-free (−6±30%, n=4, *p*=0.3) and AD-treated (+8±15%, n=6, *p*= 0.1) MDD subjects (Fig. 4B).

3.5. Immunodensity of neuroplastic proteins in the PFC/BA9 of subjects with MDD

Minor and nonsignificant changes were quantified in the PFC/BA9 of subjects with MDD (AD-free and AD-treated), when compared with controls (C, 100%), for the contents of PSD-95 (a regulator of structural plasticity; C: 96±7%, n=10; MDD: 83±7%, n=9), Arc (a regulator of cytosleleton dynamics; C: 96±5%, n=10; MDD: 81±8%, n=10), and spinophilin (a regulator of cytoskeletal functions; C: 97±10%, n=10; MDD: 89±15%, n=9) (representative immunoblots for these proteins not shown).

4. Discussion

The upregulation of cortical CB_1 receptor and unchanged CB_2 receptor (immunodensities of mature glycosylated receptor forms) as well as the increased activity of neuroprotective mTOR kinase, with unaltered pro-apoptotic JNK1/2 and specific neuroplastic markers (PSD-95, Arc, spinophilin), in subjects with MDD (AD-free/treated) support their involvement in the complex pathophysiology of MDD (a chronic relapsing disorder of depressive episodes) and in the different molecular mechanisms of AD drugs. These positive data in MDD brains (upregulation of CB_1 receptor and mTOR signaling in the PFC/BA9) are in line with previous studies which showed (with other technical approaches) increases in receptor density (^{3}H-CP55,940 radioligand agonist binding sites) and/or function (agonist-stimulated ^{35}S-GTPγS binding at Gαi/o proteins) of cortical CB_1 receptors in depressed suicides, most of them under various AD treatments at the time of death (Mato et al., 2001, 2018; Hungund et al., 2004; Choi et al., 2012; reviewed in Urigüen et al., 2013).

A remarkable feature of the present study is the demonstration of an increased content of cortical glycosylated CB_1 receptor protein (54/64 kDa) in MDD, mainly in AD-treated depressed suicides (Fig. 1). However, the specific functional significance of N(asparagine)-glycosylation of CB_1 protein in the human brain is not yet clear (see Discussion in Ruehle et al., 2017, and other references therein), although it could be involved in a better efficiency in CB_1 signaling through receptor dimerization (i.e. disulfide-linked CB_1 dimers; see López de Jesús et al., 2006). Therefore, the current findings complement previous results measuring CB_1 radioligand agonist binding sites in depressed brains (Hungund et al., 2004, Mato et al., 2018; reviewed in Urigüen et al., 2013), and together suggest an enhanced endocannabinoid signaling in the PFC/BA9 of subjects with MDD. In contrast, preclinical studies have reported controversial studies in animal models of depression such as a deficent CB_1 receptor function in the mouse brain (reviewed in Hillard and Liu, 2014), but the reason for the discrepancy is not known, although it could reflect major neurochemcial differences and/or regulatory mechanisms between species (rat/mouse brains vs human brain). However, it is also important to note that mice lacking CB_1 receptors (CB_1 knockout mice) were shown to display a depressive-like phenotype (induced by the forced swim test) with an increased reactiveness to stress (forced swim test for 14 days), indicating the involvement of this major CB receptor in the neural mechanisms of animal models of depression (Aso et al., 2011).

On the other hand, recent preclinical studies have reported positive pharmacodynamic interactions between CB_1 and CB_2 receptor ligands and atypical ADs (tianeptina, agomelatine) (Poleszak et al., 2020a) and that selective CB_2 drugs can potentiate the activity of conventional ADs in some behavioral tests (forced swim and tail suspension) in mice (Poleszak et al., 2020b). It therefore appears that, although the immunocontent of CB_2 receptors was not significantly modulated in MDD brains (AD-free or AD-treated), this CB receptor could also have a role in the actions of AD drugs in mice.

Concerning the cellular pathways associated with CB receptors, an early conflicting postmortem study (Jernigan et al., 2011) on the status of neuroprotective mTOR in MDD brains (PFC/BA10; n=12; AD-free subjects) did not assess the indispensable activity of the kinase (i.e. ratio p-Ser2448 TOR/t-TOR immunocontents) and instead it only reported a significant decrease (32%) in the total (t) amount of TOR immunoreactivity, concluding that mTOR signaling is compromised in MDD (Jernigan et al., 2011). In the present study and in marked contrast, the basal activation of mTOR kinase (p-Ser2448 TOR/t-TOR) was found significantly upregulated in AD-free (33%) and in AD-treated (25%) MDD subjects (note that t-TOR was found unaltered as expected for any enzyme regulation by phosphorylation) (see Fig. 4A, right and left), indicating a parallelism between the upregulation of CB_1 receptors and the activation of downstream mTOR in the PFC/BA9 of subjects with MDD. In this context, further studies are needed to unravel the real relevance of mTOR signaling (a kinase associated with neuroprotection and neuroplasticity) in the pathophysiology of MDD and in the mechanism of action of various classes of ADs (reviewed in Abelaria et al., 2014; Ignácio et al., 2015). Notably, rapid AD drugs such as ketamine have been shown to activate mTOR-related signaling (see Ignácio et al., 2015 and other referenes therein).

In the present study, the basal cortical activation of pro-apoptotic JNK1/2 kinase (i.e. the ratio p-Thr183/Tyr185/t-JNK) in AD-free and AD-treated MDD subjects was not different to that quantified in matched controls, suggesting that this kinase, which is a key mediator of excitotoxic damage and neuronal death by apoptosis (Yang et al., 1997; Keller and García-Sevilla, 2015), was not engaged in the PFC/BA9 of the current cohort of depressed subjects. Recently, however, it has been reported (Martín-Hernández et al., 2018) that various AD treatments in subjects with MDD significantly increased (compared with matched controls and with AD-free MDD subjects) the content of p-JNK (by 56%) in postmortem PFC/BA9 of depressed subjects (to note that in this study total kinase JNK immunoreactivity was not

assessed and therefore the ratio of real activity p-JNK/t-JNK was not reported), which was suggested to indicate an overactivation of this pro-apoptotic pathway (Martín-Hernández et al., 2018). In this context, preclinical studies have reported that acute and chronic treatments with conventional AD drugs (e.g. desipramine and fluoxetine) enhanced the expression of the phosphorylated form of the effective anti-apoptocic factor Fas-associated death domain protein (i.e. the ratio p-FADD/FADD ratio) in rat brain cortex (García-Fuster and García-Sevilla, 2016).

On the other hand, MDD and schizophrenia have been shown to differentially regulate CB receptors in the brain. Thus and in contrast to the finding of the present MDD study (CB_1 receptor upregulation in PFC/BA9), a reduced activity of CB_1 receptor (mRNA and/or immunoreactive protein) has been reported in the PFC/BA9 and PFC/BA46 of subjects with schizophrenia (Eggan et al., 2008; Urigüen et al., 2009; Muguruza et al., 2019), not only when the data were compared with those obtained in matched controls but also when the results were analyzed and compared in parallel with a cohort of MDD subjects in the same postmortem study (Eggan et al., 2010). Furthermore, recent PET studies in subjects with schizophrenia (including patients with first-episode psychosis) have reported a reduced CB_1 receptor availability in various brain regions such as the cingulate cortex, hippocampus, striatum and thalamus (Ranganathan et al., 2016; Borgan et al., 2019). It is also relevant to note that a lower functioning of CB_2 receptors has been associated with an increased susceptibility to schizophrenia in postmortem human brain and in a mouse model of psychosis (Ishiguro et al., 2010). Therefore, it can be concluded that the two major psychiatric syndromes, MDD and schizophrenia, are associated with an opposite regulation of the CB_1 and/or CB_2 receptor systems in the brain. Thus, cortical CB_1 and CB_2 receptors could induce (after the expected simultaneous activation by endoCBs) opposing actions in MDD and schizophrenia to finally moduate specific functions (see also Rodríguez-Muñoz ert al., 2017).

4.1 Limitations

There are some potential limitations in this study. Neurochemical analyses in postmortem human brains, despite major confounding variables such as long postmortem delays leading to protein degradation, remain a relevant approach to investigate relevant alterations in psychiatric syndromes such as MDD (review in McCullumsmith and Meador-Woodruff, 2011). Although this study was carefully designed to evaluate in brain (PFC/BA9) the basal status of CB_1/CB_2 receptors and associated mTOR and JNK1/2 signaling, as well as the effects of AD treatments, the reported results should be taken with some caution, mainly because of the relatively reduced number of subjects included in the AD-free (n=9) and AD-treated (n=14) subgroups, as well as the different number of total control subjects (n=19) and total MDD subjects (n=23) which precluded a perfect one to one matching design of experimental subjects (see comments on Sample Size and Prior Medicacion Exposure in McCullumsmith and Meador-Woodruff, 2011). Furthermore, the subgroup of AD-treated subjects with MDD had been medicated with a heterogeneous variety of AD drugs (citalopram, duloxetine, fluoxetine, mirtazapine or venlafaxine) with different mechanisms of action, which could result in different modulatory effects on CB receptors and associated signaling. Finally, the majority of subjects with MDD had committed suicide and therefore the results should be understood in the context of suicide victims which most probably did not respond to the received AD medications.

5. Conclusion

The upregulation of CB_1 (but not CB_2) receptor immunodensity with increased activity of neuroproetective mTOR kinase in PFC/BA9 of subjects with MDD further supports the

contribution of this CB receptor and associated signaling in the pathophysiology of MDD and in the molecular mechanisms of AD drugs.

Author statements

Contributors

M.J.G.-F and J.A.G.-S. collected and analyzed pre- and post-mortem data related to human brain samples, and designed the experimental protocols of the study. G.S. and E.H.-H. performed the immunoblot experiments, analyzed the data and prepared the table and figures under the supervision of M.J.G.-F and J.A.G.-S. J.A.G.-S. wrote the first version of the manuscript and all authors contributed to the writing of the final version and gave their approval to the manuscript.

Role of funding sources

The study was supported in part by Redes Temáticas de Investigación Cooperativa en Salud–Red de Trastornos Adictivos (RETICS-RTA, RD12/0028/0011 and RD16/0017/0010), Instituto de Salud Carlos III (ISCIII, MINECO/FEDER), Spain. E.H.-H. was supported by a predoctoral fellowship (FPI/2102/2018) from Consejería de Innovación, Investigación y Turismo del Gobierno de las Islas Baleares y del Fondo Social Europeo.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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Table 1

Demographic data of healthy controls (C) and subjects with clinical diagnosis of major depressive disorder (MDD): gender (F, female; M: male), age (yr, years), postmortem interval (PMI; h: hours), brain pH, and neuron-specific enolase-2 (NSE-2) content. These cohorts of subjects have been used in previous postmortem studies (García-Fuster al., 2014; Keller and García-Sevilla, 2017).

Groups and subgroups of subjects (n)	Gender (F/M)	Age (yr)	PMI (h)	Brain pH	NSE-2 (% C)
Healthy controls (C, n=19)	11/8	53±4	24±3	6.46±0.04	100±5
Major depressive disorder (MDD, n=23)	14/9	56±4	20±2	6.42±0.03	109±8
Antidepressant (AD-free, n=9)	7/2	64±5	24±4		
Antidepressant (AD-treated, n=14)	7/7	51±5	18±3		

Data (age, PMI, brain pH and NSE-2) are mean ± SEM values of n subjects. In a few cases and due to a lesser healthy control availability (n=19), compared with MDD (n=23), the same control was matched with two MDD (AD-free and AD-treated; same sex and very similar age, PMI, brain pH, and NSE-2 content (see Methods). Healthy controls died of accidental or violent causes (heart attack, traffic trauma, drowning, homicide) and had a negative toxicology in blood and/or urine samples for psychotropic drugs (including cannabis), except benzodiazepines (oxazepam, midazolam) in two subjects. MDD subjects mainly died of violent suicide (hanging, jumping, gun shot, drug overdose). AD drugs detected in blood and/or urine: citalopram, duloxetine, fluoxetine, mirtazapine, venlafaxine.

Table 2

Antibodies used for the detection and quantification of cannabinoid (CB_1 and CB_2) receptors and other target proteins in postmortem human brain (PFC/BA9).

Protein	Antigen	Host	Catalog	Batch	Company
CB1	Human CB1 receptor (461-472 residues)	Rabbit	23703	877720	Abcam, UK
CB2	Human CB2 receptor (20-33 residues)	Rabbit	101550	424681-1	Cayman, USA
CB2	Human CB2 receptor (228-242 residues)	Rabbit	ACR-002	AG-01	Alomone labs, Israel
p-mTOR	Human p-mTOR (p-Ser2448)	Rabbit	2971	14	Cell Signaling, USA
mTOR	Human mTOR	Rabbit	2972	7	Cell Signaling, USA
p-SAPK/JNK	Human SAPK/JNK (p-Thr183/Tyr185)	Rabbit	9251	14, 21	Cell Signaling, USA
SAPK/JNK	Human SAPK/JNK (GST fusion protein)	Rabbit	9252	12	Cell Signaling, USA
PSD-95	Recombinant rat PSD-95	Rabbit	MAB1596	LV1581097	Merck Millipore, USA
Spinophilin	Human spinophilin	Rabbit	AB5669	LV1585250	Merck Millipore USA
Arc	Human Arc	Rabbit	sc-15325	B2309	Santa Cruz, USA
Enolase-2	Human enolase-2	Rabbit	H9536	1	Cell Signaling, USA
β-actin	Human β-actin (2-16 residues)	Mouse	A1978	118K4827	Sigma Aldrich, USA

Figure legends

Fig. 1 (**A**) Immunodensity of CB_1 receptor glycosylated forms (total 54-64 kDa forms, and individual 54 kDa and 64 kDa forms), normalized to that of β-actin, in the prefrontal cortex (PFC/BA9) of all subjects with major depressive disorder (MDD, n=23), and subgroups of antidepressant (AD)-free (n=9) and antidepressant (AD)-treated (n=14) subjects, expressed as mean ± SEM (bars) percentages of the corresponding matched control group (100%, n=19). **p*=0.02; ***p*=0.004 when compared with matched controls (one-sample *t*-test). Below: representative immunoblots of cortical CB_1 receptor and β-actin (PFC/BA9) for representative experiments which included different samples of control (C; C1-C8), MDD AD-free (F; F1-F5), and MDD AD-treated (T; T1-T6) matched subjects. The molecular masses (kDa) of target proteins were estimated from referenced standards. (**B**) Individual contents of CB_1 receptor (total 54-64 kDa glycosylated forms) in PFC/BA9 of pairs of MDD subjects (n=23, black circles; right, mean value, black circle) and matched controls (n=19, white circles), normalized to that of β-actin and expressed as % of the pool of controls (left, white circle). **p*=0.03. This classic pairwise comparison better visualized the upregulation of cortical CB_1 receptor in subjects with MDD (regardless of AD treatment). Pairwise comparison is any process of comparing entities in pairs to judge which of each entity has a greater amount of some quantitative property, or whether or not the two entities are identical (control values vs experimental values).

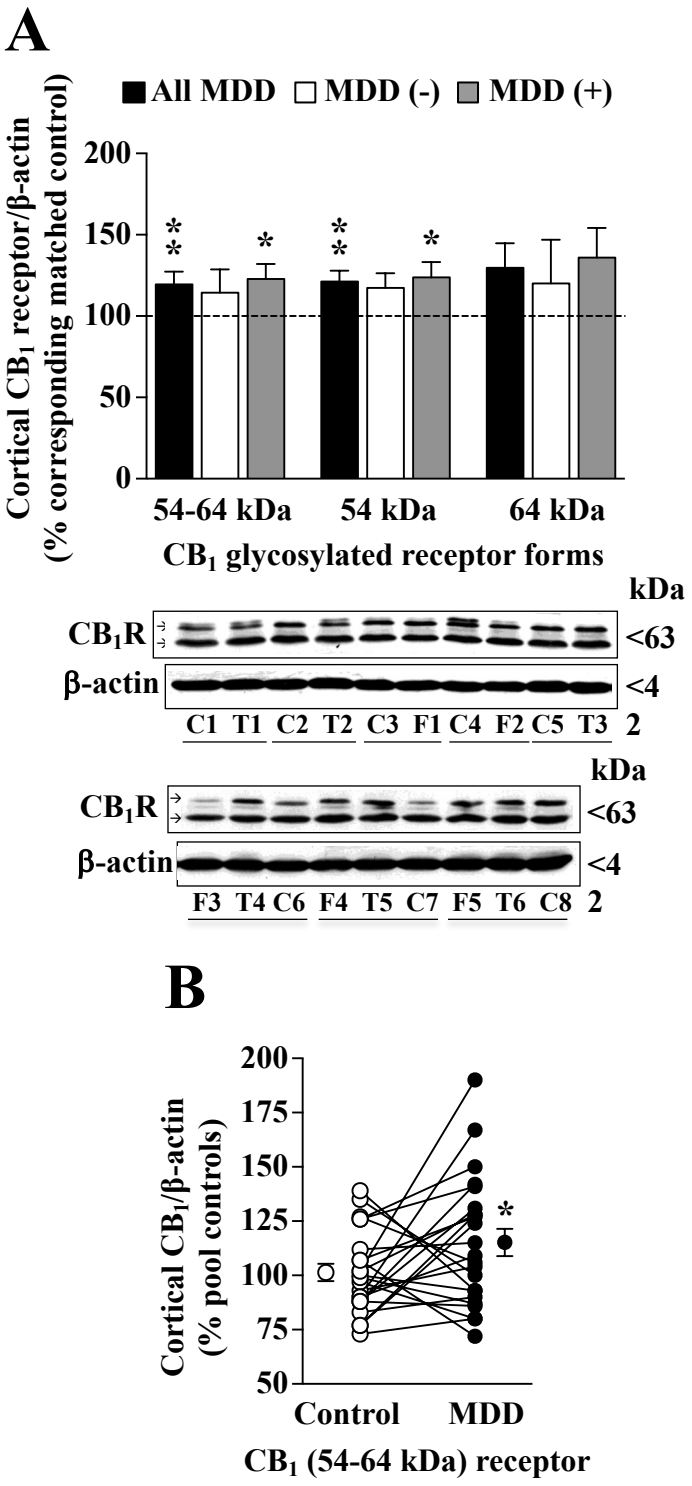

Figure 1 Salort et al

Fig. 2 (**A**) Immunodensity of CB_2 receptor glycosylated form (≈45 kDa), normalized to that of β-actin, in the prefrontal cortex (PFC/BA9) of all subjects with major depressive disorder (MDD, n=23), and subgroups of antidepressant (AD)-free (n=9) and antidepressant (AD)-treated (n=14) subjects, expressed as mean ± SEM (bars) percentages of the corresponding matched control group (100%). One-sample *t*-test did not detect significant changes (increases) in cortical CB_2 immunoreactive content in MDD (p=0.10-0.23). Below: representative immunoblots of cortical CB_2 receptor and β-actin (PFC/BA9) for representative experiments which included different samples of control (C; C1-C8), MDD AD-free (F; F1-F5), and MDD AD-treated (T; T1-T6) matched subjects. The molecular masses (kDa) of target proteins were estimated from referenced standards. (**B**) Individual contents of CB_2 receptor (≈45 kDa glycosylated forms) in PFC/BA9 of pairs of MDD subjects (n=23, black circles; right, mean value, black circle) and matched controls (n=19, white circles), normalized to that of β-actin and expressed as % of the pool of controls (left, white circle). This classic pairwise comparison better visualized the lack of changes (p=0.63) of cortical CB_2 receptor in subjects with MDD (regardless of AD treatment). Pairwise comparison is any process of comparing entities in pairs to judge which of each entity has a greater amount of some quantitative property, or whether or not the two entities are identical (control values vs experimental values).

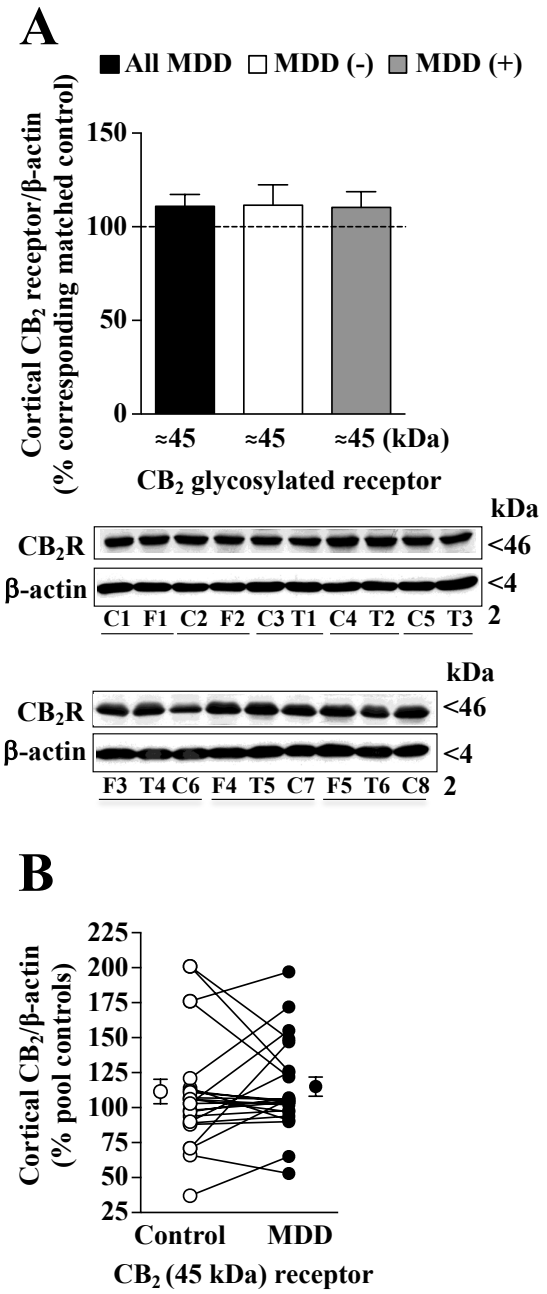

Figure 2 Salort et al

Fig. 3 (**A**) Representative autoradiograms of Western blots depicting labelling of immunodetectable phosphorylated p-Ser2448 mTOR and total t-mTOR (left panel, arrows) as well as phosphorylated p-Thr183/Tyr185 JNK1/2 and total t-JNK1/2 (right panel, arrows) with specific p- and t- antibodies in the human brain (PFC/BA9, female, 30 year-old,

postmortem interval, PMI, 15 h). The specificity of the antibodies was assessed in dephosphorylating experiments with calf intestinal mucosa alkaline phosphatase (AP). Total cortical homogenate (40 μg protein) was incubated at 30°C for 15 min in the absence (C, control samples) or presence of AP (95 units). Other samples containing AC were also incubated with 100 mM sodium pirophosphate (IC, inhibited controls). See further details in Methods, Section 2.5. The apparent molecular masses of p- and t-kinases were determined by calibrating the blots with prestained molecular weight markers (kDa) as shown on the left hand side. (**B**) Correlation analyses and representative immunoblots for the effect of PMI (5-79 hours) on the content of p-mTOR and t-mTOR (left panels), and p-JNK1/2 and t-JNK1/2 (right panels) in the PFC/BA9 of healthy subjects (12 men; age: 24-47 years). Note that for p-mTOR and p-JNK1/2 very low values were detected (gray circle) only for the phosphorylated (p-) enzymes in one (same) subject, and these p-values were not included in the corresponding correlation study. Data were expressed as integrated optical density (IOD) units. Below: representative immunoblots for TOR and JNK enzyme species (phosphorylated and total kinases) along the PMI (5-79 hours) in PFC/BA9 of healthy subjects. These PMI experiments were repeated with similar results. The molecular masses (kDa) of p- and t-kinases were estimated from referenced standards.

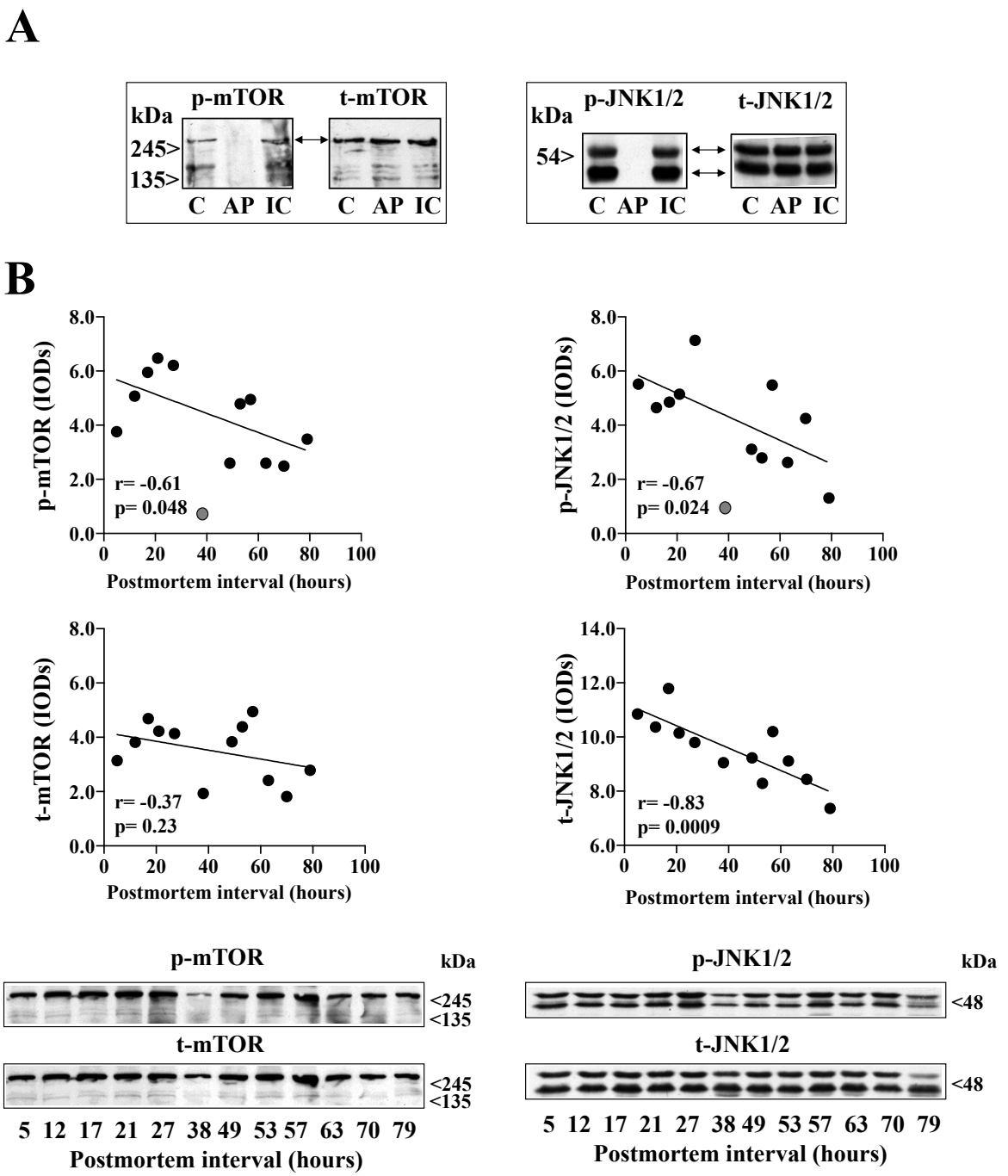

Figure 3 Salort et al

Fig. 4 (A, left) Activation of neuroprotective mTOR kinase (p-Ser2448 mTOR/t-mTOR) in the prefrontal cortex (PFC/BA9) of subjects with major depressive disorder (MDD, n=18), and subgroups of antidepressant (AD)-free (n=8) and antidepressant (AD)-treated (n=10) subjects, expressed as mean ± SEM (bars) percentages of the corresponding matched control

group (100%). *p=0.03; **p=0.002 when compared with matched controls (one-sample t-test). **(A, right)** Immunocontents of cortical mTOR forms (p-Ser2448 mTOR and t-mTOR) in MDD AD-free (p-TOR increased by 33% and t-TOR decreased by 6%) and AD-treated (p-TOR increased by 2% and t-TOR decreased by 8%) expressed as mean ± SEM (bars) percentages of the corresponding matched control group (100%). Below: representative immunoblots of cortical p-mTOR and t-mTOR which included different samples of control (C; C1-C4), MDD AD-free (F; F1-F2), and MDD AD-treated (T; T1-T2) matched subjects. The molecular masses (kDa) of target proteins were estimated from referenced standards. **(B, left)** Activation of pro-apoptotic JNK1/2 kinases (p-Thr183/Tyr185/t-JNK) in the prefrontal cortex (PFC/BA9) of subjects with major depressive disorder (MDD, n=10), and subgroups of antidepressant (AD)-free (n=4) and antidepressant (AD)-treated (n=6) subjects, expressed as mean ± SEM (bars) percentages of the corresponding matched control group (100%, **n=4-10**). One-sample t-test did not detect significant changes in cortical JNK1/2 activation in MDD (p=0.1-0.3). **(B, right)** Representative immunoblots of cortical p-JNK1/2 and t-JNK1/2 which included different samples of control (C; C1-C3), MDD AD-free (F; F1-F2), and MDD AD-treated (T; T1-T2) matched subjects. The molecular masses (kDa) of target proteins were estimated from referenced standards.

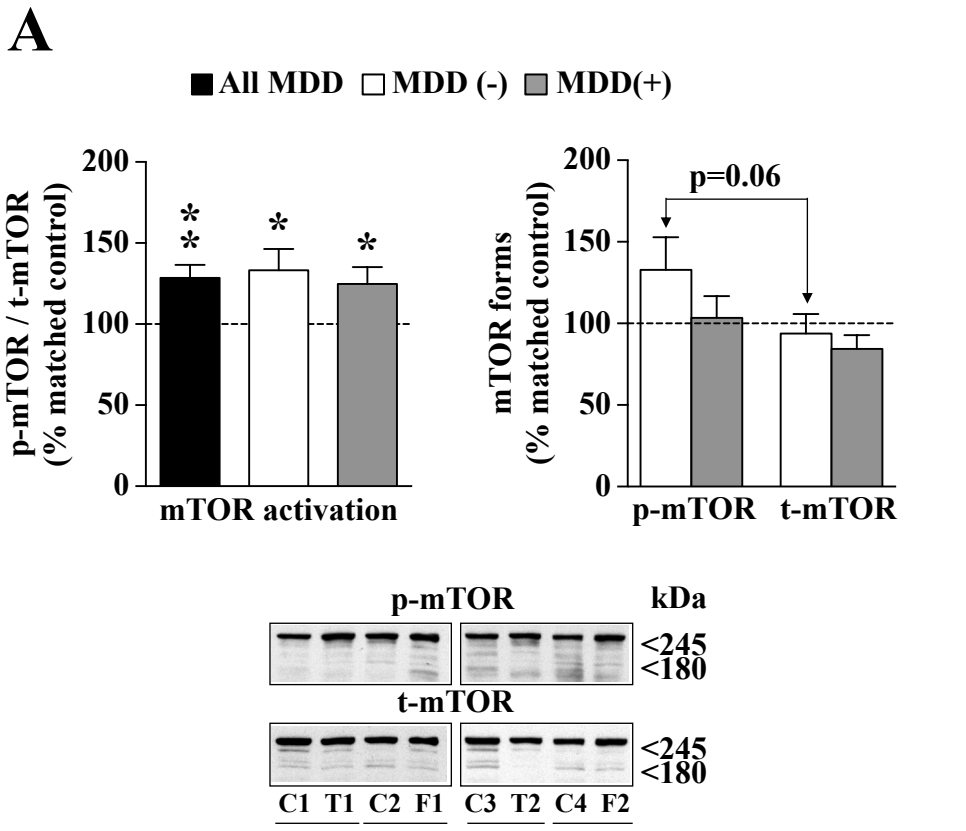

Figure 4 Salort et al