

AChE and RACK1 Promote the Anti-Inflammatory Properties of Fluoxetine

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Abstract Selective serotonin reuptake inhibitors (SSRIs) show anti-inflammatory effects, suggesting a possible interaction with both Toll-like-receptor 4 (TLR4) responses and cholinergic signaling through as yet unclear molecular mechanism(s). Our results of structural modeling support the concept that the antidepressant fluoxetine physically interacts with the TLR4–myeloid differentiation factor-2 complex at the same site as bacterial lipopolysaccharide (LPS). We also demonstrate reduced LPS-induced pro-inflammatory interleukin-6 and tumor necrosis factor alpha in human peripheral blood mononuclear cells preincubated with fluoxetine. Furthermore, we show that fluoxetine intercepts the LPS-induced decreases in intracellular acetylcholinesterase (AChE-S) and that AChE-S interacts with the nuclear factor kappa B (NFκB)-activating intracellular receptor for activated C kinase 1 (RACK1). This interaction may prevent NFκB activation by residual RACK1 and its interacting protein kinase PKCβII. Our findings attribute the anti-inflammatory properties

of SSRI to surface membrane interference with leukocyte TLR4 activation accompanied by intracellular limitation of pathogen-inducible changes in AChE-S, RACK1, and PKCβII.

Keywords Antidepressant · Cholinergic · Inflammation · Toll-like receptor · Acetylcholinesterase (AChE) · Receptor for activated C kinase 1 (RACK1)

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the first choice of treatment for patients with major depression (Santaguida et al. 2012), who frequently show enhanced activation of the pro-inflammatory cytokine interleukin-6 (IL-6) and of nuclear factor kappa B (NFκB) following antigen challenge (Dantzer et al. 2008). Clinical observations further suggest that peripheral immune activation via pro-inflammatory cytokine release is an underlying cause of this psychiatric condition and that pro-inflammatory cytokines contribute to its major symptoms. Supporting this notion, depression often accompanies inflammatory disease (such as irritable bowel syndrome, type 2 diabetes, arthritis, and autoimmune disorders) (Leonard 2010). Treatment with the SSRI fluoxetine reduces serum IL-6 levels in patients with depression and decreases the interferon gamma (INF-γ)/IL-10 ratio of in vitro human blood samples (Sluzewska et al. 1995), both consistent with an anti-inflammatory effect. Furthermore, nonresponders to SSRI medications continue to exhibit raised IL-6 levels, suggesting that nonresponse to treatment may be linked to a failure of SSRI to reduce IL-6 in these patients (O'Brien et al. 2007). Based on this cumulative information, we surmised that antidepressant treatment is capable of suppressing increased levels of innate immune responses that may contribute to depression.

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The cholinergic anti-inflammatory route (Tracey 2002) recently emerged as most relevant to this picture. Cells of the immune system can produce and respond to multiple neurotransmitters including acetylcholine (ACh) (Prado et al. 2013), which suppresses the release of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α), IL-1 β , and IL-18 (Borovikova et al. 2000). Stimulation of the vagus nerve results in the release of ACh that activates the $\alpha 7$ subunit of nicotinic receptors, attenuating the secretion of pro-inflammatory cytokines from tissue macrophages by intercepting the activation of NF κ B (Rosas-Ballina and Tracey 2009; Russo and Taly 2012). However, the SSRI fluoxetine was shown to directly block the nicotinic receptor (Maggi et al. 1998), which should interfere with the anti-inflammatory response of ACh; this response, in turn, may be effectively antagonized by overexpression of the ACh-hydrolyzing enzyme acetylcholinesterase (AChE). Therefore, we wished to assess these seemingly inverse effects of antidepressants on inflammation.

Pattern recognition receptors such as Toll-like receptors (TLRs), a family of evolutionarily conserved immune sensing receptors, have a crucial role in early host defense against invading pathogens. Individual TLRs trigger specific biological responses by selectively recruiting distinct adaptor molecules and by providing specific immunological responses targeted to the infecting microbes (Kawai and Akira 2010). Toll-like receptor 4 (TLR4) detects lipopolysaccharides (LPS) from Gram-negative bacteria, and thus, is a key activator of inflammation. TLR4 is located on the cell membrane of myeloid immune cells and upon LPS binding and interaction with its co-receptor MD-2 triggers two pathways: the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which involves the early phase of NF κ B activation leading to the production of inflammatory cytokines and the TRIF dependent pathway, which activates the IRF3 response leading to the production of type 1 interferons and the expression of IFN-inducible genes and a later phase of NF κ B activation also leading to inflammatory cytokine expression (Kagan and Medzhitov 2006; Kawai and Akira 2010). Correspondingly, stress exposure was shown to increase TLR4 and MD-2 mRNA and protein levels in the brain's cortex (Garate et al. 2011). Subsequent NF κ B activation leads to increased production of inflammatory cytokines (Kawai and Akira 2010). These cytokines can penetrate through the blood–brain barrier further activating neurons and microglia, thereby exacerbating the stress response (Zimmerman et al. 2012). Taken together, these reports suggest that blocking the inflammatory response may interrupt this vicious cycle and may also contribute to the antidepressant activity of SSRIs.

While SSRIs are largely believed to manipulate serotonin levels within brain synapses, recent data indicate that fluoxetine selectively inhibits both endosomal TLR signaling (Hutchinson et al. 2010; Sacre et al. 2010) and the nicotinic muscle $\alpha_1\beta_1\gamma\delta$ nAChR and neuronal $\alpha_2\beta_4$ or $\alpha_3\beta_4$ nAChRs

(Maggi et al. 1998). The first predicts that fluoxetine can suppress inflammation in muscle and brain, whereas the second indicates that it may have an inverse effect. This potential competition turned our interest to the intracellular level.

Receptor for activated C kinase 1 (RACK1) is an intracellular regulator of both NF κ B and AChE. RACK1 is a member of the tryptophan–aspartate repeat (WD repeat) family containing seven internal WD40 motifs, which shares a common role in scaffolding protein complexes (Adams et al. 2011). It was originally identified based on its ability to bind to activated protein kinase C (PKC) (Adams et al. 2011; Ron et al. 1994), which in turn activates NF κ B (Chen et al. 1998; Leppanen et al. 2008). RACK1 recruits its partner proteins and facilitates their interaction with various cytoplasmic proteins and transmembrane receptors, thus providing a platform for subcellular mobilization and subsequent physiological responses (Ron et al. 1999; Sklan et al. 2006). When bound to the stress-responsive “readthrough” AChE-R variant, RACK1 is implicated in stress responses and behavior (Birikh et al. 2003). However, the major AChE variant, AChE-S, was not yet tested for potential interaction with RACK1. Therefore, we decided to explore the possibility that the anti-inflammatory properties of fluoxetine are due to combined extra- and intracellular processes involving both the cholinergic and TLR4 signaling pathways and to study its effects on inflammation, AChE-S and RACK1.

Materials and Methods

Computational Modeling The crystal structure of the human TLR4-human MD-2-*Escherichia coli* LPS Ra complex was obtained from the RCSB Protein Data Bank (PDB ID: 3FXI) (Park et al. 2009). Fluoxetine-R structure was taken from (PDB ID: 3GWV). The chemical structures of all other components were extracted via DeepView/Swiss-PdbViewer v4.0.1 for docking purposes (Guex and Peitsch 1997). Modified PDB files were opened with AutoDockTools 1.5.4 (Sanner 1999); hydrogen atoms were added and files were resaved in pdbqt format. The other screened molecules were downloaded from the UCSF ZINC database (Irwin et al. 2012) in a Mol2 format: Zolofit (ZINC:01853550), citalopram (ZINC:03794601), trazodone (ZINC:00538483), nefazodone (ZINC:00538065), venlafaxine (ZINC:00896698), duloxetine (ZINC:01536779), escitalopram (ZINC:21985875), acetylcholine (ZINC:03079336). The files were opened with AutoDockTools 1.5.4 and saved in pdbqt format with natural rotations allowed. Docking of the ligands was conducted at least six times using Autodock Vina 1.1.2 (Trott and Olson 2010). Autogrid center was set to 3.485, -7.805, and -4.5; 126 grid points with 1.0 Å spacing expanding in all directions to cover the entire TLR4–MD-2 complex. Illustration of AChE–RACK1 interaction was created after docking the

monomers of recombinant human AChE (PDB ID: 3LII) and human RACK1 (PDB ID: 4AOW) using ZDOCK 3.0.2 (Dvir et al. 2010; Pierce et al. 2011; Ruiz Carrillo et al. 2012).

Cell Cultures Peripheral blood mononuclear cells (PBMC) preparation was as in Shaked et al. (2009). TNF- α and IL-6 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience Systems kits, 88-7346-22, 88-7066-22, 88-7926-22, 88-7010-22).

Western Blot Analysis Immunodetection was carried out with primary antibodies against PKC β II (sc-109, sc-210, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 and RACK1 (610178, BD Transduction Laboratories, Erembodegem, Belgium) diluted 1:1000; secondary horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Jackson Laboratories, West Grove, PA) diluted 1: 20,000 and enhanced chemiluminescence (ECL, EZECL, Biological Industries, Israel).

Immunofluorescence Human PBMC were layered on poly-L-lysine coated microscope slides by centrifugation (300 rpm, 3 min), fixed in 4 % paraformaldehyde for 20 min, permeabilized in 0.1 % Triton X-100 for 4 min, blocked in 5 % horse serum for 1 h, and incubated with primary antibodies overnight at 4 °C. Cy3-conjugated secondary antibodies were added (1 h, RT). Coverslips were rinsed twice with PBS and once with DDW and then mounted for confocal microscopy (FluoroMount-G, Electron Microscopy Sciences, Washington, DC). Primary antibodies used were against RACK1 (610178, BD Transduction Laboratories) and AChE (sc-6431, Santa Cruz). Slides were scanned using the Olympus FV-1000 confocal microscope. Image analysis was performed using ImageJ.

Cloning and Purification of RACK1 *E. coli* BL21(DE3) ps⁺ cells were transformed with the pDest-RACK1 plasmid. A single colony of the transformed cells was picked from a Luria Bertani (LB) medium agar plate containing 100 μ g/ml ampicillin and served to inoculate a 2-ml liquid culture of the same medium. One ml of this culture was used to inoculate 100 ml of LB medium. The culture was grown (37 °C, 200 rpm) to OD \approx 0.6 at 600 nm after which 0.3 mM isopropyl β -D-1-thiogalactopyranoside was added and the culture grown further (30 °C, 200 rpm, 9 h). Cells were pelleted from this culture (4 °C, 6,000 g, 20 min), the supernatant discarded, and the pellet washed with PBS. Cells were then lysed with buffer followed by French press processing. Lysate was pelleted (4 °C, 12,000 g, 30 min) and supernatant filtered (0.2 μ m) and loaded onto equilibrated Q-Sepharose FF anion exchange column. RACK1 fractions were identified by

immunoblot analysis in products of the NaCl gradient: 2CV 21 mM, 7CV 21–41 mM, 2CV 41–118 mM, and 1CV 118–1,000 mM. RACK1-containing fractions were pooled and protein kept at 4 °C in the presence of 0.2 M NaCl and 10 mM TCEP (pH 8.0).

Cross-Linking Photo-induced cross-linking was adapted from the method of described in Fancy and Kodadek (1999). Purified human recombinant AChE-S (Sigma, C1682), AChE-R (Protalix Biotherapeutics, Israel), and RACK1 (see above) were used. One micromolar protein was added to a buffer containing 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl. Pyridyl ruthenium, tris(2,2'-bipyridyl)-ruthenium(II) chloride hexahydrate (Ru(II)bpy₃²⁺) was then added to a final concentration of 125 μ M, followed by ammonium persulfate (APS) to a final concentration of 2.5 mM. After illumination with visible light for 30–60 s, reactions were stopped by adding 4 \times Laemmli sample loading buffer, heated for 20 min at 60 °C, and separated by standard SDS-PAGE.

Results

SSRIs Interact with TLR4–MD-2

To search for the molecular basis of the association between antidepressant treatment and reduced pro-inflammatory cytokine levels, we first performed an in silico screen of potential interactions between TLR4 and several subclasses of antidepressants: SSRI, serotonin antagonists and reuptake inhibitors (SARI), and serotonin–norepinephrine reuptake inhibitor (SNRI). TLR4 is the receptor for bacterial LPS and lipid A, its toxic moiety. However, TLR4 requires MD-2, a secreted molecule, to functionally interact with LPS (Park et al. 2009). Furthermore, a third protein, CD14, was shown to participate in LPS signaling leading to the activation of NF κ B and the production of pro-inflammatory cytokines. Therefore, we modeled the interaction of different antidepressant compounds such as the SSRI molecules fluoxetine and Zoloft (Fig. 1a) using the crystallographic structure of the human TLR4–MD-2 complex (PDB ID: 3FXI) and the AutoDock Vina software. Affinity energy scores were calculated for each interaction model (see Table 1). A great majority of the top models predicted powerful affinity of the tested SSRIs for two identical binding sites on the two arms of the TLR4–MD-2 complex (Fig. 1b) which were independently identified as the LPS binding pockets of this dimer (Akashi-Takamura and Miyake 2008; Shimazu et al. 1999) (Fig. 1c). ACh served as a negative control and showed no affinity to the tested site with as low as 7 % accuracy of predicted interactions. A

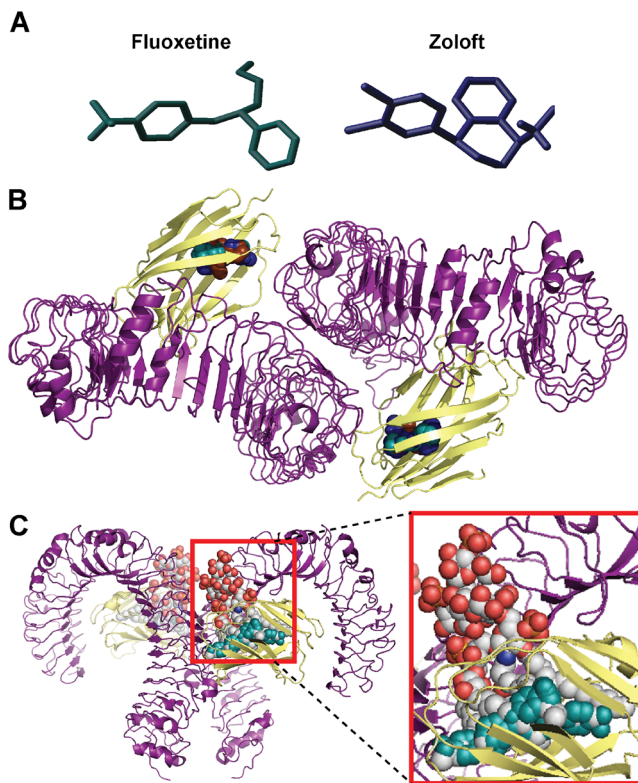


Fig. 1 Modeling of SSRI binding to TLR4–MD-2. **a** Three-dimensional structure of fluoxetine and Zoloft. **b** Fluoxetine and Zoloft are predicted to bind to the same two sites on the TLR4–MD-2 complex (cyan and blue) similar to the known TLR4 inhibitor pethidine (red). **c** Modeling predicts that fluoxetine (cyan) interacts with TLR4–MD-2 within the LPS binding site (gray with red balls indicating the LPS’s phosphate groups) of the TLR4–MD-2 dimer complex (purple and yellow sticks). Right: magnification of the interaction area

Table 1 Ratio of fits in the known agonist binding site

Molecule	Number of models in the LPS binding site out of the top 30 modeling results	Number of models in the LPS binding site out of the general modeling results	Number of models from the top 25 highest binding affinity
Fluoxetine	23/30 (77 %)	32/54	23/25
Zoloft	26/30 (87 %)	28/54	21/25
Citalopram	16/30 (53 %)	27/54	13/25
Escitalopram	20/30 (67 %)	22/54	19/25
Venlafaxine	13/30 (43 %)	15/54	12/25
Duloxetine	12/30 (40 %)	16/54	15/25
Trazodone	15/30 (50 %)	18/54	11/25
Nefazodone	20/30 (67 %)	24/54	18/25
Acetylcholine (negative control)	2/30 (7 %)	6/54	2/25
Pethidine (positive control)	25/30 (83 %)	36/54	24/25

known agonist of TLR4, the synthetic opioid pethidine which interacts with the same binding site (Hutchinson et al. 2010), served as a positive control for the modeling (red molecule in Fig. 1b). To correct for the effect on these scores of the molecular weight of the modeled compounds, we estimated the strength of the modeling outcome by comparing the fractions of the top modeling predictions for each molecule. In these analyses, 77 and 87 % predicted interactions for fluoxetine and Zoloft out of the top modeling results showed potent affinity for the LPS binding site of the TLR4–MD-2 complex. In comparison, antidepressants from other classes, such as SNRI, venlafaxine, and the SARI trazodone showed lower affinity to that site. Of all the modeled compounds, fluoxetine scored the greatest number of models from the top 25 binding affinity (23/25). Therefore, all further tests were performed with this antidepressant.

Fluoxetine Suppresses LPS Inducible Increases in Pro-Inflammatory Cytokines

Computational modeling alone could not predict the nature of the interaction between fluoxetine and the TLR4–MD-2 complex (i.e., agonistic or antagonistic) and the resulting biological effects. To address these issues, we studied the LPS-induced pro-inflammatory cytokines with or without preincubation of human PBMC with a physiologically relevant dose of 10 μM fluoxetine (Koran et al. 1996). To mimic the short-term consequences of bacterial infection under chronic fluoxetine treatment, we treated PBMC with 10 μM fluoxetine for 23 h followed by exposure to LPS for 1 h (Fig. 2a). ELISA analyses indicated that fluoxetine prevented the LPS-induced elevation of the pro-inflammatory cytokines TNF-α and IL-6 (Fig. 2b; ANOVA **p* < 0.001, *n* = 3 biological replicates). This is compatible with the anti-

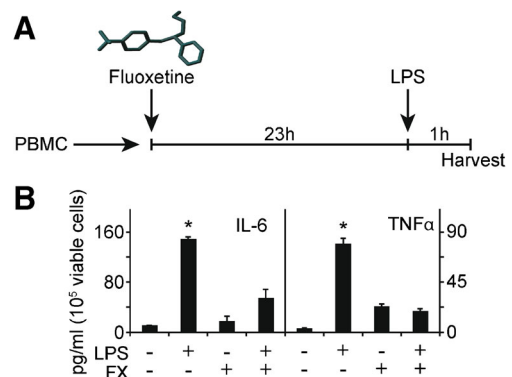


Fig. 2 Fluoxetine inhibits induction of pro-inflammatory cytokines production following LPS exposure. **a** The experimental procedure; PBMC incubated with 10 μM fluoxetine for 23 h was then exposed to LPS for 1 h and harvested. **b** Cytokine levels in the medium of PBMC exposed or not to LPS with or without fluoxetine pretreatment. Error bars represent standard deviation and asterisk indicates *p* < 0.001 (Student’s *t* test, *n* = 3)

inflammatory effects of fluoxetine in LPS-treated microglial cells (Liu et al. 2011).

ACHe-RACK1 Interaction

We have previously shown involvement of both hydrolytic and nonenzymatic functions of AChE in hematopoietic stress responses (Grisaru et al. 2006; Pick et al. 2006). Specifically, we found LPS exposure to promote production of the “readthrough” AChE-R variant which we discovered to interact intracellularly with the NF κ B-activating PKC β II cargo protein RACK1 through its unique C-terminal domain (Birikh et al. 2003; Sklan et al. 2006). However, the large majority isoform of AChE in PBMC is the “synaptic” AChE-S, much of which remains intracellular (Gilboa-Geffen et al. 2012; Soreq and Seidman 2001). To test if RACK1 interacts with AChE-S as well, we cloned, expressed, and purified recombinant human RACK1 by a two-step column purification procedure (Fig. 3).

We then used photo-induced cross-linking, shown schematically in Fig. 4a, to investigate the interaction of RACK1 with purified recombinant human AChE-S and AChE-R. Photo-induced cross-linking of unmodified proteins is an oxidative cross-linking method which employs the high-valent transition metal complex Ru(II)bpy $_3^{2+}$ and an electron acceptor such as APS. When a mixture containing the proteins of interest and these two components is illuminated briefly with visible light, Ru(II)bpy $_3^{2+}$ is photo-oxidized and extracts an electron from amino acids such as tyrosine or cysteine, leading to formation of radical species that attack adjacent groups. This process culminates in the formation of a direct covalent bond, which constitutes cross-linking of closely interacting proteins. In contrast to traditional cross-linking methods, photo-induced cross-linking is rapid, generates a high yield of cross-linked products, and does not require external cross-linking reagents, limiting the formation of artifactual cross-linking products. Cross-linked proteins were detected by immunoblotting. RACK1 in the non-illuminated sample is found in monomer form (36 kDa MW) (Fig. 4b, lane 1), but cross-linking showed that much of it exists in solution as

oligomer (lane 2). RACK1 incubation with AChE-R (lanes 5–7) lead to the formation of an additional high-molecular weight complex containing RACK1 (lanes 6–7) not seen with RACK1 alone (illustration is shown in

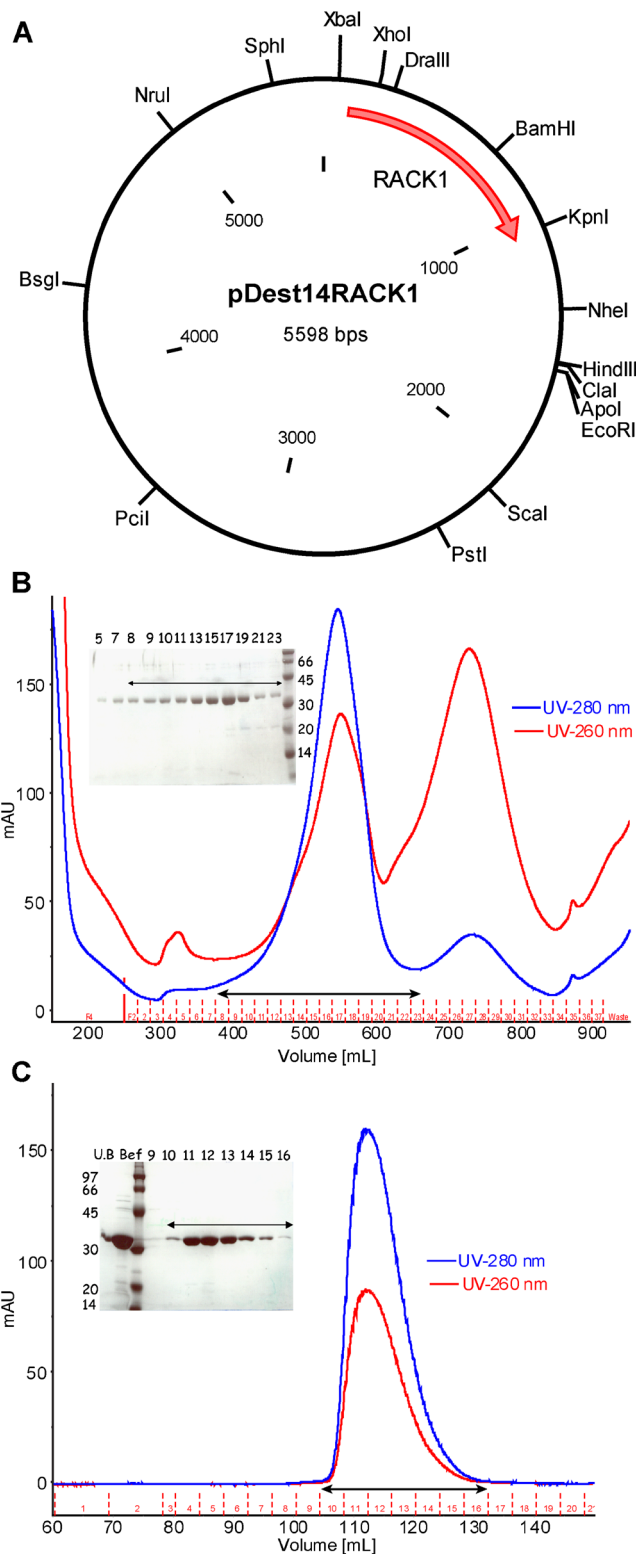


Fig. 3 Cloning and purification of recombinant human RACK1. **a** ▶ Scheme of the pDEST14 bacterial expression vector containing the human RACK1 insert. **b** Step one of RACK1 purification using Q-Sepharose FF gel filtration chromatography. The absorptions at 260 and 280 nm are shown in red and blue, respectively, as a function of the elution volume. The inset shows a Western blot detected with antibody against RACK1 of fractions 8–23 (marked with an arrow on the gel and the chromatography trace). **c** Step two of RACK1 purification using Superdex 75 gel filtration chromatography. Absorptions and insets are as above with the majority of purified RACK1 eluting in fractions 11–14

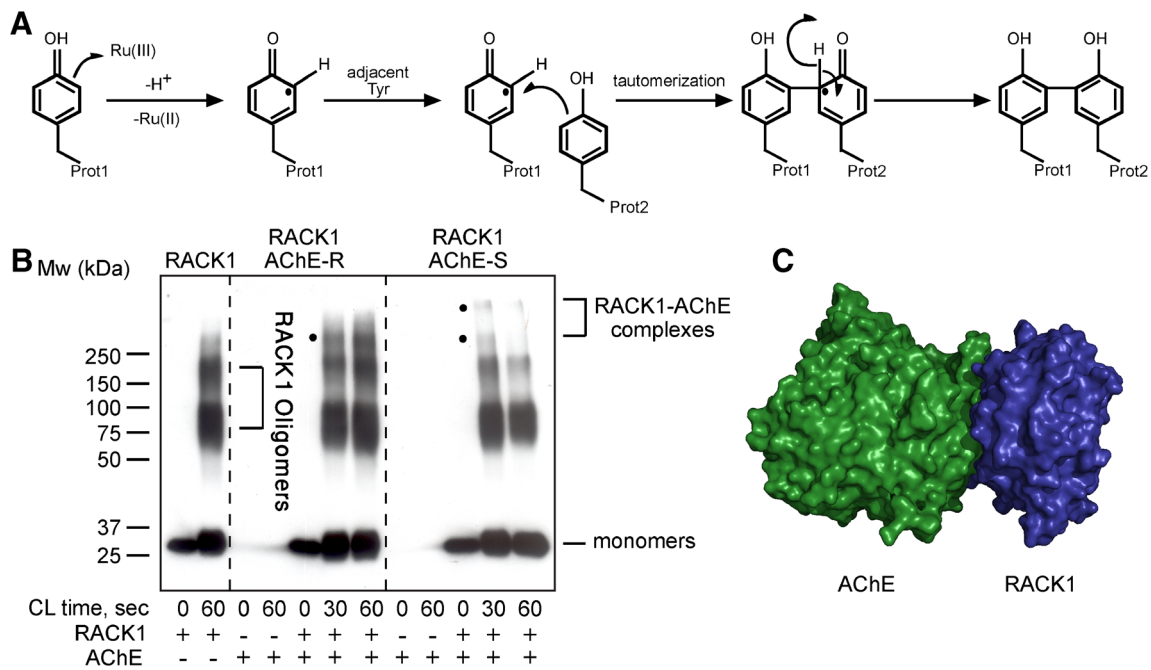


Fig. 4 AChE interaction with RACK1. **a** Scheme of photo-induced cross-linking [modified from Fancy and Kodadek (1999)]. **b** RACK1 and recombinant human AChE-R or AChE-S were cross-linked for the stated times (CL time), separated by SDS-PAGE, and detected with an

antibody to RACK1. In the presence of both AChE variants, additional high-molecular forms with RACK1 are observed. **c** Illustration of AChE–RACK1 interactions

(Fig. 4c) and compatible with our previous results (Sklan et al. 2006). Importantly, RACK1 incubation with AChE-S (lanes 10–12) induced the formation of at least two heavier RACK1–AChE-S high-molecular weight forms (lanes 11–12) not observed in lanes loaded with RACK1 alone. AChE-S in comparison to AChE-R can form homomeric dimers and tetramers which can explain the formation of additional bands over those seen following cross-linking with AChE-R (Dvir et al. 2004, 2010). The existence of such tight interactions between RACK1 and both AChE isoforms could lead to retention of AChE within cells and interfere with their secretion (Perry et al. 2007).

(Fig. 5b). Immunodetection demonstrated the intracellular spread of retained AChE (Fig. 5c).

Fluoxetine Suppresses LPS-Induced Decreases in Intracellular AChE

Fluoxetine can also limit inflammation by potentiating the cholinergic pathway (Tracey 2002). Supporting this notion, we found that exposure of PBMC to LPS lead to an increase in the AChE targeted microRNA-132 (Shaked et al. 2009), whereas fluoxetine treatment at 10 μM leads to a threefold reduction in microRNA-132 (Fig. 5a), suggesting enhanced AChE production regardless of LPS presence. Correspondingly, LPS treatment led to ca. 50 % reduction in intracellular AChE levels, while fluoxetine completely prevented such reduction

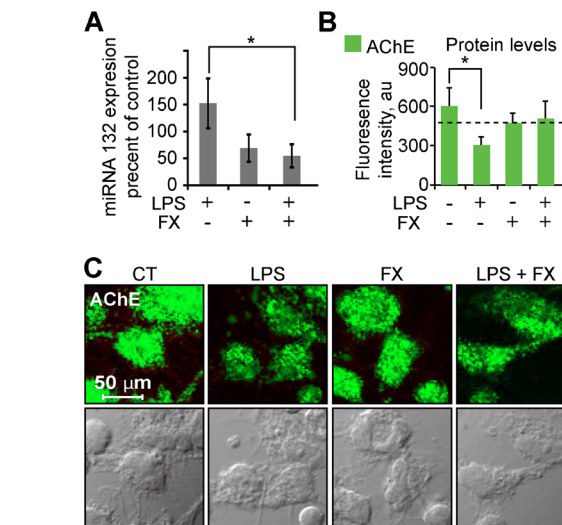


Fig. 5 Fluoxetine suppresses LPS-induced decreases in intracellular AChE. **a** AChE targeted microRNA-132 levels showing fluoxetine-induced reduction in microRNA-132 levels. Error bars representing SD and asterisk indicating $p < 0.005$ (ANOVA, $n = 3$). **b** Quantification of immunofluorescence signal with error bars representing SD and asterisk indicates $p < 0.005$. **c** Immunofluorescence staining for AChE in human PBMC under the conditions shown in Fig. 5b, showing fluoxetine prevents LPS-induced reduction of AChE intracellular levels

Fluoxetine Suppresses the LPS-Mediated Activation of RACK1 and PKC β II

Under LPS exposure, TLR4–MD-2 activation mediates enhancement of the cargo protein RACK1 that leads to mobilization of PKC β II (Birikh et al. 2003; Mochly-Rosen et al. 1991), enabling feed-forward activation of NF κ B by PKC (Chen et al. 1998; Leppanen et al. 2008). Intracellular AChE levels were retained in fluoxetine-treated and LPS-exposed cells, further suggesting potentiation of AChE–RACK1–PKC β II interactions (Sklan et al. 2006). Supporting this notion, immune labeling of PBMC exposed to LPS demonstrated RACK1 overexpression (ANOVA 2.07E-21, post hoc analysis with Dunnett T3 correction $p < 0.001$ for all groups) (Fig. 6a, b). However, fluoxetine pretreatment, both alone and following LPS exposure, prevented this increase in RACK1. In addition, we found that fluoxetine-induced decrease in the PKC β II protein levels of treated PBMC, both when added alone and under LPS exposure (Fig. 6c), suggesting that fluoxetine also blocks inflammatory response through the PKC pathway.

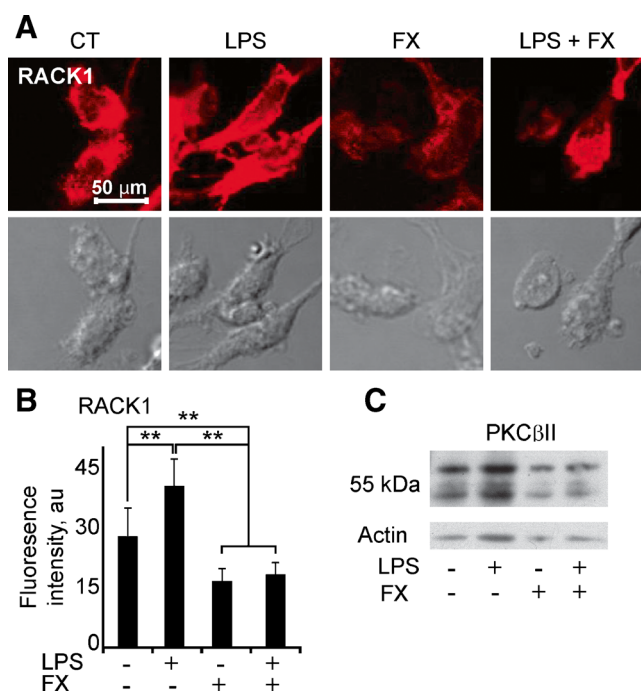


Fig. 6 Fluoxetine suppresses LPS-induced elevation of RACK1 and PKC β II. **a** Immunofluorescence staining for RACK1 in human PBMC. **b** Quantification of fluoxetine-induced reduction of RACK1 levels with error bars representing SD. Double asterisks indicate $p < 0.001$ (ANOVA, $n = 3$). **c** PKC β II protein levels in human PBMC under the noted conditions determined by Western blot; note fluoxetine-mediated suppression in PKC β II protein levels

Discussion

We found that the anti-inflammatory effects of fluoxetine involve both extracellular and intracellular pathways. At the extracellular level, suppression by fluoxetine of TLR4 activation can limit inflammatory response whereas blockade of α 7nAChR activation might have the inverse effect of limiting the anti-inflammatory effect of α 7nAChR activation. At the intracellular level, our findings add AChE, RACK1, and PKC β II to the list of signaling proteins by which fluoxetine induces its NF κ B-mediated anti-inflammatory properties. Retained AChE can balance fluoxetine blockade of α 7nAChR by “trapping” RACK1 and preventing RACK1-mediated NF κ B activation via PKC β II. Together, these checks and balances sustain the TLR4 response to LPS under native conditions and limit it under fluoxetine treatment ensuring suppressed inflammation. This complexity can explain the disputed effect of fluoxetine on inflammation in the literature (Liu et al. 2011; Sacre et al. 2010).

RACK1 interacts with a large number of proteins either directly or as part of a larger complex taking significant role in shuttling, anchoring, and stabilizing proteins (Adams et al. 2011). It can promote or suppress the activity of its binding partners such as PKC β II and maintain their active conformation (Ron et al. 1994, 1999; Stebbins and Mochly-Rosen 2001). As a scaffold protein, RACK is essential for distinct cellular activities such as the cAMP/PKA pathway (Adams et al. 2011; Bird et al. 2010; He et al. 2010) and the translation machinery (Nilsson et al. 2004). In addition, in the CNS, RACK1 interacts with ion-gated and ligand-gated channels regulating NMDAR (Thornton et al. 2004) and other channels’ functions (Brandon et al. 2002; Brandon et al. 1999). Therefore, RACK1–AChE–S interactions can have additional biological effects beside the mediation of NF κ B activation via PKC β II.

The PKCs are important mediators of immune intracellular signaling (Loegering and Lennartz 2011). In our current work, we limited our experiments to PKC β II; PKC β II knockout mice lose the capacity for contextual fear response (Weeber et al. 2000), highlighting the importance of this specific kinase to the interrelationship between anxiety and inflammation. However, the PKC family comprises of at least ten mammalian serine/threonine protein kinases with a broad range of tissue distribution and differential cellular localization (Saito et al. 2002). For example, PKC ζ mediates activation of the transcription factors activator protein-1 and NF κ B in response to TCR/CD28 co-stimulation in a cell type (T cell lines)-dependent manner (Coudronniere et al. 2000). Also, thymocytes from PKC α -overexpressing transgenic mice exhibit accelerated cell proliferation and IL-2 production in response to TCR stimulation (Iwamoto et al. 1992). PKC β knockout mice have reduced splenic B cells, a significantly lower number of B-1 lymphocytes and low levels of serum IgM and IgG3 (Leitges et al. 1996), and PKC δ has a role in NF κ B activation during early B cell

development (Saijo et al. 2003). Additionally, PKC ϵ was shown to be an important mediator of macrophage IL-4-induced NO production (Sands et al. 1994) to play a similar role in mediated LPS signaling in dendritic cells (Valledor et al. 2000) and to be critical for LPS-induced IKK and NF κ B activation as well as production of TNF- α and IL-12 in monocyte-derived dendritic cells (Aksoy et al. 2002). Our study may thus shed light on only a small part of this complex picture.

It has long ago been suggested that depression is associated with an excess of brain ACh (Tarsy et al. 1972). Neuroimaging studies show increased concentrations of the ACh precursor choline in the CNS of depressive patients (Steingard et al. 2000) that were reversed after recovery from depression (Charles et al. 1994). Furthermore, glucocorticoids trigger ACh release in the hippocampus, prefrontal cortex, and nucleus accumbens of rats (Del Arco et al. 2007) and injection of corticotropin releasing hormone into the nucleus accumbens shell of rats increases local extracellular ACh levels while causing aversive behaviors, including swim depression, anhedonia, and anxiety (Chen et al. 2012). Interestingly, a knock-down of AChE in the hippocampus was suggested as an animal model of depression-like behavior (Mineur et al. 2013), and on the other hand, fluoxetine was examined for protection from excess of ACh (Bertrand et al. 2008). Within the brain, we have recently demonstrated that superfusion of cerebral arterioles with therapeutically effective doses of fluoxetine induces consistent, dose-dependent vasodilation mediated by muscarinic cholinergic pathways (Ofek et al. 2012). Thus, both the nicotinic and the muscarinic arms of the cholinergic pathway are involved in depression and pathogen response at several interactive levels. This dual outcome of the long-term usage of SSRI antidepressants should be addressed.

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