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The extracts of Fructus Akebiae, a preparation containing 90% of the active ingredient hederagenin: Serotonin, norepinephrine and dopamine reuptake inhibitor

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ABSTRACT

Fructus Akebiae is a traditional Chinese herbal extract that has been used for the treatment of depressive disorders in China. Previous studies demonstrated that Fructus Akebiae extracts (FAE) displayed a potent antidepressant-like activity in animal behavior tests and found that the specific active ingredient from the extracts of Fructus Akebiae is hederagenin. However, the underlying mechanism is unknown. Here we provide evidences that FAE enhances the signaling of central monoamines via inhibition of the reuptake of the extracellular monoamines including serotonin (5-HT), norepinephrine (NE) and dopamine (DA). In rat brain membrane preparations and HEK293 cells transfected with human serotonin transporter (SERT), NE transporter (NET) and DA transporter (DAT), we found that FAE displayed marked affinity to rat and cloned human monoamine transporters in *ex vivo* and *in vitro* experiments, using competitive radio ligand binding assay. In uptake assays using rat synaptosomes and transfected cells, FAE was found to significantly inhibit all three monoamine transporters in a dose- and time-dependent manner, with a comparable or better potency to their corresponding specific inhibitors. In contrast, FAE (10 μM), showed no significant affinity to a variety array of receptors tested from CNS. In support of our uptake data, *in vivo* microdialysis studies showed that administration of FAE (12.6, 25, 50 mg/kg) significantly increased extracellular concentrations of 5-HT, NE and DA in frontal cortex of freely moving rats. Taken together, our current study showed for the first time that FAE is a novel triple inhibitor of monoamine transporters, which may be one the mechanisms of its antidepressant activity.

1. Introduction

Depression is a common prevalent psychiatric disorder with high morbidity and mortality (Schechter et al., 2005). The current antidepressants in use include tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs), and serotonin and norepinephrine reuptake inhibitor (SNRIs). All these antidepressants exert their effect by increasing the levels 5-HT and/or NE, with known weaknesses such as slow onset and severe side effects (Gumnick and Nemeroff, 2000) (Fava, 2003). Therefore, exploring more promising antidepressants with fewer side effects has been a desirable effort. In the past decades, there has been a growing interest in the therapeutic effects of natural products on mental disorders. In particular, the antidepressant effects of a variety of traditional Chinese medicines (TCM) such as Chinese St. John's Wort herb, marinade root, gingko, valerian, areca seed, used individually or in the formulated prescription, have received a great amount of attention, in that these natural products showed no or very little side effect (An et al., 2008; Dar and Khatoon, 2000; Luo et al., 2000; Li et al., 2003).

Fructus Akebiae has a long history in China of being used in the treatment of mental disorders. Our previous studies found out that the main chemical compositions of FAE are triterpenoid saponins, with hederagenin being the active ingredient. We previously reported an extraction method by which hederagenin could be purified from the extracts of Fructus Akebiae with a purity of approximately 95% (Yang et al., 2011) (Fig. 1). Using behavioral tests sensitive to antidepressant drugs, we demonstrated that acute and sub-chronic administration of the Fructus Akebiae extracts (FAE) produced antidepressant-like effects, as evidenced by decreases in the duration of immobility in forced swim and tail suspension tests in mice, and the reversal of chronic unpredicted mild stress-induced inhibition of sucrose consumption in rats (Zhou et al., 2010). In addition, the extracts decreased levels of plasma adrenocorticotrophic hormone and serum corticosterone in rats exposed to chronic unpredicted mild stress. Both behavioral and biochemical effects of the extracts were

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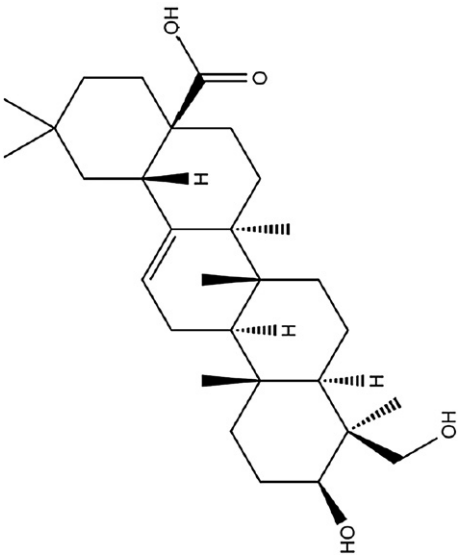

Fig. 1. The structure of Fructus Akebiae extracts.

comparable to that of the proven antidepressant escitalopram (Zhou et al., 2010). Yet, the monoaminergic mechanism of the FAE remains unclear.

As the monoaminergic system is one of the most important targets in the pathophysiology and therapy of depression (Elhwuegi, 2004; Millan, 2004), our current studies were designed to explore the effect of FAE on monoaminergic systems. Our previous study found that FAE has high affinity to SERT, NET and DAT, compared to other potential targets using receptor-screening tests (data not shown). Here, we used a series of competitive radio ligand binding and transporter inhibition bioassays to determine FAE affinities to the rat and cloned human (h) SERTs, NETs, and DATs. We also evaluated the affinity of FAE to numerous other targets (D_1, D3, D2, D4, D5, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2a}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6, 5-HT_7, α_{1A}, α_{1B}, α_{2A}, β_1, M_1, M_2, M_3, M_4, M_5, μ, κ, δ, A_1, A_{2A}, H_1, H_2, H_3, H_4) to determine its selectivity. In addition, we tested the effect of FAE on the transport activities of three monoamines, 5-HT, NE and DA, using transporter-transfected HEK cell line as well as rat brain synaptosomal preparations. Furthermore, to characterize the *in vivo* effect of FAE on monoaminergic systems, we observed the extracellular levels of 5-HT, NE, and DA in the prefrontal cortex using microanalysis in freely moving rats administered with FAE.

2. Materials and methods

2.1. FAE preparation

2.1.1. Sample preparation

Five hundred grams of Fructus Akebiae dry fruit powder was defatted twice by ultrasonication in petroleum ether (1000 ml) at room temperature. The solvent was volatilized, and the coarse powder was extracted twice by recirculation for 1 h with 2 l of 80% ethanol (EtOH). The extract was concentrated under reduced pressure into 500 ml, and was placed overnight at the room temperature before filtration. H_2O (800 ml) was added to the filtrate followed by extraction with 3 times with 800 ml of ethyl acetate (EtOAC) and 4 times with 800 ml of H2O-saturated n-butanol (n-BuOH). n-BuOH was recycled under reduced pressure and the general saponin was obtained. A total of 5 g of general saponin was degraded for 3 h with 60 ml of 2 mol/L HCl in 45% EtOH at 100 °C and filtered, followed by washing off acid with H_2O and dried in vacuo, resulting in crude crystal. The crude crystal was dissolved in 300 ml of hot 70% EtOH and decolored by heating-recirculation with 1 g of activated carbon for 0.5 h, followed by filtration at hot temperature. The filtrate was concentrated for 12 h at 4 °C under reduced pressure to obtain clustered crystal. The crystal was filtered and washed off chloridion with H_2O, followed by drying in vacuo into white powder. The yield of final extract was approximately 0.5% (w/w). The powder was stored at 4 °C until analysis.

2.1.2. HPLC analysis

For HPLC analysis, 8 mg FAE was dissolved in 10 ml of methanol and filtered through a 0.2 μm nylon membrane prior to injection into HPLC. The hederagenin standard solution was prepared by dissolving 2 mg hederagenin in 10 ml of methanol and filtering through a nylon membrane. HPLC analysis was carried out using Agilent 1100 series HPLC systems linked to both diode array and multiple wavelength detectors (Agilent, USA). Samples were separated using an Agela C18 column (4.6 mm × 250 mm, i.d. 5 μm, Agela Technologies, USA) which was maintained at 25 °C. The mobile phase was used according the following ratio: CH3OH–H2O–CH3COOH–(C2H5)3N (87:13:0.04:0.02, v/v). For each run, 20 μl of the sample solution was injected. The solvent flow was 0.8 ml/min, and the detection wavelength was 210 nm. Chemstation software was used to control the instrument and for data acquisition and processing.

2.2. Binding assay

2.2.1. Rat monoamine transporters

Male SD (Sprague–Dawley) rats were sacrificed by cervical dislocation and their brains rapidly removed and then dissected on ice. Cerebral cortex, hypothalamus and striatum were homogenized in 4 ml. assay buffer (50 mM Tris–HCl buffer pH 7.4.) and centrifuged at 40,000 *g* for 10 min at 4 °C. The pellets were centrifuged twice at 20,000 g and then resuspended in 4 ml of assay buffer. The samples were incubated at 37 °C for 20 min to remove endogenous 5-HT, followed by two centrifugations as previously described by Wong et al. (1993b). Use Bio-Rad protein assay reagents (1:5 ratio) were used to analyze the protein concentration of synaptosomal suspension. The membrane samples (containing 50 μg protein per sample) were incubated with varied concentrations of drugs, including [^{3}H]-citalopram (1.2 nM, for SERT), [^{3}H]-nisoxetine (1.0 nM, for NET) or [^{3}H]-win35428 (1.0 nM, for DAT) in 50 mM Tris Cl, pH 7.4, 150 mM NaCl (300 mM NaCl for nisoxetine), respectively. Non-specific binding was determined by including separate samples of 1 μM fluoxetine, 10 μM desipramine and 10 μM nomifensine for SERT, NET and DAT, respectively.

2.2.2. Human monoamine transporters

Membranes were prepared from HEK293 cells stably expressing hSERT, hNET or hDAT as described previously (Blakely et al., 1998; Tatsumi et al., 1997). Briefly, [^{3}H] citalopram (1 nM), [^{3}H] nisoxetine (2 nM), or [^{3}H] win35, 428 (2 nM) (Perkin-Elmer Life Sciences, Boston, MA) was used to label human 5-HT, NE, or DA transporters, respectively. Non-specific binding was determined by including separate samples of 1 μM fluoxetine, 10 μM desipramine or 10 μM nomifensine for SERT, NET and DAT, respectively.

2.2.3. Other potential binding targets

FAE (10 μM) was tested for numerous other potential binding targets by using standard receptor binding procedures (Bymaster et al., 2001; Millan et al., 2001b). The tested targets include D_1, D3, D2, D4, D5, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2a}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6, 5-HT_7, α_{1A}, α_{1B}, α_{2A}, β_1, M_1, M_2, M_3, M_4, M_5, μ, κ, δ, A_1, A_{2A}, H_1, H_2, H_3, H_4.

2.3. Uptake assay

2.3.1. Rat synaptosomes

Male SD (Sprague–Dawley) rats weighing 180–250 g were sacrificed by asphyxiation with carbon dioxide and cervical dislocation followed by decapitation. The brains were removed quickly and the

cerebral cortex, hypothalamus, and striatum were dissected on ice. All dissected tissues were homogenized using Teflon-glass tissue homogenizer in 4 ml ice-cold sucrose (0.32 M). The homogenized tissue was centrifuged at 2000×*g* for 10 min at 4 °C. The synaptosomal pellets were resuspended with 8 ml ice-cold assay buffer pH 7.4 (10 mM HEPES, 133 mM NaCl, 4.85 mM KCl, 1.2 mM KH2PO4, 1.5 mM MgSO4, 1.5 mM $CaCl_2$, 11.1 mM Glucose). Use Bio-Rad protein assay reagents (1:5 ratio) were used to analyze the protein concentration of synaptosomal suspension. Uptake of 5-HT, NE and DA was determined as described previously (Wong et al., 1993a). The compounds to be tested were dissolved in DMSO and further diluted with assay buffer, and tested over a 10-log unit concentration range in duplicate. Thirty to 50 μg synaptosomes per sample (in a total volume of 200 μl) was preincubated at 37 °C in a shaking water bath for 10 min. Vehicle or modifiers were then added for 10 to 20 min. Uptake was initiated by adding [^{3}H] 5-HT (20 nM), [^{3}H] NE (20 nM), or [^{3}H] DAT (50 nM; PerkinElmer Life and Analytical Sciences). After a 10-min incubation at 37 °C, uptake was terminated by filtration through (polyethylene mine-coated) GF/B Whatman filters using a Brandel Cell Harvester (Brandel, Gaithersburg, MD). Nonspecific uptakes for DAT, SERT, and NET were determined using 10 μM nomifensine, 10 μM citalopram, and 10 μM desipramine, respectively.

2.3.2. Transporter-transfected cell lines

The uptake of 5-HT, NE, and DA into HEK293 cells stably expressing human transporters was determined using the methods modified as previously described (Millan et al., 2008; Janowsky et al., 1986). Briefly, culture medium was removed from cells and the cells were washed once with phosphate-buffered saline (PBS). Krebs-Ringers-HEPES (KRH) assay buffer (100 μl/well, containing 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM MgSO4, 1.2 mM KH2MgSO4, 10 mM HEPES, pH7.4) was then added. Varied concentrations of drugs were administered into cells in a 24-well plate. The uptake was initiated by the addition of [^{3}H] 5-HT (50 nM), [^{3}H] NE (80 nM), and [^{3}H] DA (50 nM) (Perkin-Elmer Life Sciences, Boston, MA). Uptake was terminated by five washes using cold KRH assay buffer after 10 min incubation at 37 °C, and cells were solubilized using 400 μl of scintillation fluid and counted on a Packard Top Count scintillation counter.

2.4. In vivo microdialysis studies

The effect of FAE on extracellular monoamine levels in frontal cortex was evaluated using *in vivo* microdialysis coupled with high performance liquid chromatography (Carboni et al., 1989; Matthews et al., 2005). Briefly, male Sprague–Dawley rats were anesthetized with 3% halothane using calibrated vaporizer and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Each rat was cannulated in the medial prefrontal cortex (mPFC) (anterior 3.2, lateral 0.5, ventral 2.0) relative to bregma and skull according to the atlas of Paxinos et al. (1997). The probe was secured to the skull with dental cement and stainless steel screws. After surgery, the animals were treated with daily penicillin (10,000 U, s.c.) to prevent infection and were housed in individual test cages. After recovery from surgery, the animals were subjected to microanalysis: a microanalysis probe (CMA/12, 2.0 mm membrane) was inserted into the guide cannula to replace the dummy cannula. The probe was perfused at 2 μl/min with artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl2, and 1.0 mM MgCl2 pH 7.4). After at least 2 h of equilibration, dialysate samples were collected every 30 min into collection tubes added with chloric acid (20 mM, 10 μl/tube, to prevent the degradation of monoamines). The steady baseline of each neurotransmitter was established before drug treatment by collecting the first four samples. After baseline sampling was completed, animals were orally administered FAE (6.25, 12.5, 25 mg/kg) or vehicle (0.2% Tween 80 and 0.5% methylcellulose dissolved in water) at the end of the sixth sampling. Three hours after FAE administration, dialysis samples were collected every 20 min and analyzed using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) to determine the levels of NE, 5-HT, and DA. At the end of each experiment, animals were euthanized, and the probe placement was verified histologically. Data from rats with incorrect probe placement were discarded. The HPLC system consisted of a micro bore reverse-phase column (particle size 5 μm, 150×4.6 mm; Model C-18, DIKMA Technologies Ltd., Beijing, China), an Agilent 1100 pump (flow rate 1.0 ml/min; Agilent Technologies, Palo Alto, CA, USA) and a Hewlett-Packard HP 1049A glassy carbon amperometric detector (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of 85 mM citrate, 100 mM sodium acetate, 0.9 mM octyl sodium sulfate, 0.2 mM EDTA, and 15% methanol, pH 3.7. External standard curves were used to quantify the amounts of NE, 5-HT, DA in each sample calculated by area under curve (AUC). The volume of injection was 50 μl. The detection limit of the assay was 20 pg/sample.

2.5. Animals and materials

Male Sprague–Dawley rats (2 months old, 180–250 g) were obtained from the Laboratory Animal Centre of Southern Medical University (Guangzhou, China) and were acclimated to the facility for 1 week before use in the experiments. The animals were housed at 22±1 °C with a 12:12 h light/dark cycle (lights on at 7:00 a.m.), and were given ad libitum access to water and food. All procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Fructus Akebiae was obtained from Beijing Tongrentang Medicine Facility, Beijing, China (batch #: 701001532–1). The plant was harvested from Jiangsu Province, China. Hederagenin standard preparation was obtained from Shanghai Tauto Biotech Co., Ltd, China (batch #: 08072522). Desipramine (catalog number D-3900), fluoxetine (catalog number F-132), paroxetine (catalog number P-1372), and nomifensine (catalog number M-2017) were purchased from Sigma-Aldrich (St. Louis, MO). Radio ligands ([^{3}H] NE, catalog number NET-048, 5–15 Ci/mmol; [^{3}H] nisoxetine, catalog number NET-1084, 85.5 Ci/mmol; [^{3}H] WIN-35, 428, catalog number NET-1033, 85.6 Ci/mmol; and [^{3}H] hydroxytryptamine creatinine sulfate, catalog number NET-498, 25–35 Ci/mmol) and scintillation cocktail (Ultima Gold, catalog number 6013329) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

2.6. Statistical analysis

All data for inhibition curves were analyzed by nonlinear regression using Prism software (GraphPad Software Inc., San Diego, CA) to determine IC_{50} values. For the microdialysis experiments, two-way repeated measures ANOVA followed by Tukey's test was used to compare the percentage increase over baseline between groups, time, and treatment as independent factors, and time as the repeated factor.

3. Results

3.1. Preparation of FAE

With the method employed in the present study, the yield of extracts from Fructus Akebiae was about 0.5%. The content of hederagenin in the FAE was calculated through peak area ratio between its area in test solution and hederagenin area in the hederagenin standard solution according to Pharmacopoeia of Chinese. HPLC analysis showed that the enrichment of hederagenin (Fig. 1) in the FAE was 90.48%.

3.2. Binding of FAE to rat monoamine transporters

To evaluate the binding capacity of FAE to monoamine transporters, the binding assays were conducted using rat brain tissues. We found that FAE showed a high affinity for rat monoamine transporters including

Table 1
Binding affinity of FAE as compared with reference antidepressants agents at monoamine transporters.

	Species	Radioligand	Tissues	K_i values (nM)	
				FAE	Comparator
SERT	Rat	[^{3}H]-citalopram	Cerebral cortex	3.89 ± 0.18	13.68 ± 0.53 nM (fluoxetine)
SERT	Human	[^{3}H]-citalopram	HEK293 cell line	0.65 ± 0.04	4.92 ± 0.12 (fluoxetine)
NET	Rat	[^{3}H]-nisoxetine	Hypothalamus	0.22 ± 0.04	10.82 ± 0.71 (desipramine)
NET	Human	[^{3}H]-nisoxetine	HEK293 cell line	0.14 ± 0.03	5.71 ± 0.64 (desipramine)
DAT	Rat	[^{3}H]-win35,428	Striatum	2.87 ± 0.54	37.28 ± 1.54 (nomifensine)
DAT	Human	[^{3}H]-win35,428	HEK293 cell line	1.03 ± 0.04	15.83 ± 0.94 (nomifensine)

K_i value = $IC_{50}/(1 + L/K_D)$, where L equals concentration of radioligand added. Comparators are fluoxetine (selective serotonin reuptake inhibitor), desipramine (norepinephrine reuptake inhibitor), and nomifensine (dopamine reuptake inhibitor). Data presented were generated in a minimum of two separate assays conducted on different test days. Each test concentration was run in triplicate and presented as mean ± S.E.M.

SERT, NET and DAT (Table 1). For SERT, the binding of [^{3}H]-citalopram to rat cerebral cortical membranes was competed by FAE and fluoxetine. Our results showed that FAE was 4.5 fold more potent than fluoxetine in binding to rat SERT (K_i values for FAE: 3.89 ± 0.18; Fluoxetine: 13.68 ± 0.53 nM, Table 1; Fig. 2A). In evaluation of FAE binding capacity to DAT, [^{3}H]-win35428 (1.0 nM) was applied to the brain membrane preparation, and the binding was competed using FAE or DAT specific ligand nomifensine. We found that the affinity of FAE to rat DAT was 13 fold higher than nomifensine (the K_i values for FAE and nomifensine were 2.87 ± 0.54 and 37.28 ± 1.54 nM, respectively; Table 1; Fig. 2B). Similarly, the affinity of FAE to rat NET was found to be 50 fold more potent than desipramine, a known specific inhibitor of NET. The K_i values for FAE and desipramine were 0.22 ± 0.04 and 10.82 ± 0.71 nM, respectively (Table 1; Fig. 2C). In comparison of the affinity of FAE to the three tested monoamine transporters, we found that FAE binds to NET with the highest affinity (0.22 nM) followed by DAT (2.87 nM) and SERT (3.89 nM).

3.3. Binding of FAE to human monoamine transporters

To study whether FAE has similar affinity to human monoamine transporters as that from rat, we used HEK293 cells transfected with SERT, NET or DAT and tested FAE capacity to compete with specific inhibitors of different transporters. As shown in Table 1, FAE showed high affinity to human monoamine transporters, consistent with our results from rats. FAE and fluoxetine dose-dependently competed binding of the SERT radio ligand [^{3}H]-citalopram to membranes from cells transfected with hSERT. The K_i values for FAE and fluoxetine were 0.65 ± 0.04 and 4.92 ± 0.12 nM, respectively (Table 1; Fig. 3A). The binding of [^{3}H]-Win35, 428 to hDAT was competed by FAE and nomifensine with K_i values of 1.03 ± 0.04 and 15.83 ± 0.94 nM, respectively (Table 1; Fig. 3B). FAE and desipramine competed binding of the NET radiolabeled [^{3}H]-nisoxetine to membranes from cells transfected with the hNET with K_i values of 0.14 ± 0.03 and 5.71 ± 0.64 nM, respectively (Table 1; Fig. 3C). Similar to rat monoamine transporter, FAE affinity for hNET was higher than that for hSERT and hDAT.

3.4. FAE inhibits monoamine uptake in rat synaptosomes

To study the effect of FAE on monoamine transport activity, rat brain synaptosomes were prepared and treated with FAE in a varied dosage. Our results showed that FAE inhibited all three transporters (SERT, DAT and NET) in a dose-dependent manner (Fig. 4A–C). The K_i value for uptake of [^{3}H]-5-HT into rat cerebral cortical synaptosomes was 3.04 ± 0.84 and 84.59 ± 1.84 nM for FAE and fluoxetine, respectively (Table 2; Fig. 4A). FAE and desipramine inhibited uptake of [^{3}H]-NE into hypothalamic synaptosomes with K_i values of 0.86 ± 0.34 and 4.58 ± 0.24 nM, respectively (Table 2; Fig. 4B). FAE also inhibited [^{3}H]-DA uptake in rat striatal synaptosomes with K_i values of 2.88 ± 0.24 and 40.54 ± 0.21 nM, respectively (Table 2; Fig. 4C). Similar to its binding profile, FAE exhibited a more potent inhibition on these transporters

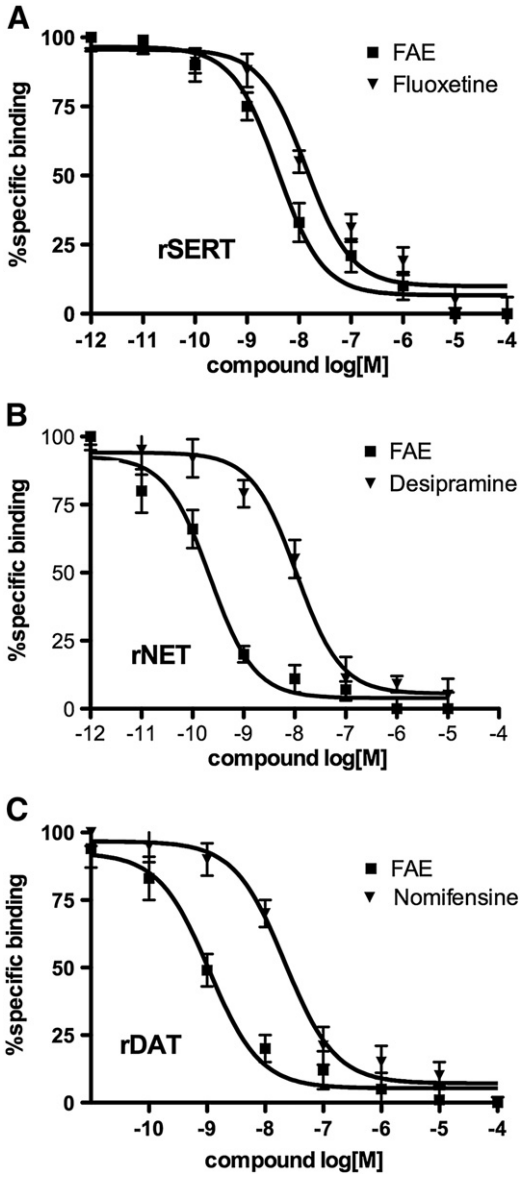

Fig. 2. FAE competes for the binding of radioligands specific to the rSERT (A) rNET(B) and rDAT (C). For each transporter bioassay, a known comparator was used [fluoxetine (5-HT reuptake inhibitor); desipramine (NE reuptake inhibitor); and nomifensine (DA reuptake inhibitor)]. The IC_{50} value was generated from each of these curves and used to generate the K_i values. Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The K_i values for FAE and the comparators are shown in Table 1.

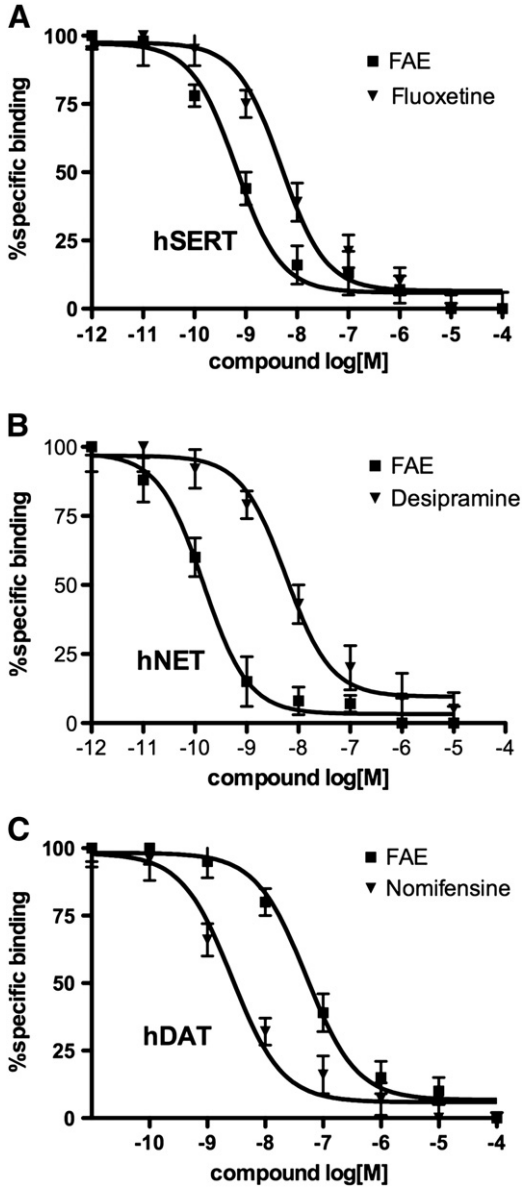

Fig. 3. FAE competes for the binding of radioligands specific to the cloned human SERT (A) NET (B) and DAT (C) expressed in HEK293 cell lines. For each transporter bioassay, a known comparator was used [fluoxetine (5-HT reuptake inhibitor); desipramine (NE reuptake inhibitor); and nomifensine (DA reuptake inhibitor)]. The IC_{50} value was generated from each of these curves and used to generate the K_i values. Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The K_i values for FAE and the comparators are shown in Table 1.

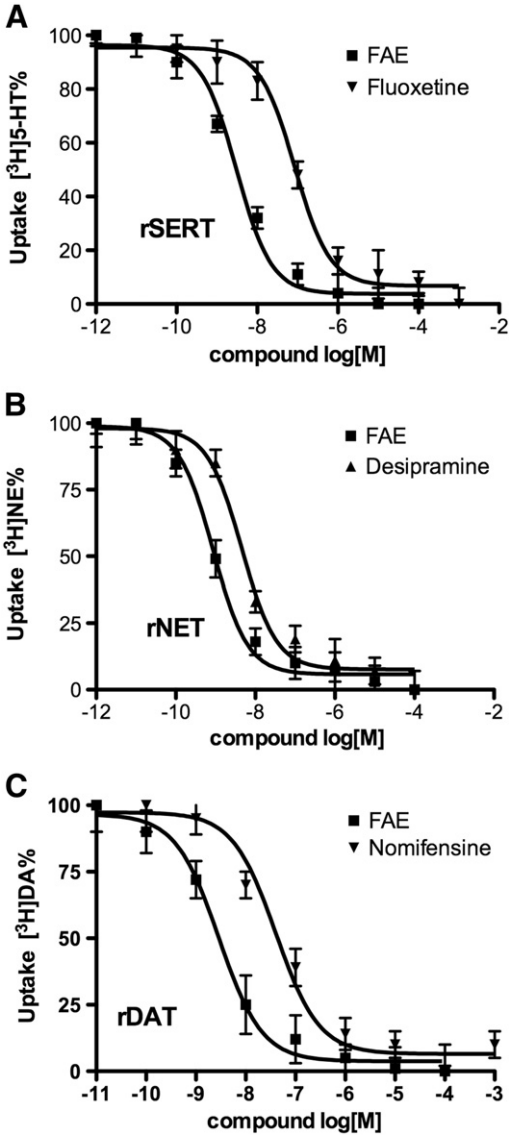

Fig. 4. Functional activity of FAE demonstrates inhibition of radiolabeled uptake of serotonin (A) norepinephrine (B) and dopamine(C) in rat synaptosomes. For each uptake transporter bioassay, a known comparator was used [fluoxetine (5-HT reuptake inhibitor); desipramine (NE reuptake inhibitor); nomifensine (DA reuptake inhibitor)]. Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The IC_{50} values for FAE are shown in Table 2.

than their corresponding specific inhibitors; and FAE has a better effect on NET than SERT and DAT (Table 2).

3.5. FAE inhibits uptake activities of human monoamine transporters

HEK cells were transfected with hSERT, hDAT or hNET and were treated with FAE at varied dosages. Consistent with the results from rat synaptosomes, we found FAE also significantly inhibited the activities of NET, SERT and DAT in these transfected cell lines. The K_i values for FAE to inhibit the uptake of [^{3}H]-5-HT, [^{3}H]-5-NE, and [^{3}H]-DA into cells expressing the corresponding human recombinant transporters were 1.34 ± 0.04, 0.15 ± 0.02 nM and 1.09 ± 0.12 nM, respectively (Table 2; Fig. 5). Again, these data demonstrated that FAE inhibits transport activities of NET with higher potency than SERT and DAT.

3.6. FAE selectively bound to NET, SERT and DAT

To determine whether FAE might also bind to other potential targets, we tested the binding capacity of FAE (10 μM) to numerous protein targets including G-protein coupled receptors (D_1, D3, D2, D4, D5, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2a}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6, 5-HT_7, α_{1A}, α_{1B}, α_{2A}, β_1, M_1, M_2, M_3, M_4, M_5, μ, κ, δ, A_1, A_{2A}, H_1, H_2, H_3, H_4). As shown in Table 3, except NET, SERT and DAT, FAE did not show significant affinity to all tested targets, suggesting that the binding of FAE is selective for the three monoamine transporters.

3.7. FAE administration induced an increase in monoamine level in microdialysate from rat frontal cortex—in vivo study

To evaluate the effect of FAE on central extracellular monoamine levels, we administered FAE orally to rats and studied monoamine levels using *in vivo* microdialysis. We found that acute administration of FAE led to a significant increase in 5-HT level in the frontal cortex in

Table 2
Influence of FAE as compared with reference antidepressant agents on the uptake of [^{3}H] serotonin (5-HT), [^{3}H] norepinephrine (NE), and [^{3}H] dopamine (DA) in rat cerebral synaptosomes or intact cells expressing human transporters.

	Species	Radioligand	Tissues	IC_{50} values (nM)	
				FAE	Comparator
SERT	Rat	[^{3}H]-5-HT	Mid brain	3.04 ± 0.84	84.59 ± 1.84 (fluoxetine)
SERT	Human	[^{3}H]-5-HT	HEK293 cell line	1.34 ± 0.04	25.02 ± 0.30 (fluoxetine)
NET	Rat	[^{3}H]-NE	Frontal cortex	0.86 ± 0.34	4.58 ± 0.24 (desipramine)
NET	Human	[^{3}H]-NE	HEK293 cell line	0.15 ± 0.02	1.08 ± 0.48 (desipramine)
DAT	Rat	[^{3}H]-DA	Striatum	2.88 ± 0.24	40.54 ± 1.21 (nomifensine)
DAT	Human	[^{3}H]-DA	HEK293 cell line	1.09 ± 0.12	26.76 ± 1.34 (nomifensine)

Data IC_{50} values are presented as mean ± S.E.M. IC_{50} of three independent determinations, each performed in triplicate. Data presented were generated in a minimum of two separate assays conducted on different test days.

a dose- and time-dependent manner (Treatment: F (5,214) = 24.0, P<0.0001; time: F (7,346) = 69.70, P<0.0001). Post hoc tests showed that FAE above concentrations 12.5 mg/kg induced an increase in 5-HT at all time points compared with vehicle, peaking 1 h post FAE administration. At the peak time point (1 h after treatment), the maximal effect of FAE was observed at a dose of 25 mg/kg (Fig. 6A). Similarly, acute administration of FAE induced a dose- and time-dependent increase in NE level in the frontal cortex, peaking 1 h after FAE administration at 50 mg/ml (Treatment, F (3,15) = 21.280, P<0.0001; time, F (4,159) = 74.35, P<0.0001). Post hoc tests showed that all doses including 6.25 mg/kg of FAE induced increases in NE level at all time points (Fig. 6B). DA levels in the frontal cortex were also found to be elevated after administration of FAE (Treatment, F (1,29) = 36.07, P<0.0001; time, F (4,545) = 81.75, P<0.0001; Fig. 6C). Post hoc tests showed that at 12.5 mg/kg or above, FAE induced an increase in DA level at all time points in a dose-dependent manner, peaking at 1 h after FAE administration. The maximal effect of FAE was observed at a dose of 50 mg/ml (Fig. 6C).

4. Discussion

Monoaminergic neurotransmission in the central nervous system has been shown to be involved in the pathogenesis and the therapeutic targets of depression and other psychiatric disorders (Cools et al., 2005; Hirschfeld, 2000). Decreases in brain concentrations of 5-HT, NE and DA were reported in patients with stress and depression, suggesting a dysregulation of monoaminergic systems (Aan het Rot et al., 2009; Ruhe et al., 2007). Although most currently prescribed antidepressants act on serotonergic and noradrenergic neurotransmission, a growing number of studies suggest that the dopaminergic system may also be an important therapeutic target for the treatment of depression (D'Aquila et al., 2000; Nutt et al., 2006; Shaw et al., 2007). It was shown that the levels of dopamine and its metabolite homovanillic acid were decreased in depressed patients compared to normal individuals (Hamner and Diamond, 1996; Papakostas, 2006; Mitani et al., 2006). Some studies report that the dopamine D2/D3 receptor binding is increased and dopamine transporter activity is decreased in depressed patients (Klimek et al., 2002; Shah et al., 1997). A recent study examined the effects of the treatment using duloxetine, a serotonin and norepinephrine reuptake inhibitor (SNRI), in combination with the bupropion, a dopamine and norepinephrine reuptake inhibitor, in patients with major depressive disorder who had not achieved symptom remission with either treatment alone. The combination of these drugs resulted in a significant improvement in depressive symptoms in these patients, suggesting that a drug targeting serotonergic, noradrenergic, and dopaminergic neurotransmission simultaneously would likely be more effective in treating depression (Mischoulon et al., 2000; Dutta et al., 2008; Shaw et al., 2007; Papakostas et al., 2006).

In our present study, we found that FAE bound to NET, SERT and DAT with high affinity, and potently inhibited 5-HT, NE and DA reuptake in both rat synaptosomal preparations and intact cell line expressing human transporters, suggesting that FAE is novel triple reuptake inhibitor. We conducted our assays using both rat brain synaptosomes and a human transporter-transfected cell line to evaluate effect of FAE and clarifying whether there is an issue with species

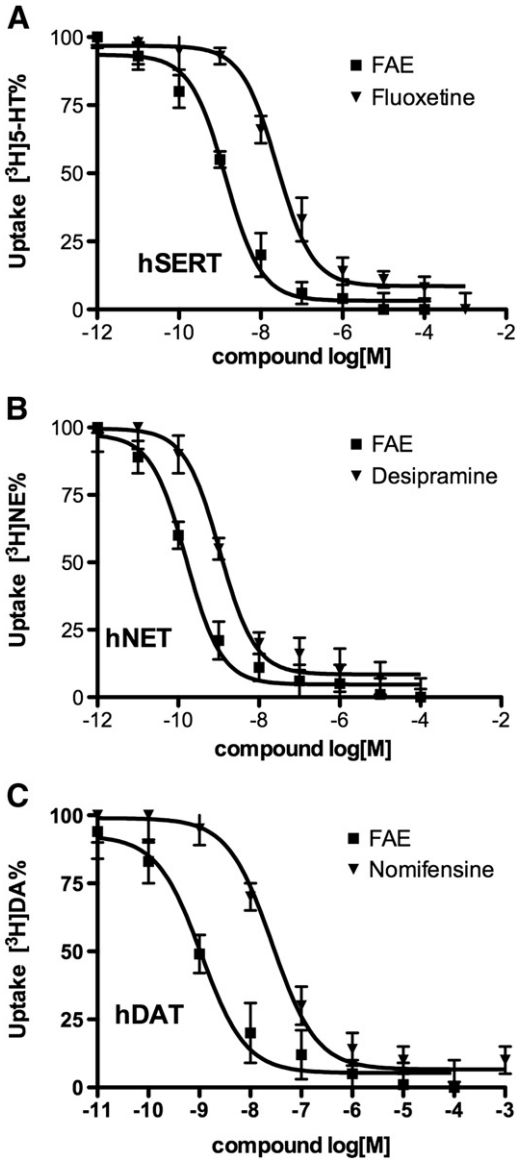

Fig. 5. Functional activity of FAE demonstrates inhibition of radiolabeled uptake of serotonin (A) norepinephrine (B) and dopamine(C) in cloned human transporters expressing in HEK293 cell lines. For each uptake transporter bioassay, a known comparator was used [fluoxetine (5-HT reuptake inhibitor); desipramine (NE reuptake inhibitor); nomifensine (DA reuptake inhibitor)]. Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The IC_{50} values for FAE are shown in Table 2.

Table 3
Binding affinity of FAE for CNS receptors.

Targets	Species	Tissue	Radioligand (nM)	% Inhibition of binding at 10 μM FAE	IC_{50} values for FAE (nM)
D_1	Human	HEK293 cell line	[^{3}H]-SCH23390(1)	77.50%	3989 ± 185
D_3	Human	CHO cell lines	[^{3}H]-spiperone(1)	63.30%	3254 ± 212
D_2	Human	HEK293 cell line	[^{3}H]-spiperone(1)	32.50%	> 10,000
D_4	Human	HEK293 cell line	[^{3}H]-spiperone(1)	55.50%	2514 ± 221
D_5	Human	HEK293 cell line	[^{3}H]-SCH23390(1)	32.50%	> 10,000
5-HT_{1A}	Human	HEK293 cell line	[^{3}H]-8-OH-DPAT(2)	72.30%	4125 ± 632
5-HT_{1B}	Human	HEK293 cell line	[^{3}H]-LSD(6)	55.40%	5455 ± 263
5-HT_{1D}	Human	HEK293 cell line	[^{3}H]-LSD(10)	44.21%	> 10,000
5-HT_{2a}	Human	HEK293 cell line	[^{3}H]-ketanserin(1)	71.23%	4525 ± 263
5-HT_{2C}	Human	CHO cell lines	[^{3}H]-mesulergine(1)	44.68%	> 10,000
5-HT_3	Human	HEK293 cell line	[^{3}H]-BRL3694	12.33%	> 10,000
5-HT_{5A}	Human	HEK293 cell line	[^{3}H]-LSD(1)	68.99%	4201 ± 322
5-HT_6	Human	HEK293 cell line	[^{3}H]-LSD(1)	72.69%	5011 ± 321
5-HT_7	Human	HEK293 cell line	[^{3}H]-LSD(4)	81.66%	5654 ± 412
α_{1A}	Rat	Frontal cortex	[^{3}H]-parzosin(1)	28.23%	> 10,000
α_{1B}	Human	HEK293 cell line	[^{3}H]-parzosin(1)	17.22%	> 10,000
α_{2A}	Rat	Cortex	[^{3}H]-clonidine(2)	70.22%	5442 ± 268
β_1	Human	HEK293 cell line	[^{3}H]-DHA(1)	51.23%	3985 ± 212
M_1	Human	CHO cell line	[^{3}H]-NMS(1)	41.58%	> 10,000
M_2	Human	HEK293 cell line	[^{3}H]-NMS(1)	38.22%	> 10,001
M_3	Human	CHO cell line	[^{3}H]-NMS(1)	66.23%	4521 ± 301
M_4	Human	CHO cell line	[^{3}H]-NMS(1)	45.33%	> 10,000
M_5	Human	CHO cell line	[^{3}H]-NMS(1)	36.87%	> 10,001
μ	Human	CHO cell line	[^{3}H]-dipnorphine(1)	15.66%	> 10,002
κ	Human	CHO cell line	[^{3}H]-dipnorphine(1)	58.44%	4385 ± 312
δ	Human	CHO cell line	[^{3}H]-dipnorphine(1)	41.55%	> 10,000
A_1	Human	CHO cell line	[^{3}H]-DPCPX(1)	11.23%	> 10,001
A_{2A}	Human	HEK293 cell line	[^{3}H]-CGS21680(10)	6.33%	> 10,002
H_1	Human	CHO cell line	[^{3}H]-pyrilamine(2)	24.35%	> 10,003
H_2	Human	HEK293 cell line	[^{3}H]-tiotodine(1)	30.22%	> 10,004
H_3	Human	CHO cell line	[^{3}H]-hestamine(1)	59.33%	4385 ± 322
H_4	Human	CHO cell line	[^{3}H]-histamine(1)	65.25%	5185 ± 256

The concentration of FAE for primary binding experiments was 10 μM, and % inhibition (mean of 4 determinations) is shown. Compound exhibiting > 50% inhibition at any receptor was evaluated in secondary binding assays with full concentration curves to derive the IC_{50} (mean ± S.E.M. for 4 determinations).

difference. Previous studies reported conflicting results regarding the affinities of SSRIs including citalopram, paroxetine and fluoxetine to rat and human SERTs (Owens et al., 1997; Table 1; Blakely et al., 1991; Owens et al., 1997). Thus, it pointed out the importance of determining the drug affinities in both species (Sur et al., 1998). In our current studies, we found that FAE demonstrated equivalent high affinity for both rat and human SERTs and showed a 4 and 7-fold higher affinity than fluoxetine in rat SERT and human SERT binding assays. In addition, our results demonstrate that FAE also potently bound hNET and rat NET with high affinity: we found a 50 and 40 fold higher affinity of FAE than desipramine in rat NET and hNET binding assays. Furthermore, we found FAE is able to bind to DAT with relative high affinity in both rat and human species. Comparing the affinities of FAE to the three-monoamine transporters, we found the affinity to NET is the highest, followed by SERT and DAT (NET > SERT > DAT). As seen in Table 1, FAE had a higher affinity for the human NET, SERT and DAT than many current antidepressants (including desipramine, fluoxetine and nomifensine).

Consistent with the results from binding assays, our data from functional studies for the transporters showed a similar pattern in the capacity of FAE to inhibit the monoamine reuptake using rat synaptosomes and intact cells expressing the human transporters. We found that FAE is most potent in inhibiting NE uptake, followed by 5-HT and DA uptake. As seen in Table 2, FAE is much more potent than other transporter modulators that have highly stereo selective nature of the binding to these monoamine transporters.

Non-selective interaction of uptake inhibitors with neuronal receptors may increase the potential of side effect. Previous studies showed that citalopram and clomipramine displayed a modest affinity to 5-HT_{2C} sites with antagonist properties (Millan et al., 2001a) (Poirier and Boyer, 1999). Reboxetine was also found to have mild affinity for h5-HT_{2C} receptors. While blockade of 5-HT_{2C} sites favorably influences mood, it may also elicit hyperphagia (Millan et al., 2000). Moreover, antagonism by clomipramine of H_1 receptors contributed to weight gain, and the action on H_1 and M_1 receptors was the cause of its cardiovascular autonomic and sedative side effects (Owens et al., 1997). In our current studies, we screened a series of potential targets that FAE might interact with, but we found FAE displayed no or very weak affinity to all of them including receptors for 5-HT, NE, DA, histamine, and muscarine, in particular, suggesting FAE might be a novel potent antidepressant without the side effects that the classical ones have.

In support of our results from *in vitro* studies, *in vivo* microdialysis studies showed that the administration of FAE significantly increased the levels of NE, 5-HT, and DA in the prefrontal cortex of rats at doses (25 and 50 mg/kg) that were effective in the mouse forced-swim and tail-suspension tests (Zhou et al., 2010). These results suggest that elevated extracellular monoamines were the consequence of the functional blockade of the monoamine transporters. The increases in monoamine levels were observed at the 60-min time point after administration of FAE, consistent with the results of the forced-swim and tail-suspension tests. Taken together, our results suggest that FAE is a novel and potent triple reuptake inhibitor that displays a significant impact on depression-like behaviors in animals.

In conclusion, FAE is a novel potent triple reuptake inhibitor that can bind to all three-monoamine transporters (NET, SERT and DAT) with high affinities. FAE mainly contain Hederagenin, which is a biopesticide, indicating that the safety and toxicity of FAE need to be further evaluated. We also realize that further work is needed to evaluate the clinical efficacy and potential long-term side effects of FAE.

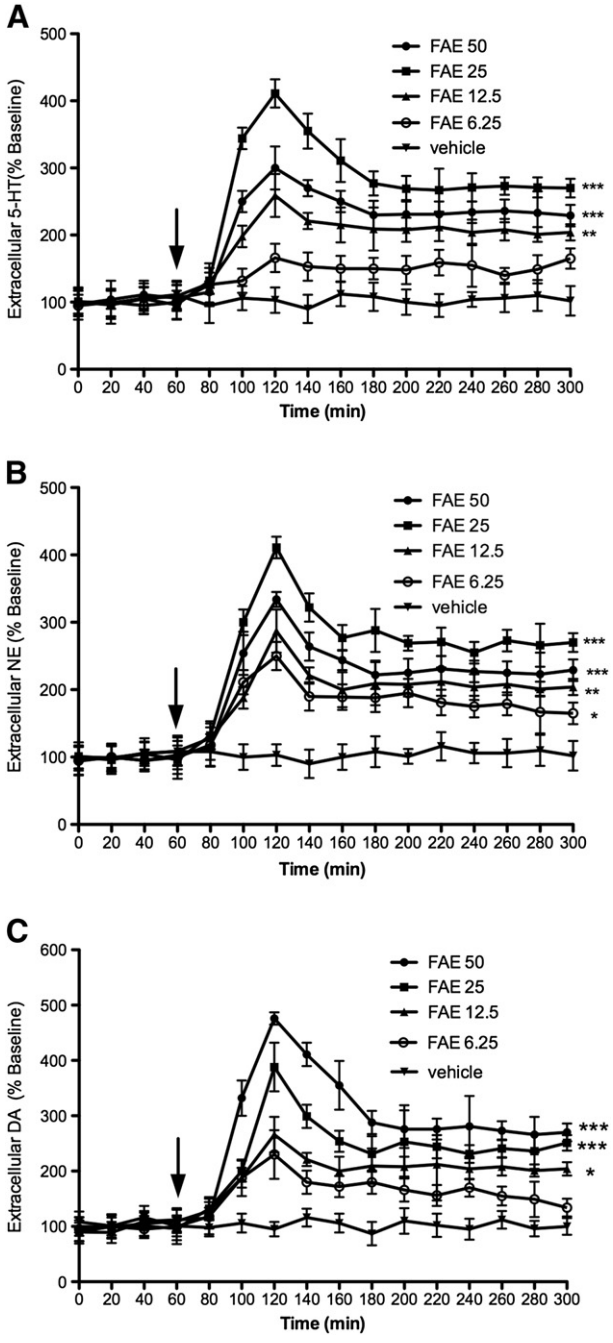

Fig. 6. Effect of FAE on extracellular 5-HT, NE and DA levels in the frontal cortex of rats measured by micro dialysis (n = 4–6 per group). Data points represent mean ± S.E.M. of 5-HT, NE and DA levels expressed relative to basal pretreatment values (define as 100%). Arrow indicates time of drug administration. FAE was administered via oral gavage in a volume of 1 ml/kg to rats. Asterisks (*, $P<0.05$) indicate significant differences compared with saline.

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