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## ORIGINAL ARTICLE

# CAPON Is a Critical Protein in Synaptic Molecular Networks in the Prefrontal Cortex of Mood Disorder Patients and Contributes to Depression-Like Behavior in a Mouse Model

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## Abstract

Aberrant regulation and activity of synaptic proteins may cause synaptic pathology in the prefrontal cortex (PFC) of mood disorder patients. Carboxy-terminal PDZ ligand of NOS1 (CAPON) is a critical scaffold protein linked to synaptic proteins like nitric oxide synthase 1, synapsins. We hypothesized that CAPON is altered together with its interacting synaptic proteins in the PFC in mood disorder patients and may contribute to depression-like behaviors in mice subjected to chronic unpredictable mild stress (CUMS). Here, we found that CAPON-immunoreactivity (ir) was significantly increased in the dorsolateral PFC (DLPFC) and anterior cingulate cortex in major depressive disorder (MDD), which was accompanied by an upregulation of spinophilin-ir and a downregulation of synapsin-ir. The increases in CAPON and spinophilin and the decrease in synapsin in the DLPFC of MDD patients were also seen in the PFC of CUMS mice. CAPON-ir positively correlated with spinophilin-ir (but not with synapsin-ir) in mood disorder patients. CAPON colocalized with spinophilin in the DLPFC of MDD patients and interacted with spinophilin in human brain. Viral-mediated CAPON downregulation in the medial PFC notably reversed the depression-like behaviors in the CUMS mice. These data suggest that CAPON may contribute to aspects of depressive behavior, possibly as an interacting protein for spinophilin in the PFC.

**Key words:** major depression, NOS1AP, spinophilin, stress, synapsin

## Introduction

Mood disorders, that is, major depressive disorder (MDD) and bipolar disorder (BD), are among the most common and severe psychiatric disorders. Despite their high prevalence and huge cost for society, the underlying disease mechanisms have so far, remained elusive. Different lines of investigation have indicated the involvement of the prefrontal cortex (PFC) in MDD and BD. Neuroimaging studies have shown remarkable decreases in gray matter volume/thickness, metabolic activity and blood flow (Drevets et al. 2008; Rigucci et al. 2010), and postmortem studies have shown reduced neuronal and glia cell densities (Rajkowska et al. 1999, 2001; Cotter et al. 2001, 2002; Rajkowska and Miguel-Hidalgo 2007; Lucassen et al. 2014), in the dorsolateral PFC (DLPFC) and anterior cingulate cortex (ACC) of MDD and BD patients.

Recently, it has been shown that synaptic pathology is also prominent in brain tissue of mood disorder patients, for example, a loss of synapses in the DLPFC of MDD patients (Kang et al. 2012), decreases in dendritic length in the DLPFC of BD patients (Konopaske et al. 2014), and reductions in spine density in the hippocampus of mood disorder patients (Rosoklija et al. 2000) and in the DLPFC of BD patients (Konopaske et al. 2014). These findings have inspired researchers to investigate the neurotransmitter/neuromodulator alterations in mood disorders that may be the basis of the changes in PFC activity (Gao and Bao 2011; Bao et al. 2012). Our series of studies have found expression changes for a number of genes related to the production/release of glutamate, GABA, stress hormone, retinoid, and nitric oxide (NO) in DLPFC and/or ACC of mood disorder patients (Gao et al. 2013; Qi et al. 2013, 2015; ; Zhao et al. 2015, 2016).

Exposure to chronic unpredicted mild stress (CUMS) is widely used as an animal model for depression-like behaviors (Pollak et al. 2010) and also in these models, synaptic abnormalities have been described (Licznarski and Duman 2013; Qiao et al. 2016). Interestingly, chronic treatments with traditional antidepressant drugs could reverse the changes in synaptic morphology induced by CUMS (Duman and Duman 2015; Chen et al. 2016).

The protein synapsin is believed to anchor synaptic vesicles to the cytoskeletal framework of the presynaptic terminal (Cesca et al. 2010). Synapsin knockout mice have a normal life span but exhibit a decreased number of synaptic vesicles (Corradi et al. 2008), while synapsin mRNA levels were found to be reduced in the DLPFC in mood disorders (Kang et al. 2012; Schmidt et al. 2015). Spinophilin is a cytoskeletal protein present in the postsynaptic density (PSD) (Muly et al. 2004). Spinophilin-deficient mice show a marked increase in spine density during development (Feng et al. 2000) and a lower level in anxiety- and depression-like behaviors (Wu et al. 2017). In addition, spinophilin protein levels showed a trend to an increase in the DLPFC in mood disorders (Koh et al. 2003). Therefore, we propose that alterations in synaptic proteins like synapsin and spinophilin might be implicated in the etiology of mood disorders like depression.

The synapsin-binding protein carboxy-terminal PDZ ligand of NOS1 (CAPON) was first identified in the rat brain and is also known as NOS1AP (NOS1 adaptor protein) (Jaffrey et al. 1998). CAPON has at least 3 isoforms in human brain: CAPON-L, CAPON-S, and CAPON-S' (Wang et al. 2016). At the presynaptic site, CAPON links to nitric oxide synthase 1 (NOS1) through the PDZ (PSD-95/disks-large/ZO-1) ligand motif and binds to synapsin I through the phosphotyrosine-binding (PTB) domain, thus

forming a ternary complex (Jaffrey et al. 2002). At the postsynaptic site, CAPON competes with PSD-95, a scaffold protein between NMDA receptor and NOS1, in its interaction with the sole PDZ domain of NOS1 (Jaffrey et al. 1998; Li et al. 2015). Interestingly, spinophilin also contains a PDZ domain (Allen et al. 1997) and interacts with PSD-95 in the striatum (Baucum et al. 2010), indicating a possible interaction between CAPON and spinophilin at the postsynaptic site. These data suggest that CAPON may serve as a critical adaptor protein in the synaptic molecular networks at both presynaptic and postsynaptic sites.

Linkage and association studies have shown that *capon* is an attractive candidate gene for schizophrenia susceptibility (Wang et al. 2016), while 8 single-nucleotide polymorphisms (SNPs) of the *capon* gene have further been associated with depression-related phenotypes within schizophrenia (Cheah et al. 2015). In addition, an intronic SNP of *capon* (rs386231) was significantly associated with the severity of depression in post-traumatic stress disorder (Lawford et al. 2013). Our earlier work showed that the expression of NOS1, a CAPON-binding protein, was significantly reduced in the ACC in depressive patients (Gao et al. 2013). These studies suggested that CAPON may be relevant to depression. Yet, very little is known about the protein levels and functions of CAPON in the PFC in relation to mood disorders.

Here, we hypothesized that CAPON may be altered together with its interacting synaptic proteins in the PFC of mood disorder patients and can contribute to depression-like behaviors in mice. To test this, we measured CAPON, synapsin and spinophilin levels in postmortem samples of the DLPFC and ACC of mood disorder patients and in the PFC and cingulate cortex of mice subjected to CUMS paradigm. The association of CAPON with synapsin and spinophilin was analyzed in human brain tissues. In addition, we investigated the effects of viral-mediated CAPON knockdown in the medial PFC on CUMS-induced depression-like behaviors in mice.

## Materials and Methods

### Brain Material

Brain material was obtained from the Netherlands Brain Bank (NBB, director Dr I. Huitinga) following permission for a brain autopsy and for the use of the brain material and clinical data for research purposes. The mood disorder patients had been diagnosed during their lifetime in psychiatric clinics according to Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria to have an MDD or a BD. The diagnosis was confirmed postmortem by a board-certified psychiatrist, based on the DSM-IV criteria and the extensive medical records of the NBB. The control (CTR) subjects had not suffered from a primary neurological disorder, other psychiatric diseases, or alcohol abuse. The mood disorder patients did not abuse alcohol either. Drug-abusing subjects were also excluded. The absence of neuropathological changes, both in the patients with mood disorders and in the CTRs, was confirmed by systematic neuropathological investigation (van de Nes et al. 1998). The material consisted of paraffin-embedded tissues of DLPFC (MDD,  $n = 16$ ; BD,  $n = 12$ ; CTR,  $n = 16$ ) and ACC (MDD,  $n = 16$ ; BD,  $n = 14$ ; CTR,  $n = 16$ ). The localization of DLPFC and ACC are corresponding to Brodmann area 9 and 24, respectively, as determined by experienced neuropathologists. The MDD group and the BD group were well matched with the CTR group for age, postmortem delay, fixation time, pH value in cerebrospinal fluid (CSF pH),

and brain weight. The clock time of death and month time of death were also matched between MDD and CTR or between BD and CTR, because there are widespread circadian and circannual molecular fluctuations in the human brain (Lim et al. 2017; Bao and Swaab 2018). Detailed clinicopathological information and *P*-values of parameter matches are given in Supplementary Table 1.

### Antibody Specificity

We used the same antibodies as described before in literature on CAPON (Xu et al. 2005; Carrel et al. 2015; Hernandez et al. 2016), spinophilin (Sweet et al. 2009; Egbujo et al. 2015; Shelton et al. 2015; Mi et al. 2017; Brocos-Mosquera et al. 2018), and synapsin (Marker et al. 2013; Kim et al. 2015). The specificity of these antibodies was further confirmed in our human brain tissues by western blot. As shown in Supplementary Figure 1, the rabbit anti-CAPON antibody (Santa Cruz Bio.) recognized CAPON-S (30 kDa), CAPON-L (55 kDa) and the phosphorylated CAPON (75 kDa), which was consistent with the manufacturer's instructions. Both rabbit (Millipore, ) and mouse (Santa Cruz Bio.) antispinophilin antibodies detected an appropriate band at ~130 kDa and a weak band at ~100 kDa, the latter was considered as a cleaved form of the protein (Erdozain et al. 2016; Brocos-Mosquera et al. 2018). The rabbit antisynapsin antibody (Cell Signaling Technology) mainly recognized synapsin I at the expected size (~72 kDa), although it showed a weak cross-reaction with synapsin II (~55 kDa).

### Immunohistochemistry

We used a similar protocol as described in our previous study (Gao et al. 2013). All the sections from the CTR, BD, and MDD subjects were stained at the same time for each molecule. In brief, antigen retrieval was performed with microwave in sodium citrate buffer (pH 6.0) for CAPON and synapsin I or Tris-HCl buffer (pH 9.0) for spinophilin. The sections used for spinophilin staining were incubated with tris-buffered saline (TBS) milk (5% milk powder, pH 7.6) at room temperature (RT) for 60 min to reduce nonspecific staining. Then, the sections were incubated with rabbit anti-CAPON antibody at 1:50 in supermix (0.5% Triton, 0.25% gelatin in TBS) or rabbit antisynapsin antibody at 1:400 in supermix or rabbit antispinophilin antibody at 1:500 in TBS containing 0.5% Triton X-100 overnight at 4°C. The next day, the sections were incubated with biotinylated goat antirabbit IgG (Vector Laboratories), followed by incubation with a ABC Elite kit (Vector Laboratories). Finally, the sections were incubated for 10 min in 3',3'-diaminobenzidine-tetrahydrochloride (DAB)-nickel substrate solution [0.5 mg/mL DAB, 2.2 mg/mL ammonium nickel sulfate, 0.01% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in TBS].

### Double-Labeling of CAPON and Spinophilin in the DLPPFC

Spinophilin and CAPON signals were sequentially detected by immunohistochemistry on the DLPPFC sections from 2 MDD patients. Spinophilin staining was performed using a monoclonal antibody (1:50, Santa Cruz Bio.) with the same protocol as mentioned above except for developing brown color in DAB substrate solution (0.5 mg/mL DAB, 0.01% H<sub>2</sub>O<sub>2</sub> in TBS). The sections were then incubated with the rabbit anti-CAPON antibody (1:50, Santa Cruz Bio.) at RT for 1 h followed by overnight incubation at 4°C. The next day, the sections were incubated with antirabbit alkaline phosphatase antibody (1:200, Jackson lab)

and developed blue color in NBT/BCIP substrate solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.34 mg/mL NBT, 0.175 mg/mL BCIP, 0.24 mg/mL levamisole). After washing in 100% methanol for 5 min to remove brown discoloration, the sections were cover-slipped using Kaiser's glycerin (Merck Chemicals).

### Microscopy and Image Analysis

All the images were collected under a Zeiss Axioskop microscope (Zeiss) with neofluar objectives (Zeiss) and a motorized XYZ stage, black-and-white camera (Sony). Optical density analysis was performed with the software Image Pro 6.3 (Mediacybernetics) plus home-developed macros, with the identities of all the subjects unknown to the investigator.

The method of determining the integrated optical density (IOD) has been described in our previous study (Gao et al. 2013). Briefly, the collected images were transformed into optical density (OD) images by use of a standard transformation curve. The collected images included an intact gyrus from DLPPFC or ACC, in which the gray matter or different layers (I, II-III, IV, and V-VI) were delineated based upon adjacent sections with thionine staining. All big blood vessels were excluded from the area of interest. OD values >3 times (spinophilin), 2 times (CAPON), or 2.5 times (synapsin) the background value were considered a positive signal, a level that was determined by a pilot study for the threshold value. The IOD was calculated by multiplying the percentage of the positive area by the OD of positive signals.

Double-stained images were captured by a Nuance FX camera (PerkinElmer) under a Zeiss Axioskop microscope with Plan-NEOFLUAR objectives (Zeiss). The spectral analyses were performed using the Nuance multispectral imaging system as described previously (Siljee et al. 2013; Ten Kulve et al. 2016). Pseudocolored images were generated using the Nuance software to enhance color separation and visualization of colocalization.

### CUMS Model

Animals were housed in an air-conditioned room at a temperature of 25–27°C, with food and water ad libitum, except when specified otherwise. Prior to the experiments, the animals were allowed one week to adapt to their new circumstances. We established the CUMS model in male ICR mice (20–25 g) according to the protocol described in our earlier paper (Gao et al. 2014). After exposure to different kinds of stressors for 4 consecutive weeks, the CUMS mice showed similar changes as reported previously (Zhou et al. 2011), that is, low hair scores, longer immobility time in forced swim test (FST) and tail suspension test (TST), and elevated corticosterone levels in plasma as measured by an enzyme-linked immunosorbent assay (DSL) (Supplementary Fig. 2), confirming this model.

All mice were sacrificed by rapid decapitation between 09:00 and 11:00 h. The whole brain was taken out, and the PFC and cingulate cortex were dissected (Chiu et al. 2007). All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011, USA), and all experimental protocols were approved by the Animal Care Committee of Xuzhou Medical University.

### Protein Extraction and Western Blot

Total protein was extracted from the tissues and then subjected to western blot as we have described before (Gao et al.

2016, 2017). The same antibodies used in the human studies were employed to detect CAPON (1:500), synapsin (1:800) and spinophilin (Millipore, 1:800) in animal tissues.  $\beta$ -actin (Santa Cruz Bio., 1:1500) was used as a protein-loading control (Zhang et al. 2018). Band densities were quantified using Image J software (National Institutes of Health). The relative protein levels were determined by normalizing the densitometry value of proteins of interest to that of  $\beta$ -actin.

### Coimmunoprecipitation

Human cerebral cortex tissues were homogenized in cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors. The extract was centrifuged at 10 000–13 000 *g* to remove insoluble material. The supernatant was precleared with Protein A/G PLUS-Agarose (Cell Signaling Technology) at 4°C for 1 h and then subjected to immunoprecipitation (IP) with appropriate IgG at 4°C overnight. Protein A/G PLUS-Agarose beads were added for 2 h of incubation. Immunoprecipitated proteins were eluted with protein-loading buffer and subjected to western blot analysis.

### Adeno-Associated Virus Preparation and Delivery

We used a previously validated siRNA targeting the CAPON transcript (Carrel et al. 2009) and an unrelated sequence as a negative control (scramble, 5'-TTCTCCGAACGTGTCACGT-3'). Each siRNA was converted to shRNA and its DNA was subcloned into the pAAV-SYN-eGFP-U6-shRNA vector (OBiO). The shCAPON-AAV and scramble-AAV particles were produced and purified in OBiO. The viral titers ( $2.0 \times 10^{13}$  v.g./mL) were determined by real-time quantitative PCR with specific primers for the WPRE sequence as follows (5'-3'): TTACGCTATGTGGATAC GC (forward) and AGAGACAGCAACCAGGAT (reverse).

Mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g) and were placed into a stereotaxic frame (RWD Life Science). A total of 0.5  $\mu$ L of viral solution was injected bilaterally into the prelimbic cortex (PrL) using the following coordinates: AP = +2.50 mm, ML =  $\pm$ 0.45 mm, DV = -1.75 mm and into the infralimbic cortex (IL) using the following coordinates: AP = +1.90 mm, ML =  $\pm$ 0.45 mm, DV = -2.5 mm. The needle was maintained in the place for an additional 5 min to facilitate the diffusion of the virus and then slowly withdrawn. After 2 weeks of recovery from the surgery, all the mice were subjected to the above-mentioned CUMS protocol, the hair score was observed weekly during the CUMS treatment. The behavioral testing commenced after exposure to different kinds of stressors for 6 consecutive weeks. The whole brains were taken out following behavior tests. Viral injection sites were confirmed by GFP fluorescence.

### Forced Swim Test

The FST was performed according to the literature (Can et al. 2012). Briefly, Mice were placed individually into Plexiglas cylinders (40 cm height, 18 cm diameter) filled to a height of 15 cm water (25°C) and exposed to a 15-min test swim. Immobility time was recorded during the last 5-min period of the test by an experimenter blind to treatment groups. The water in the cylinder was changed between animals. A mouse was considered to be immobile when it floated in an upright position

and made only small movements to keep its head above the water.

### Tail Suspension Test

The TST was performed as previously described (Doucet et al. 2013). Mice were suspended by their tail from a metal rod using adhesive tape attached 0.5–1 cm from the base of their tail. The rod was fixed 5 cm from the surface of a table. Mice were considered immobile only when they hung passively and completely motionless. The total duration of immobility during a 6-min test was calculated.

### Hair Score

The hair score was assessed weekly according to a previously reported method (Zhou et al. 2007), using a scale from 1 to 3: A health state was noted one and a damaged state with piloerection and/or dirty fur was noted 3. Intermediate state was noted 2.

### Statistical Analysis

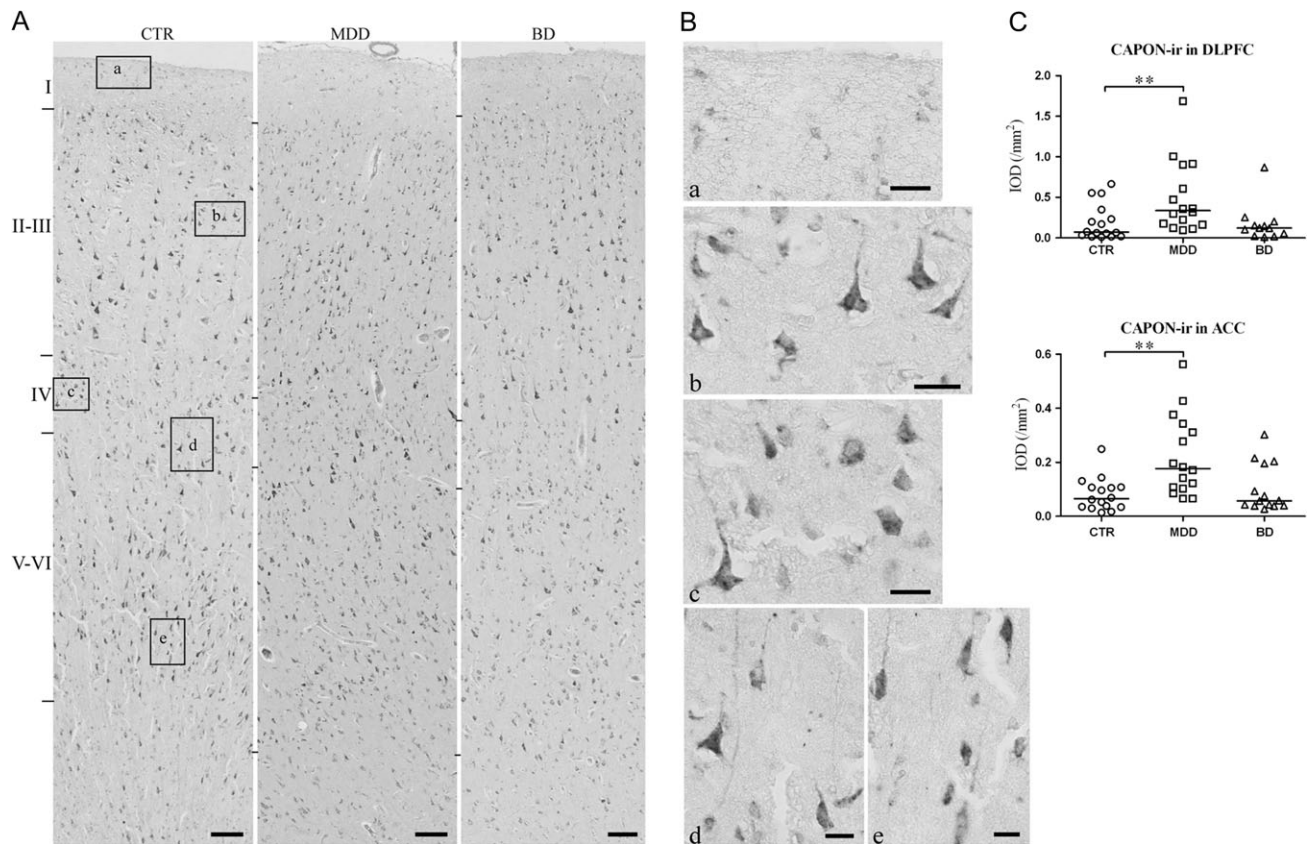
The differences among CTR, MDD, and BD groups were first evaluated by the Kruskal–Wallis test and, if significant, were further evaluated by the Mann–Whitney *U* test. The differences in clock time or month of death (circular parameters) between groups were tested with the Mardia–Watson–Wheeler test (Batschelet 1981). The comparison between 2 groups in animal studies was performed by the Student's *t* test. The correlation was examined with the Spearman test. Statistical analyses were performed using SPSS version 13.0 (SPSS Inc.). Tests were 2-tailed and values of *P* < 0.05 were considered to be statistically significant.

## Results

### Changes of CAPON-, Spinophilin-, and Synapsin-ir in the DLPFC and ACC of Mood Disorder Patients

CAPON-ir was present in all the layers of the DLPFC gray matter (Fig. 1A), such as the glial cells in Layer I, the small pyramidal neurons in Layers II–III, the small round neurons (probably interneurons) in Layer IV, and the pyramidal neurons with long fibers in Layer V–VI (Fig. 1B). CAPON-ir staining had a similar distribution in the ACC as in the DLPFC (data not shown). There was a significant difference in the CAPON-ir IOD among the 3 groups, that is, CTR, MDD, and BD (*P* = 0.005 for DLPFC; *P* = 0.001 for ACC), which was found to be due to the significant increase in the MDD group compared with the CTR group (*P* = 0.007 for DLPFC; *P* = 0.001 for ACC, Fig. 1C).

Spinophilin punctate staining was evident in Layer I in the DLPFC (Fig. 2A,B). In Layers II–VI, spinophilin-ir appeared to be localized in the neuronal cytoplasm and processes, with much stronger staining in the MDD group compared with the CTR group (Fig. 2A–C). Indeed, we found a significant difference in the spinophilin-ir IOD among the 3 groups in the DLPFC gray matter (*P* = 0.026), which was mainly based on the MDD group (*P* = 0.046) and not on the BD group (Fig. 2D). The significant increase in spinophilin-ir IOD in the DLPFC gray matter in MDD group showed no layer-specific difference, and it was characterized by a synergistic increase in spinophilin-ir level in Layers I, II–III, IV, and V–VI (Supplementary Fig. 3). Spinophilin-ir staining had a similar distribution in the ACC as in the DLPFC (data not shown). No significant difference was found in the



**Figure 1.** Changes of CAPON-ir in the DLPFC and ACC in mood disorders. (A) The laminar structure of the cortex was determined using adjacent sections with thionine staining. CAPON-ir was distributed throughout the entire gray matter in the DLPFC of CTR, MDD, and BD subjects. The morphology of CAPON-ir cells in MDD and BD patients appeared to be comparable with the controls. (B) The higher magnification of the boxed areas in A. Note that the CAPON-ir staining was present in glial cells in layer I (a), in the major dendrites and soma of pyramidal neurons in layers II–III and V–VI (b, d, e), and in small rounded neurons in layer IV (c). (C) Quantitative analysis showed that the CAPON-ir IOD was significantly increased in the gray matter of DLPFC and ACC of MDD patients. Scale bar: A, 100  $\mu\text{m}$ ; B, 12  $\mu\text{m}$ . \*\* $P < 0.01$ .

spinophilin-ir levels in the ACC gray matter among the 3 groups ( $P = 0.239$ ), though the spinophilin-ir IOD showed a trend toward an increase in the MDD group compared with the CTR group ( $P = 0.060$ ) (Fig. 2D).

Synapsin punctate staining was distributed throughout the entire gray matter in the DLPFC (Fig. 3A), and it was much stronger in Layer I (Fig. 3A,B). Synapsin-ir staining had a similar distribution in the ACC as in the DLPFC (data not shown). There was no significant difference in the synapsin-ir IOD among these groups either in the DLPFC ( $P = 0.109$ ) or in the ACC ( $P = 0.584$ ), but a trend toward a decrease in the synapsin-ir levels in the DLPFC was seen in the MDD group relative to the CTR group ( $P = 0.058$ , Fig. 3C). It should be noted that the percentage of synapsin-negative area was comparable between BD and CTR ( $P = 0.591$ ) and between MDD and CTR ( $P = 0.917$ ).

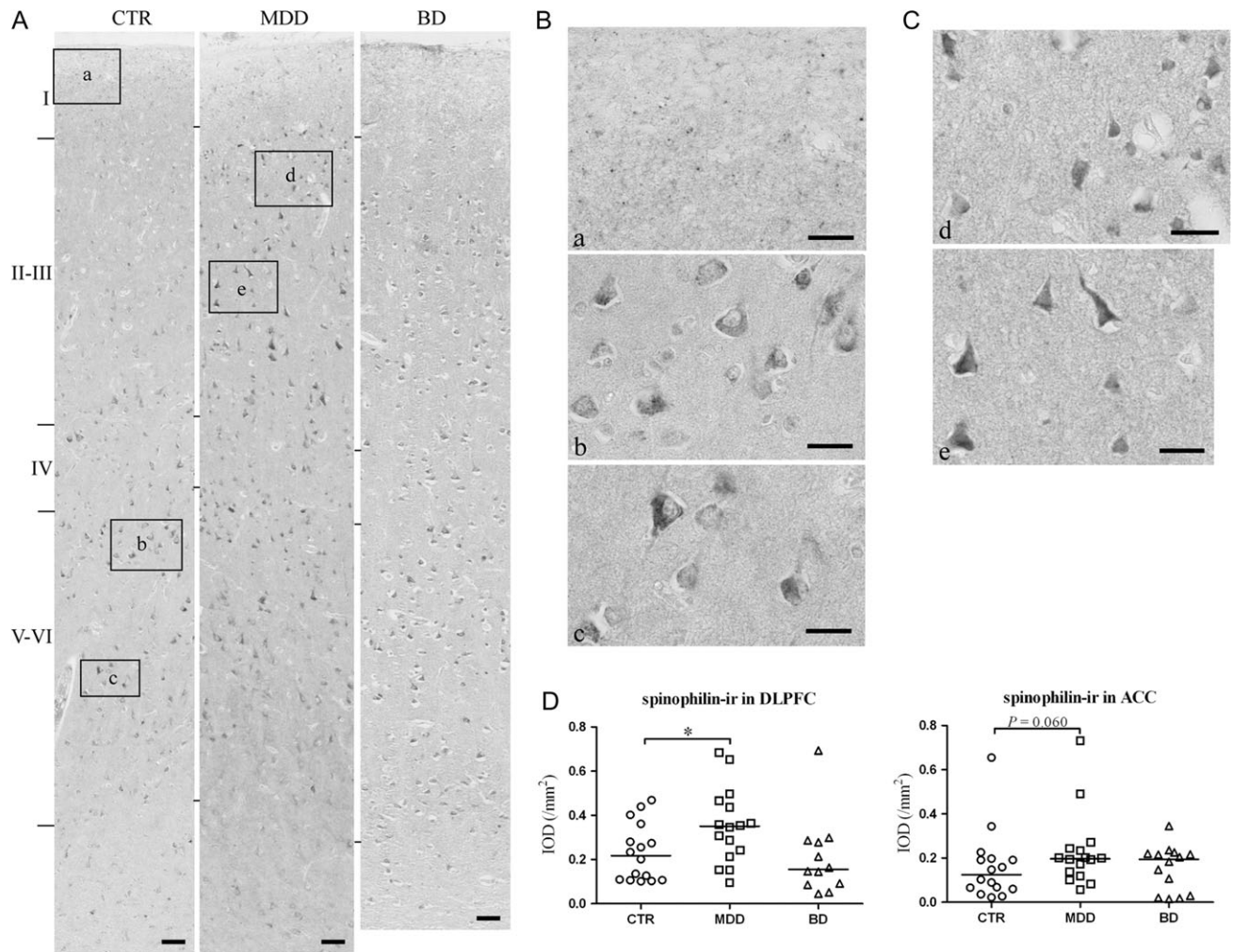
#### Analysis of Possible Confounding Factors

As shown in Supplementary Table 1, CTR–MDD and CTR–BD are matched for age, clock time of death, month time of death, post-mortem delay, fixation time, pH value in CSF, and brain weight. The correlations of CAPON-, spinophilin-, and synapsin-ir levels with age, postmortem delay, fixation time, pH value in CSF, and brain weight are provided in Supplementary Table 2. We found no significant correlations except for a significantly negative correlation between CAPON-ir levels and PMD in the DLPFC of the

control subjects ( $\text{Rho} = -0.670$ ,  $P = 0.005$ ) and a significantly positive correlation between spinophilin-ir levels and pH value in CSF in the DLPFC of the BD patients ( $\text{Rho} = 0.839$ ,  $P = 0.001$ ). The relationship of CAPON-, spinophilin-, and synapsin-ir levels with sex, suicide attempt/thoughts, and age of onset is provided in Supplementary Table 3. The possible influence of antidepressants or mood stabilizers' treatments on our measurements is provided in Supplementary Table 4. We found no significant effects of these confounders such as sex, suicide, age of onset and medicine on our data, except that bipolar patients taking mood stabilizers had a significant higher synapsin-ir level in the DLPFC than bipolar patients who were not taking mood stabilizers. It should be noted that the sample size of the subgroups was relatively small and that the subgroups were not well matched anymore for the putative confounding factors as mentioned in Supplementary Table 1.

#### Association of CAPON with Synapsin and Spinophilin

Since the changes of CAPON-, spinophilin-, and synapsin-ir levels were quantified in the same cohort, we subsequently analyzed the correlations among them in both DLPFC and ACC. There was a significantly positive correlation between CAPON and spinophilin in the DLPFC in the pooled group ( $\text{Rho} = 0.574$ ,  $P < 0.001$ , Fig. 4A), which was based on both MDD patients ( $\text{Rho} = 0.606$ ,  $P = 0.013$ , Fig. 4B) and BD patients ( $\text{Rho} = 0.685$ ,  $P = 0.014$ ,



**Figure 2.** Changes of spinophilin-ir in the DLPFC and ACC in mood disorders. (A) The laminar structure of the cortex was determined using adjacent sections with thionine staining. Spinophilin-ir was observed to distribute throughout the entire gray matter in the DLPFC of CTR, MDD, and BD subjects. The morphology of spinophilin-ir cells in MDD and BD patients appeared to be comparable with the controls. (B, C) The higher magnification of the boxed areas in A. In Layer I, a punctuated spinophilin staining was evident (a). In Layers II-III, spinophilin-ir was stronger in the MDD patients than in the CTR and it was present in the small interneurons or glial cells and in the cytoplasm of pyramidal neurons (d, e). In Layers V-VI, spinophilin-ir was also observed in neuronal soma and processes (b, c). (D) Quantitative analysis showed that the spinophilin-ir IOD was increased in the gray matter of DLPFC and ACC of MDD patients. Scale bar: A, 100  $\mu\text{m}$ ; B and C, 12  $\mu\text{m}$ . \* $P < 0.05$ .

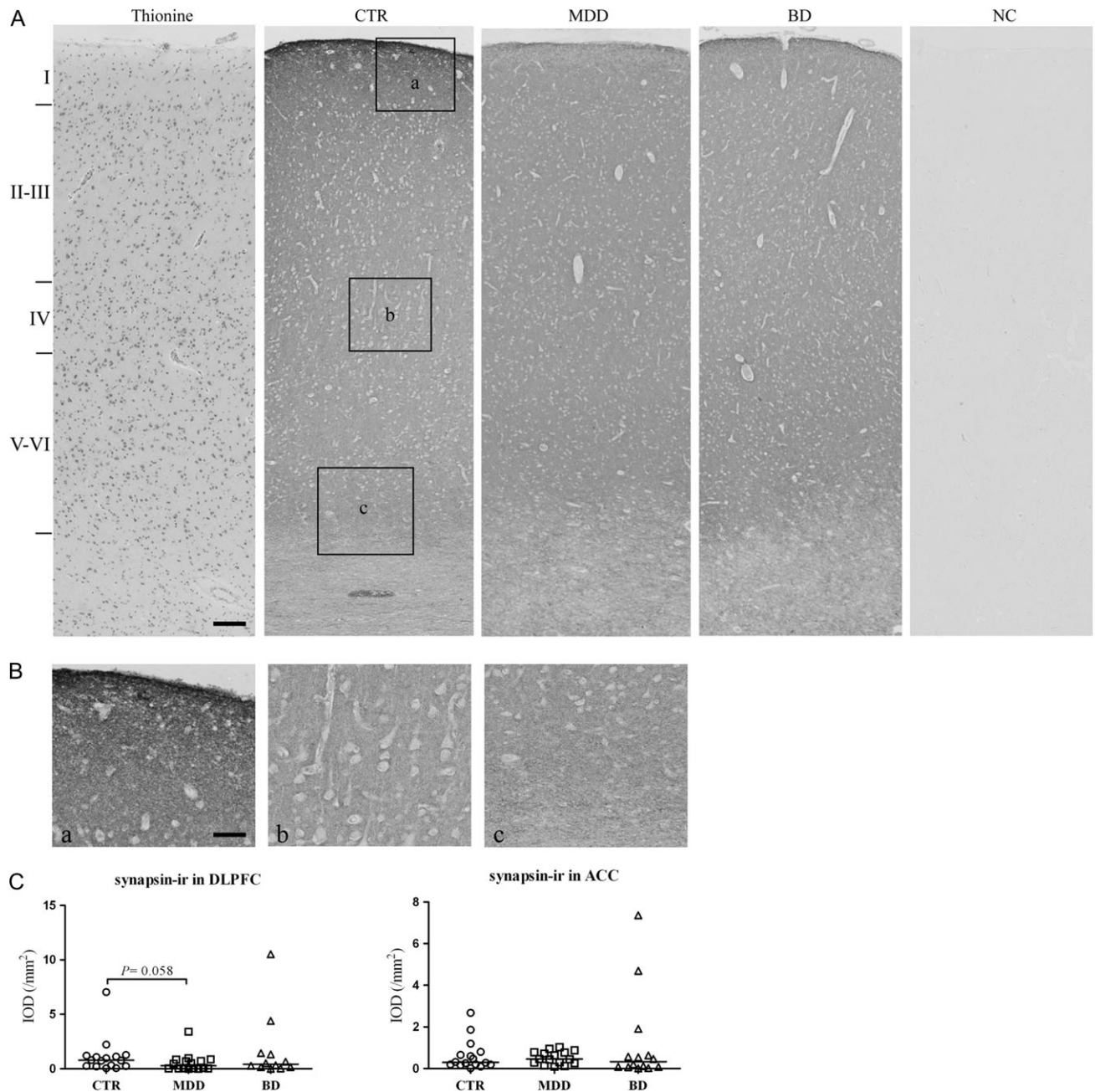
Fig. 4C), but not on the controls ( $Rho = 0.321$ ,  $P = 0.226$ ). CAPON was also positively associated with spinophilin in the ACC in the pooled group ( $Rho = 0.473$ ,  $P = 0.001$ , Fig. 4D), but the correlation did not reach statistical significance when we analyzed separately in the individual group. No correlations were found between CAPON and synapsin or between spinophilin and synapsin in the DLPFC or in the ACC either for the CTR or MDD or BD group. These data indicated that the high levels of CAPON and spinophilin in DLPFC may together participate in the pathology of major depression. Thus, we focused on the association of CAPON and spinophilin in the subsequent experiments.

The colocalization of CAPON and spinophilin was studied in DLPFC sections using a sequential immunocytochemical method. As shown in Figure 5, CAPON-ir (blue) and spinophilin-ir (brown) were coexpressing in some neurons in both Layer II/III (Fig. 5A) and Layer V (Fig. 5B), which was indicated by the yellow color. In Layer II/III, 36% of area was positive for CAPON within spinophilin-ir area; 24% of area was positive for spinophilin within CAPON-ir area. In Layer V, 30% of area was positive for

CAPON within spinophilin-ir area; 19% of area was positive for spinophilin within CAPON-ir area. Furthermore, we performed coimmunoprecipitation to identify the interaction between CAPON and spinophilin in human brain tissues. IP with the CAPON polyclonal antibody showed that spinophilin was associated with CAPON (Fig. 5C). CAPON was immunoprecipitated by the spinophilin monoclonal antibody (Fig. 5D). These findings indicated that CAPON-spinophilin formed a complex in the PFC neurons.

### Changes of CAPON, Spinophilin and Synapsin in the PFC of CUMS Mice

As mentioned in the Materials and Methods, we successfully established the CUMS model in mice (Supplementary Fig. 2). The protein levels of CAPON, spinophilin, and synapsin were measured by western blot in the PFC and cingulate cortex of CTR and CUMS mice. CUMS caused a significant increase in the CAPON ( $P = 0.010$ ) and spinophilin ( $P = 0.001$ ) protein levels and



**Figure 3.** Changes of synapsin-ir in the DLPFC and ACC in mood disorders. (A) The laminar structure of the cortex was determined using adjacent sections with thionine staining. Synapsin-ir was distributed throughout the entire gray matter in the DLPFC of CTR, MDD, and BD subjects. The morphology of punctated synapsin-ir in MDD and BD patients showed no significant changes compared with the controls. NC, negative control, without primary antibody incubation. (B) The higher magnification of the boxed areas in A. Note that the synapsin punctate staining was much stronger in Layer I than in other layers (a-c). (C) Quantitative analysis showed that the synapsin-ir IOD showed a trend to a decrease in the DLPFC gray matter of MDD patients. Scale bar: A, 100  $\mu$ m; B, 12  $\mu$ m.

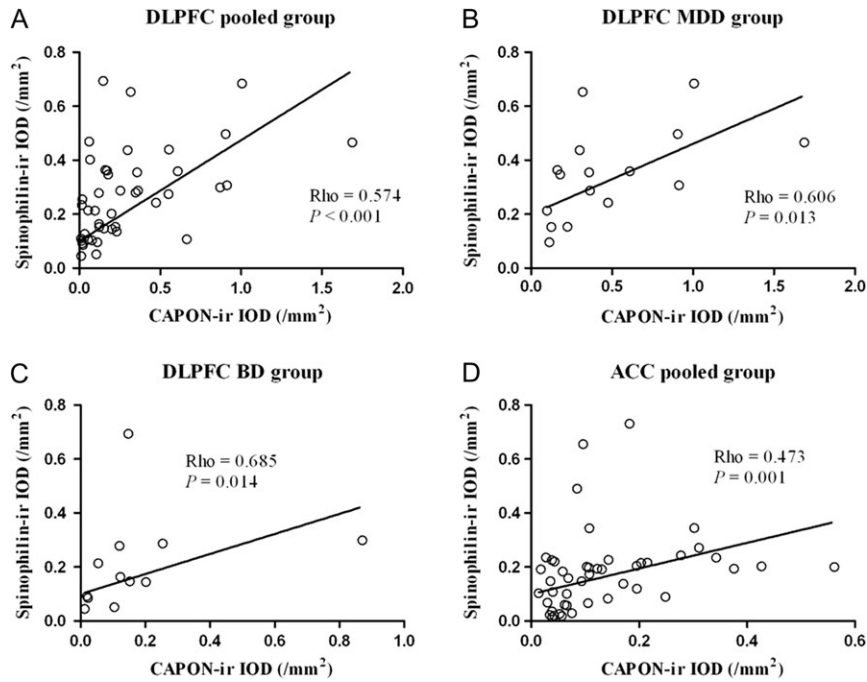
a significant decrease in the synapsin level ( $P = 0.034$ ) in the PFC (Fig. 6A,B). In the cingulate cortex, CAPON was significantly decreased ( $P = 0.002$ ) and synapsin significantly increased ( $P < 0.001$ ), but spinophilin showed no significant changes in the CUMS mice (Fig. 6A,C).

#### Effects of CAPON Downregulation on Depressive Behavior in CUMS Mice

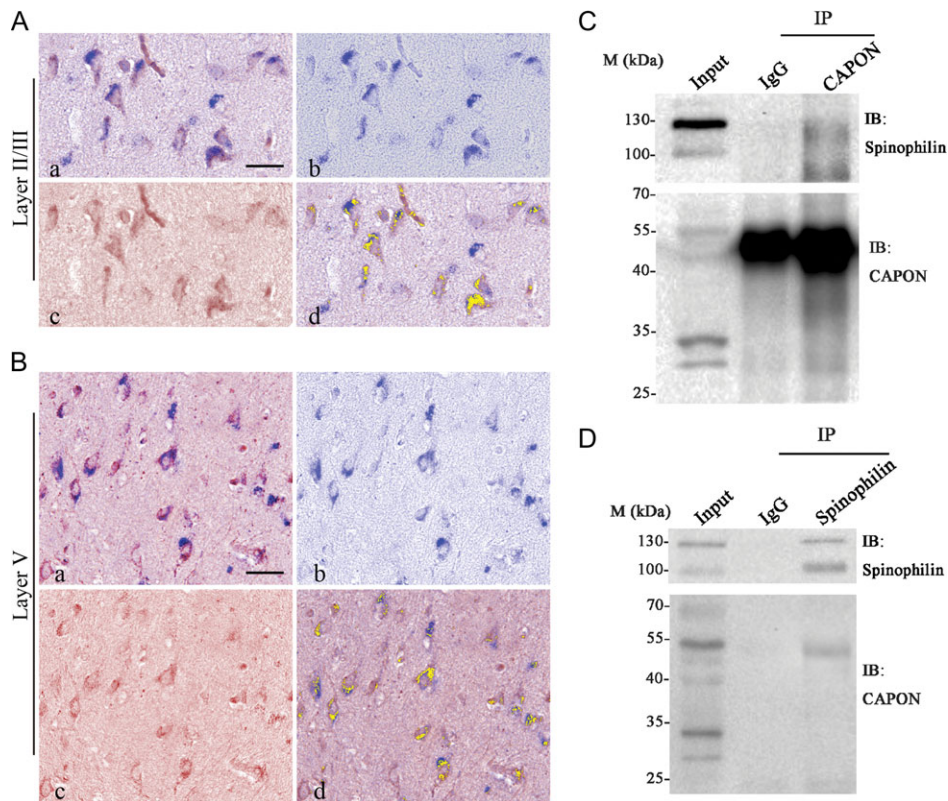
A previously validated shRNA sequence was used to silence CAPON (Carrel et al. 2009), and the downregulation efficiency

was assessed in vitro by western blot (Fig. 7A). We infused adeno-associated virus (AAV)-mediated scramble- or CAPON-shRNA into the medial PFC of CUMS mice. As shown in the mouse brain atlas and the GFP fluorescence images, the infected areas were mainly in the medial PFC including both PrL and IL (Fig. 7B). The downregulation of CAPON-ir level in the infected area was further validated in vivo by immunohistochemistry (Fig. 7C). The hair score showed a gradual reduction in both shCAPON and scramble groups following CUMS treatment. The CUMS mice with shCAPON-AAV had higher hair scores ( $P = 0.001$  at 4 week;  $P = 0.015$  at 5 week;  $P = 0.005$  at

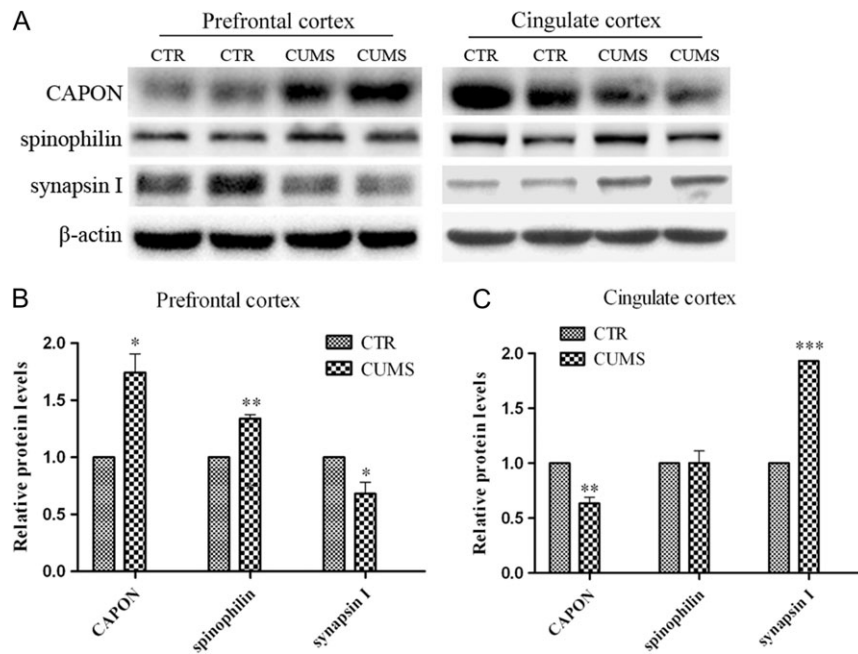




**Figure 4.** Correlation analyses for CAPON- and spinophilin-ir levels in the DLPFC and ACC. (A–C) The CAPON-ir IOD significantly correlated with the spinophilin-ir IOD in the DLPFC pooled group, which was mainly based upon the MDD and BD groups, but not upon the CTR group. (D) There was a significant correlation between CAPON- and spinophilin-ir IODs in the ACC-pooled group, but the significance disappeared when correlation analyses were performed in the individual group, for example, MDD, BD, or CTR group.



**Figure 5.** Colocalization and interaction analyses between CAPON and spinophilin in the DLPFC. (A–B) CAPON and spinophilin were sequentially detected in the DLPFC of an MDD patient by immunohistochemistry. In both Layers II–III (A) and Layer V (B), CAPON signals (b, blue) and spinophilin signals (c, brown) were separated from the original image (a). The colocalization of CAPON and spinophilin are shown in d (yellow). Scale bar: 50  $\mu\text{m}$ . (C–D) coimmunoprecipitation was performed in human brain extract to identify the interaction between CAPON and spinophilin. IP with the CAPON polyclonal antibody showed that spinophilin was associated with CAPON (C). Note that the CAPON-L band overlapped with the heavy chain of IgG at  $\sim 55$  kDa. Conversely, CAPON was immunoprecipitated by a spinophilin monoclonal antibody (D). Ten percent of lysates (1 mg protein) were loaded as an Input.



**Figure 6.** Changes of CAPON, spinophilin, and synapsin protein levels in the cortex of CUMS mice. Western blot was used to measure the protein levels of CAPON, spinophilin, and synapsin in 2 brain cortical areas of CTR and CUMS mice ( $n = 6$  for each group). (A) Representative blot images for CAPON, spinophilin, and synapsin.  $\beta$ -actin was used as a loading control. (B) Quantification graphs showed that CAPON and spinophilin protein levels were significantly increased, and that synapsin I level was significantly decreased in the PFC of CUMS mice. In the cingulate cortex, the CAPON protein level was significantly decreased, the synapsin I protein was significantly increased in the CUMS mice. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

8 week) and shorter immobility time in both FST ( $P = 0.019$ ) and TST ( $P = 0.006$ ) than those with scramble-AAV (Fig. 8A–C). The locomotor activity showed no significant difference in the CUMS mice between shCAPON and scramble groups (Fig. 8D).

## Discussion

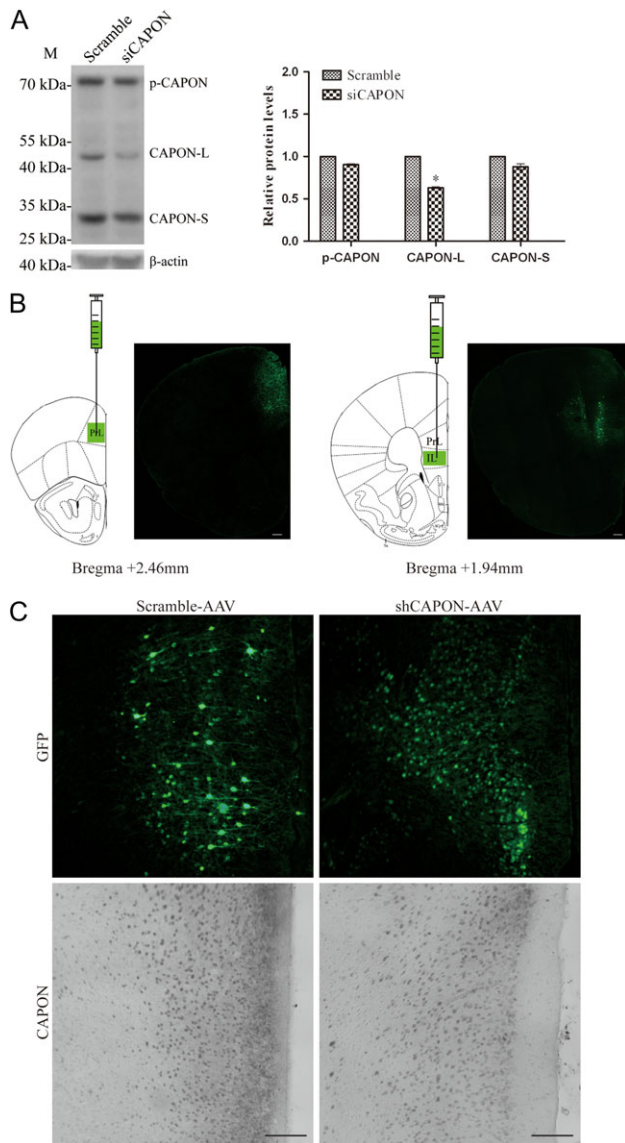
In the present study, we reported an upregulation of CAPON-ir levels in the PFC in both MDD patients and CUMS animals, which was accompanied by an increase in the spinophilin-ir levels and a reduction in the synapsin-ir levels. CAPON-ir was closely associated with spinophilin-ir, as determined by the correlation and colocalization analyses in the DLPPFC of MDD patients. In addition, coimmunoprecipitation experiments revealed an interaction between CAPON and spinophilin in human cerebral cortex. More importantly, downregulation of CAPON in the PFC reversed the CUMS-induced depressive behaviors in mice. These results suggested that, as an interacting protein for spinophilin, CAPON may be implicated in synaptic pathology that could underlie aspects of depression.

CAPON has at least 3 isoforms in human brain. CAPON-L consists of a PTB domain and a PDZ ligand motif (Xu et al. 2005; Carrel et al. 2009), whereas CAPON-S only contains a PDZ ligand motif, CAPON-S' is the truncated version of CAPON-S (Hadzimichalis et al. 2010) with unknown functions at present. It was reported that mRNA levels of CAPON-S (but not CAPON-L) significantly increased in the DLPPFC in schizophrenia and BD patients (Xu et al. 2005). Here, we used a polyclonal antibody that recognized both CAPON-L and CAPON-S in human brain and found that CAPON-ir levels showed no significant changes in either the DLPPFC or the ACC in BD patients, suggesting that the *capon* gene polymorphisms may result in the elevation of CAPON-S mRNA. Indeed, 3 SNPs within *capon* gene were found to be related to schizophrenia (Brzustowicz et al. 2004). The

increased expression of CAPON-S mRNA as found in schizophrenia and BD was significantly associated with genotype at all the 3 SNPs (Brzustowicz et al., 2004; Xu et al., 2005).

We for the first time reported that CAPON-ir levels were significantly increased in both the DLPPFC and ACC in MDD patients. However, the immunohistochemistry analysis cannot differentiate between CAPON-L and CAPON-S. Further Western blot analysis should be used to determine which isoforms of CAPON are responsible for the increase using frozen PFC samples of MDD patients. The elevated CAPON levels were also seen in the PFC in the CUMS mice, indicating an important role of PFC CAPON in depression phenotype. In contrast, we found a significant decrease in the CAPON levels in the cingulate cortex in CUMS mice. One possible explanation might be that cingulate cortex in rodents is not exactly homologous to ACC in human. In addition, cingulate cortex and PFC receive overlapping projections from the basal forebrain, amygdala, hypothalamus, and brainstem in rodents, but there are some important differences among divisions (Hoover and Vertes 2007). For example, the amygdala distributes more heavily to the PFC than to the cingulate cortex, which may also account for the discrepancy between PFC and cingulate cortex in the CAPON alterations.

We measured the total amount of CAPON protein by western blot in the CUMS mouse cortex including both gray matter and white matter, while the CAPON-ir levels were quantified in the gray matter of brain cortex in mood disorder patients. Since CAPON-ir is also present in the glial cells as shown in Figure 1 and as reported previously (Li et al. 2008; Jiang et al. 2010), the CAPON expression levels in the white matter may contribute to the discrepancy between human and animal studies. Therefore, further quantitative immunohistochemical analysis should be performed in the future to determine which cell types are responsible for the changes of CAPON protein in the mouse cortex following the CUMS protocol.



**Figure 7.** Identification of CAPON downregulation efficiency and virus injection sites. (A) The CAPON siRNA (siCAPON) was transfected into mouse neuronal cells. Scramble siRNA was used as a negative control. Western blot analysis showed that the siCAPON significantly downregulated the long-form CAPON (CAPON-L) protein, without affecting the phosphorylated CAPON (p-CAPON) and the short-form CAPON (CAPON-S) levels. \* $P < 0.05$ . (B) The injection sites, that is, PrL and IL, were indicated in the mouse brain atlas and were confirmed by the GFP fluorescence. (C) Immunohistochemistry showed that the CAPON-ir level was lower in the area infected by shRNA AAV particles (shCAPON-AAV) than that in the area infected by the scramble-AAV particles. Scale bar: 100  $\mu$ m.

Spinophilin is a cytoskeletal protein present in the neuronal dendritic spines (Muly et al. 2004). Loss of spinophilin increased the spine density during development (Feng et al. 2000), and ameliorated anxiety- and depression-like behaviors in mice (Wu et al. 2017). In the same cohort used for CAPON measurements, we found that MDD patients had a significant increase in the spinophilin-ir levels in the DLPFC, which was consistent with the trend toward the elevation of spinophilin protein levels in the DLPFC of MDD patients as determined previously by slot blot (Koh et al. 2003). An upregulation of spinophilin protein was also found in the PFC of CUMS mice. In addition, previous studies

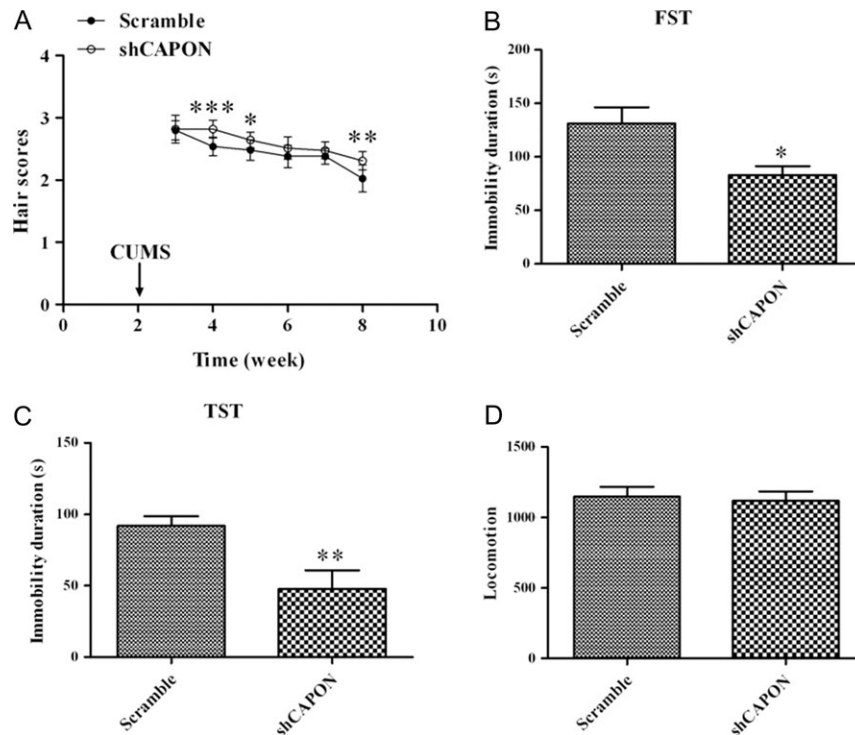
showed an increased spinophilin expression at both mRNA and protein levels in the hippocampus of chronic stress animals (Orlowski et al. 2012; Kaastrup Muller et al. 2015). These data suggest that spinophilin levels are elevated in the PFC and hippocampus during stress and depression.

Synapsin is a presynaptic protein anchoring synaptic vesicles to the presynaptic terminals (Cesca et al. 2010). In agreement with the previous studies showing the reduced synapsin I mRNA in the DLPFC in MDD (Kang et al. 2012; Schmidt et al. 2015), we found a trend toward the reduction in the synapsin-ir levels in the DLPFC of MDD patients, which was also reflected in the PFC of CUMS mice. In addition, synapsin I mRNA and protein levels were significantly decreased in the hippocampus of mice subjected to chronic mild stress (Elizalde et al. 2010; Liu et al. 2015). These accumulated data demonstrate that stress and depression induce the reduction of synapsin in the PFC and hippocampus. Since synapsin ( $-/-$ ) mice exhibits a decreased number of synaptic vesicles (Corradi et al. 2008), the reduced synapsin levels might be related to the abnormality in synaptic transmission in MDD.

At the presynaptic site, CAPON formed a complex with synapsin I and NOS1, contributing to NO-mediated synaptic functions (Jaffrey et al. 2002). However, we did not find any correlations between CAPON-ir and synapsin-ir in the DLPFC or in the ACC. One possible reason could be that the interaction between CAPON and synapsin was identified in rat brain extracts and has so far not been confirmed in human brain tissues. Another possibility is that CAPON may affect the presynaptic modulation via other pathways than in association with synapsins, a possibility that deserves further investigation.

At the postsynaptic site, CAPON competes with PSD-95 for binding the sole PDZ domain of NOS1 (Jaffrey et al. 1998; Li et al. 2015), suggesting the existence of CAPON and PSD-95 in the same complex. In addition, spinophilin was found to be present in the PSD and interacted with PSD-95 (Baucum et al. 2010). We therefore proposed that CAPON and spinophilin may collaboratively participate in the dendritic pathology in mood disorders. This is supported by the following evidence: 1) we found a positive correlation between CAPON and spinophilin in the DLPFC in both MDD and BD patients; 2) we found a colocalization of CAPON and spinophilin in some DLPFC neurons in MDD patients; 3) we identified an interaction between CAPON and spinophilin in human brain. Overexpression of CAPON decreased the number of the matured spines, the length and the branch of dendrites in cultured cortex neurons (Candemir et al. 2016). Spinophilin knockout mice showed an increased spine density during development (Feng et al. 2000). Therefore, the collaborative increases in CAPON and spinophilin may lead to loss of synapses in the DLPFC (Kang et al. 2012) or decreased spine density in the hippocampus of mood disorder patients (Rosoklija et al. 2000) and in the DLPFC of BD patients (Konopaske et al. 2014) that might be related to anxious or depressive symptoms in mood disorder patients. However, further studies are needed to clarify how CAPON regulates spinophilin and/or the interaction between them in animal models for depression.

As an adaptor protein for NOS1, CAPON may also function through NOS1-NO signaling in regulating anxiety and/or depression phenotype. Our previous study showed that NOS1-ir levels decreased in the ACC and that NO concentration were reduced in CSF of mood disorder patients (Gao et al. 2013). Here, we found that CAPON-ir levels increased in the ACC in MDD. We performed a correlation analysis in the overlapping patients and found a significantly positive correlation between CAPON-ir IOD and NOS1-ir cell density ( $Rho = 0.800$ ,  $P = 0.003$ ,



**Figure 8.** Effects of CAPON downregulation on depressive behaviors in CUMS mice. Hair score, FST, and TST were used to assess the CUMS-induced modifications in mice ( $n = 9$  for the scramble group and  $n = 14$  for the shCAPON group). CUMS treatment induced a gradual reduction in the hair score in both CAPON-shRNA (shCAPON) and scramble control groups. At the end of the second week, the third week, and the sixth week following CUMS protocol, CUMS mice with shCAPON-AAV injection showed significant higher hair scores compared with the scramble group. Silencing CAPON significantly decreased the immobility duration in both FST and TST, while the locomotion ability showed no significant differences. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

$n = 11$ ) and a significantly negative correlation between CAPON-ir IOD and CSF NO levels ( $Rho = -0.569$ ,  $P = 0.034$ ,  $n = 14$ ).

In mice exposed to CUMS or corticosterone, significant increases in the CAPON protein level and its association with NOS1 were found in the hippocampus (Zhu et al. 2014). Infecting the hippocampus with CAPON-overexpressing virus increased the CAPON-NOS1 interaction. Interrupting the CAPON-NOS1 interaction as reported previously (Zhu et al. 2014) or downregulating CAPON in the current study rescued the CUMS-induced anxiety-/depression-like behaviors. These data further supported the concept of CAPON as a negative regulator of NOS1-NO system (Jaffrey et al. 1998; Courtney et al. 2014) by which it participated in the pathophysiology of stress and depression. NO modulates synaptic transmission, and structural and functional plasticity (Cossenza et al. 2014). For example, NOS inhibition leads to disturbances in dendrite morphology and to a reduction in synapse number in the PFC during rat brain development (Sanchez-Islas and Leon-Olea 2004). Therefore, the overexpression of CAPON may cause abnormalities in synaptic morphology and function and thus contribute to depression-/anxiety-like behaviors through downregulating the activity of NOS1-NO system.

Some limitations of this study should be mentioned. Here, we found no significant effects of antidepressant use for MDD patients or mood stabilizers use for BD patients on the CAPON-, spinophilin-, or synapsin-ir levels either in DLPFC or in ACC, except for a significant increase in the synapsin-ir levels in BD patients taking mood stabilizers compared with BD patients not taking mood stabilizers (Supplementary Table 4). In agreement with this preliminary analysis outcome, previous studies also showed that chronic treatment with antidepressant or

electroconvulsive therapy decreased the spinophilin levels and increased the synapsin levels in the hippocampus of mice (Kaastrup Muller et al. 2015; Liu et al. 2015). Consequently, if antidepressant treatments would have interfered with our measurements, this would rather have led to an underestimation of the observed differences between controls and mood disorder patients. Nevertheless, we cannot completely rule out possible effects of antipsychotic drug use on our data. For example, chronic treatments of haloperidol reduced the spinophilin protein levels in the DLPFC and hippocampus of monkey (Lidow et al. 2001; Koh et al. 2003) and in dissociated rat hippocampal neurons (Critchlow et al. 2006), whereas the atypical antipsychotic medicine clozapine administration significantly increased the spinophilin expression in primary hippocampal neurons of rats (Critchlow et al. 2006). We did not analyze the numbers of CAPON- or spinophilin-positive cells as we did for the NOS1-positive cells in the previous study (Gao et al. 2013) because the CAPON-ir IOD had a significantly positive correlation with the number of CAPON-positive cells ( $Rho = 0.882$ ,  $P < 0.001$ ) in a pilot study ( $n = 11$ ), suggesting that the IOD value as an objective and less time-consuming method could accurately reflect the CAPON- and spinophilin-ir cell densities.

## Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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## Notes

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