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ORIGINAL ARTICLE

Psychiatric disturbances regulate the innate immune system in CSF of conscious mice

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Environment may affect brain activity through cerebrospinal fluid (CSF) only if there are regulatory molecules or cascades in CSF that are sensitive to external stimuli. This study was designed to identify regulatory activity present in CSF, better elucidating environmental regulation of brain function. By using cannulation-based sequential CSF sampling coupled with mass spectrometry-based identification and quantification of proteins, we show that the naive mouse CSF harbors, among 22 other pathways, the innate immune system as a main pathway, which was downregulated and upregulated, respectively, by acute stressor (AS) and acute cocaine (AC) administrations. Among novel processes and molecular functions, AS also regulated schizophrenia-associated proteins. Furthermore, AC upregulated exosome-related proteins with a false discovery rate of 1.0×10^{-16}. These results suggest that psychiatric disturbances regulate the neuroimmune system and brain disorder-related proteins, presenting a sensitive approach to investigating extracellular mechanisms in conscious and various mouse models of psychiatric disorders.

INTRODUCTION

Neuroimmune systems, especially the innate immune system, are being increasingly implicated in mental disorders.[1–4] More specifically, the immune systems are postulated to prune dendrites and help maintain neuronal plasticity in the brain.[5–7] The fact that neuronal plasticity are subject to environmental regulations suggests that these neuroimmune systems be also sensitive to environment, which awaits experimental evidence for validation.

Cerebrospinal fluid (CSF) has been used as a source of biomarkers in humans with neuropsychiatric disorders and as an avenue to deliver therapeutic agents directly into CNS in rodent models.[8–12] As extracellular molecules in CSF bath brain cells and modulate cellular plasticity constantly,[13] monitoring the dynamics of these molecules in rodent models may unravel the brain mechanisms underlying behavior and pathogenesis. However, such a monitoring method is missing at present.

Mice are primary biomedical models and research tools and the ability to profile CSF molecules in mice will help better understand the molecular mechanisms for brain function and disorder. Mouse models for human neuropsychiatric disorders allow easy genetic modifications through different approaches and technologies.[14–18] In most cases, CSF is used as a drug delivery system by implanting intracerebroventricular (ICV) cannulae through skull into the ventricles in brain.[19,20]

Here we report a stereotaxic approach coupled with protein profiling analysis of sequential multiple CSF samples from the same group of live C57BL/6J mice, dissecting environmental regulation of multiple pathways in CSF of conscious mouse brain.

MATERIALS AND METHODS

Animals and stereotaxic surgery

Adult male mice (25–30 g, C57BL/6 from Charles River Laboratories, Wilmington, MA, USA) were subjected to standard stereotaxic surgery for the implantation of unilateral or bilateral guide cannulae. In brief, the animals were anesthetized and placed in a Kopf stereotaxic frame, and an incision was made to expose the skull. Small holes were drilled and the guide cannula (Plastics One, Roanoke, VA, USA) was lowered 0.5 mm above the lateral ventricles (ICV) and cemented with dental acrylic cement mixed with cyanoacrylate glue to secure the cannula to the skull. Cannula dummies (Plastics One) are inserted into the guide cannulas to keep them patent. The coordinates used for ICV in mm was Ant. ±3.5, Vert. ±1.3 and Lat. ±1.0 according to the atlas of Slotnick and Leonard.

### Histological verification of cannula placement

Seven days after surgery, mice were anesthetized with ketamine/xylazine solution and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.1. The brains were carefully removed and blocked to obtain the forebrain region. The forebrain was postfixed for 48 h, cryoprotected in 20% sucrose overnight and then rapidly frozen in dry ice. A complete set of coronal serial sections was cut through the forebrain at 30 mm, mounted on microscope slides and thionin counterstained to identify the site where the cannula was placed.

Acute stressor (AS) and acute cocaine (AC) treatment

Approximately 30 min after the first withdrawal of CSF, each of nine mice was placed into warm water in a 4-l beaker (three-fourth of the volume filled), followed by forced swimming for 15 min. Wet mice were all dried out under a warm lamp (60 W) and 30 min later withdrawn for a second CSF sample. Next day, each mouse was intraperitoneally injected with cocaine (single dose at 20 mg kg^{-1}), followed by a third CSF withdrawal 30 min later.

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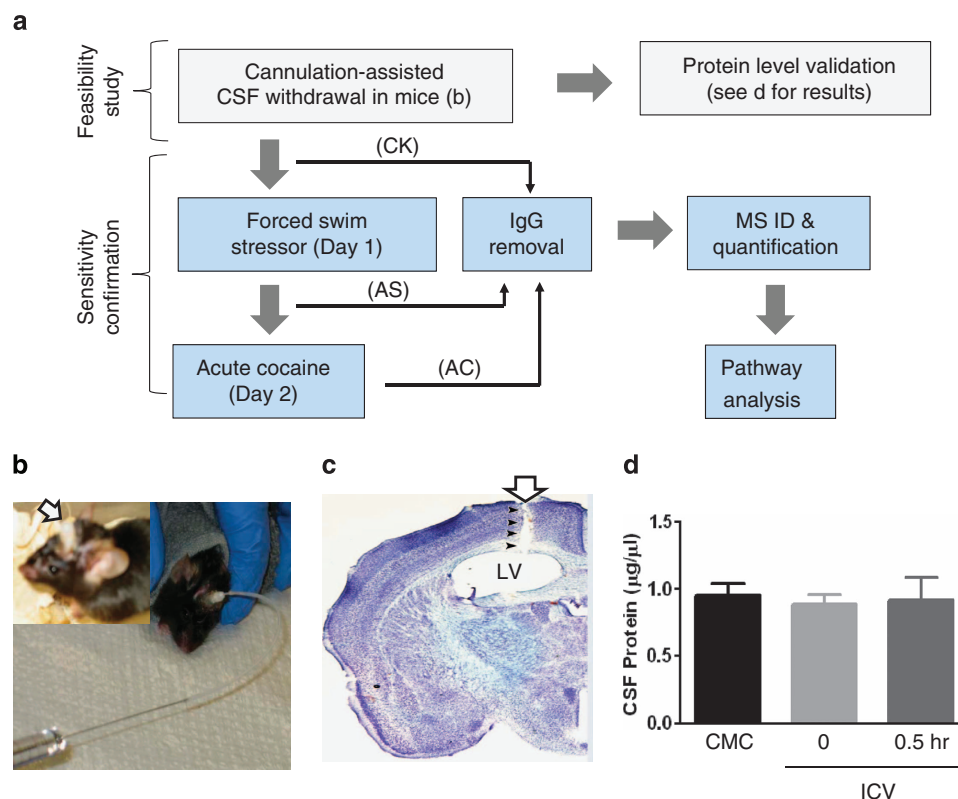
Figure 1. Cannulation-based repeated sampling of cerebrospinal fluid (CSF) from intracerebroventricular (ICV) of mouse brain. (**a**) Study design. Notice that feasibility study and sensitivity confirmation used two different groups of mice. (**b**) ICV cannulation and CSF withdrawal. Insert, a close-up of an installed ICV cannula indicated by an open arrow. (**c**) Postsurgery verification of cannula installed to ICV, based on coronal sectioning of the brain. Open arrow, drilled hole for guide cannula; arrowhead, hole directed into lateral ventricle (LV). (**d**) Constant protein content in CSF withdrawn from mouse cerebellomedullary cistern (CMC) or ICV with a 0.5-h interval, based on Invitrogen's NanoOrange quantification technology.

CSF sampling

Sampling from the cerebellomedullary cistern (CMC) followed a published procedure.[21] To withdraw CSF from the ICV cannula, tubing (~2 cm in length, Plastics One) was connected to the internal (Plastics One) of the cannula at one end and to a 10-μl Hamilton syringe at the other end. Negative pressure was placed on the internal by gently pulling the plunger of the syringe. Each withdrawal was ~2 μl, about 0.5 h before or after treatment. Collected CSF was transferred into an ice-cold tube, followed by −20 °C storage before further processing by mass spectrometry (MS) analysis for identification and quantification of proteins.

MS-based protein profiling

CSF was pooled from nine mice for each condition. We used Pierce's Antibody-Based Albumin/IgG Removal Kit to deplete albumin and IgG before MS analysis at Bioproximity LLC (Chantilly, VA, USA). The samples were run on the Thermo LTQ Velos (San Jose, CA, USA; a dual-pressure linear ion trap mass spectrometer). The Velos utilized the same S-lens as the Q-Exactive to focus the ion cloud as it entered the mass spectrometer and improved overall sensitivity. The linear ion trap enabled very fast sequencing speed of 15 MS/MS scans in 1.8 s. Collision-induced fragmentation was used to fragment peptides for sequence analysis. CID produced peptide ladders from both N- and C-terminal ends of the peptide, allowing for complementary and confirmatory sequence interpretation resulting in high confidence assignments. The limit of identification sensitivity of MS was ~1 fmol.[22]

MS data analysis

Parsimony type was used to classify protein forms that could have overlapping peptide sequences.[23] PepHits were used to normalize for load variation and normalized PepHits were used to calculate a sensitivity value: base 2 log of ratio (acute stressor (AS)/control (CK), acute cocaine (AC)/AS or AC/CK) for condition-related difference. sensitivity value larger than 1

(twofold upregulation) or less than −1 (twofold downregulation or 0.5-fold) are arbitrarily cutoffs for biological significances.

Pathway analysis

We used the frequently updated MetaCore software/database to map pathways (Thomson Reuters, New York, NY, USA).[24] Proteins with biologic significance per sensitivity values were analyzed for pathway regulations or subcellular localizations with significance or enrichment score ($-\log_{10}P$-value). In Enrichment by Protein Function interactome analysis, P-values reflect the probability to have that many or more objects of that protein class in an experiment than would be expected by chance (with a positive Z-score). If a Z-score is negative, the P-value represents the probability to have that many or fewer objects of that protein class than would be expected by chance. Protein function categorization was performed by listing of Network Objects or based on Public Ontologies (GO Molecular Functions), both implemented in MetaCore.

Interactions between AS and AC were evaluated by carrying out the interactions between datasets analysis of AS- or AS-regulated proteins (also implemented in MetaCore). This algorithm was to examine whether the objects contained in one dataset had significant numbers of interactions with the other active datasets. A P-value described the probability of the observed ratio of connections with the occurring merely by chance. A Z-score gave a measure of how saturated the potential number of interactions between datasets were. The higher the Z-score the more saturated the object's connections were with objects from the dataset.

RESULTS

The purpose of this study was to map out regulatory pathways in the CSF that may not only mirror but also modulate brain activity. We were focused on pathway analysis because molecular pathways have central roles in both biologic and disease
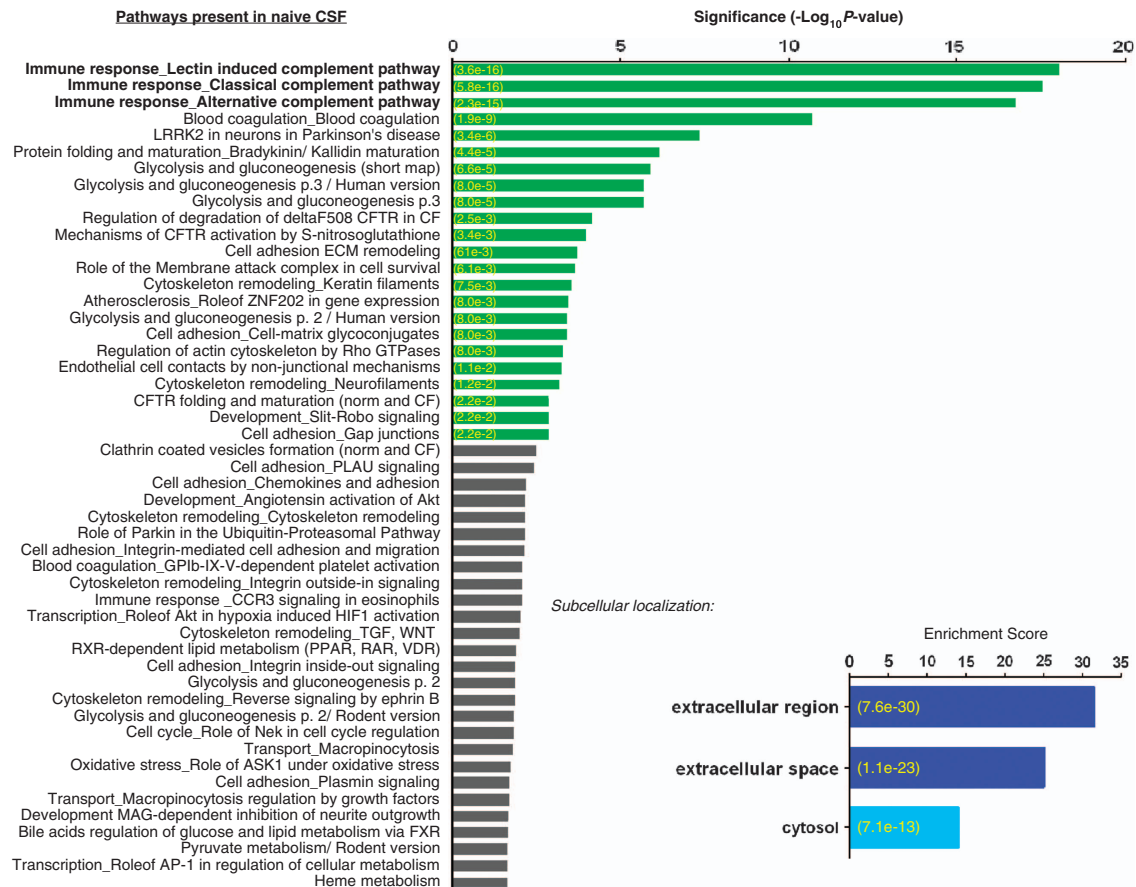
Figure 2. Pathways present in naive cerebrospinal fluid (CSF). Green bar, 23 significant pathways; dark gray bar, not significant. Insert, top two localizations of CSF proteins to extracellular regions. Parenthesis on bar, false discovery rate (FDR).
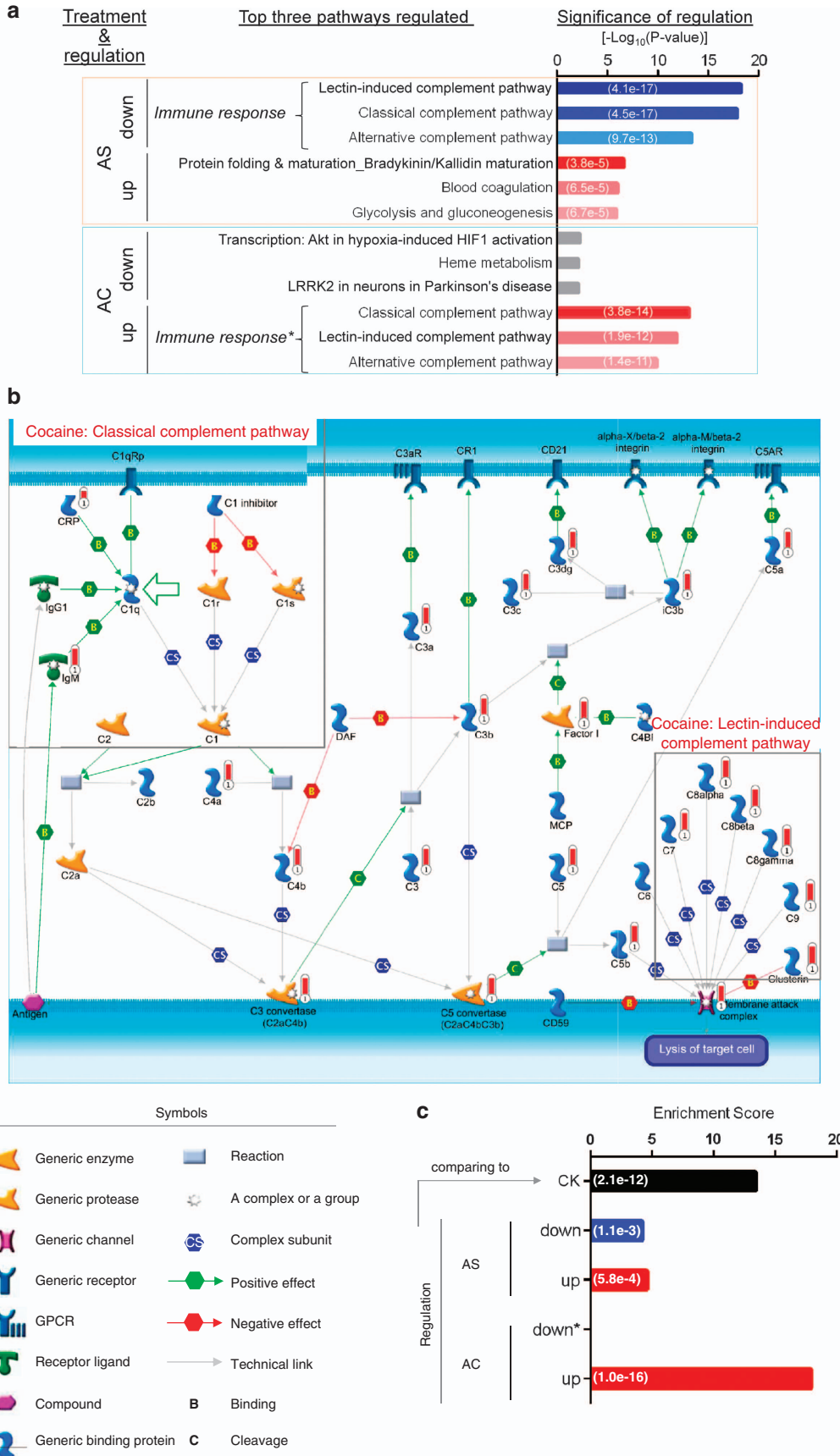
mechanisms.[25–32] The study design is outlined in Figure 1a. In adult mice, after stereotaxic surgery and unilateral or bilateral cannulation, ICV was used for repeated CSF sampling (Figure 1b) for the determination of dynamics of protein composition in the extracellular space. The correct position of cannula ICV implantation was confirmed by histological examination (Figure 1c, where slightly large appearance of the ventricle could be due to the cannula placement). We found that the average protein level from an ICV sample (~2 µl) was neither different from one in the next sample that was collected 0.5 h later, nor different from one in a CMC sample (Figure 1d). These data suggest that 2 µl sampling does not significantly affect protein contents in the subsequent samples from the same mice. Next, we carried out protein profiling analysis of CSF samples from mouse brain before (CK) and after AS, and after AC by MS analysis for identification and quantification of these proteins.

As a result, a total of 268 proteins were detected in the CSF samples at the sensitivity of ~1 fmol; 134 (50.0%) were regulated by AS and 185 (69.0%) were regulated by AC, comparing with CK (see Supplementary Table 1 for a complete list of regulated proteins). Those regulated were mainly peptidase inhibitor activity (see Supplementary Figure 1). Among the AS-regulated proteins, 14 were known ligands ($P = 0.0000742$, Z-score = 4.934), 10 were proteases ($P = 0.0282$, Z-score = 2.297) and 34 were known enzymes ($P = 0.0120$, Z-score = 2.474), based on enrichment by protein function interactome analysis. Among the AC-regulated proteins, 17 were known ligands ($P = 0.000179$, Z-score = 4.585), 13 were proteases ($P = 0.0298$, Z-score = 2.205) and 45 were known enzymes ($P = 0.0212$, Z-score = 2.19). In all, 62.2% of the AS-upregulated proteins were also upregulated by AC (representing 37.0% of its own); 42.3% of the AS-downregulated proteins were

also downregulated by AC (representing 52.4% of its own; see Supplementary Table 1 for details). On the basis of these enrichments by protein function, both environments regulated more enzymes than ligands or proteases. Only 188 of the proteins were detected in the naive CSF.

Systematic analysis of the 188 proteins revealed 23 significant pathways in the naive CSF. Among the 23 significant pathways in the naive CSF, the complement pathways of the innate immune system represent the major ones (Figure 2). They included the lectin-induced ($P = 9.59 \times 10^{-19}$, false discovery rate (FDR) = 3.6×10^{-16}), classical ($P = 3.10 \times 10^{-18}$, FDR = 5.8×10^{-16}) and alternative ($P = 1.86 \times 10^{-17}$, FDR = 2.3×10^{-15}) complement pathways. These findings suggest that this immune system may be pruning synapses constantly to facilitate synaptic plasticity in the CNS.[7] Other pathways with less significances were blood coagulation, LRRK2 ($P = 4.83 \times 10^{-8}$), protein folding ($P = 7.39 \times 10^{-7}$) and glycolysis ($P = 1.29–2.04 \times 10^{-6}$) pathways.[33,34] Cell surface matrix and cytoskeleton configurations were also reflected by the CSF pathways. Majority of the 188 proteins were known localized to extracellular region ($P = 1.30 \times 10^{-29}$; Figure 2, insert), consistent with the fact that these were sampled from the extracellular space inside the brain (ICV). In addition to these pathways, responses to wounding ($P = 1.34 \times 10^{-39}$) and stress ($P = 1.71 \times 10^{-35}$) are the top processes, and endopeptidase inhibitions ($P = 3.86 \times 10^{-46}$) or enzyme activity ($P = 4.32 \times 10^{-42}$) regulations are the top molecular functions in the ICV[35,36] (Supplementary Figure 2).

Complement pathways were regulated by both AS and AC but in two opposite directions. Fifteen-minute forced swimming downregulated the complement pathways the most and by contrast, AC upregulated the complement pathways the most by comparing with CK (Figure 3a). As evidenced for the significance

a

Treatment & regulation	Top three pathways regulated	Significance of regulation [-Log₁₀(P-value)]

Figure panel a: Treatment & regulation vs Top three pathways regulated vs Significance of regulation [$-\log_{10}(P\text{-value})$]

AS down — *Immune response*
- Lectin-induced complement pathway (4.1e-17)
- Classical complement pathway (4.5e-17)
- Alternative complement pathway (9.7e-13)

AS up
- Protein folding & maturation_Bradykinin/Kallidin maturation (3.8e-5)
- Blood coagulation (6.5e-5)
- Glycolysis and gluconeogenesis (6.7e-5)

AC down
- Transcription: Akt in hypoxia-induced HIF1 activation
- Heme metabolism
- LRRK2 in neurons in Parkinson's disease

AC up — *Immune response**
- Classical complement pathway (3.8e-14)
- Lectin-induced complement pathway (1.9e-12)
- Alternative complement pathway (1.4e-11)

b

Cocaine: Classical complement pathway

Cocaine: Lectin-induced complement pathway

Symbols:
- Generic enzyme
- Generic protease
- Generic channel
- Generic receptor
- GPCR
- Receptor ligand
- Compound
- Generic binding protein
- Reaction
- A complex or a group
- CS Complex subunit
- Positive effect
- Negative effect
- Technical link
- B Binding
- C Cleavage

c

Enrichment Score

comparing to CK (2.1e-12)

Regulation
AS down (1.1e-3)
AS up (5.8e-4)
AC down*
AC up (1.0e-16)

of regulations, the P-values were 5.43×10^{-19} for AS down-regulation and 1.56×10^{-13} for AC upregulation of the lectin-induced pathway; 1.20×10^{-18} for AS downregulation and 1.37×10^{-14} for AC upregulation of the classical pathway; and 3.84×10^{-14} for AS downregulation and 1.38×10^{-16} for AC upregulation of the alternative pathways (Figure 3a). In this study, we did not sample the CSF immediately before the AC, as a control for the AS 'hangover' effects, but when compared with the naive CSF, AC still upregulated these complement pathways the most (Supplementary Figure 3). AS upregulated the protein folding (Bradykinin/Kallidin maturation), blood coagulation and glycolysis pathways all with smaller significances ($P = 2.26$–12.1×10^{-7}). AC downregulated none of the pathways in a significant manner (the latest pathway datasets indicated that AC might downregulate C5, which however is not confirmed yet).

Particular proteins in the regulated complement pathways are indicated in the pathway map (Figure 3b). The AS-downregulated proteins included C3, C4, C5, Factor 1, and the C3 and C5 convertases and the membrane attach complex in the lectin-induced complement pathway. The AC-upregulated proteins included CRP and IgM in the classical pathway, and C7, C8, C9 and clusterine in the lectin-induced complement pathway. This profiling method may help us to better understand how complement pathways are regulated, contributing to the dynamics of circuitry in the conscious CNS.[6,37]

In addition to the 6 AS-regulated and 3 AC-regulated pathways, AS regulated 36 other pathways (20 downs and 16 ups) and AC upregulated 20 other pathways (Figure 2 and Supplementary Figure 3). Most of them were novel, including cell adhesion remodeling and extracellular cytoskeleton. AS upregulated processes such as the Kallikren-kinin (inflammation) system, cytoskeleton rearrangement and AC upregulated the Kallikren-kinin system as well, along with interleukin-6 signaling and integrin-mediated cell matrix adhesion. AC upregulated endopeptidase regulators significantly ($P = 3.49 \times 10^{-45}$), whereas AS downregulated these molecular functions ($P = 3.49 \times 10^{-22}$, data not shown). These findings suggested that the CSF pathways were sensitive to extensive regulations by environment, which may underlie an extracellular interaction between environment and brain circuitry.

Majority of the regulated proteins have not been characterized intensively yet. Supplementary Figure 4 shows six of them that were differentially affected by AS and AC. For example, Serpina3C and malate dehydrogenase 1 protein levels were downregulated and upregulated (to 0.3-fold and 5.91-fold) by AS only. Interestingly, the human ortholog Serpina and the human malate dehydrogenase 1 were both associated with Schizophrenia,[38–41] perhaps underlying stressors as a risk factor for this brain disorder. The inflammatory response protein Mug1 and the Felty syndrome-related Eef1a1 protein levels were largely upregulated (3.35-fold and 13.24-fold) by AC only. The copper-carrying protein ceruloplasmin and serum amyloid A4 levels were first downregulated (0.41-fold and 0.1-fold) by AS and then upregulated (2.80-fold and 2.70-fold) by AC. In addition, the levels of cathelicidin antimicrobial peptide were downregulated to 0.17-fold by AS but upregulated to 2.82-fold by AC. The cytoskeletal protein Tubb5 was (up 11.1-fold) regulated by AC only. The GABAA receptor modulator[42] diazepam-binding inhibitor's levels were upregulated to 5.54-fold by AS but downregulated to 0.48-fold by AC. The

levels of candidate signaling protein contactin associated protein-like 5A were upregulated to 5.17-fold by AS but downregulated to 0.1-fold by AC. Although CSF proteins might have unusual composition, these findings imply new pathways of brain response to changing environment, warranting future investigation of their roles in cellular function of the brain.

More interestingly, AC upregulated proteins associated with extracellular vesicular exosomes, with a high enrichment score of 17.9 and a FDR of 1.0×10^{-16}, with little downregulation of proteins in this category (Figure 3c). In contrast, AS exerted both up- and downregulations with much smaller enrichment scores (4.6 and 4.2). These findings suggest that AC could alter another type of intercellular communication, in addition to neurotransmission.

Finally, interaction analysis showed that all four data sets (up- or downregulations of AS or AC) were overconnected; none were underconnected (Supplementary Table 2). Specifically, AC-upregulated proteins were most significantly overconnected with AS-sensitive proteins (Z-score > 11.4), as well as AC-downregulated ones (Z-score = 9.8). By contract, AC-downregulated proteins were least significantly overconnected with AS-sensitive proteins (Z-score < 5.2). Such an overconnected pattern suggests that cocaine-stimulated brain activity be closely related to stress pathways, which merits further study.

DISCUSSION
By using a simple and sensitive method to monitor molecular composition in CSF of conscious mouse brain, we observed, for the first time, that the innate immune system represented the main pathways inside the CSF and that AS and cocaine could both regulate the innate immune system in the brain but in two opposite directions. In addition, we show that environment may regulate exosome-related protein levels in CSF.

To our knowledge, this is the first in vivo study on CSF proteins in intact conscious brains of adult mice. Altered concentrations of proteins or small molecules in CSF have been demonstrated for psychiatric disorders such as schizophrenia, bipolar disorder and depression.[43–46] In fact, many novel proteins are present in the CSF but little is known about their modes of action and function yet. In this study, we report that in both AS- and AC-treated animals more proteins were upregulated and fewer proteins were downregulated (Supplementary Table 1). These results suggest that novel CSF activity remains to be discovered for their role and impact on CNS function and dysfunction. Some of these proteins with known intracellular activity (such as the AS- and AC-upregulated Eukaryotic Translation Initiation Factor 4A/Eif4a1 or RAP1, GTP-GDP Dissociation Stimulator 1/Rap1gds1) may have been released into extracellular space by subcellular mechanisms such as formation of extracellular vesicles[47–49] including exosomes (Figure 3c). Therefore, it will be interesting to examine which of these molecules, including proteins, genetic materials and small molecules, in the CSF are confined to the extracellular vesicles because these extracellular vesicles may affect intracellular activity of brain cells. In addition, the innate immune system may have had an important role in environmental regulations of neuronal activity including morphologic neuroplasticity. One limitation of this study design lies in lack of control for hangover effects of AS on the AC results. However, the AC upregulation of the innate

Figure 3. Cerebrospinal fluid (CSF) pathways regulated by acute stressor (AS) and acute cocaine (AC). (**a**) Top three pathways downregulated (down) or upregulated (up) by AS (upper portion/brown rectangle) or by AC (lower portion/aquamarine rectangle). Color bars, statistically significant; gray bar, not significant. (**b**) Pathway map for AS-downregulated lectin-induced complement pathway. Upleft insert, unique proteins of AC-upregulated classical complement pathway; downright insert, unique proteins of AC-upregulated lectin-induced complement pathway. Red thermometer, significantly regulated proteins. (**c**) Upregulation of exosome-related proteins by AC. Asterisk, not listed among top 50 categories; parenthesis on bar, false discovery rate (FDR). In all, 11.6% of the AC-upregulated proteins were exosome related compared with 2.1% of the AC downregulated, 6.1% of the AS upregulated or 7.7% of the AS downregulated; see Supplementary Table 1 for details. Enrichment score ($-\log_{10}P$-value) of regulation is based on comparison with control (CK; black bar).

immune system holds true based on comparison with either CK or AS (Supplementary Figure 3).

In this study we have shown that the lateral ventricles in adult mouse brain, after stereotaxic surgery and cannulation, unilaterally or bilaterally, can be utilized for repeated CSF sampling for the determination of and alterations in protein dynamics in the brain. Previous continuous CSF sampling in mice from the brain and cisternum magnum had been controversial because of low volume and production rate in mice compared with rats and humans. However, with new and sensitive protein detection technology, we report constant CSF protein content of 1 µg µl^{-1} sensitivity from the multiple CSF sampling from the lateral ventricles. Further studies will be necessary to localize brain-area-specific alterations associated with the global molecular protein dynamics predicted by this model. That approach will be much straightforward dissection of brain regions to determine associated protein dynamics with specific function for further analysis. This will provide a powerful tool to determine the roles of such identified proteins and transcription factors and specific brain regions in neuropsychiatric disorders.

As mentioned above, this study at its current stage has major limitations. The controls used were not necessarily the best controls, particularly considering the serial nature of the design and the preliminary nature of the development of the approach: stressed versus non-stressed and cocaine versus saline-treated would have been much better comparisons. As of the limited design, we are unable to evaluate yet the time courses of the regulations, in particular.

In summary, we have used stereotaxic surgical approaches coupled with protein profiling analysis of sequential multiple CSF sampling in conscious mouse models for neuropsychiatric disturbances and demonstrated the potentials of monitoring molecular dynamics in nervous system extracellular space in mouse brain. This method, when combined with site-specific investigation of neurobiological mechanisms in the brain, provides a powerful tool to screen and identify the screen-associated real-time changes in nonsynaptic signal transmission in such animal models of brain disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ESO and ZCL designed and performed the animal experiments including CSF withdrawal; ESO proof-read the manuscript; NS provided laboratory technical assistance during stereotaxic cannulation and withdrawal of CSF. ZCL conceived the idea, developed the project, analyzed the data and wrote the manuscript.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)