Homer expression in the hippocampus of an animal model of attention-deficit/hyperactivity disorder

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Abstract. Attention-deficit/hyperactivity disorder (ADHD) is a pervasive neurobehavioral disorder. We previously demonstrated differential expression of some isoforms of Homer, a family of scaffolding proteins localized to the postsynaptic density of glutamatergic excitatory synapses, in the spontaneous hypertensive rat (SHR), which is the most frequently used animal model of ADHD. Since these changes were observed in the prefrontal cortex (PFC), a critical structure in ADHD, it was hypothesized that these Homer isoforms may play a role in ADHD. The present study aimed to extend these findings to the hippocampus, which has direct connections to the PFC and subserves attention and cognition, two functions that are disturbed in ADHD. Hippocampal mRNA and protein expression of several Homer isoforms were investigated in both SHR and control Wistar-Kyoto (WKY) rats using reverse transcription-polymerase chain reaction and Western blotting, respectively. Both mRNA and protein for Homer 1a and Homer 2a/b, but not Homer 1b/c, were expressed at significantly lower levels in the hippocampus of SHR compared to WKY rats. The effects of methylphenidate (MPH) on spatial learning and memory in SHRs were also examined using the Morris water maze and on hippocampal expression of Homer isoforms. MPH improved spatial learning and memory and up-regulated hippocampal expression of Homer 1a and Homer 2a/b, but not Homer 1b/c, in SHRs. The animal model of ADHD may have altered expression of Homer 1a and Homer 2a/b in the hippocampus, in addition to the PFC. Future studies will focus on elucidating the specific mechanisms of Homer 1a and Homer 2a/b in ADHD.

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Introduction

Attention-deficit/hyperactivity disorder (ADHD; MIM143465) is a pervasive neurobehavioral disorder affecting approximately 5% of children and adolescents, and 3% of adults (1). Children with this disorder are characterized by inattentiveness, hyperactivity and impulsivity (2-4). ADHD is a highly heritable developmental disorder resulting from complex gene-gene and gene-environment interactions. However, the neural basis of ADHD is unknown. The spontaneously hypertensive rat (SHR) is currently the most widely used animal model of ADHD and has good face validity for the disorder. In particular, these rats show impaired sustained attention without notable sensory problems, as well as motor impulsiveness and hyperactivity, relative to control Wistar-Kyoto (WKY) rats (3,6-12). In addition, SHRs exhibit brain pathology similar to ADHD, including reduced brain volumes relative to controls, specifically in the prefrontal cortex, occipital cortex and hippocampus (13). Therefore, this model has been widely used to investigate the neural basis of ADHD.

The Homer protein family of scaffolding proteins, which is localized to the postsynaptic density (PSD) of glutamatergic excitatory synapses, has recently received a great deal of attention regarding its role in cognitive and behavioral function. Converging evidence indicates a potential role of Homer in the behavioral pathologies associated with neuropsychiatric disorders, including addiction and/or alcoholism, depression, anxiety, epilepsy and schizophrenia (14). The Homer family is the product of three independent mammalian genes (Homer 1-3) (15,16), including immediate early gene isoforms (Homer 1a, ania-3) and constitutively-expressed isoforms (Homer 1b-g, Homer 2a/b and Homer 3). We previously reported that Homer 1a and 2a/b are differentially expressed in the prefrontal cortex of SHR compared to control WKY rats (17). Accordingly, we speculate that these Homer isoforms may contribute to the etiology and development of ADHD.

Previous studies have demonstrated that ADHD may involve the abnormal development and functioning of entire neural networks, not restricted to the prefrontal cortex (PFC), but also involving the striatum and hippocampus (18,19). In addition to the core symptoms of ADHD (inattentiveness, hyperactivity and impulsivity), children with this disorder often struggle with deficits in executive functioning, working

Table I. RT-PCR parameters for the different Homer isoforms.

Gene name	Primer sequence	MW (bp)	Cyling (X)	Temperature (˚C)
Homer 1a	F: 5'-ATTGAGCCACTATCTGCC-3' R: 5'-TTACACCCACGGTCTTCC-3'	521	28	54
Homer 1b/c	F: 5'-ACAGCCACAAGACAGAGC-3' R: 5'-CATCCTGACAAACCCGAG-3'	405	28	54
Homer 2a/b	F: 5'-CTTCTGTAATGCGGGTGC-3' R: 5'-CCCAGTTCATAGGGTTGC-3'	221	30	54
β-actin	F: 5'-TAAAGACCTCTATGCCAACACAGT-3' R: 5'-CACGATGGAGGGGCCGGACTCATC-3'	241	24	54

and visuospatial memory, temporal processing and difficulty tolerating delayed rewards (19-23). The hippocampus is directly connected to the PFC and subserves attention and cognition, two functions that are disturbed in ADHD (4,19,24-28). Therefore, in the present study we investigated the differential expression of several Homer isoforms (1a, 1b/c and 2a/b) in the hippocampus of SHR and control WKY rats. In addition, since methylphenidate (MPH) is currently one of the most commonly prescribed psychoactive stimulants for ADHD treatment and has been used as a tool to investigate ADHD pathogenesis, we examined the effects of MPH on spatial learning and memory, and on the hippocampal expression of Homer isoforms.

Materials and methods

This study involved two independent experiments. In Experiment 1, SHR (n=8) and WKY rats (n=7) were sacrificed to investigate hippocampal Homer mRNA and protein expression using RT-PCR and Western blotting, respectively. In Experiment 2, SHR received MPH or saline (n=10 per group) prior to testing on the Morris water maze, and were then sacrificed for the assessment of hippocampal Homer mRNA and protein expression as in Experiment 1.

Subjects and housing. Experimental animals were obtained at 4 weeks of age from Shanghai SLAC Laboratory Animal Co., Ltd. The colony room was illuminated on a 14/10-h light/dark cycle and the temperature was maintained at 22-24˚C. Food and water were available *ad libitum*. Animals were allowed 1 week to acclimatize to laboratory conditions before experiments began. All procedures were conducted in accordance with the guidelines for animal care and experimentation of Nanjing Medical University.

RT-PCR. Both sides of the hippocampus were dissected out, and one was processed for the isolation of RNA and the other for protein. Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) was performed on 2 μg of RNA extract and 4 μl of 0.05 μg/μl oligo(dT)15 primer at 70˚C for 5 min. The RT reaction was performed in 40-μl volumes containing 400 units Moloney murine leukemia virus (M-MLV) reverse transcriptase, 5X M-MLV buffer and 40 units of RNase inhibitor (Promega, Madison, WI, USA) at 42˚C for 1 h, followed by inactivation of RTase at 95˚C for 10 min. The PCR assay was performed in 20-μl volumes containing 1 μl of sample DNA extract and 19 μl of reaction mixture (10X *Taq* Buffer, 25 mM $MgCl_2$, 10 mM dNTP, 10 pmol/μl of each primer, 5 U/μl of *Taq* DNA polymerase; Invitrogen) per reaction. Amplification reactions were carried out in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) with preliminary denaturation at 95˚C for 5 min, followed by 24-30 repeated cycles (X) of denaturation at 94˚C for 30 sec, annealing at 54˚C for 40 sec and primer extension at 72˚C for 30 sec, with a final extension at 72˚C for 7 min (Table I). To verify that there was no DNA contamination in the mRNA extracts, RT-PCR assays were also performed on the same concentrations of mRNA without RTase. DNA bands were quantified by agarose electrophoresis densitometry and differential display RT-PCR gels were analyzed for quantification using a UVP imaging system. Density values for Homer 1a, 1b/c and 2a/b were expressed as a percentage of the density of the internal standard β-actin.

Western blot analysis. Tissue samples were transferred into 1 ml of RIPA buffer (1% NP-40, 25 mmol/l Tris-HCl, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin), homogenized by sonication and then incubated on ice for 15 min. The homogenate was centrifuged at 15,000 x g at 4˚C for 10 min. The supernatant was then transferred into ice-cold sample tubes and stored at -20˚C until analysis. Protein concentrations were determined using the Bradford assay. Equal amounts of protein (100 μg/20 μl per lane) were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) (Invitrogen). Proteins were subsequently transferred to polyvinylidene fluoride membranes (Sartorius, GM) and exposed to blocking buffer (5% nonfat dry milk in PBS with 0.1% Tween-20) at room temperature for 1 h. The blots were incubated overnight at 4˚C in primary antibody incubated in bovine serum albumin: anti-goat Homer 1a or Homer 2a/b (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rabbit β-actin (1:1,000 dilution; Beijing Biosynthesis Biotechnology, Beijing, China). This was followed by a 1-h incubation at room temperature in a donkey anti-goat horseradish peroxidase-linked secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology) for Homer 1a and

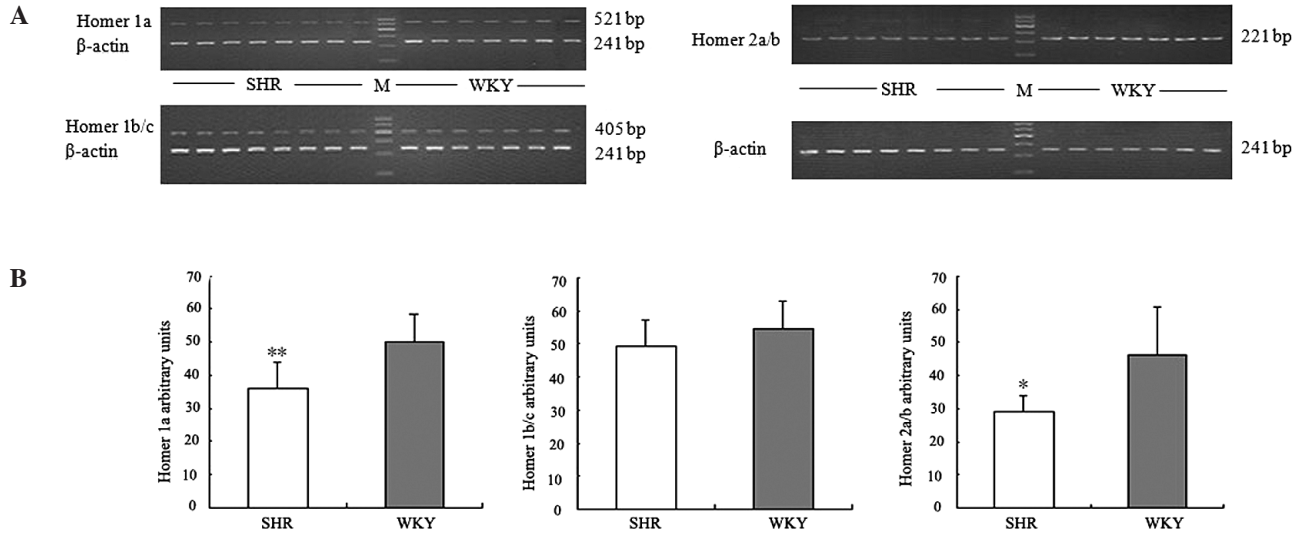

Figure 1. Hippocampal expression of Homer mRNA in SHR and WKY rats. (A) Homer 1a, Homer 1b/c and Homer 2a/b mRNA. (B) Quantification of Homer mRNA expressed as a percentage of the internal standard β-actin. *p<0.05 relative to WKY rats by independent t-test. Data represent the means ± SD. n=7-8 per group. M, marker.

Homer 2a/b or a goat anti-rabbit secondary antibody (1:3,000 dilution; Santa Cruz Biotechnology) for β-actin. Immunoblots were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) for 10-15 min to enhance the faint Homer band, and images were obtained on a Kodak Image Station 2000R system. Kaleidoscope pre-stained standards (Bio-Rad, Hercules, CA, USA) and MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using Kodak 1D Image Analysis software. Density values of Homer 1a and 2a/b proteins were expressed as a percentage of the density of the internal standard β-actin.

Drugs. MPH (Daiichi Industrial Co., Ltd., Suzhou, Japan) was dissolved in 0.9% sterile saline (SAL) and administered perorally via gavage. Rats received 2 mg/kg/BW of MPH or an equal volume of SAL twice daily (bid) at 10:00 and 14:00 for 14 days (29,30).

Behavioral assessment. A Morris water maze was used to evaluate the effect of MPH on spatial learning and memory in SHRs 24 h after their last MPH injection (31). The Morris water maze consisted of a cylindrical tub (130 cm diameter, 50 cm depth) that was filled with water (23±2˚C) to 11 cm below the rim. The water was rendered opaque by the addition of black, non-toxic paint. The pool was divided into four quadrants of equal area, which were arbitrarily called northeast, southeast, southwest and northwest. A circular platform (9 cm diameter) was submerged 1 cm below the water surface in the middle of one quadrant (the target quadrant) with its center located 30 cm from the perimeter of the maze. A closed-circuit television camera mounted onto the ceiling directly above the center of the pool was used to record swim trajectories and other parameters, which were then subjected to an electronic image analyser (HVS Image Ltd., Twickenham, Middlesex, UK).

Animals underwent an acquisition trial for 3 consecutive days as they learned to find a hidden platform that was consistently placed in one quadrant of the maze. Animals began each trial in a different cardinal position of the maze, requiring the use of constant extra-maze spatial cues to find the platform. A maximum latency of 60 sec to find the platform was allotted for each trial and there was a 30 min rest period between trials. Rats that failed to locate the platform within the allotted time were manually guided to it and placed on the platform. All rats were allowed to remain on the platform for 30 sec at the conclusion of each trial. A daily latency for each rat was calculated as the average of the 3 acquisition trials for that day. Latency and the total swim distance were used to evaluate spatial learning and memory (32).

Statistical analysis. Statistical analysis was performed using independent t-tests with significance set at $p<0.05$. Data are presented as the means ± SD.

Results

Differential mRNA and protein expression of Homer isoforms in the hippocampus of SHR and WKY rats. Expression of Homer 1a and 2a/b mRNA was significantly lower in SHR than in WKY rats, as shown in Fig. 1. However, no significant difference was found in the expression of Homer 1b/c mRNA. Similarly, expression of Homer 1a and 2a/b proteins was significantly lower in SHR compared to WKY rats, as shown in Fig. 2. It should be mentioned that the expression of Homer 1b/c protein was examined in our pre-experiment by Western blotting. Unfortunately, we did not obtain any bands, due to the primary antibody. Ideally, this will be investigated if we find a suitable antibody in future.

Effects of MPH on spatial learning and memory in SHRs. Following 14 days of repeated treatment, rats receiving MPH performed significantly better in the Morris water maze than rats receiving SAL. In particular, rats treated with MPH showed faster latencies to locate the platform and shorter total swim distances, as shown in Fig. 3.

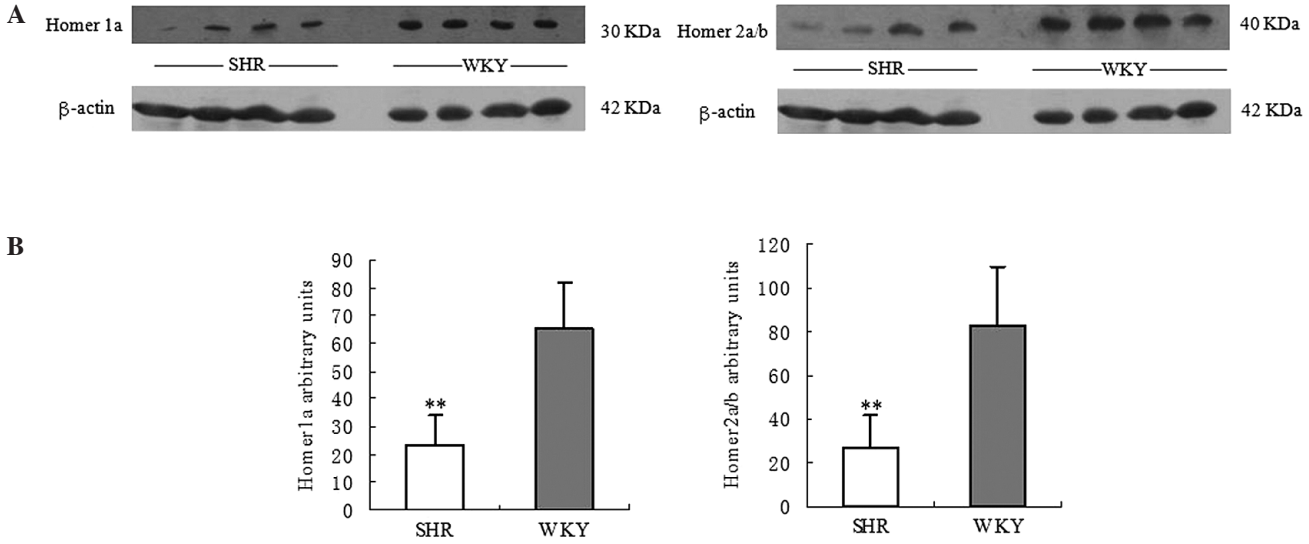

Figure 2. Hippocampal expression of Homer protein in SHR and WKY rats. (A) Homer 1a and Homer 2a/b. (B) Quantification of Homer protein expressed as a percentage of the internal standard β-actin. *p<0.05 relative to WKY rats by independent t-test. Data represent the means ± SD. n=7-8 per group.

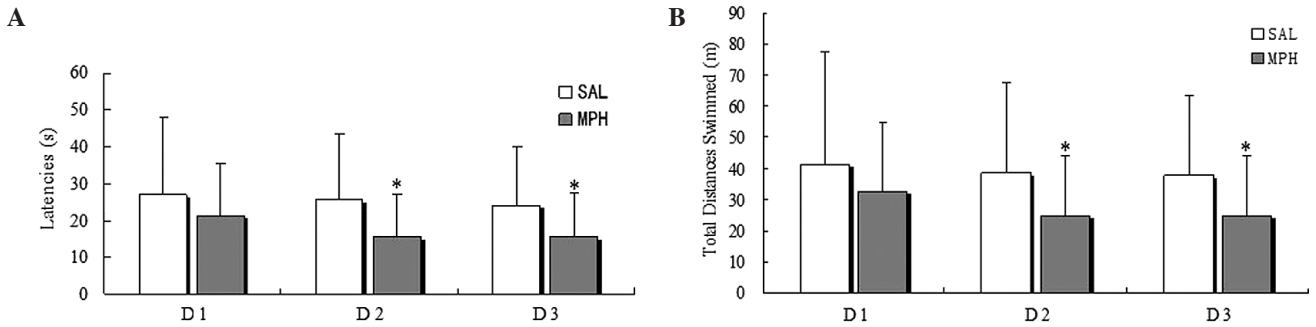

Figure 3. Effect of repeated MPH on spatial learning and memory in SHRs. (A) Latency to reach the platform. (B) Total swim distance. MPH (2 mg/kg/bodyweight) or an equal volume of SAL was administered twice daily for 14 days. *p<0.05 and **p<0.01 relative to SAL treatment by independent t-test. Data represent the means ± SD. n=10 per group. D 1-3, days 1-3 of acquisition.

Effects of MPH on Homer mRNA and protein expression in SHRs. Compared to the SAL-treated SHR group, the MPH-treated SHR group had significantly higher hippocampal expression of Homer 1a and Homer 2a/b mRNA, but not Homer 1b/c mRNA, as shown in Fig. 4. The MPH-treated group also exhibited enhanced hippocampal expression of Homer 1a and Homer 2a/b protein, as shown in Fig. 5.

Discussion

SHRs display behavioral and neurochemical features of ADHD. In this study, they were obtained at 4 weeks of age and were allowed 1 week to acclimatize to laboratory conditions before experiments began. Therefore, they were 5 weeks old at the initiation of the experiments, a stage equivalent to periadolescence in human. In a previous study, we found that mRNA and protein for Homer 1a and Homer 2a/b, but not Homer 1b/c, were expressed at a significantly lower level in the PFC of SHR compared to WKY rats. In the present study, these findings were extended to the hippocampus, indicating that hippocampal dysfunction may also be involved in the etiology and pathogenesis of ADHD. A widely accepted hypothesis for ADHD involves dysfunction of the PFC (33-36), which is connected directly to the ventral hippocampus and indirectly to the dorsal hippocampus via the thalamus. Thus, the hippocampus appears to share many functional characteristics with the PFC, and both have been implicated in mnemonic and attentional processes. Magnetic resonance imaging studies of children with ADHD have repeatedly found reduced PFC volumes (18,37,38), particularly of the inferior aspect (34). The PFC-hippocampal circuit regulates a variety of processes that have been implicated in the pathophysiology of ADHD, including attention, memory and emotion (39-41). For instance, Plessen *et al* found that children and adolescents with ADHD had larger hippocampal volumes than did healthy controls, primarily due to increases in the anterior portions of the hippocampus. Larger volumes tended to be associated with less severe ADHD symptoms (42), suggesting that enlarged anterior portions of the hippocampus may represent a neural compensation for dysfunctional PFC activity. In addition, rats with X-ray-induced hippocampal damage showed cognitive improvement in response to amphetamine, a stimulant drug used to treat ADHD (43). Therefore, we speculate that Homer dysfunction may exist in both the PFC and hippocampus of children with ADHD.

Stimulants were initially prescribed for ADHD in 1937, and MPH is currently one of the most commonly prescribed psychoactive stimulants for its treatment (44-46). MPH

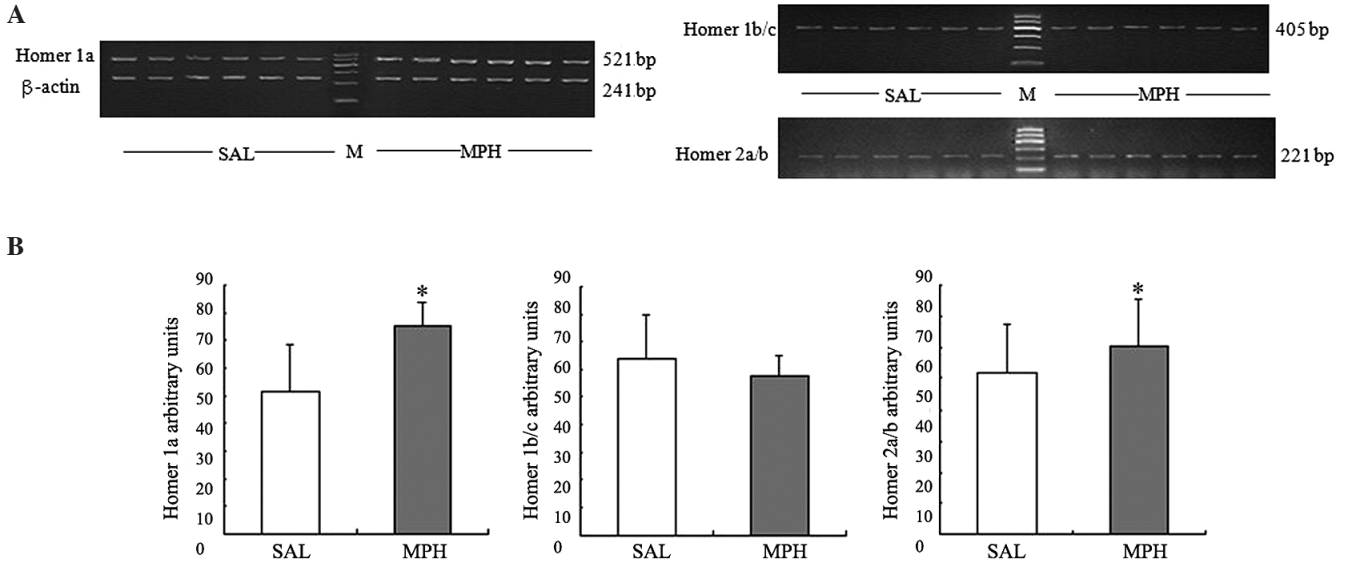

Figure 4. Effect of repeated MPH on hippocampal expression of Homer mRNA. (A) Homer 1a, Homer 1b/c and Homer 2a/b mRNA. (B) Quantification of Homer mRNA expressed as a percentage of the internal standard β-actin. *p<0.05 relative to SAL by independent t-test. Data represent the means ± SD. n=10 per group. M, marker.

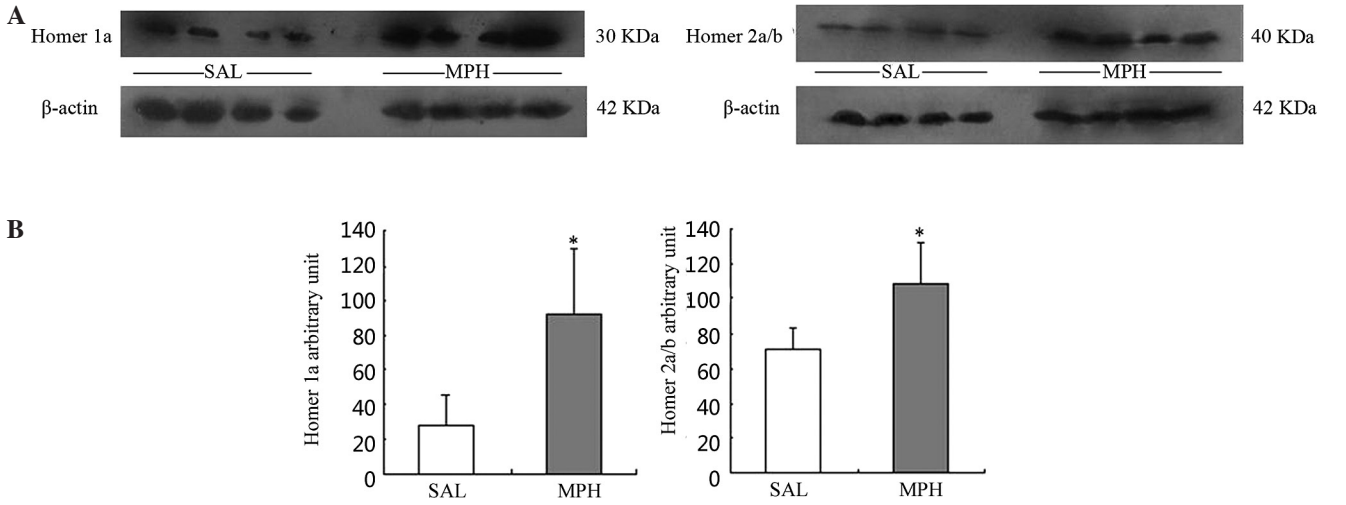

Figure 5. Effect of repeated MPH on hippocampal expression of Homer protein. (A) Homer 1a and Homer 2a/b protein. (B) Quantification of Homer protein expressed as a percentage of the internal standard β-actin. *p<0.05 relative to SAL by independent t-test. Data represent the means ± SD. n=10 per group.

improves academic performance (47,48), working (49) and visual (50) memory, nonverbal (51) and visuospatial (52) learning, and reading skills (53), which may explain why, in addition to its medicinal use, it is used illicitly by healthy students as a study aid (54). In addition to being a first-line treatment for ADHD, MPH has also been used as a tool to investigate its pathogenesis. Hence, the effects of repeated low-dose MPH on spatial learning and memory were examined, as well as expression of Homer isoforms in the hippocampus of the SHR. Reports regarding the effects of MPH on learning and memory have been inconsistent. The majority of studies have investigated MPH-induced facilitation of attention and the improved learning and memory that are presumed to result (33,55,56). Few reports exist of the adverse effects of high-dose MPH on learning and memory (57), and different dosages and routes of administration have diverse effects. An MPH dose of 2 mg/kg/body weight and the peroral route of administration were selected, as this dose is considered to be clinically relevant in rodents via ingestion (29,55,77). Accordingly, we observed that repeated treatment with MPH improved learning and memory in SHRs, similar to its demonstrated clinical effect. Furthermore, MPH unregulated mRNA and protein expression of Homer 1a and 2a/b, but not Homer 1b/c, in the hippocampus of SHRs. These data therefore provide further evidence that Homer 1a and Homer 2a/b may be involved in the etiology and pathogenesis of ADHD.

Homer proteins are predominantly localized at the PSD in mammalian neurons and act as adaptor proteins for many PSD proteins. They regulate signal transduction, synaptogenesis and receptor trafficking, in addition to maintaining and regulating extracellular glutamate levels in limbo-corticostriatal brain regions. In mammals, the Homer family of adaptor proteins includes Homer 1, 2 and 3, all of which have several isoforms that result from alternative splicing. These

Homer isoforms share a similar structure, which includes a conserved amino-terminal Enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain that binds to a proline-rich sequence found in numerous proteins, including: Group 1 metabotropic glutamate receptors (58), inositol-1,4,5-tripho-sphate receptors (16,58), ryanodine receptors (59,60), transient receptor potential canonical-1 ion channels (61) and the NMDA glutamate receptor scaffolding protein Shank (61,62). Homer isoforms are primarily classified into long and short isoforms. Homer 1a is one of the short isoforms, which lack the carboxy-terminal domain; they are expressed in an activity-dependent manner as immediate-early gene products, possibly disrupting Homer clusters by competitive binding to target proteins. Homer 2a/b is one of the long isoforms, which are constitutively expressed and consist of two major domains: the amino-terminal EVH1 domain, and the carboxy-terminal self-assembly domain containing a coiled-coil structure and leucine zipper motif. Multimers of long Homer proteins, coupled through their carboxy-terminal domains, are thought to form protein clusters with other PSD proteins, which are bound through their amino-terminal domains. Such Homer-mediated clustering probably regulates or facilitates cross-talk between target proteins.

Converging preclinical observations indicate a potential role for both short and long Homer isoforms in behavioral pathologies associated with neuropsychiatric disorders, such as addiction and/or alcoholism, depression, anxiety, epilepsy and schizophrenia. Homer 1 knockout mice exhibit pronounced learning deficits during acquisition of both the Morris water maze and radial arm maze, indicating poor reference and working memory (64,65) Activity-dependent re-organization of central synapses is thought to play significant roles in learning and memory. When short hairpin RNA was introduced to specifically block expression of endogenous Homer 1a, activity-induced changes were almost completely suppressed. Homer 2 knockout mice did not exhibit abnormalities in attention or cognitive processing, but exhibited a hyperactive phenotype similar to chronic cocaine-treated rats (66), and overexpression of Homer 2 reduced cocaine-induced hyperactivity (67). Therefore, Homer protein is a new target for pharmacogenomics in behavioral disorders. Homer 1a and Homer 2a/b appear to play a role in ADHD, though perhaps through different pathways.

Homer 1a and Homer 2a/b are known to regulate metabolic glutamate receptor signal transduction. Levels of extracellular glutamate in the PFC and nucleus accumbens are reduced in Homer 1 and 2 knockout mice (68,69), and this effect can be reversed in Homer 2 knockout mice by expressing the Homer 2 gene using adenovirus-associated virus (70). The glutamate system is also known to be altered in ADHD: i) imaging has demonstrated that the activity of glutamate projection from the PFC to the striatum is reduced in ADHD patients (71); ii) drugs enhancing glutamate transmission reduce hyperactivity in dopamine transporter knockout mice (72); iii) dopamine hypofunction may result from a defect in the glutamate-stimulated release of dopamine in the nucleus accumbens shell of SHR, which also causes hyperactivity and impulsiveness in this model (73); and iv) n-methyl-d-aspartate receptor subunit dysfunction at hippocampal glutamatergic synapses may be one of the underlying mechanisms leading to abnormal behavior in SHRs, and possibly in human ADHD (74). Therefore, Homer 1a and 2a/b may play a significant role in the pathogenesis of ADHD through regulation of the glutamate neurotransmitter system. In addition, previous studies have reported that Homer 1a and Homer 2a/b may contribute to the regulation of the dopamine signaling pathway. Nicotine acts as an indirect dopamine agonist, inducing Homer 2a/b expression in some regions that project to the frontal cortex (75). Normal mice that ingest high levels of alcohol show increased dopamine release, while Homer 2 knockout mice exhibit no change in dopamine release. Homer 2-mediated overexpression in both wide-type and knockout mice by adeno-associated virus reverses this phenomenon (76). Cocaine, which blocks dopamine reuptake and increases extracellular dopamine, can also induce Homer 1a expression (77). Dysfunction of the dopamine system is considered to be an important factor in the incidence of ADHD. Accordingly, Homer 1a and 2a/b may contribute to ADHD through effects on both the glutamate and dopamine systems.

In conclusion, the Homer protein family has received increasing attention due to its unique molecular properties, which allow both the clustering and functional modulation of a plethora of different binding proteins in the postsynaptic membrane. We speculate that the pathogenesis of ADHD is associated not only with abnormal transmission in the presynaptic membrane, but also abnormal processes in the postsynaptic membrane. Therefore, Homer 1a and Homer 2a/b, as the major regulatory proteins of signal transduction in postsynaptic membranes, may play an important role in the pathogenesis of ADHD. The current data provide novel information for the broad identification of genes involved in the mechanisms of ADHD. Further study of Homer proteins, especially Homer 1a and 2a/b, is warranted to provide insight into their specific roles in the etiology of ADHD.

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