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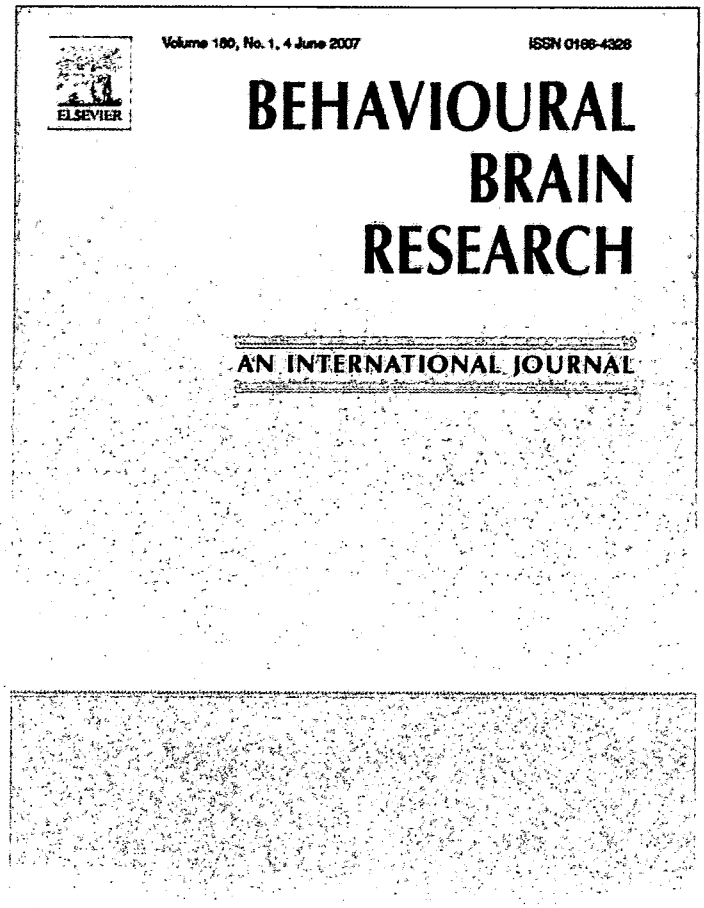

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Research report

Enriched environments influence depression-related behavior in adult mice and the survival of newborn cells in their hippocampi

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Abstract

Major depression is a highly prevalent mental disorder and environmental factors have been strongly implicated in its pathophysiology. Clinical studies have demonstrated that stress or depression can lead to atrophy and cell loss in the hippocampus. Studies of animal models of depression have suggested that reduced neurogenesis in the adult hippocampus might contribute to such structural changes and to the behavior of these animals. On the other hand, increased hippocampal neurogenesis can be induced by the administration of antidepressants or electroconvulsive seizure, suggesting that increased neurogenesis might be related to the treatment of depression. Thus, an enriched environment (EE), which also enhances neurogenesis, is expected to have therapeutic effects on depression-related behaviors. To investigate the effects of an EE during adulthood on these behaviors, we subjected adult mice housed in an EE for five weeks to behavioral tests. In an open field test, EE mice exhibited a decrease in the distance traveled and an increase in the amount of time spent in the center. The startle response was smaller in EE mice than in control mice. EE mice also showed reduced immobility time in a forced swim test. The immobility time in EE mice was approximately half that observed in mice treated with a tricyclic antidepressant, imipramine. In our experimental condition, increased survival of newborn cells was observed in EE mice by 5-bromo-2′-deoxyuridine (BrdU)-labeled immunohistochemistry. Double-staining of BrdU and a mature neuron marker, NeuN, revealed that the majority of surviving cells were neurons. Our results suggest that EE, which enhanced the survival of newborn neurons, shows beneficial effects on behavioral despair and habituation to a novel environment.

Keywords: Enriched environment; Depression; Antidepressant; BrdU; Forced swim test; Neurogenesis

1. Introduction

Major depressive disorder is a common mental disease with a lifetime prevalence of ~20% [41]. Although genetic susceptibility has been considered to be involved in the disorder, severe stressors have also been associated with a substantial increase in its risk [22,26]. In rodents, models of depression rely on responses to stress, and show particular aspects of the disorder; for example, cognitive or attentional impairment and abnormalities in psychomotor activity [9,32]. Moreover, stressors produce dendritic atrophy, death or endangerment of hippocampal neurons [27], and inhibit neurogenesis in the adult hippocampus [12,15]. This evidence suggests that stress-induced structural and/or functional alterations in the hippocampus have important

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roles in the pathogenesis of depression. Indeed, brain imaging studies have reported that hippocampal volume is decreased in patients with depression [3,36].

Recent studies have reported that neurons are produced in the hippocampus throughout the lifetime of animals, including humans [13]. The number of new granule neurons generated each month is 6% of the total granule cell population [5]. The axons of adult-generated hippocampal neurons extend into the CA3 region [18,39], and newborn cells mature into functional neurons in the hippocampus [39]. A substantial reduction in the number of newly generated cells impairs hippocampal-dependent forms of associative memory formation [37]. Moreover, adult hippocampal neurogenesis is enhanced by the administration of electroconvulsive seizure [23,25] or antidepressant treatment [25,35]. These findings indicate that adult-generated neurons may contribute in a significant manner to the functions of the hippocampus, and that increased neurogenesis may underlie the therapeutic effects of treatments for depression.

Enriched environments (EEs) also increase the number of new neurons in the adult dentate gyrus [20], and reduce spontaneous apoptotic cell death in the hippocampus [42]. It has been reported that EEs modify hippocampal functions such as long-term potentiation, through cAMP-dependent protein kinase [11], and hippocampus-dependent memory [4,11,20]. These findings suggest that environmental enrichment is also expected to have effects on depression-related behaviors. A preweaning EE paradigm is known to reduce the immobility time in a forced swim test [24], a variable that is considered to reflect "behavioral despair" [34]. However, the effects of environmental enrichment during adulthood are poorly understood. Thus, we performed a behavioral analysis of adult mice housed in an EE.

2. Materials and methods

2.1. Animals

Inbred C57BL/6J female mice were used in this study and were housed in a temperature-controlled room under a 12 h light–dark cycle (light on at 8:00 a.m.) with ad libitum access to food and water. The control condition consisted of three mice in a common cage housing (190 mm × 260 mm × 125 mm). The EE condition consisted of three mice in a large rodent cage (350 mm × 400 mm × 180 mm) containing three toys – a running wheel, plastic tube and wooden swing – in addition to several wooden environmental enrichment products (Tapvei Oy, Kortteinen, Finland), which were changed once per week. For each experiment, we used the batches of mice, which were housed in each condition from 10 weeks of age. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

2.2. Experimental design for behavioral tests

Behavioral tests were carried out with mice that had been housed in the control or EE condition for five weeks at start of testing (15 weeks old). Mice were maintained in their housing condition throughout all tests. All behavioral tests were performed between 9:00 a.m. and 7:00 p.m.. We prepared seven independent batches of mice for behavioral tests ($n = 234$); one batch consisted of the same number of EE and control mice (EE: $n = 9$ or 18, control: $n = 9$ or 18). Neurological tests (in five batches), wire hang tests (in five batches), open field tests (in four batches), elevated plus maze tests (in three batches), social interaction tests (in three batches), startle response/prepulse inhibition tests (in two batches), and forced swim tests (no treat: in two batches; imipramine treat: in three batches), were conducted. The variances in the data among the batches were analyzed statistically (see Section 2.12). To minimize carryover effects of experimental manipulation on behavior, at least one day of rest was allotted between tests.

2.3. Neurological/motor function tests

The righting, whisker twitch, eye blink and ear twitch reflexes were evaluated. A number of physical features, including the presence of whiskers and bald patches, were also recorded.

Neuromuscular strength and balance were examined with the wire hang test. A mouse was placed on a wire cage lid, and then the lid was inverted and held at a height of approximately 30 cm above the cage litter. Latency to fall onto the bedding was recorded, with a 60 s cutoff time.

2.4. Open field test

Locomotor activity was measured using an open field test. It was recorded during the first exposure to the open field apparatus (50 cm × 50 cm × 40 cm; O'hara & Co., Tokyo, Japan). The illumination level was 22 lx at the floor of the open field. The apparatus was cleaned with water after each trial. The field was divided by software (see below) into 16 equal-sized squares containing 4 central areas and 12 peripheral areas. Horizontal activity, time spent in the central area and the number of fecal boli were recorded. Data were collected for 30 min. Data acquisition and analysis were performed automatically, using Image OF software (see Section 2.9).

2.5. Startle response/prepulse inhibition test

Response to acoustic startle and prepulse inhibition (PPI) of the startle response were measured using a startle reflex measurement system (O'hara & Co., Tokyo, Japan). A test session began by placing a mouse in a Plexiglas cylinder and leaving the mouse undisturbed for 10 min. The cylinder was cleaned with water after each trial. The startle stimulus was white noise for 20 ms at 110 or 120 dB, and the background noise level was 70 dB. The prepulse sound was presented 100 ms before the startle stimulus, for 20 ms, and its intensity was 74 or 78 dB. Four combinations of prepulse and startle stimuli were used (74/110, 78/110, 74/120 and 78/120 dB). Six blocks of six different trial types (four trial types with the combinations of prepulse and startle stimulus and two startle stimulus only trials) were presented in a pseudorandom order such that each trial type was presented once within a block. The average intertrial interval was 15 s (range, 10–20 s). The startle response was recorded for 500 ms starting with the onset of the startle stimulus, and the maximum startle amplitude (I_{max}) was determined. After the test, average values of I_{max} were calculated for the six trial types. The %PPI was defined as $[\{I_{max}\text{ (startle stimulus)}\} - \{I_{max}\text{ (prepulse and startle stimulus)}\}]/I_{max}\text{ (startle stimulus)} \times 100$.

2.6. Social interaction test

A pair of mice was placed simultaneously at opposing corners in the open field apparatus (50 cm × 50 cm × 40 cm; O'hara & Co., Tokyo, Japan) and allowed to explore freely for 10 min. We performed a social interaction test in a familiar situation with dim light [14]. The pair of mice had been housed in same environment condition but different cages. The illumination level was 22 lx at the floor of the open field. Mice were familiar with the apparatus because they had previously performed the open field test for 30 min in the same apparatus. The apparatus was cleaned with water after each trial. Total duration of contacts, number of contacts and total distance traveled were measured. Analysis was performed automatically using Image SI software (see Section 2.9).

2.7. Elevated plus maze test

The elevated plus maze consisted of two open arms (25 cm × 5 cm) with 3-mm-high ledges and two enclosed arms of the same size, with 15-cm-high transparent walls (O'hara & Co., Tokyo, Japan). The arms and central square

were made of white plastic and were elevated to a height of 34 cm above the floor. Arms of the same type were arranged at opposite sides to each other. A mouse was placed in the central square of the maze (5 cm × 5 cm), facing one of the enclosed arms. Behavior was recorded during a 10 min test session. The illumination level was 40 lx at the central square of the maze. The maze was cleaned with water after each trial. For data analysis, we used the following three measures: the percentage of time spent in the open arms, the percentage of open arm entries and the total number of arm entries. Data acquisition and analysis were performed automatically, using Image EP software (see Section 2.9).

2.8. Forced swim test

The apparatus consisted of a Plexiglas cylinder (22 cm height × 11.5 cm diameter). A modified forced swim test [8], in which the cylinder was filled with water (23–25 °C) up to a height of 17 cm, was used. A mouse was placed inside the cylinder, and the immobility time was recorded over a 10 min test period. The illumination level was 100 lx on the desk that the cylinder was placed on. Data acquisition and analysis were performed automatically, using Image FZ software (see Section 2.9). To estimate antidepressant-like effects of environmental enrichment, mice were administered intraperitoneally with imipramine, a tricyclic antidepressant (10 mg/kg; Sigma, St. Louis, MO) or saline, 30 min before the tests. We randomly divided the mice in a batch into four groups (EE + imipramine, EE + saline, control + imipramine and control + saline), and performed forced swim tests. Imipramine was dissolved in sterile saline.

2.9. Behavioral data analysis

Behavioral data from the open field tests, startle response/prepulse inhibition tests, social interaction tests, elevated plus maze tests and forced swim tests were automatically analyzed as described previously [29,30]. Briefly, behaviors were monitored by a color charged-coupled device camera (Watec Co., Ltd., Yamagata, Japan) that was connected to a Macintosh computer. Images were captured at one or two frames per second. All applications used for the behavioral studies (Image OF, Image EP, Image SI, Image FZ) were run using a Macintosh computer. These applications were based on the public domain NIH Image program (developed at the U.S. National Institute of Mental Health and available on the Internet at http://rsb.info.noh.gov/nih-image/) and were modified for each test by one of the authors (Tsuyoshi Miyakawa, available through O'hara & Co., Tokyo, Japan).

2.10. Immunohistochemistry

Mice were housed in a control or EE condition from 10 weeks of age, and injected with 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO, 75 mg/kg, i.p.) at 15 weeks of age. BrdU is incorporated into DNA as bromouracil during the S phase of the cell cycle, to label newly generated cells. BrdU was dissolved in 0.9% NaCl and sterile-filtered at 0.2 μm. The solution was adjusted to a pH of 7.4 using 1N NaOH. Mice were maintained in their housing conditions until they were sacrificed at day one (proliferation paradigm) or four weeks (survival paradigm) after injection. Thirty mice (proliferation paradigm: $n = 18$; survival paradigm: $n = 12$) were used for histological analyses, and these were separate from the animals used for behavioral tests.

Mice were anaesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde overnight at 4 °C, and stored in a 30% sucrose solution. Coronal freezing microtome sections were stored in cryoprotectant (25% ethylene glycol, 25% glycerin, 0.05 M phosphate buffer, 0.01% sodium azide) at −20 °C until use.

Sections were immunostained as described previously with some modifications [16,17]. Free-floating sections were thoroughly rinsed with TBS (0.05 M Tris-base, 0.15 M NaCl adjusted to pH 7.4 using HCl). DNA was denatured by incubating sections in 2N HCl for 2 h at room temperature. Diaminobenzidine staining was performed with a monoclonal rat anti-BrdU antibody (1:200; Accurate Chemical & Scientific Corporation, Westbury, NY), an anti-rat biotinylated secondary antibody (1:200; Vector Laboratories, Inc., Burlingame, CA) and avidin-biotin-peroxidase complex (Vector Laboratories, Inc.). For double-immunofluorescence staining, combinations of the following primary antibodies were used: a monoclonal rat anti-BrdU antibody (1:200), a monoclonal mouse anti-neuronal nuclei (NeuN) antibody (1:200; Chemicon International, Inc., Temecula, CA) or a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Chemicon). Alexa 555 anti-rat, and Alexa 488 anti-rabbit or Alexa 488 anti-mouse (highly cross-adsorbed) IgG (H + L) (Molecular Probes, Inc., Eugene, OR) secondary antibodies (all generated in goat) were used.

2.11. Stereological analysis

Every sixth section was selected from serial coronal sections (40 μm) through the entire hippocampus of each animal and processed for immunostaining. Hippocampal sections were analyzed for immunopositive cells in the granule cell layer (GCL) and the subgranular zone (SGZ: within two cell body diameter from the edge of the GCL). The total number of BrdU-positive cells was determined in 9.9 ± 0.2 slices per mouse (not lower than eight slices). Cells were counted at 400-fold magnification using an optical microscope (Axiovert 200, Zeiss, Tokyo, Japan). The total number of BrdU-labeled cells in the dentate gyrus was estimated by multiplying the number of cells counted in every sixth section by six. For double-staining, slices were analyzed on a confocal laser-scanning microscope (Fluoview BX50; laser, Argon 488 nm and HeNe 543 nm; Olympus, Tokyo, Japan). At least 50 BrdU-labelled cells per animal were randomly selected from throughout the dentate gyrus and analyzed for the coexpression of BrdU and NeuN or GFAP. We measured GCL volume (EE: $n = 6$, control: $n = 6$) as described previously, with minor modifications [4,20]. Briefly, a one-in-six series of adjacent sections stained with Mayer's Hematoxylin solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 3 min was used to trace the GCL area. The GCL images were captured with a 10-fold objective (Axiovert 200 controlled by Slide Book™ 3.0 (Intelligent Imaging Innovations, Inc., Denver, CO)). The stained GCL area was traced and the number of pixels within this area was automatically measured using Slide Book™ 3.0. The reference volume was estimated by summing the traced granule cell areas for each section multiplied by the distance between sections sampled (240 μm).

2.12. Statistical analysis

Statistical analysis was conducted using SPSS 11.0J for Windows (SPSS Japan, Inc., Tokyo, Japan). The variances in the data from different batches of mice were analyzed by two-way ANOVA. No significant batch-environmental interaction effect on behaviors was observed. Where significant batch effects on behavioral data (time spent in the center area in open field test, startle response, and total immobility time during the test in EE and control mice administered with imipramine or saline) were not found, we present total results for all of the batches. Where significant batch effects on behavioral data were observed, we show data from a representative batch analyzed by a two-tailed t test. Fisher's exact test was used to compare EE and control mice for general health (physical characteristics, sensory/motor reflexes and motor test). Time courses of distance traveled in the open field test and immobility time in the forced swim test were analyzed by repeated measures ANOVA. Values in the table and figures are expressed as means ± standard error of the mean (S.E.M.).

3. Results

3.1. General health in EE mice

An EE had no significant effect on either physical characteristics (whiskers and fur) or sensory-motor reflexes (eye blink, ear twitch, whisker response and righting reflex) (all p values >0.05) (Table 1). EE mice weighed 2% more than control mice ($p < 0.001$), and exhibited a longer latency to fall in the wire hang test ($p = 0.009$) (Table 1); however, there were no significant correlations between any of the measurements from the behavioral tests and weight or latency to fall (all p values >0.05).

Table 1
General physical characteristics and sensory/motor function in control and EE mice

	EE mice	Control mice	p value
Physical characteristics			
Weight (g)	22.0 ± 0.09	21.6 ± 0.09	<0.001
Whiskers (% with)	96.3	95.8	1.00
Fur (% with normal fur)	100	95.1	0.12
Sensory/motor reflex			
Eye blink (% with normal response)	95.1	97.5	0.68
Ear twitch (% with quick response)	97.5	100	0.50
Whisker twitch (% normal response)	93.6	88.2	0.19
Righting reflex (% normal response)	100	100	1.00
Motor test			
Wire hang (latency to fall; s)	60.0 ± 0.0	56.0 ± 1.3	0.009

Data represent the means ± S.E.M. (enriched environment, $n = 81$; control, $n = 81$).

3.2. Locomotor activity, anxiety and social behaviors in EE mice

We first examined the effect of an EE in the open field test (Fig. 1A and B). The distance traveled was shorter in EE mice (time course in Fig. 1A, environmental effect, $F(1,34) = 7.94$, $p = 0.008$; total distance during the test in Fig. 1B, $t(124) = 4.00$, $p < 0.001$). We then compared time spent in the center of the open field apparatus, between EE and control mice. EE mice spent significantly more time in the center than control mice did (Fig. 1C, $t(124) = 2.62$, $p = 0.010$). There was no significant difference between EE and control mice in the number of fecal boli during the open field test (data not shown).

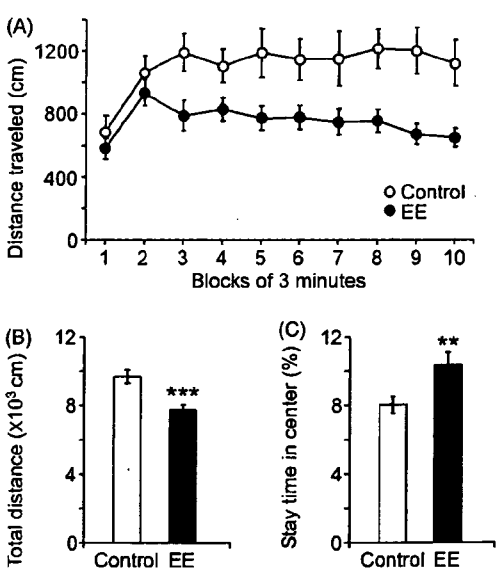

Fig. 1. Locomotor activity of EE mice in the open field test. (A) Time course of distance traveled. Representative data for four independent batches of mice are shown (EE: $n = 18$, control: $n = 18$). (B) Total distance traveled. (C) Time spent in the central area. Results from four batches of mice are shown in (B) and (C) (EE: $n = 63$, control: $n = 63$). Data represent means ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$.

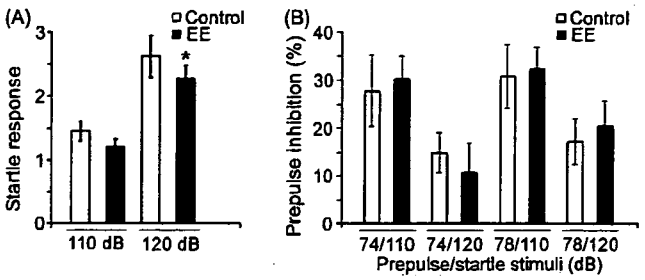

Fig. 2. Startle response and the prepulse inhibition in EE mice. (A) Startle response. Results from two batches of mice are shown (EE: $n = 36$, control: $n = 35$). (B) Prepulse inhibition of the startle response. Representative data for two independent batches of mice are shown (EE: $n = 18$, control: $n = 18$). Data represent means ± S.E.M. $^{*}p < 0.05$.

We also examined startle reactivity in EE mice. The response to 120 dB in EE mice was smaller than that in control mice (Fig. 2A, $t(69) = 2.17$, $p = 0.034$), and similar results were observed in the responses to 110 dB (Fig. 2A, $t(69) = 1.57$, $p = 0.12$). However, there was no significant difference in PPI between EE and control mice (Fig. 2B, 74/110 dB: $t(34) = 0.28$, $p = 0.78$, 78/110 dB: $t(34) = 0.57$, $p = 0.57$, 74/120 dB: $t(34) = 0.19$, $p = 0.85$, 78/120 dB: $t(34) = 0.47$, $p = 0.64$).

In the social interaction test, EE mice showed a significant decrease in the number of social contacts compared with control mice (Fig. 3A, $t(16) = 2.47$, $p = 0.034$); however, there was no significant difference in the total duration of contacts between EE and control mice (Fig. 3B, $t(16) = 1.79$, $p = 0.093$). Moreover, the total distance traveled was also less in EE mice (Fig. 3C, $t(16) = 3.18$, $p = 0.012$). The number and total duration of contacts per distance traveled did not differ significantly between EE and control mice (data not shown).

We then performed the elevated plus maze test. No significant differences between EE and control mice were found in the three parameters examined: the percentage of time spent in the open arms (Fig. 3D, $t(34) = 0.29$, $p = 0.78$), the percentage of open arm entries (Fig. 3E, $t(34) = 0.80$, $p = 0.43$) and the total number of arm entries (Fig. 3F, $t(34) = 0.42$, $p = 0.68$).

3.3. Depression-related behavior in EE mice

In this test, EE mice spent significantly less immobility time than control mice (time course in Fig. 4A, environmental effect, $F(1,52) = 8.75$, $p = 0.005$; total immobility time during the test in Fig. 4B, $t(52) = 2.96$, $p = 0.005$). As antidepressants are known to decrease immobility time [10,34], we compared the immobility times between EE mice and mice administered antidepressants. The time courses of immobility time for all cases of drug-treated mice, are shown in Fig. 4C. We confirmed that an EE reduced the total immobility time when treated with saline (Fig. 4D, $t(52) = 3.84$, $p < 0.001$). Control mice treated with imipramine showed a significant decrease in immobility compared with EE mice treated with saline (Fig. 4D, $t(52) = 2.69$, $p = 0.010$). The total immobility time was similar for EE and control mice after imipramine administration (Fig. 4D, $t(52) = 0.009$, $p = 0.993$).

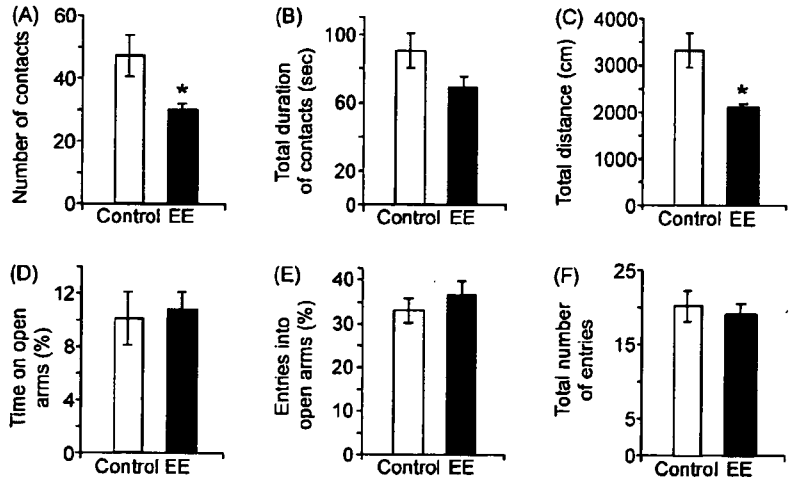

Fig. 3. Social interaction and emotional behavior in the elevated plus maze test in EE mice. The total number of contacts (A), total duration of contacts (B) and distance traveled (C) in the social interaction test are shown. Representative data of three independent batches of mice are shown (pairs of EE mice: $n=9$, pairs of control mice: $n=9$). The percentage of time spent in the open arms (D), the percentage of open arm entries (E) and the total number of arm entries (F) in the elevated plus maze test are shown. Representative data for three independent batches of mice are shown (EE: $n=18$, control: $n=18$). Data represent means ± S.E.M. $^{*}p<0.05$.

3.4. Newborn cells in the dentate gyri of EE mice

We examined newborn cells in the dentate gyri of EE mice by immunohistochemistry of BrdU labeling. Surviving BrdU-immunoreactive cells were observed in the dentate gyri of EE (Fig. 5A) and control mice (Fig. 5B) sacrificed four weeks after BrdU injection. To determine the phenotype of these BrdU-positive cells, we performed immunofluorescent labeling with BrdU and the neural marker NeuN (Fig. 5C), or the glial marker GFAP (Fig. 5D). We counted the double labeled cells with BrdU and NeuN or GFAP and found that environmental enrichment did not affect the differentiation of newly generated cells towards either a neuronal (EE, 90.6 ± 3.3%, $n=3$; control, 89.0 ± 2.4%, $n=3$; $p=0.72$) or glial fate (EE, 7.7 ± 1.7%, $n=3$; control, 6.4 ± 0.7%, $n=3$; $p=0.53$). Most surviving BrdU-positive cells in the dentate gyri of mice housed in either environmental condition expressed NeuN, but not GFAP. Quantitative analysis by counting the number of BrdU-labeled cells confirmed an increase in the number of surviving BrdU-positive cells in EE mice (Fig. 5E, EE: 548.5 ± 41.3, control: 386.7 ± 60.2, $t(10)=3.64$, $p=0.005$). Moreover, the volumes of the dentate gyri of EE mice were similar to those of control mice (Fig. 5F, EE: 0.238 ± 0.009 mm^3, control: 0.227 ± 0.003 mm^3, $t(10)=1.16$, $p=0.27$), and the proliferation of newborn cells

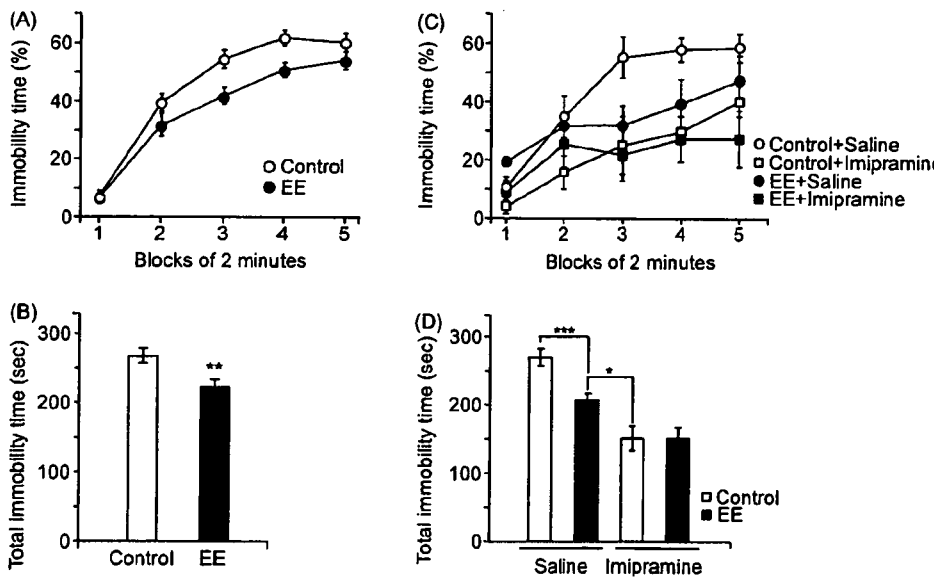

Fig. 4. Immobility time in the forced swim test in EE mice. Time courses of immobility time (A) and total immobility time during the test (B) in EE and control mice (EE: $n=27$, control: $n=27$). (C) Effect of imipramine (10 mg/kg) on the time course of immobility time. Representative data for three independent batches of mice are shown (EE + saline: $n=9$, control + saline: $n=9$, EE + imipramine: $n=9$, control + imipramine: $n=9$). (D) Total immobility time during the test in EE and control mice administered with imipramine or saline. Results from three batches of mice are shown (EE + saline: $n=27$, control + saline: $n=27$, EE + imipramine: $n=27$, control + imipramine: $n=27$). Data represent means ± S.E.M. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

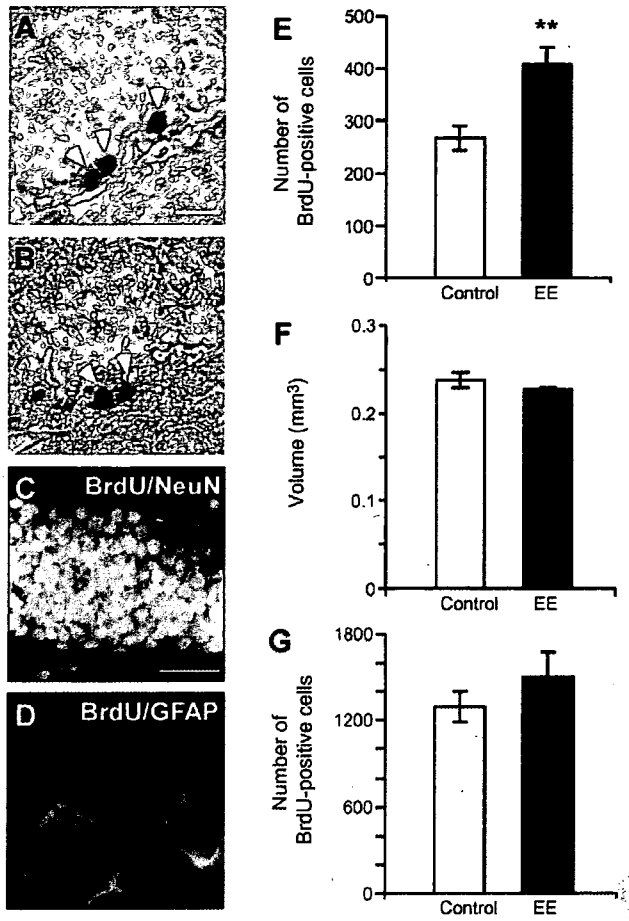

Fig. 5. Newborn cells in the dentate gyri of EE mice. Photomicrographs of BrdU-positive cells in the dentate gyrus of an EE (A) and a control (B) mouse, four weeks after BrdU injection (survival paradigm), are shown. Arrow heads indicate BrdU-positive cells. Scale bar = 25 μm. Surviving cells were double-labeled with BrdU and NeuN (C) or GFAP (D). Merged images are shown. Scale bar = 50 μm. (E) The number of BrdU-positive cells in the survival paradigm (EE: $n = 6$, control: $n = 6$). (F) The volume of dentate gyri in the survival paradigm (EE: $n = 6$, control: $n = 6$). (G) The number of BrdU-positive cells in the proliferation paradigm (EE: $n = 11$, control: $n = 7$). Data represent means ± S.E.M. $^{**}p < 0.01$.

in the dentate gyri of mice sacrificed one day after BrdU injection was not significantly changed by environmental enrichment (Fig. 5G, EE: 1300.9 ± 158.2, control: 1094.6 ± 121.5, $t(16) = 0.93$, $p = 0.37$).

4. Discussion

Our results support the hypothesis that environmental enrichment during adulthood has beneficial effects on behaviors in mice. The distance traveled by EE mice was shorter than that traveled by control mice (Fig. 1A and B), indicating a reduction in locomotor activity in a novel environment during an open field test, as previously reported [2,21,40]. EE mice showed a longer latency to fall off in a wire hang test (Table 1) and a reduction in total immobility time in a forced swim test (Fig. 4A and B). These results suggest that the decrease in locomotor activity is not due to loss of motor function. In addition, locomotor activity was not significantly different between two groups within a few minutes of the start of recording; however, the activity gradually became lower in EE mice (Fig. 1A). This reduction in locomotor activity in EE mice could be a result of a quick habituation to a novel environment. One reason for this might be that EE mice had been exposed to changing stimuli in their housing environment (i.e., several toys and wide cage).

Our results showed that EE mice spent more time in the center of the open field apparatus (Fig. 1C), a behavior that is thought to reflect reduced anxiety [7]. However, in this study, environmental enrichment failed to improve the anxiety-like behavior observed in the elevated plus maze (Fig. 3D–F). This is in disagreement with results obtained in mice housed in EEs from an early age or over a longer period [1,19]. As the degree of conflict might be more in the elevated plus maze test than in free-exploration in the open field test [6,7], our EE paradigm might be insufficient to reduce the anxiety in more conflictive situations. In this study, EE mice showed a significant decrease in the number of social contacts (Fig. 3A–C); however, it is likely that this decrease is a consequence of the decreased locomotor activity in EE mice. The total duration of social contacts was not significantly different between the two groups. Thus, it is unlikely that EE mice are less social. The results of the social interaction test (Fig. 3A–C) and startle response test (Fig. 2A) also disagreed with those in mice housed in EEs from an early age and/or over a longer period [31,40]. The nature of EE protocols varies widely between laboratories; however, the timing and duration of environmental enrichment might be important parameters for the effects on behaviors [33]. In addition, there was no significant difference in PPI between EE and control mice (Fig. 2B), suggesting that exposure to an EE during adulthood did not affect sensory motor gating, at least in our paradigm. The forced swim test is known to assess "behavioral despair" in rodents by the measurement of immobility time [34]. Reduced immobility time in the forced swim test was found in EE mice (Fig. 4). We confirmed in a preliminary experiment that imipramine did not affect motor activity (data not shown). It is unlikely that this phenomenon is due to the hyperactivity of the EE mice, as they showed decreased locomotor activity in the open field test (Fig. 1A and B). Thus, it suggests that the EE enhanced the tolerance against an inescapable, stressful situation. The beneficial effect of environmental enrichment on depression-related behavior was also shown in rats reared in an EE during the first month of life [24]. Our result showed that this effect could be also achieved in mice housed in an EE during adulthood. Experience-dependent plasticity is induced by EE [33]; thus, EE might also induce an alteration in depression-related behavior in adult rodents.

The enhanced neurogenesis in adult animals housed in an EE is thought to be a potential mechanism mediating the behavioral effects of EEs [20,33,38]. We found enhanced survival of newborn cells in the dentate gyri of EE mice (Fig. 5A, B and E), as reported previously [20]. As the volume of the dentate gyrus was similar between control and EE mice (Fig. 5F), the enhanced survival of newborn cells might not be affected by the volume of the dentate gyrus. Our results show that EE can affect depression-related behavior, and that EE enhances the

survival of newly generated cells, most of which differentiate into neurons, consistent with a previous study [20]. However, it was impossible to show a direct relationship between the altered behaviors and the increase in newborn cells induced by an EE. In a recent study, the reduction in adult-generated cells in the dentate gyrus due to methylazoxymethanol acetate, a toxin that inhibits cell proliferation, prevents the EE-induced improvement in hippocampal-dependent memory [4], suggesting that EE-induced neurogenesis may have an important role in behavioral outputs. On the other hand, the arrest of adult hippocampal neurogenesis does not attenuate the effect of EE on spatial learning and anxiety-like behavior [28]. An investigation into a causal relationship between increased neurogenesis and the behavioral effects of EEs is underway. Exposure to an EE increases the gene expression of several growth factors and neurotransmitters [38]. The effects of environmental enrichment can be achieved through multiple pathways, including neurogenesis-independent mechanisms. Further studies are necessary to understand the mechanism underlying the effects of environmental enrichment.

Acknowledgements

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POSTSYNAPTIC DENSITY: A KEY CONVERGENT SITE FOR SCHIZOPHRENIA SUSCEPTIBILITY FACTORS AND POSSIBLE TARGET FOR DRUG DEVELOPMENT

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CONTENTS

Summary

Many studies have supported roles for both genetic and environmental factors in the etiology of schizophrenia. A major hypothesis at present is that schizophrenia is a polygenic disorder where alterations in a set of genes lead to impaired neurodevelopment, which in turn results in altered neurotransmission. Several neurotransmitters, including glutamate, dopamine, serotonin and gamma-amino butyric acid (GABA), have been implicated in schizophrenia, and, as such, there is a growing interest in trying to elucidate the mechanisms whereby alterations in the function of schizophrenia susceptibility gene products can lead to disturbance in signaling at synapses. In this article, we will summarize what is known about schizophrenia susceptibility factors that reside at postsynaptic density (PSD), a unique postsynaptic site where signals from neurotransmitters converge. PSD may be a promising target for novel classes of drugs to treat schizophrenia.

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Introduction

Schizophrenia is a complex neuropsychiatric condition affecting about 1% of the population worldwide. Its etiology remains unclear, although genetic and epidemiological studies suggest that the interplay between genetic and environmental factors during the early stages of neurodevelopment could contribute to its pathogenesis (1, 2). Recently, systematic linkage and association studies have provided a list of susceptibility genes that include *neuregulin (NRG)-1, dysbindin, disrupted-in-schizophrenia 1 (DISC1), neuronal nitric oxide synthase (nNOS)* and *carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (CAPON)* (3–5) (Table I).

Brain imaging studies of patients and neuropathological approaches have revealed that schizophrenia patients present with enlarged lateral ventricles, decreased brain volumes and altered synaptic morphology including decreased dendritic spines and arborizations (3, 6–12). These neuroanatomical disturbances have led some to postulate that impaired neurodevelopment may lead to altered neurotransmission in schizophrenia. Interestingly, the majority of genetic susceptibility factors for schizophrenia localize to postsynaptic density (PSD), a unique postsynaptic site, where they may converge and modulate signals downstream of various neurotransmitter receptors (Fig. 1). Of all the neurotransmitters thought to contribute to the pathophysiology of schizophrenia, glutamate has received the most attention. This is because numerous studies have demonstrated that hypoglutamatergic signaling is associated with psychosis. Some studies have found that phencyclidine, a *N*-methyl-*D*-aspartate (NMDA) receptor antagonist, induces schizophrenia-like behaviors in humans (13). In addition, studies in mice, rats and monkeys treated with phencyclidine have shown that these animals displayed behavioral and/or neuroanatomical deficits that resemble those seen in schizophrenia patients (14–16). Thus, PSD linked to NMDA receptors may be a site of convergence for the disease pathways, and, as such, it may be an important target for new drugs to treat schizophrenia. Once we would have identified signaling pathways altered in schizophrenia and their phenotypic outcomes, we can design agents to alleviate or treat these phenotypic insults.

In this review, we will focus on genetic susceptibility factors for schizophrenia that may play a role at PSD associated with NMDA receptors, and discuss potential therapeutic interventions at this site. Our discussion will be centered on the function of these gene products as well as genetic studies linking alterations in these genes to abnormal phenotypes in humans.

Neuregulin-1 and ErbB4

Function

NRG-1 belongs to the family of proteins containing an epidermal growth factor–like motif that is known to activate ErbB1–4 receptors tyrosine ki-

Table I: Susceptibility genes for schizophrenia.

Gene	Chromosomal locus	Function
CAPON	1q22	Regulates the coupling of nNOS to the NMDA receptor via PSD-95
DISC1	1q42	Neurites outgrowth
		Neuronal migration
		Axonal elongation
		Modulates cyclic AMP pathway
Dysbindin	6p22.3	Modulates dopamine release
		Stimulates glutamate release
		Upregulates presynaptic proteins
		Neurotrophic effect through Akt signaling pathway
NRG-1	8p22	Neuronal migration and differentiation
		Modulates $GABA_A$ receptor expression
		Recruits CHRNA7 to synapses
		Regulates expression and plasticity of NMDA receptor
nNOS	12q24	Second messenger of NMDA receptor
		Modulates dopamine and serotonin neurotransmission

NMDA = *N*-methyl-*D*-aspartate; Akt = protein kinase B; GABA = gamma-amino butyric acid.

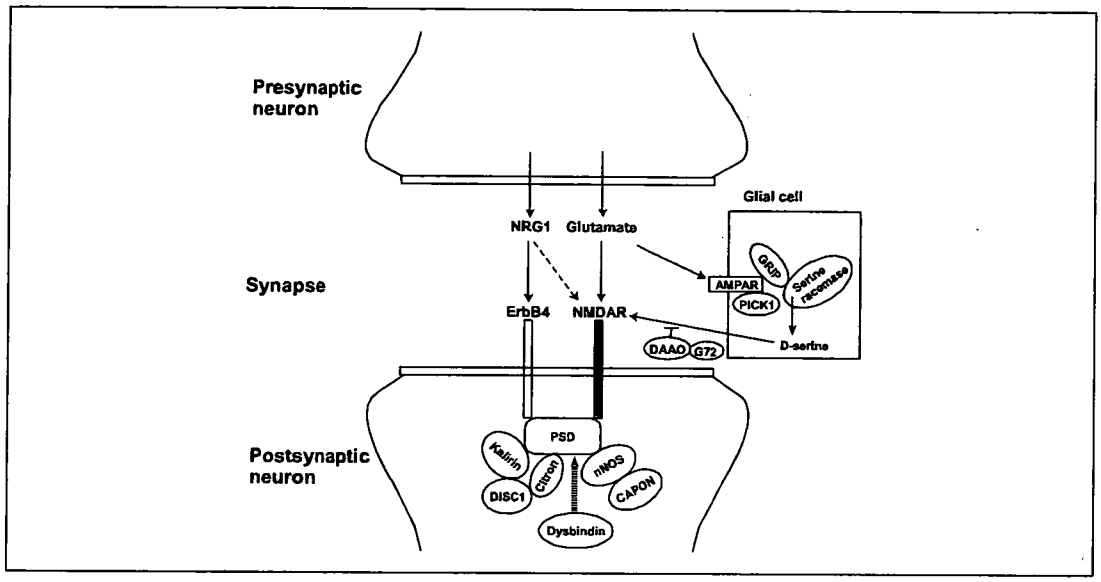

Fig. 1. Schematic representation of schizophrenia gene products localizing at postsynaptic density (PSD). Neuregulin (NRG)-1, dysbindin, disrupted-in-schizophrenia 1 (DISC1), neuronal nitric oxide synthase (nNOS) and carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (CAPON) are schizophrenia susceptibility gene products that reside at PSD associated with *N*-methyl-*D*-aspartate receptor (NMDAR). GRIP = glutamate receptor-interacting protein; AMPAR = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor; PICK1 = protein interacting with C kinase 1.

nase (17, 18). NRG-1 has nine known splicing variants that are all expressed in human brain (19). The NRG-1 receptor, ErbB4, has been well characterized. Of most importance, ErbB4 occurs in PSD where it interacts with PSD95, an anchoring protein of the NMDA receptor (20, 21). ErbB4 has several isoforms that are expressed in the brain (22). Splicing variants containing exon 16 encode for ErbB4 isoforms carrying a metalloprotease-sensitive extracellular domain referred to as JM-a. Splicing variants containing exon 26 encode for a cytoplasmic domain containing a phosphotidylinositol-3 kinase (PI3K) binding site referred to as CYT-1.

NRG-1 regulates the expression of three receptors that have been implicated in the pathophysiology of schizophrenia: NMDA, gamma-amino butyric acid (GABA) and nicotinic acetylcholine receptors (23–26). Studies using cerebellar slice cultures have shown that a NRG-beta isoform stimulates the expression of NR2C, an NMDA receptor subunit (27). As discussed below, studies with human tissue have demonstrated a novel function of NRG-1–ErbB4 interaction.

Human studies

Ten independent groups found a genetic association between the *NRG-1* gene and schizophrenia (28–37). A recent study reported for the first time that an NRG-1 variant in individuals at high risk for schizophrenia is associated with impaired activation of frontal and temporal lobes, lower intelligence quotient and increased susceptibility for psychotic symptoms (38).

A study using autopsied human brains reported that type I NRG-1 mRNA is increased in the dorsolateral prefrontal cortex of schizophrenia patients (39). Similar results were obtained in the hippocampus of a much larger and separate sample of schizophrenia patients (40).

Studies using postmortem brains from schizophrenia patients also reported a decrease in the number of oligodendrocytes (41–45). Consistent with these reports, microarray studies have also reported a general reduction in the expression of oligodendrocyte- and myelinization-associated genes in schizophrenia patients (46–48). Since NRG-1 plays an important role in oligodendrocytes development, NRG-1 dysfunction could lead to poor myelination and thus impaired synaptic for-

mation due to improper oligodendrocyte development.

Even though numerous genetic studies in schizophrenia patients have found that the *NRG-1* gene is associated with schizophrenia, we still do not know how alteration in NRG-1 function can lead to the disorder. A study by Hahn *et al.* (49) using postmortem brains from schizophrenia patients suggests that upregulation of NRG-1–ErbB4 signaling suppresses NMDA receptor activation more in schizophrenia patients than in healthy controls. Other studies supporting an association between enhanced NRG-1–ErbB4 signaling and schizophrenia include a report showing a genetic interaction between the Icelandic *NRG-1* haplotype and ErbB4 (37). Another study reported that two ErbB4 isoforms, JM-a and CYT-1, were overexpressed in the dorsolateral prefrontal cortex of schizophrenia patients from their Ashkenazi cohort (50). CYT-1 is a known activator of the PI3K–protein kinase B (Akt) pathway that has been implicated in some cases of schizophrenia.

DISC1

Function

The *DISC1* gene was originally identified at the breakpoint on chromosome 1 resulting from a balanced chromosomal translocation between chromosomes 1 and 11 in a large Scottish family with hereditary psychiatric disorders, including schizophrenia (51, 52). DISC1 is multifunctional and has several subcellular distributions (53). In proliferating cells and developing neurons, a pool of DISC1 forms a complex with dynein, nuclear distribution element-like, and lissencephaly 1 protein at the centrosome (54). Disruption of this complex following suppression of DISC1 expression by RNA interference leads to improper neuronal migration and abnormal dendritic arborizations in the cerebral cortex *in vivo* (54). DISC1 may also function to modulate the cyclic AMP (cAMP) pathway by binding to phosphodiesterase 4B, which itself was found to be disrupted by a balanced chromosomal translocation in two patients with schizophrenia (55). The cAMP pathway is known to modulate learning, memory and mood, and, as such, the interaction between DISC1 and PDE4B suggests that DISC1 may play a role in synaptic plasticity (56–58).

In adult brains, DISC1 turns out to be enriched in PSD and the nucleus. A study demonstrated by immunoelectron microscopy that DISC1 was present in about 8% of axon terminals in the frontal and parietal cortex of healthy subjects (59) (Fig. 2). Furthermore, yeast two-hybrid studies have shown that DISC1 interacts with numerous PSD proteins, including Citron (60, 61).

Human studies

Association of DISC1 with schizophrenia in general has also been supported by various genetic studies (53, 62). For example, a *DISC1* haplotype was found to be undertransmitted in a North American Caucasian population with schizoaffective disorder (63). The same study also reported that haplotype blocks located within exons 1 and 9 are associated with various neuropsychiatric disorders, including schizophrenia, and that the Phe607 allele is overtransmitted in patients with schizoaffective disorders. Another study showed that the Ser704 DISC1 allele is overtransmitted among schizophrenia patients, and that in healthy individuals this allele was associated with defective hippocampal formation and function (64). In addition, several studies have shown that the Ser704Cys DISC1 polymorphism is associated with impaired cognitive function (64–66).

Another study reported that an asymptomatic carrier of the Scottish mutation presented with a deficit in P300 event-related potential, which indicates impairment in higher processes such as memory and attention (67).

Deficits in spatial and working memory function as well as reduced gray matter volume in the dor-

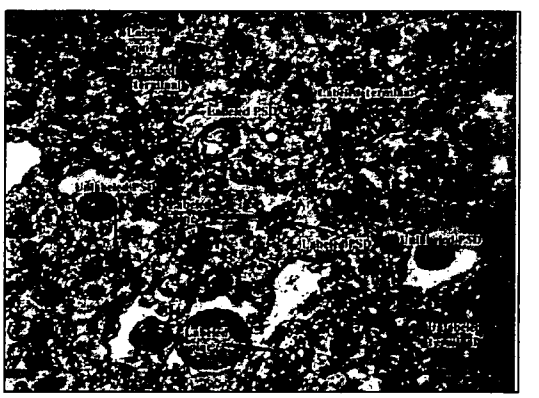

Fig. 2. Electron micrograph showing DISC1 immunoreactivity in various postsynaptic densities (PSD). Reproduced from ref. 59 with permission from John Wiley & Sons, Inc. © 2006.

solateral prefrontal cortex were found in individuals who carry the Finnish *DISC1* risk haplotype, HEP1 (68). Another *DISC1* haplotype, HEP3, has been associated with impaired visual memory and attention (69).

In postmortem brains, we reported that disturbance in the nuclear pool of DISC1 is observed in schizophrenia patients as well as in alcohol/substance abuse patients (70).

Dysbindin

Function

Dysbindin was originally found as a binding partner of alpha- and beta-dystrobrevin, which are causative genes of Duchenne muscular dystrophy (71). Dystrobrevins exist in the dystrophin-associated protein complex that is important for proper muscle function (72). Lack of dystrophin leads to alterations in neuronal membranes that in turn can cause cognitive deficits in affected individuals (73).

Little is known about the functions of dysbindin in neurons. It has been shown that suppression of dysbindin expression in PC12 cells resulted in an increase of dopamine release (74). Another study showed that dysbindin might influence exocytotic glutamate release via upregulation of the presynaptic machinery molecules (75). The same study also reported that dysbindin promotes neuronal viability through PI3K–Akt signaling. A recent study demonstrated that dysbindin interacts with snapin, and the two proteins colocalize in presynaptic sites rich in synaptic vesicle membrane as well as in PSD (76). More studies are needed to elucidate a possible role for dysbindin at PSD.

Human studies

Numerous studies have reported an association between genetic variants of dysbindin and schizophrenia in several populations (75, 77–83).

Some studies have shown that dysbindin single-nucleotide polymorphisms (SNPs) are associated with impairments in general cognitive ability, frontal brain function, as well as more severe negative symptoms in healthy subjects and patients with schizophrenia (84–86). For instance, a study conducted on 213 patients with schizophrenia or schizoaffective disorder found that dysbindin genetic variants that have been associated with schizophrenia can affect intelligence (87). Another study found an association between a dysbindin risk haplotype and spatial working memory (88).

Studies with human autopsied brains have revealed alterations in levels of dysbindin mRNA and protein. One study reported that dysbindin expression is reduced in the hippocampus of schizophrenia patients (89). This reduction was more prominent in the inner molecular layer of the dentate gyrus and correlated with an increase in vesicular glutamate transporter-1. These results suggest that altered glutamatergic signaling may lead to improper hippocampal formation. Another study found decreased dysbindin mRNA levels in the prefrontal cortex and midbrain of schizophrenia patients (90).

nNOS and CAPON

Function

The neuronal form of nitric oxide synthase, also known as neuronal nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPH-d), is the major source of nitric oxide in the central nervous system (91, 92). Nitric oxide is known to function as a second messenger downstream of the NMDA receptor (93). Signal downstream of the NMDA receptor is mediated in part by nNOS, since calcium entering through NMDA receptor channels binds to the calmodulin–nNOS protein complex. CAPON, a new risk factor for schizophrenia, is an adapter protein mediating the coupling of nNOS to the NMDA receptor via PSD95 protein (94).

Human studies

nNOS gene polymorphism has been associated with schizophrenia (95–97). A study showed that the alternative NOS1 exons 1c and 1f are associated with psychosis and altered prefrontal cortex function (96). Another study conducted on 215 Japanese patients with schizophrenia and 182 healthy subjects found an association between an SNP located in exon 29 of the *nNOS* gene and schizophrenia (97). However, another study reported that they did not find an association between a CA dinucleotide repeat polymorphism in the 3'-UTR exon 29 of the nNOS gene and schizophrenia in a Chinese cohort (98).

A study reported various alterations in the morphology as well as the number of NOS-positive neurons in the striatum of schizophrenia patients as measured by immunohistochemistry (99). Another study reported that schizophrenia patients have a reduced number of NADPH-d–positive neurons in the hippocampal formation and neocortex of the lateral temporal lobe (100). This study al-

so found that in schizophrenia patients, the number of NADPH-d–positive neurons was increased in the white matter of the lateral temporal lobe as well as in parahippocampal white matter. There has also been a report of altered distribution of NADPH-d–positive neurons in the frontal lobe of schizophrenia patients (101).

One study reported that patients with schizophrenia and those with bipolar disorder have increased expression of CAPON in the dorsolateral prefrontal cortex (102). According to the authors of this study, one of the consequences associated with increased expression of CAPON is NMDA receptor hypofunction due to disturbance of the interaction between nNOS and NMDA receptor.

Conclusions

NRG-1, DISC1, dysbindin, nNOS and CAPON are examples of key schizophrenia susceptibility factors that could modulate glutamatergic signaling at PSD. Alteration in these factors may mediate the disease-associated disturbance of NMDA–glutamatergic signaling. Thus, agents that can modulate signaling involving these factors at PSD to enhance overall glutamate signaling could potentially be efficacious drugs to treat schizophrenia. Findings from human genetic studies have allowed scientists to make genetically engineered mice displaying schizophrenia-like symptoms, which can be used to elucidate the mechanisms whereby schizophrenia susceptibility genes contribute to its pathophysiology as well as to screen novel classes of drugs for the treatment of schizophrenia.

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