表1　事業対象者

	年齢	学歴	障害名	手帳	日中の所属先
Aさん	20代	大卒	アスペルガー障害	知的（B2）	地域活動支援センター
Bさん	30代	大卒	アスペルガー障害	精神（2級）	アルバイト
Cさん	30代	大学院卒	アスペルガーの疑い	精神（2級）	地域活動支援センター
Dさん	40代	普通高卒	広汎性発達障害	精神（2級）	デイケア
Eさん	50代	大卒	広汎性発達障害	精神（2級）	地域活動支援センター
Fさん	20代	大卒	アスペルガー障害	精神（2級）	地域活動支援センター
Gさん	40代	普通高卒	広汎性発達障害	知的（B2）	地域活動支援センター
Hさん	20代	大卒	高機能自閉症	知的（B2）	地域活動支援センター
Iさん	20代	大学中退	高機能自閉症	知的（B2）	福祉就労
Jさん	30代	大卒	アスペルガー障害	精神（2級）	地域活動支援センター

2 サポートホーム事業の概要

サポートホーム事業は、発達障害の人が主に在宅から、地域でのひとり暮らしを目指すための準備段階として、最長二年間、仮のひとり暮らしを経験する場である（前頁・図1参照）。暮らしの場は、グループホーム・イオプレイス（1Kアパートタイプ）とした。支援者は各部屋を訪問し、地域でのひとり暮らしに向けたスキルアップ、相談、アセスメントをおこない、その準備から地域移行後のフォローアップまでをおこなっている（前頁・図2、3参照）。

サポートホーム事業の対象者はモデル事業時を含め表1の通りである。また、入居の条件として、横浜市在住であり、就労または日中の活動場所に安定的に通えている発達障害の人を対象としている。

3 サポートホームでの関わり

(1)「折り合い点」を設ける

共同生活とは異なり、ひとり暮らしとなると、たとえば、食事・入浴・就寝など何時からという決まりがない。人と合わせる必要が基本的にはないため、これが正解というライフスタイルは本人が決めていくことになる。生活に偏りがある場合には、健康面、衛生面などの視点から支援者の考えを提案していく。本人、支援者どちらが正解ということではなく、本人の考えや行動を否定しないかたちで支援者が提案をすることで「折り合い点」を設け、望ましい状態に近づけていくことを関わり方のベースとしている。

(2)介入のための「三つの視点」

サポートホームでは、折り合い点を目標とした介入型の関わりをすることで、今は問題とされていなくても、将来的に想定される課題を抽出し「予防する」ことをひとつの目的としている。つまり、予防的であるために、問題となる前からの介入が必要なのである。ただし、支援者の思いつきや感覚で介入しないよう、以下の三つの視点をもっての関わりとしている。

一つ目は、「生活のしやすさ」である。これは、家事全般のスキルアップや、やるべきことの優先順位のつけ方、効率的な時間の使い方などを教えていくことで、生活がしやすくなるというところに繋げていく視点である。たとえば、部屋を片づけず散らかっているため、よく物をなくし混乱してしまう人に対して、何度言ってもやらない、怠けている、やる気がないと判断するのではなく、いつ・どのように・どのくらいの頻度でやればいいか知らないだけ、という視点で関わる。片づけをするという行動を具体的に教えていくことで、物をなくすことや混乱が減り、結果本人の生活のしやすさに繋げていくのである。

二つ目は、「生活の豊かさ」で、広がりのある選択肢の中から、自分の希望や意思に沿って物事を選べるように関わっていく視点である。

ここで事例を紹介したい。

大学卒業後、地域活動支援センターに週五日フルタイムで通うAさんは、PCを使ったデータ入力等、問題なくこなせるようになった。日中の安定があり、次のステップとして生活面の自立を考え、サポートホーム入居に至った。

入居初日、Aさんが夕食は自炊をしたいと希望したため、一緒にスーパーへ同行した。Aさんは、スーパーに入るなり、「肉と魚と野菜をバランスよく」と言

いながら、店内を数十分回って選んだものは、ハム（肉）・しらす（魚）・キュウリ（野菜）の三つであった。Aさんは、「これで肉と魚と野菜です」とレジに向かおうとした。確かに、肉・魚・野菜であったが、私は総菜コーナーを見てみることをAさんに提案した。惣菜コーナーに行くと、Aさんはビックリした表情で、「これはいいですね」と言って、ハムを取り下げメンチカツを、しらすを取り下げ白身魚のフライをかごに入れた。

二日目、同様にスーパーに行くと、真っ先に昨日の総菜コーナーへ行き、メンチと白身魚のフライを選んで購入した。

三日目、同様にスーパーへ行くと、同じ総菜コーナーへ一目散に向かった。そこで、私は、「栄養バランスを考えた食事が大事」というAさんの〝知識としての言葉〟を受けて、野菜の入っている鶏の照り焼きを提案した。Aさんは、数秒考えた後、私の提案を受け入れてくれた。

四日目、同様にスーパーへ行くと、Aさんは、「メンチにしようか、鶏の照り焼きにしようか」と二択で迷っていた。

買い物初日に、こちらの提案を受け入れ、メニューを決めることができた。二日目には、自主的に選ぶ経験をした。三日目にまた同じものを選ぼうとしたときに、新たな選択肢の提案をし、受け入れてくれた。四日目にはこれまでに選んだものの中からの二択になった。予防的な介入がなければ、Aさんは、メンチや白身魚のフライが好きな人、こだわっている人、というレッテルをはられていたであろう。

四日目から二択になり、三択・四択……と選択肢を広げていく関わりを続けていくことで、Aさんは手ごねのハンバーグを作れるまでになった。現在はひとり暮らしに移行し三年目であるが、「お買い得商品」を見てその日のメニューを決められるまでに至っている。

三つ目は、「社会とのつながり」である。発達障害の人は、困り感の違いなどその特性から、ひとり暮らしになると社会的な孤立を招きかねない。そのためにも、家族以外の第三者との関わりを通じて、困ったときにまわりの支援者に頼る経験を、生活の場であるサポートホームで積み上げていく必要がある。また、将来的に関わる頻度は減らしても関わり続ける必要があることは明らかであろう。

4 サポートホーム事業を通じて

サポートホームでの経験が、生活する上での得意不得意や「自分を知る」機会となり、初めて自分に合った将来の生活環境を自分で選択できるようになると考えられる。一方、関わる側にとっては、経験により積み上がる部分と積み上がりにくい部分がわかることで、地域でひとり暮らしするための支援ネットワーク構築に繋げていくことができるのである。

5 教えられるものとしての「生活」

学齢期を過ぎると「生活」は暗黙のもと、すでに学び終えていると思われている節がある。学んでいない場合、「それくらい言えばわかる、できる」と思われ、できない場合は、これまでの親のしつけや本人の責任とされてしまうこともあるだろう。

発達障害の人の特性の一つに、暗黙知の学習困難があると思われる。前述のAさんは家族とスーパーに買い物に行く経験は何度もしていた。しかし、意図的には教えられてはこなかったのである。勉強や仕事と同様、「生活」も教えられてこなければ、できるようにはならないのである。勉強や仕事とは違い「生活」は、学ぶ機会が与えられにくく、学齢期から意図的に教えていく必要があることは、成人期の彼らとの関わりから教えられるところである。

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Molecular Autism

RESEARCH Open Access

Serum microRNA profiles in children with autism

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Abstract

Background: As regulators of gene expression, microRNAs (miRNAs) play a key role in the transcriptional networks of the developing human brain. Circulating miRNAs in the serum and plasma are remarkably stable and are suggested to have promise as noninvasive biomarkers for neurological and neurodevelopmental disorders. We examined the serum expression profiles of neurologically relevant miRNAs in autism spectrum disorder (ASD), a complex neurodevelopmental disorder characterized by multiple deficits in communication, social interaction and behavior.

Methods: Total RNA, including miRNA, was extracted from the serum samples of 55 individuals with ASD and 55 age- and sex-matched control subjects, and the mature miRNAs were selectively converted into cDNA. Initially, the expression of 125 mature miRNAs was compared between pooled control and ASD samples. The differential expression of 14 miRNAs was further validated by SYBR Green quantitative PCR of individual samples. Receiver-operating characteristic (ROC) analysis was used to evaluate the sensitivity and specificity of miRNAs. The target genes and pathways of miRNAs were predicted using DIANA mirPath software.

Results: Thirteen miRNAs were differentially expressed in ASD individuals compared to the controls. MiR-151a-3p, miR-181b-5p, miR-320a, miR-328, miR-433, miR-489, miR-572, and miR-663a were downregulated, while miR-101-3p, miR-106b-5p, miR-130a-3p, miR-195-5p, and miR-19b-3p were upregulated. Five miRNAs showed good predictive power for distinguishing individuals with ASD. The target genes of these miRNAs were enriched in several crucial neurological pathways.

Conclusions: This is the first study of serum miRNAs in ASD individuals. The results suggest that a set of serum miRNAs might serve as a possible noninvasive biomarker for ASD.

Keywords: Autism spectrum disorder, microRNA, complementary DNA, microarray, quantitative PCR

Background

Autism spectrum disorder (ASD) refers to a group of heterogeneous neurodevelopmental disorders characterized by impairments in communication and social interaction, and restricted, repetitive and stereotypic patterns of behavior [1]. According to a recent estimate, 1 in 88 individuals has ASD [2]. ASD is largely genetic in origin, with most data supporting a polygenic epistatic model [3,4]. However, owing to the heterogeneous nature of this disorder, classical genetic studies have not necessarily been successful in identifying suitable candidate genes for ASD. In addition to the genetic factors, environmental factors also play a vital role in predisposing individuals to ASD [5]. In recent years, epigenetic mechanisms, which act at the interface of genes and the environment, have been identified as a potential contributor to the pathogenesis of several neurodevelopmental abnormalities such as ASD [6]. Epigenetic factors control heritable changes in gene expression without changing the DNA sequence [7].

MicroRNAs (miRNAs) have recently emerged as prominent epigenetic regulators of a variety of cellular processes, including differentiation, apoptosis and metabolism [8]. miRNAs are a class of small (approximately 21 nucleotides) noncoding transcripts that can modulate cellular messenger RNA (mRNA) and protein levels by interacting with specific mRNAs, usually at the 3′ untranslated region (UTR), resulting in mRNA degradation or repression of translation [9,10], through partial sequence complementation [11]. MiRNAs are abundantly present in the brain,

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and have been found to play crucial roles in several facets of brain function, particularly in neuronal plasticity and neuronal development [12].

So far, three studies have determined the expression of miRNAs in lymphoblastoid cell cultures of ASD patients, and one study has identified dysregulated miRNAs in the cerebellar cortex of ASD patients. Abu-Elneel *et al.* first observed an altered expression of miRNAs and their brain-specific targets in the postmortem cerebellar cortices of autism subjects [13]. Studies in lymphoblastoid cells have implicated brain-related miRNAs and their targets in the pathophysiological conditions underlying autism [14-16].

MiRNAs have also been found to be present in the extracellular fluids such as plasma, serum, saliva, and urine of humans in detectable concentrations [17-20]. In particular, serum miRNAs, which may be derived from circulating blood cells, are known to be remarkably stable, reproducible and resistant to the actions of RNase [21], suggesting the potential efficacy of serum miRNAs as noninvasive biomarkers for ASD. Therefore, in the present study we performed miRNA expression profiling of serum samples from individuals with ASD who had never received drug treatment.

Methods

This study was approved by the Ethics Committee of Hamamatsu University School of Medicine, Japan. A detailed description of the study was given to all participants and their parents before enrollment. Blood samples were collected from the donors after obtaining written informed consent.

Subjects

In this study, we included 55 subjects with ASD (age = 11.29 ± 2.45 years (mean ± SD); range = 6 to 16 years; 48 males and 7 females) and 55 age- and sex-matched typically developed control subjects (age = 11.3 ± 2.37; range = 6 to 16; 41 males and 14 females). There were no significance differences in the age ($P = 0.9685$) or sex ($P = 0.089$) distribution between the control and ASD groups (Table 1). All of the participants were Japanese. The diagnosis of ASD was made on the basis of the Diagnostic and Statistical Manual, Fourth Edition, Text Revision (DSM-IV-TR; American Psychiatric Association, 2000) criteria. The Autism Diagnostic Interview-Revised (ADI-R) [22] was conducted by experienced child psychiatrists who are licensed to use the Japanese version of the ADI-R. Participants having comorbid psychiatric illnesses were excluded by means of the Structured Clinical Interview for DSM-IV (SCID) [23]; any additional psychiatric or neurological diagnosis was also excluded. None of the participants had received any drug treatment for ASD.

Typically developed individuals (control group) were recruited through advertisements in local newspapers. Control group participants underwent a comprehensive assessment of their medical history; those with neurological or other medical disorders were excluded. The SCID was also conducted to screen all participants for any past or present mental illness. None of the control participants were diagnosed with any neuropsychiatric condition.

Serum separation

Blood samples were collected between 11:00 am and noon from each subject by venipuncture, and the samples were kept for 30 min at room temperature. All protocols for serum separation were completed within 1 h of drawing blood. Serum was separated by centrifugation at 3,500 rpm for 10 min at room temperature. Hemolyzed samples were excluded from the study at this stage. The clear supernatant was collected into RNase/DNase-free microfuge tubes in 200 μl aliquots and stored at −80°C until use.

RNA extraction and cDNA synthesis

Total RNA, including miRNA, was extracted from 200 μl serum by using an MiRNeasy Serum/Plasma Kit (QIAGEN GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. Briefly, five volumes of QIAzol lysis reagent was added to the sample; a synthetic spike-in control, *Caenorhabditis elegans* miR-39 (1.6×10^8 copies/μl), was added to the lysed samples for internal normalization. After adding an equal volume of

Table 1 Clinical and/demographic variables of individuals with autism and of control subjects

Clinical/demographic	Autism (n = 55)	Control (n = 55)	*P* value
Age	11.29 ± 2.45 (6 to 16)	11.31 ± 2.37 (6 to 16)	0.9685[a]
Sex			
Male	48	41	0.089[b]
Female	7	14	
ADI-R			
Domain A score, social	17.7 ± 7.62 (10 to 29)		
Domain BV score, communication	12.8 ± 5.62 (8 to 25)		
Domain C score, stereotype	4.1 ± 2.84 (3 to 12)		

Values are expressed as the mean ± SD (range).[a]*t*-test, [b]chi-square test. *ADI-R*, Autism Diagnostic Interview-Revised.

chloroform, the samples were centrifuged for 15 min at 12,000 g at 4°C. The upper aqueous phase was mixed with 1.5 volumes of 100% ethanol, transferred to a spin column, centrifuged, washed, and eluted in 14 μl RNase-free water.

Two microliters of each RNA sample was used for cDNA synthesis using the miScript II RT kit (QIAGEN). The reverse-transcription reaction mix (20 μl) was prepared using Hispec buffer (for selective conversion of mature miRNAs into cDNA), nucleics mix, RT mix and RNase-free water. The reaction mixture was incubated for 60 min at 37°C, followed by denaturation for 5 min at 95°C. Each cDNA was further diluted to 220 μl with RNase-free water and stored at −20°C until use.

microRNA screening

Initial screening was done using the Human Neurological Development & Disease miRNA PCR array (SABiosciences, Frederick, MD, USA), which contains 84 miRNA assays, and a custom-made array (SABiosciences) with 41 miRNA assays (see Additional files 1 and 2). For this purpose, 40 samples were chosen from 55 ASD subjects at random, and 40 matched control samples were selected. All the miRNAs included in the arrays have previously been reported to play a role in various aspects of brain development and function and/or in several neuropsychiatric conditions such as ASD. Both arrays included *C. elegans* miR-39 primer assays for internal normalization, snoRNA/snRNA (SNORD48, SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2) PCR control assays, positive PCR control (PPC) assays, and miRNA reverse transcription control (miRTC) assays.

RNA from age- and sex-matched control (n = 40) and ASD (n = 40) samples were pooled separately to generate four pools per group, with each pool consisting of 10 samples. Then, cDNA prepared from 3 μl of the pooled RNA was used for array screening. The expression of miRNAs was detected and quantified by means of SYBR Green reverse-transcription quantitative PCR performed with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Normalization

The data sets were first calibrated using a *C. elegans* miR-39 assay, which detected the spike-in control that was added to the serum samples during RNA extraction. This calibration would have resolved any differences in recovery that may have occurred during the purification procedure, or any differences in amplification efficiency. Due to the very low expression of snoRNA/snRNA PCR controls in the serum, three alternate normalization strategies, as recommended by the manufacturer, were used for data normalization. These were (i) normalization to the whole plate Ct mean, (ii) normalization to the plate Ct mean of commonly expressed (Ct < 30) miRNAs, and (iii) normalization to the Ct mean of at least four invariant miRNAs with little (<1) Ct variation between samples. In the third strategy, miR-125b-5p, miR-126-5p, miR-140-5p and miR-191-5p were chosen for the data normalization of the neurological array, whereas miR-103a-3p, miR-21-5p, miR-23a-3p, and miR-25-3p were chosen for the custom array. Consistent results were obtained using all three strategies.

Data analysis

An Excel-based miRNA PCR Array Data Analysis tool (SABiosciences; http://pcrdataanalysis.sabiosciences.com/mirna) was used for data analysis. SABiosciences makes use of the ΔΔCt method for the relative quantification of miRNAs. Student's *t*-test was used to examine any differential expression of miRNAs between the ASD and control groups; values of $P < 0.05$ were considered to indicate statistical significance (GEO Accession Number: GSE58850).

Quantitative reverse-transcription PCR

SYBR Green qPCR, performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems), was used for the validation of differentially expressed miRNAs (the accession ID and mature miRNA sequence are given in Additional file 3). In this validation experiment, all the samples (55 subjects with autism and 55 controls) were examined individually. Ten microliters of qPCR reaction mixture was prepared with a universal primer, primer assay and RNase-free water. All the qPCR reactions were performed in triplicate with the following cycling conditions: 95°C → 15 min, followed by 40 cycles of 94°C → 15 sec, 55°C → 30 sec and 70°C → 30 sec.

The Ct values of nine miRNAs (miR-101-3p, miR-106b-5p, miR-151a-3p, miR-195-5p, miR-19b-3p, miR-27a-3p, miR-320a, miR-328, and miR-489) were in the range of 25–30, while the remaining five miRNAs (miR-130a-3p, miR-181b-5p, miR-433, miR-572, and miR-663a) had Ct values in the range of 30 to 35.

Normalization

A randomly chosen control sample was amplified in each plate and used as an interplate calibrator to correct for the experimental differences among consecutive PCR runs. The qPCR data was first subjected to interplate calibration, followed by *C. elegans* miR-39 (detection of the spike-in control) calibration. The expression of miR-16, an miRNA highly abundant in the red blood cells, was analyzed in each sample to examine the extent of hemolysis in the serum. Any sample that showed significant hemolysis (the value of Ct invariant miRNA - Ct miR16 was greater than 5) was omitted from further analyses. Finally, the qPCR data were normalized to the average of three invariantly expressed miRNAs, let-7a, miR-

191-5p and miR-103a-3p. Among these, miR-191-5p and miR-103a-3p were selected on the basis of their performance as invariant miRNAs in the neurological array and custom array, respectively, while let-7a has been widely reported as an invariant miRNA in blood. The fold change in gene expression between the control and ASD groups was determined by the ΔΔCt method of relative quantification.

Statistical analysis

All statistical calculations were performed with PASW Statistics 18 software (IBM, Tokyo, Japan). Student's *t*-test and chi-square test were used to examine any variability in the distribution of age and sex, respectively, across the control and ASD groups. Any differential expression of miRNAs between the control and ASD groups was determined by Mann–Whitney test. The relationship between the expression of miRNA and ADI-R subscores was evaluated by Spearman's correlation coefficient. Analysis of covariance (ANCOVA) was used to control for potential covariates such as age and sex. Receiver Operating Characteristics (ROC) curve analysis was used for evaluating the diagnostic power of miRNAs.

Enrichment pathway analysis and target gene prediction

The DIANA mirPath v2.0 (http://diana.cslab.ece.ntua.gr/pathways/) functional annotation tool was used to predict the target genes and altered pathways of differentially expressed miRNAs. This tool predicts the miRNA targets based on DIANA-microT-CDS and/or experimentally verified targets from TarBase v6 (manually curated, experimentally validated miRNA-gene interactions database).

Results

microRNA screening

Ct values of the PPC controls were 19 ± 2 across all samples, indicating the uniformity of reaction conditions. The differences between the Ct values of PPC and miRTC were calculated as <7, indicating that there was no inhibition of the reverse-transcription reaction.

In the preliminary array screening, we observed an altered expression of 14 miRNAs in the ASD samples compared to those of controls (Figure 1). MiR-151a-3p, miR-181b-5p, miR-320a, miR-328, miR-433, miR-489, miR-572 and miR-663a were downregulated, while miR-101-3p, miR-106b-5p, miR-19b-3p, miR-195-5p, miR-130a-3p and miR-27a-3p were upregulated.

Confirmation with quantitative PCR

The differential expression of the 14 miRNAs was further validated by SYBR Green qPCR. We observed consistent results for all miRNAs except miR-27a-3p (Figure 2). miR-151a-3p (ΔΔCt = −2.01, P = 8.29E-06),

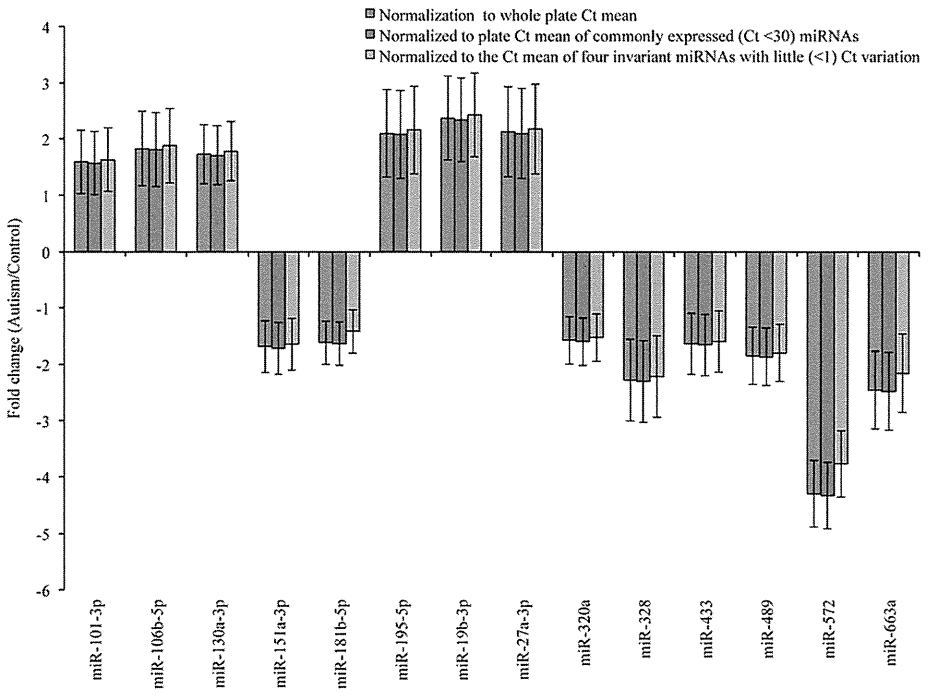

Figure 1 Results from preliminary screening experiments. The Ct values obtained were normalized and calculated by three different strategies. miR-151a-3p, miR-181b-5p, miR-320a, miR-328, miR-433, miR-489, miR-572 and miR-663a were downregulated while miR-101-3p, miR-106b-5p, miR-19b-3p, miR-195-5p, miR-130a-3p and miR-27a-3p were upregulated. Bars represent the fold change in subjects with autism as compared to controls.

MiR-181b-5p (ΔΔCt = −3.39, P = 1.04E-10), miR-320a (ΔΔCt = −2.47, P = 5.02E-12), miR-328 (ΔΔCt = −2.28, P = 4.33E-06), miR-433 (ΔΔCt = −2.33, P = 0.0001), miR-489 (ΔΔCt = −2.10, P = 1.25E-06), miR-572 (ΔΔCt = −2.47, P = 2.66E-08) and miR-663a (ΔΔCt = −2.06, P = 0.00002) were downregulated, while miR-101-3p (ΔΔCt = 1.43, P = 0.003), miR-106b-5p (ΔΔCt = 1.30, P = 0.008), miR-130a-3p (ΔΔCt = 2.35, P = 1.89E-09), miR-195-5p (ΔΔCt = 1.43, P = 0.0016) and miR-19b-3p (ΔΔCt = 1.87, P = 6.88E-09) were upregulated in the ASD individuals.

When the samples from the ASD individuals were examined for correlations between the expression of each miRNA and each of the three domains assessed by ADI-R (Domain A score, social; Domain score BV, communication; Domain C score, stereotype), none of the miRNA expressions was correlated with any of the domains (see Additional file 4).

The effects of age and sex on miRNA expression were examined by ANCOVA. The difference in the expression of miRNAs between the ASD and control groups remained significant even after adjusting for the effects of age and sex (see Additional file 5).

Receiver operating characteristic analysis

Receiver operating characteristic (ROC) curve analysis was used to evaluate the predictive power of differentially expressed miRNAs to distinguish between ASD individuals and controls. The analysis showed significant diagnostic values of these 13 differentially expressed miRNAs for ASD (Figure 3). High values for sensitivity, specificity and the area under the curve (AUC) were observed for five miRNAs: miR-181b-5p, miR-320a, miR-572, miR-130a-3p and miR-19b-3p (see Additional file 6).

Enrichment pathway analysis and target gene prediction

Using DIANA mirPath software, we found that the predicted target genes of the differentially expressed miRNAs could be involved in diverse vital neurological pathways (Figure 4). By a thorough analysis of the target genes and the pathways involving them, a total of 600 predicted genes and 18 neurological pathways were found (see Additional file 7). The top ten neurological pathways were those involved in axon guidance, TGF-beta signaling, MAPK signaling, adherens junction, regulation of actin cytoskeleton, oxidative phosphorylation,

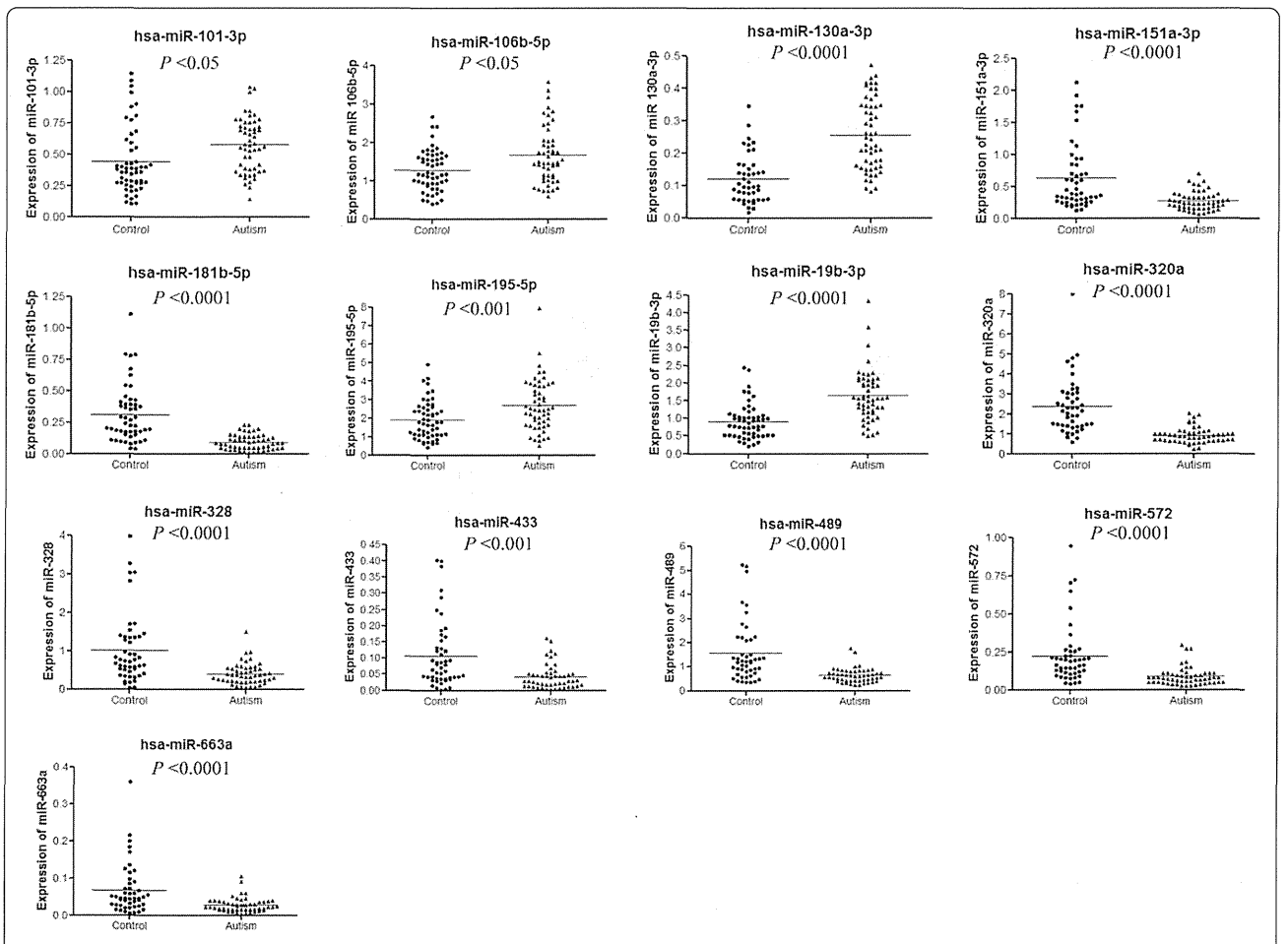

Figure 2 Scatter plot showing the differential expression of 13 microRNAs in autistic subjects.

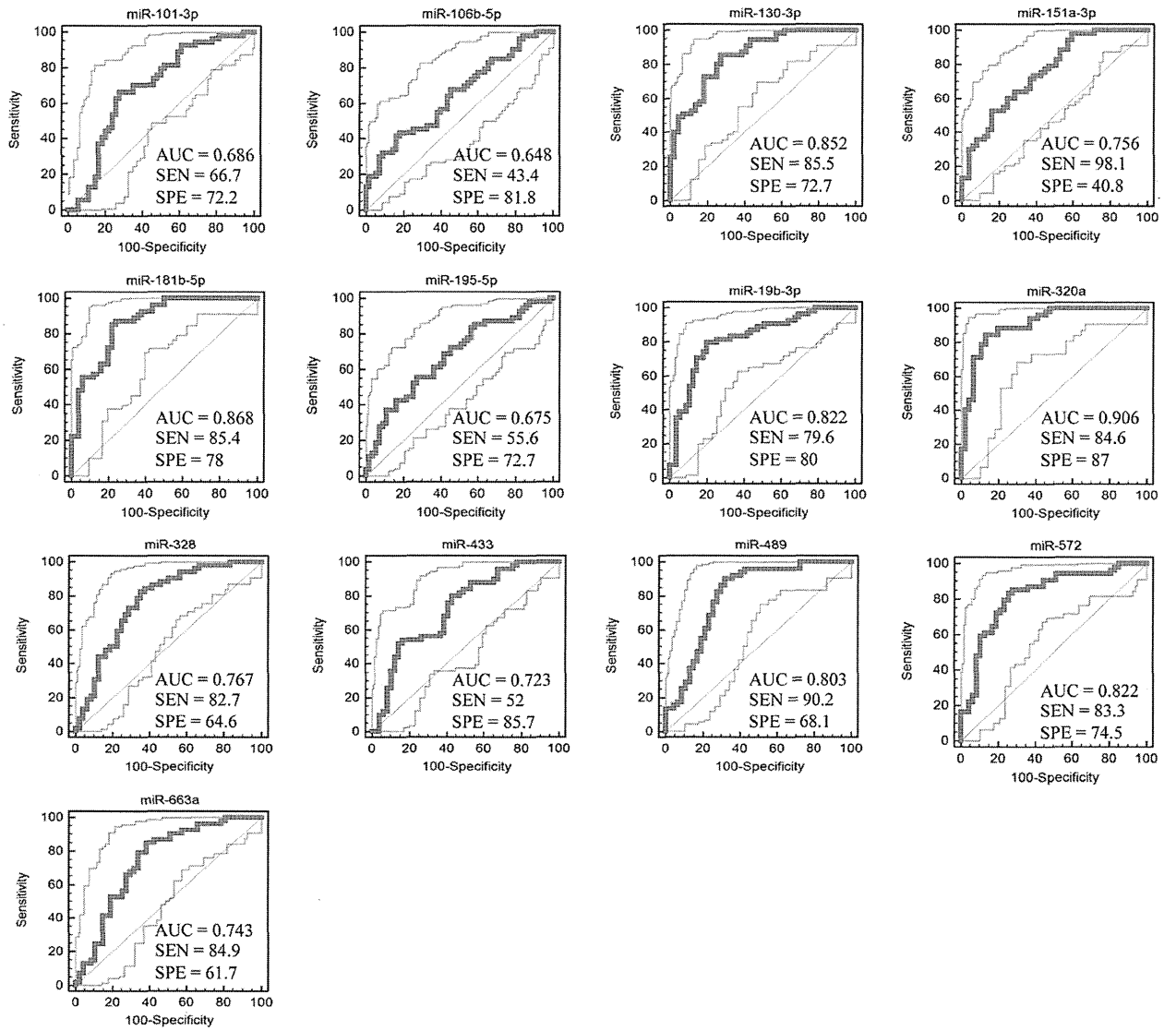

Figure 3 Receiver operating characteristic (ROC) curve of 13 microRNAs. AUC, area under the ROC curve; SEN, sensitivity; SPE, specificity.

hedgehog signaling, focal adhesion, mTOR signaling and Wnt signaling. No specific pathways were observed for miR-572.

Discussion

This is the first report on serum miRNAs in subjects with ASD. Altered expression of 13 miRNAs (downregulation of 8 miRNAs; upregulation of 5 miRNAs) was observed in our ASD subjects. Previous reports have shown a differential expression pattern of miRNAs in the postmortem brain [13] and in the lymphoblastoid cell lines of ASD individuals [14-16]. The results of the present and previous studies are summarized in Table 2, in which hsa-miR-181b-5p, hsa-miR-195-5p, hsa-miR-320a and hsa-miR-328 showed the same direction of regulation as in the brain [13] and lymphoblasts [14-16], while hsa-miR-106b-5p, hsa-miR-19b-30 and hsa-miR-663a did not. The reason for the latter differences in miRNA expression between the present and previous results is not known. The fact that the direction of alteration in the expression of hsa-miR-106b-5p in this study was the opposite of that reported in the previous postmortem study [13] suggests that the serum level of certain miRNAs may not reflect that in the brain, and thus that our findings should be treated with caution. However, it was interesting that hsa-miR-181b-5p and hsa-miR-328 in serum showed the same direction of regulation as in the brain. As mentioned above, serum miRNA expression is very stable, reproducible and resistant to RNase action [21]. In addition, an ANCOVA showed

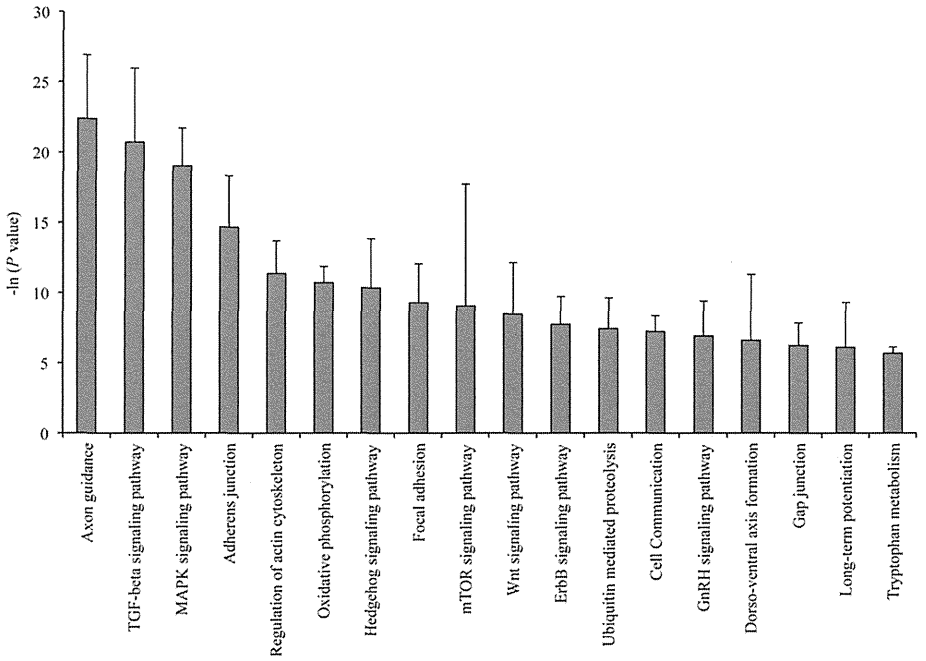

Figure 4 Neurologically relevant pathways (predicted by DIANA mirPath) of the target genes of differentially expressed microRNAs.

that confounding factors such as age and sex did not influence the results observed here. Therefore, hsa-miR-181b-5p and hsa-miR-328 in serum may become peripheral biomarkers reflecting the miRNA expression profile of individuals with ASD.

ROC curve analyses showed significant diagnostic values of 13 differentially expressed miRNAs for ASD (Figure 3). High values for sensitivity, specificity and area under the curve (AUC) were observed for five miRNAs: miR-181b-5p, miR-320a, miR-572, miR-130a-3p and miR-19b-3p (see Additional file 6). Therefore, these five miRNAs may be potential candidates for circulating miRNA-based prediction of ASD.

MiRNA can influence gene silencing via translational repression or mRNA degradation [24]. This mRNA destabilization may alter several downstream pathways and induce several noticeable effects [25]. A number of neurologically relevant pathways and target genes were identified by our enrichment analysis, with most of the target genes being involved in multiple pathways. Collectively, these results predicted several neurologically relevant canonical pathways for the target genes of the five miRNAs (miR-130a-3p, miR-19b-3p, miR-320a, miR181b-5p, and miR-572) that showed a good discriminative power in ROC analysis. Most of these genes and pathways have already been implicated in the pathogenesis of ASD [4,26-30].

The differentially expressed miRNAs in this study, which included miR-101, miR-106b, miR-130a, miR-151a, miR181b, miR-328, miR-433, miR-489 and miR-572, were previously reported to have altered expression in schizophrenia [31-35], supporting the contention that ASD and schizophrenia share common neurobiological features [36].

The detection of clinically useful noninvasive biomarkers that could allow early intervention for ASD is

Table 2 Comparison of the results of the present study and previous autism microRNA studies

miR ID	Present result	Previous report	Type of sample	Reference
hsa-miR-106b-5p	↑	↓	Brain	[13]
hsa-miR-181b-5p	↓	↓	Brain, lymphoblastoid cell line	[13,16]
hsa-miR-195-5p	↑	↑	Lymphoblastoid cell line	[15]
hsa-miR-19b-3p	↑	↓	Lymphoblastoid cell line	[14]
hsa-miR-320a	↓	↓	Lymphoblastoid cell line	[14]
hsa-miR-328	↓	↓	Brain	[13]
hsa-miR-663a	↓	↑	Lymphoblastoid cell line	[14]

(↑), upregulated, (↓), downregulated.

an important goal in ASD research. As an initial step toward this goal, our results suggest that serum miRNAs could be potential peripheral biomarkers of ASD. A limitation of this study is that the samples came from ASD individuals ranging from 6 to 16 years old, although ASD is an early-onset disorder. Therefore, to accurately evaluate the diagnostic power of circulating miRNAs in ASD, further studies on subjects of lower age will be necessary. Another limitation of the study is that we used the same sample set in both the screening and validation. It would have been more informative if we had screened an independent sample set.

Conclusions

This preliminary noninvasive study found a set of significantly differentially expressed miRNAs in the sera of children with ASD. The predicted target genes of these miRNAs were found to be associated with neurologically relevant pathways and functions.

Additional files

Additional file 1: miScript miRNA PCR Array Human Neurological Development and Disease (MIHS-107Z) 96 × 4 format.

Additional file 2: 384-Well Custom miScript miRNA PCR Array (CMIHS02055E) Template - 48 × 8 format.

Additional file 3: miRNA mature sequences with miRBase accession ID.

Additional file 4: Correlation between miRNA expression and Autism Diagnostic Interview-Revised (ADI-R) scores.

Additional file 5: ANCOVA analysis for checking the effect of age, sex, disease status and interaction between sex/status of differentially expressed miRNAs.

Additional file 6: Receiver operating characteristics (ROC) curve data showing the sensitivity and specificity of the 13 differentially expressed miRNAs.

Additional file 7: Predicted neurological pathways with the number of target genes and pathway ID.

Abbreviations

ADI-R: Autism Diagnostic Interview-Revised; ANCOVA: analysis of covariance; ASD: autism spectrum disorder; AUC: area under the curve; cDNA: complementary DNA; CI: confidence interval; Ct: threshold cycle; DSM-IV-TR: Diagnostic and Statistical Manual, Fourth Edition, Text Revision; MAPK: mitogen-activated protein kinase; miRNA: microRNA; miRTC: miRNA reverse transcription control; mRNA: messenger RNA; mTOR: mammalian target of rapamycin; PPC: positive PCR control; qPCR: quantitative polymerase chain reaction; ROC: receiver operating characteristics; SCID: Structured Clinical Interview for DSM-IV; SD: standard deviation; TGF: transforming growth factor; UTR: untranslated region.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MMV and AA carried out the molecular genetics studies and drafted the manuscript. IT and KI participated in the sequence alignment and helped to draft the manuscript. KY and TW analyzed data and performed statistical analysis. TT, MT, and TS evaluated and diagnosed participants, and are involved in revising the manuscript critically for clinical contents. KS and NM conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Perinatal asphyxia alters neuregulin-1 and COMT gene expression in the medial prefrontal cortex in rats

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ABSTRACT

Epidemiological studies suggest that perinatal complications, particularly hypoxia-related ones, increase the risk of schizophrenia. Recent genetic studies of the disorder have identified several putative susceptibility genes, some of which are known to be regulated by hypoxia. It can be postulated therefore that birth complications that cause hypoxia in the fetal brain may be associated with a dysregulation in the expression of some of the schizophrenia candidate genes. To test this, we used an animal model of perinatal asphyxia, in which rat pups were exposed to 15 min of intrauterine anoxia during Caesarean section birth, and examined the expression of mRNA of five of the putative susceptibility genes (NRG1, ErbB4, AKT1, COMT and BDNF) by real-time quantitative PCR in the medial prefrontal cortex (mPFC) and the hippocampus at 6 and 12 weeks after birth. The expression of NRG1 mRNA was significantly decreased in the mPFC, but not in the hippocampus, at 6 and 12 weeks after birth. In addition, a significant increase in COMT mRNA expression was observed in the mPFC at 12 weeks. The alteration in mRNA levels of NRG1 and COMT was not associated with a change in their protein levels. These results suggest that perinatal asphyxia may lead to disturbances in the PFC, which in turn may exert a long-lasting influence on the expression of specific genes, such as NRG1 and COMT. Our results also suggest that translational interruption may occur in this model of perinatal asphyxia.

1. Introduction

Schizophrenia is a chronic, severe, and disabling brain disorder that has affected people throughout history. Although the etiology of schizophrenia is not well understood, findings from epidemiological and neuropathological studies indicate that pathogenic processes that culminate in the development of schizophrenia are initiated early in life (Murray, 1994). The neurodevelopmental hypothesis that schizophrenia has its origin in aberrant brain development is supported by evidence that obstetric complications at or close to the time of birth contribute to the risk for the development of schizophrenia later in life (Byrne et al., 2007; Cannon et al., 2002).

The issue of perinatal asphyxia has been studied in a rodent model of global hypoxia during Caesarean section (C-section) birth (Bjelke et al., 1991). In this model, the intact uterus containing rodent pups is isolated from an anesthetized dam by a C-section on the expected day of birth and immersed in a water bath kept at 37 °C for 14–17 min to induce intra-uterine global hypoxia. Such global hypoxia can lead to alterations in central dopamine function during adulthood that are consistent with the postulated pathophysiology of schizophrenia (Boksa and El-Khodor, 2003). For instance, we have recently produced perinatal asphyxia in rat pups by exposing them to 15 min of intrauterine anoxia during C-section birth (Wakuda et al., 2008). When methamphetamine-induced locomotor activity was tested at adulthood (12 weeks after birth), it was greatly increased, accompanied by an increase of dopamine release in the nucleus accumbens. Such findings were not observed at adolescence (6 weeks after birth).

Accumulating evidence from recent studies has advanced our understanding of the neurobiology of schizophrenia. One of the major areas of progress has been the identification of putative susceptibility genes for schizophrenia, which has been made by family studies (Aoki-Suzuki et al., 2005; Chen et al., 2004; Lichtenstein et al., 2009; Yamada et al., 2004), national record linkage studies (Eaton et al., 2006; Ekelund et al., 2004; Numakawa et al., 2004), and emerging

Abbreviations: AKT1, V-akt murine thymoma viral oncogene homolog 1; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; C-section, Caesarean section; COMT, catechol-O-methyltransferase; ErbB4, v-erb-a erythroblastic leukemia viral oncogene homolog 4; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HP, hippocampus; NRG1, neuregulin-1; PBS, phosphate buffered saline; PFC, prefrontal cortex; qPCR, quantitative polymerase chain reaction.

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0278-5846/

evidence from a genome-wide association study (Schizophrenia Psychiatric Genome-Wide Association Study [GWAS] Consortium, 2011). Some of these candidate genes are responsive to hypoxia (Schmidt-Kastner et al., 2006). Neuregulin-1 (NRG1) has been one of the candidate genes most associated with an increased risk of schizophrenia (Gong et al., 2009; Munafò et al., 2008). NRG1 is a trophic factor that contains an epidermal growth factor (EGF)-like domain that signals by stimulating ErbB receptor tyrosine kinases. The functional NRG1 receptor v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErbB4) is expressed in the midbrain dopaminergic neurons in mice and primates (Abe et al., 2009; Zheng et al., 2009). Furthermore, the signaling of NRG1 and its receptor ErbB4 has been implicated in important neurodevelopmental processes of putative relevance to the aetiopathology of schizophrenia (Corfas et al., 2004; Fazzari et al., 2010; Hahn et al., 2006; Jaaro-Peled et al., 2009; Mei and Xiong, 2008). Both the catechol-O-methyltransferase (COMT) and brain-derived neurotrophic factor (BDNF) genes are also regulated by hypoxia (Schmidt-Kastner et al., 2006), and are associated with the dopamine system in the brain; COMT is an enzyme involved in the degradation of dopamine and BDNF is one of the trophic factors involved in the development of dopaminergic neurons (Baquet et al., 2005). V-akt murine thymoma viral oncogene homolog 1 (AKT1), a component of the downstream signaling pathways of both NRG1/ErbB4 and BDNF, is also one of the schizophrenia candidate genes that are regulated by hypoxia (Emamian et al., 2004; Schmidt-Kastner et al., 2006; Thiselton et al., 2008). Moreover, AKT1 mediates dopaminergic neurotransmission (Beaulieu et al., 2005). Nicodemus et al. (2010) have suggested that NRG1/ErbB4 and AKT1 signaling is implicated in the pathogenesis of schizophrenia.

To obtain insight into the delayed alterations in central dopamine function during adulthood that are consistent with the postulated pathophysiology of schizophrenia, we used an animal model of rats born by C-section and exposed to additional global hypoxia (Wakuda et al., 2008). Subsequently, we examined the mRNA expression levels of the five schizophrenia candidate genes described above (NRG1, ErbB4, AKT1, COMT and BDNF) in the prefrontal cortex (PFC) and the hippocampus (HP), in addition to their protein levels in the PFC, at two developmental periods, adolescence (6 weeks of age) and adulthood (12 weeks of age).

2. Methods

2.1. Animals and induction of perinatal asphyxia

All experiments were performed in accordance with the Guide for Animal Experimentation of the Hamamatsu University School of Medicine. Intrauterine anoxia was induced in rats delivered by C-section according to the method described originally by Bjelke et al. (1991). Pregnant female Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) within the last day of gestation were anesthetized by diethyl ether and hysterectomized. The uterus, including fetuses, was placed in a water bath at 37 °C for induction of 15 min of asphyxia. Rats that had delivered normally on the day of the experiment were used as surrogate mothers. Each surrogate mother received four pups from another surrogate mother, four C-section-delivered and four asphyxia-exposed pups. At 3 weeks after birth, male rats were selected for the experiments described below and were housed three per cage in a temperature- and humidity-controlled colony room, which was maintained on a 12-h light/dark cycle (07:00 to 19:00 h lights on) and with food and water provided ad libitum. The animals were divided into 2 groups in terms of delivery: birth by C-section alone (C group, $n = 28$) or by C-section plus 15 min of global perinatal asphyxia (A group, $n = 28$).

2.2. RNA preparation

To determine the target gene expression levels, 12 animals (6-week-old, $n = 6$; 12-week-old, $n = 6$) from each of the two groups were used. All the animals were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and rapidly decapitated. The bilateral medial PFC (mPFC) and the whole HP were dissected on ice and used for the polymerase chain reaction (PCR) quantification analysis. The region of mPFC was defined as the cingulate (Cg), prelimbic (PrL) and infralimbic (IL) cortices according to the atlas of Paxinos and Watson (1997) (Fig. 1).

Total RNAs from rat mPFC and HP tissue were isolated from brain tissues using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). The RNAs were cleaned up using RNeasy Mini Kit and DNase set (Qiagen, Hilden, Germany). The quality and quantity of RNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Yokohama, Japan). RNA with a 260/280 nm ratio in the range of 1.8–2.0 was considered high quality. The complementary DNAs (cDNAs) from the RNA preparations were synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies).

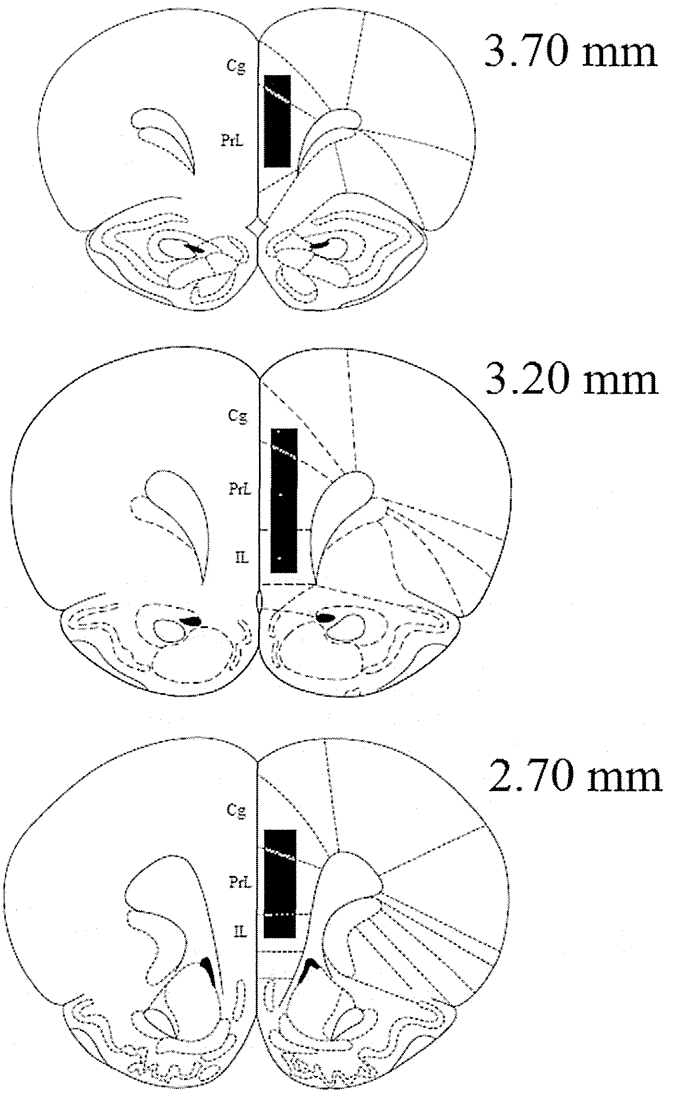

Fig. 1. Schematic representation of the rat brain regions analyzed for real-time qPCR. The brain regions of mPFC sampled for analysis are indicated by the filled rectangles. The drawings were made according to the atlas of Paxinos and Watson (1997), in a caudal to rostral distribution using the bregma (mm) as a reference point.

2.3. Real-time quantitative PCR conditions

Real-time quantitative PCR (qPCR) was performed using an ABI PRISM 7700 Sequence Detection System in combination with continuous SYBR Green detection (Applied Biosystems, Foster City, CA). Real-time qPCR was performed in a 25 μl reaction volume containing 2.5 μl cDNA, 12.5 μl SYBR Green PCR Master Mix (Qiagen), 2.5 μl each of the sense and antisense primers (10 μM), and 5 μl H_2O. The general PCR condition profile was as follows: polymerase activation at 95 °C for 15 min, followed by 50 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. After amplification, a melting curve was acquired to determine the optimal PCR conditions by heating the PCR products at 20 °C/s to 95 °C, then cooling at 20 °C/s to 60 °C. Primer sequences for qPCR amplification were designed using online Primer3 software (http://biocore.unl.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of primers and sizes of PCR amplicons are listed in Table 1.

The ΔΔCt method (comparative Ct method) (Livak and Schmittgen, 2001) was employed for the diagnostic assays. The Ct values were calculated using three kinds of housekeeping genes (glyceraldehydes-3-phosphate dehydrogenase [GAPDH], cyclophilin A and β-actin). The housekeeping genes fulfilled the criterion that the absolute value of the slope of the log input amount vs. ΔCt should be <0.1, which was selected as the internal reference value.

2.4. Western blot analysis

To determine the protein quantity, we used another set of 16 animals (6-week-old, $n = 8$; 12-week-old, $n = 8$) from each of the two groups. Total protein from the rat mPFC tissue was quantified with a Pierce BCA Protein Assay kit (Thermo Scientific, Barrington, IL). GAPDH was used as the internal control to normalize the expression of proteins. We used the following primary antibodies for western blot detection: ErbB4 (ab32375), COMT (ab126618), GAPDH (ab8245) (all from Abcam, Tokyo, Japan), NRG1 (sc-348), BDNF (sc-546) (both from Santa Cruz Biotechnology, Santa Cruz, CA), and AKT1 (#2938) (Cell Signaling Technology, Danvers, MA). Fluorescent-labeled anti-rabbit and anti-mouse secondary antibodies (Rockland, Gilbertsville, PA) were used for the detection of proteins with an Odyssey Infrared Imaging System (Li-cor Bioscience, Lincoln, NE). Representative images of the western blots are shown in Fig. 2.

2.5. Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). In the RT-PCR analysis, the values for the A group are relative to the mean of the corresponding values of the C group, which was a reference set as 100. In the Western blot analysis, the values indicate the relative protein levels normalized to GAPDH expression. Differences in the gene and protein expression levels between the A and C groups were determined by Student's *t*-test as well as two-way analysis of variance (ANOVA), in which treatment (C group and A group) and age (i.e. 6-week-old and 12-week-old) were the independent variables. In the two-way ANOVA, the interaction between treatment and age was also tested. We used SPSS version 22J (IBM Corp., Tokyo) for the statistical analyses. The level of statistical significance was set at $p < 0.05$.

3. Results

Significant changes in the mRNA levels of NRG1 and COMT were observed in the mPFC. The mRNA levels of NRG1 in the A group were significantly decreased at 6 (16% decrement) ($t = -2.24$, $df = 10$, $p = 0.049$) and 12 weeks of age (24% decrement) ($t = -2.61$, $df = 10$, $p = 0.026$), compared with those in the C group (Table 2). There were significant main effects of treatment [$F_{(1,20)} = 11.771$; $p = 0.003$] and age [$F_{(1,20)} = 30.235$; $p < 0.001$], but no significant interaction between treatment and age [$F_{(1,20)} = 0.510$; $p = 0.483$]. Compared with those of the C group, the A group rats showed a significant increase in the mRNA expression levels of COMT at 12 weeks, but not 6 weeks (48% increment) ($t = 2.80$, $df = 10$, $p = 0.019$) (Table 2). There were significant main effects of treatment [$F_{(1,20)} = 8.313$; $p = 0.009$] and age [$F_{(1,20)} = 49.079$; $p < 0.001$], but no significant interaction between treatment and age [$F_{(1,20)} = 0.412$; $p = 0.528$]. There was no significant difference in the mRNA expression levels for the remaining three genes (ErbB4, AKT1, and BDNF) at 6 or 12 weeks. In the HP, there was no overt change in the mRNA expression for the five genes tested between the A and C groups, at either 6 or 12 weeks after birth.

There was no apparent change in the protein quantity for the five genes tested between the A and C groups, at either 6 or 12 weeks after birth (Table 3).

4. Discussion

We evaluated the mRNA levels of five genes that are related to the regulation of the dopaminergic system (Abe et al., 2009; Baquet et al., 2005; Beaulieu et al., 2005; Zheng et al., 2009), NRG1, ErbB4, AKT1, COMT and BDNF, in the mPFC and HP in rats born by C-section with added global hypoxia at two developmental periods, adolescence (6 weeks of age) and adulthood (12 weeks of age). Among the five genes, apparent changes were observed in two genes, NRG1 and COMT: expression of the mRNA of NRG1 was decreased at 6 and 12 weeks, while expression of the mRNA of COMT was increased at 12 weeks after delivery. These changes were observed in the mPFC, but not the HP. Since both NRG1 and COMT are strongly implicated in

Table 1
Primer sequences and length of PCR products for each gene used in the real-time quantitative PCR.

Genes		Primer sequences (5′-3′)	Bases	Amplicon size
NRG1	Forward	GAGTCAGTTCAAGAGCCCGTTAA	1926–1948	69 bp
	Reverse	GCCATTGGGCTTGGTTCTTT	1975–1994	
ErbB4	Forward	TGATTGCAGCCGGAGTCAT	1988–2006	72 bp
	Reverse	TGACATAAACGGCAAATGTCAGA	2037–2059	
AKT1	Forward	GAACGACGTAGCCATTGTGA	45–64	101 bp
	Reverse	AGGTGCCATCATTCTTGAGG	126–145	
COMT	Forward	ATCTTCACGGGGTTTCAGTG	1272–1291	145 bp
	Reverse	GAGCTGCTGGGGACAGTAAG	1397–1416	
BDNF	Forward	AAGGCTGCAGGGGCATAGAC	1247–1266	111 bp
	Reverse	TGAACCGCCAGCCAATTCTC	1338–1357	
GAPDH	Forward	GACATGCCGCCTGGAGAAAC	805–824	92 bp
	Reverse	AGCCCAGGATGCCCTTTAGT	877–896	
Cyclophilin A	Forward	GTCTGCTTCGAGCTGTTTGC	100–119	80 bp
	Reverse	AATCCTTTCTCCCCAGTGCT	160–179	
Actin β	Forward	CGTGAAAAGATGACCCAGATCA	427–448	90 bp
	Reverse	AGAGGCATACAGGGACAACACA	495–516	

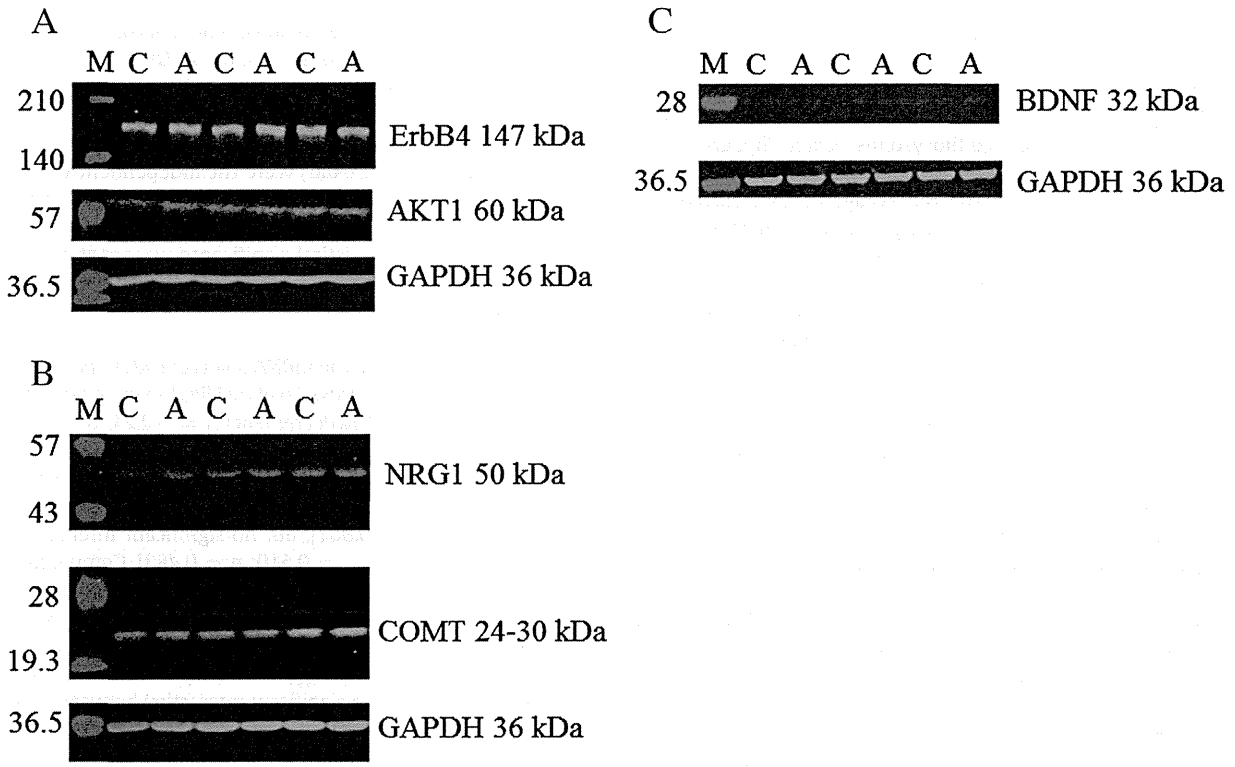

Fig. 2. A representative gel showing the protein levels of ErbB4, AKT1, NRG1, COMT, and BDNF in the mPFC brain region. GAPDH was used as the housekeeping protein. The molecular weight of the protein marker is shown in kDa. (A) 7.5% gel; ErbB4, 147 kDa; AKT1, 60 kDa. (B) 10% gel; NRG1, 50 kDa; COMT, 24–30 kDa. (C) 15% gel; BDNF, 32 kDa. M, molecular weight marker; Lane C, mPFC of the rats delivered by cesarean section (C group); Lane A, mPFC of the rats delivered by cesarean section with 15 min anoxia (A group).

schizophrenia at both the gene and transcript levels, our results further support the general concept that perinatal asphyxia interacts with the genes for schizophrenia (Schmidt-Kastner et al., 2006).

It is not known when the levels of the expression of NRG1 mRNA begin to decrease. Although there are no available reports in this regard, in the present study the reduction of the gene mRNA levels was observed at two developmental periods, i.e., 12 and 6 weeks after birth. Therefore, alterations of the expression of NRG1 mRNA may be a long-lasting phenomenon. Our data may not be comparable with those of a previous study in which 6-day-old rat pups were exposed to a hypoxia condition (an air mixture of 8% O_2, 0.03% CO_2, and balance N_2 for 120 min), and then protein levels of the NRG1α isoform in the frontal cortex were measured in 56-day-olds, and found to be elevated (Nadri et al., 2007). A similar inconsistency is found among the postmortem studies of schizophrenic patients. Bertram et al. (2007) reported NRG1α isoform protein reductions in the PFC of schizophrenic patients. However, isoform-specific increases in NRG1 mRNA expression in the PFC have also been observed in schizophrenic patients (Hashimoto et al., 2004; Law et al., 2006, 2007; Silberberg et al., 2006). The inconsistent results may be due to the use of different samples, i.e., patients or various animal models. Nonetheless, all the results from animal and patient studies seem to show that aberrant signals of NRG1 may play roles in schizophrenia, because both mutant mice deficient in NRG1 and transgenic mice overexpressing NRG1 are known to exhibit schizophrenia-like phenotypes (Ehrlichman et al., 2009; Kato et al., 2010; O'Tuathaigh et al., 2007, 2010).

In contrast to NRG1 mRNA levels in the mPFC, which were altered at 6 and 12 weeks after delivery, COMT mRNA levels in the mPFC

Table 2
Normalized relative mRNA expression in rats born by the C-section with or without global perinatal asphyxia.

Gene	mPFC C group	mPFC A group	p value	HP C group	HP A group	p value
6 weeks of age						
NRG1	100 ± 9.6	83.5 ± 6.7	.049*	100 ± 13.1	86.8 ± 7.4	.204
ErbB4	100 ± 19.1	89.3 ± 23.2	.599	100 ± 20.9	96.8 ± 17.1	.865
AKT1	100 ± 13.1	108.5 ± 8.7	.473	100 ± 5.0	101.0 ± 4.9	.917
COMT	100 ± 22.6	128.4 ± 10.7	.180	100 ± 13.8	113.6 ± 14.1	.373
BDNF	100 ± 7.2	104.0 ± 11.3	.719	100 ± 5.2	113.0 ± 6.5	.059
12 weeks of age						
NRG1	100 ± 8.5	75.8 ± 12.7	.026*	100 ± 15.6	91.5 ± 9.9	.505
ErbB4	100 ± 13.5	116.1 ± 16.8	.341	100 ± 10.0	92.1 ± 15.2	.529
AKT1	100 ± 22.1	79.9 ± 14.6	.249	100 ± 5.9	90.6 ± 5.3	.104
COMT	100 ± 16.1	148.0 ± 12.3	.019*	100 ± 14.1	95.5 ± 7.5	.689
BDNF	100 ± 11.3	114.0 ± 12.7	.293	100 ± 4.8	91.7 ± 9.5	.268

Values are expressed as the means ± SEM. $N = 6$ animals in each group. Note a statistically significant difference, $^*p < 0.05$.

Table 3
Normalized protein levels of the mPFC of rats delivered by C-section with or without global perinatal asphyxia.

Protein	mPFC C group	mPFC A group	p value
6 weeks of age			
NRG1	0.323 ± 0.014	0.321 ± 0.014	.950
ErbB4	0.558 ± 0.027	0.600 ± 0.021	.239
AKT1	0.479 ± 0.019	0.504 ± 0.009	.253
COMT	0.335 ± 0.012	0.341 ± 0.017	.768
BDNF	0.410 ± 0.009	0.398 ± 0.012	.428
12 weeks of age			
NRG1	0.324 ± 0.014	0.331 ± 0.010	.670
ErbB4	0.559 ± 0.025	0.544 ± 0.021	.651
AKT1	0.466 ± 0.021	0.450 ± 0.014	.540
COMT	0.343 ± 0.015	0.356 ± 0.011	.462
BDNF	0.386 ± 0.008	0.396 ± 0.008	.385

Values are expressed as the means ± SEM. Values indicates the relative protein levels normalized to GAPDH expression. $N = 8$ animals in each group.

increased at 12 weeks with no change at 6 weeks. These results may be compatible with our previous study employing the same perinatal model, in which we showed that methamphetamine-induced locomotor activity can be greatly increased at 12 weeks, with no change at 6 weeks after birth (Wakuda et al., 2008). Dopaminergic projections from the ventral tegmental area to the cortex exhibit marked postnatal maturation (Rosenberg and Lewis, 1995; Tseng et al., 2007). Until young adulthood, the concentration of dopamine continues to increase in the PFC (Lambe et al., 2000; Rosenberg and Lewis, 1995). The delayed occurrence of the increase in COMT mRNA levels may reflect functional impairment of the maturation of the mesocortical dopaminergic projections. However, the degree to which the disturbance of the mesocortical dopaminergic system affects the methamphetamine-induced locomotor activity (Wakuda et al., 2008) is unclear from the current study. In a previous postmortem study, COMT mRNA overexpression due to promoter hypomethylation was observed in the frontal lobes of patients with schizophrenia (Abdolmaleky et al., 2006). Since perinatal asphyxia may change the methylation patterns of gene promoters (Herrera-Marschitz et al., 2011; Kumral et al., 2013), epigenetic modulation may be involved in the increase in COMT mRNA observed in the mPFC of animals experiencing perinatal asphyxia.

In a previous study, we used the same animal model of perinatal asphyxia as used in the present study, and assessed behaviors using a methamphetamine-induced locomotion test at 6 and 12 weeks after birth. At 6 weeks, there was no change in the methamphetamine-induced locomotion. However, at 12 weeks, we found an elevation in methamphetamine-induced locomotor activity (Wakuda et al., 2008). This previous observation suggests that perinatal asphyxia can cause delayed alterations in central dopamine function. The present results suggest a possible explanation for this phenomenon. In this study, decreased NRG1 mRNA levels in the mPFC were observed at 6 and 12 weeks after birth. Since the NRG1-ErbB signaling cascade is involved in the regulation of neurodevelopment and neurotransmission, including via dopamine pathways (Roy et al., 2007), an interruption in the development of dopamine systems in the mPFC may begin at least from adolescence (at 6 weeks) and persist during adulthood (at 12 weeks). The present study also showed increased COMT mRNA levels in the mPFC at 12, but not 6, weeks after birth. Since COMT is critically involved in the degradation of synaptic dopamine (Gogos et al., 1998), an increase of dopamine turnover in the mPFC may emerge during adulthood (at 12 weeks). Taken together, these results suggest that the immaturity of the mesocortical dopamine system in the peripheral asphyxia model might arise through a disturbance of the NRG1-ErbB signaling cascade, in addition to enhancement of the COMT function during adulthood. Such a mechanism might be responsible for the delayed appearance of dopamine dysfunction, as represented by the exhibition of aberrant behavior after methamphetamine during adulthood, but not in adolescence (Wakuda et al., 2008).

The HP is one of the brain regions which have been shown to be vulnerable to hypoxia. Indeed, in our previous study using the same animal model, we observed a decrease in the number of dentate granule cells in the hippocampus at 12 weeks (Wakuda et al., 2008). New neurons are continuously generated in the dentate gyrus throughout the adult life of a variety of mammals (Eriksson et al., 1998), and all of the 5 genes tested here, NRG1, ErbB4, AKT1, COMT and BDNF, appear to increase the growth and development of newly born dentate granule cells. NRG1 induces an increase in cell proliferation and migration in the adult dentate gyrus (Mahar et al., 2011), while ErbB4 plays a fundamental role in controlling NGR1-induced migration (Gambarotta et al., 2004). AKT1-knockout mice have lower levels of cell proliferation in the dentate gyrus (Balu et al., 2012), indicating that AKT1 can activate neurogenesis in dentate granule cells. It has been proposed that dopamine modulates the maturation of newly born dentate granule cells (Mu et al., 2011). BDNF is strongly expressed in the dentate granule cells, and increases neurogenesis in the adult dentate gyrus (Scharfman et al., 2005). In spite of evidence that the 5 genes are particularly well-documented pro-proliferative factors, there was no apparent change in mRNA levels of any of the 5 genes. Therefore, the decrease in the number of dentate granule cells observed in the previous study may have been caused by factors other than the 5 genes.

Although proteins, as the end products of gene expression, are the major executors of biological processes, we observed no change in the protein levels of NRG1 and COMT in the mPFC at 6 and 12 weeks. That the mRNA levels of NRG1 and COMT were altered without any change in the protein levels could suggest that the protein translation is interrupted during steady-state measurement in perinatal asphyxia model. A similar discordance between mRNA and protein levels has been reported in schizophrenic patients (Baracskay et al., 2006). Alternatively, the protein may be less abundant by steady-state measurement in a perinatal asphyxia model. Whatever the reason, the mismatch in the mRNA and protein levels may still provide important information in regard to the pathophysiology of the perinatal asphyxia model. The decreased NRG1 mRNA levels without a corresponding change in the protein levels may have a lower rate of degradation, leading to down-regulation of the NRG1 signal, while the increased COMT mRNA levels without a change in the protein levels may have a higher rate of degradation, causing the up-regulation of the COMT signal. Such altered metabolic mechanisms in the perinatal asphyxia model might partially contribute to the potentiated responsiveness to methamphetamine (Wakuda et al., 2008). Clearly, further work on the relationship between mRNA levels and the corresponding protein expressions will be needed to gain additional insights into the detailed pathophysiology of schizophrenia.

There were limitations in the present study. The multiple comparisons of five genes in two regions and the relatively small sample size, which might have caused type I and type II errors, respectively, render the data presented here preliminary. In addition, we only tested two developmental periods, i.e., 6 and 12 weeks after birth. Given the neurodevelopmental hypothesis for schizophrenia and the fact that the genes examined here have been shown to be regulated by hypoxia (Schmidt-Kastner et al., 2006), further studies of the earlier periods of development (from birth to 6 weeks of life) would be informative.

5. Conclusion

The findings from the current study suggest that perinatal asphyxia may induce the alteration of both NRG1 and COMT mRNA expression in the mPFC, which may account for the dysfunction in the mesocortical dopaminergic system in schizophrenia. Our findings of altered gene expressions associated with exposure to hypoxia during the perinatal period may provide an explanation for the epidemiological evidence that a history of perinatal hypoxia is a risk factor for the development of schizophrenia. This animal model was thus found to be useful for revealing the pathogenesis of schizophrenia, especially at the cellular and molecular levels.

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特集2

自閉症の地域生活を支える

自閉症スペクトラムの人たちには、生涯を通じてなにかしらの支援が必要とされます。しかしながら、本人や家族は、地域のどこに、だれに、その特性や必要に合わせた支援を求めればいいのか、その入り口すら見当たらないということもあるようです。自閉症のある人たちの生活を地域でどのように支えていくのか、横浜市と狛江市、それぞれの都市のサイズに合わせた取り組みから考えてみたいと思います。

横浜市　発達障害の人へのひとり暮らしに向けた支援 ～サポートホーム事業から～

特定非営利活動法人PDDサポートセンター
グリーンフォーレスト　サポートホーム事業　コーディネーター　浮　貝　明　典

Ⅰ　「生活」と生活支援の現状と取り組み

人の一日は、「生活」という勉強や仕事などの日中活動以外の時間を合わせて初めて成り立っています。通常、一日の「生活」は家庭で担われていますが、グループホームなど家庭とは異なる「生活」の場ができ、家族ではない第三者が「生活」にかかわることで、日中活動との密接さが明らかになります。当法人の運営する地域活動支援センター「オフィスウイング」「ウイングネクスト」では、知的に遅れがないとされている高機能自閉症・アスペルガー障害の人たちの就労移行支援をおこなっており、学校卒業後の進路や、就労に向けたステップアップとして利用されています。日々のかかわりの中で、スムーズに就労に結びつく人がいる一方、本センターに安定的に通うことさえ難しい人もいます。そして、この後者の背景に「生活」が影響していることが少なくなかったのです。

発達障害に特化した社会資源の少なさ、本人とまわりの困り感の違い、自分に支援が必要だと気づきにくい特性からも、そもそも生活支援の必要性は認識されにくく、支援に繋がりにくい状況があります。そのため、問題が起きてから社会資源に繋がってくる状況も多く、問題が起きる前からかかわる予防的な支援が必要とされています。また、家族や本人から聞き取りをしていく中では、既存の入所施設や共同生活型のグループホームの利用はイメージしにくいのだけれど、何の準備もないままひとり暮らしをすることにも不安があるという状況もありました。

前述の現状と課題を踏まえ、当法人では、2009年から約２年間の横浜市発達障害者支援開発モデル事業を経て、2012年11月から横浜市より、発達障害者サポートホーム事業（以下サポートホーム事業）の委託を受け、成人期の発達障害の人の予防的な生活支援の取り組みを開始しています。

また、サポートホーム事業と連動して、厚生労働科学研究「成人期以降の発達障害者の相談支援・居住空間・余暇に関する現状把握と生活適応に関する支援についての研究（主任研究者・中京大学・辻井正次）平成24年４月～」の分担研究を受託し、NPO法人アスペエルデの会（名古屋）、滋賀県社会福祉事

業団（現・社会福祉法人グロー）と共に、発達障害の成人期以降の支援の基本的なモデルを提案するとともに、現状ある福祉サービスに、新しい効果的なメニューを提案していくことを目指しています。

II　サポートホーム事業の概要

サポートホーム事業は、発達障害の人が主に在宅から、地域でのひとり暮らしを目指すための準備段階として、最長2年間、仮のひとり暮らしを経験する場です。（図1参照）暮らしの場は、グループホーム・イオプレイス（1Kアパートタイプ）としています。支援者は各入居者の部屋を訪問し、地域でのひとり暮らしに向けたスキルアップ、相談、アセスメントをおこない、その準備から地域移行後のフォローアップまでをおこなっています。（図2参照）また、ひとり暮らしへの移行を前提としない50日以内限定の体験部屋もあります。体験部屋には、衣類と食材以外は全て用意されており、一旦実家を離れてひとりで生活するとはどういうことか、どうなる

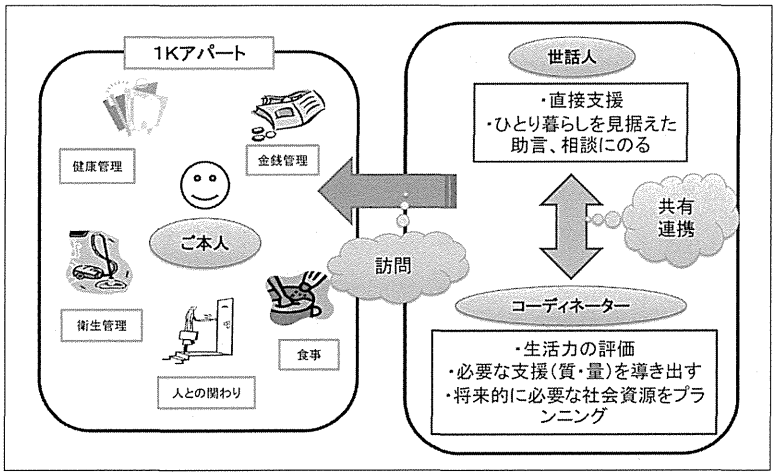

図1　GHイオプレイスでの暮らし

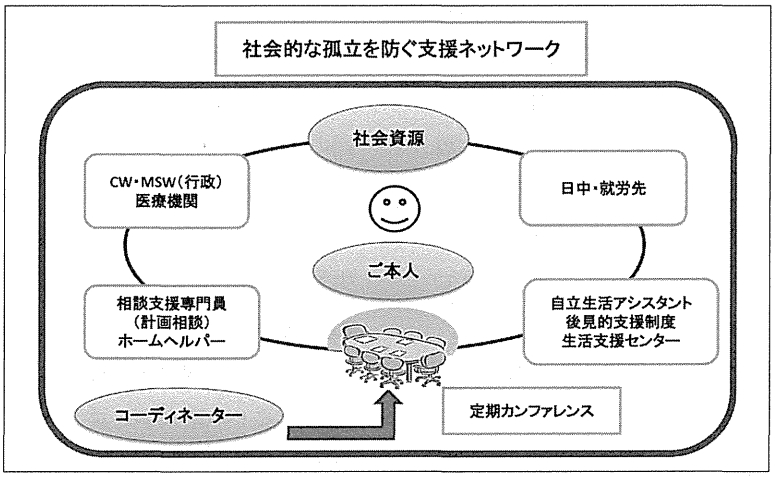

図2　地域でのひとり暮らしへ

のかをその名の通り体験できます。

サポートホーム事業の対象者は以下の通りです。（図３参照）また、入居の条件として、横浜市在住であり、就労または日中の活動場所に安定的に通えている発達障害の人、としています。

III　入居に至るまで

入居前の聞き取りをおこなうにあたって、発達障害の人にはスキル（能力）としての「できる」「できない」（CAN）ではなく、今現在「やっている」「やっていない」（DO）で聞き取る方が有効です。発達障害の人の多くは、たとえば「洗濯はできますか」と聞けば「できます」と答えます。「洗濯は週何回やっていますか」と聞くと、「やっていません」と答える人が多いのです。発達障害の人に対して、能力ではなく意欲（やる必要性を感じて行動に繋がる）の変化をみていくことが重要ではないかと思うのです。

聞き取りは、入居日の約１ヵ月前から週１程度の頻度でおこなっていきます。たとえば、朝昼夜の食生活をどうしていきたいか、自炊なのか弁当なのか外食なのか、１週間の、おおよその本人の希望や予定を聞き取ります。掃除や洗濯の頻度、予算立てなども同様に聞き取り、一緒に生活プランを立てていきます。地域で暮らすための最低限のルールやマナーは伝えますが、支援者がお膳立てしすぎたり、支援者が決めた生活を送ってもらうのではなく、本人が主体的に決めた生活をまずはしてもらうことで、本人がどんなことに困るのか、どんなところにサポートが必要なのかをみていくようにしています。

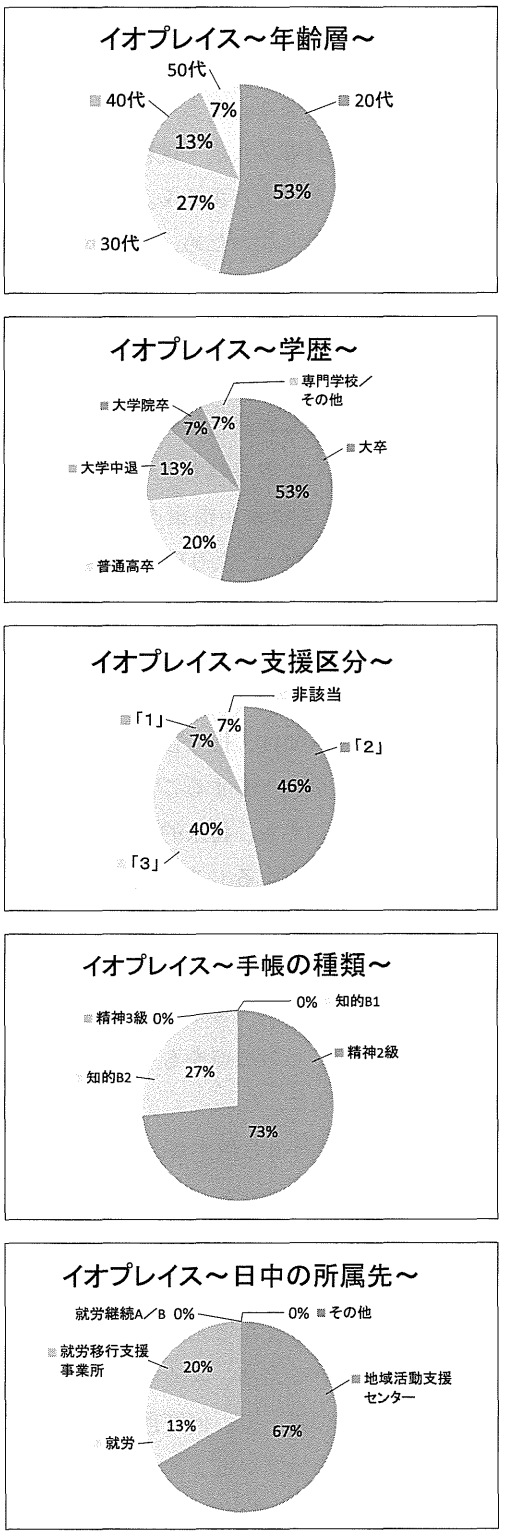

図３　イオプレイス