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ORIGINAL RESEARCH

Adolescent Mouse Takes on An Active Transcriptomic Expression During Postnatal Cerebral Development



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Abstract Postnatal cerebral development is a complicated biological process precisely controlled by multiple genes. To understand the molecular mechanism of cerebral development, we compared dynamics of mouse cerebrum transcriptome through three developmental stages using high-throughput RNA-seq technique. Three libraries were generated from the mouse cerebrum at infancy, adolescence and adulthood, respectively. Consequently, 44,557,729 (infancy), 59,257,530 (adolescence) and 72,729,636 (adulthood) reads were produced, which were assembled into 15,344, 16,048 and 15,775 genes, respectively. We found that the overall gene expression level increased from infancy to adolescence and decreased later on upon reaching adulthood. The adolescence cerebrum has the most active gene expression, with expression of a large number of regulatory genes up-regulated and some crucial pathways activated. Transcription factor (TF) analysis suggested the similar dynamics as expression profiling, especially those TFs functioning in neurogenesis differentiation, oligodendrocyte lineage determination and circadian rhythm regulation. Moreover, our data revealed a drastic increase in myelin basic protein (MBP)-coding gene expression in adolescence and adulthood, suggesting that the brain myelin may be generated since

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mouse adolescence. In addition, differential gene expression analysis indicated the activation of rhythmic pathway, suggesting the function of rhythmic movement since adolescence; Furthermore, during infancy and adolescence periods, gene expression related to axon repulsion and attraction showed the opposite trends, indicating that axon repulsion was activated after birth, while axon attraction might be activated at the embryonic stage and declined during the postnatal development. Our results from the present study may shed light on the molecular mechanism underlying the postnatal development of the mammalian cerebrum.

Introduction

Cerebral development is important in shaping the senior nervous center, which controls movements, generates feelings and facilitates other brain functions. There is an increasing interest on gene regulation during postnatal cerebral development. Traditional gene knockout assay and differentially-expressed gene (DEG) expression assay have been performed to identify key regulatory genes. For example, epidermal growth factor receptor *ErbB* was reported to regulate thyroid hormones during the cerebral development [1–4]; anti-oncogene *Pten* was found to play important roles in growth, proliferation, polarization of nerve cells and differentiation of synapse [5–9]; brain-derived neurotrophic factor (BDNF) was also illustrated to regulate the growth, development, apoptosis and reparation of nerve cells as a neurotrophin (NT) [10–13]. Moreover, lots of protein families functioned as chemotactic factors, such as netrin and slit [14–17]. Several studies on gene regulatory network using DNA chip and serial analysis of gene expression (SAGE) have also been performed to identify regulatory genes in the cerebral development [18,19]. With the development of high-throughput sequencing techniques, Allen Institute for Brain Science presented the Allen Developing Mouse Brain Atlas, describing the expression of approximately 2000 important development genes across seven embryonic and postnatal stages of mouse brain development in detail [20]. Nevertheless, no thorough study focused on the transcriptomics of the cerebral development has been performed so far. Thus to demonstrate the dynamics and regulation of the gene expression during the cerebral development, we sequenced three transcriptome libraries of the male mouse postnatal cerebrums using the RNA-sequencing (RNA-seq) technique. We analyzed the data with multiple data-mining tools, including the Web Gene Ontology Annotation Plot (WEGO), IDEG6 and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Our results suggested a remarkable trend of increase in gene expression before adolescence and the trend of decrease during the late postnatal cerebral development in the mouse. Overall, the gene expression level in adolescence was much higher than that in both infancy and adulthood. The DEG analyses from the present study would be helpful in better understanding of the gene regulation during the mouse postnatal cerebral development.

Results and discussion

The property of rmRNA-seq data

Ribo-minus RNA-sequencing (rmRNA-seq) technique was employed to obtain transcriptional profiles from the mouse cerebrum samples at a single-nucleotide resolution using the

ABI SOLiD sequencing system. After filtering, 17,319,524, 25,176,480 and 30,618,188 mapped reads were generated at three different developmental stages infancy, adolescence and adulthood, respectively. Approximately 60% of the reads were uniquely mapped to the reference genomic loci (Table S1).

Gene expression was estimated by calculating uniquely-mapped read density as reads per kilobase of exon model per million mapped reads (RPKM) [21]. Consequently, we identified 15,344, 16,048 and 15,775 genes expressed in the mouse cerebrum at infancy, adolescence and adulthood, respectively (with cutoff value of 5 reads/gene). Among them, 14,607 genes were universally expressed at all three developmental stages, whereas 218, 489 and 449 genes were uniquely expressed at infancy, adolescence and adulthood stages, correspondingly (Figure 1).

Gene expression analysis

GO functional analysis suggested that genes expressed in the cerebrum were involved in a variety of biological processes. Genes involved in basal metabolism GO terms, such as cell, organelle (cellular component, Figure S1A), cellular process, biological regulation and metabolic process (biological process, Figure S1B), were highly expressed throughout all three stages. In addition, gene expression related to binding and catalytic activities was obviously higher than other GO terms in terms of molecular function (Figure S1C), suggesting that genes involved in these two GO terms were expressed actively before adolescence. Furthermore, in terms of biological process, genes related to biological regulation and developmental process, mainly involved in the synaptic occurrence, neuronal migration

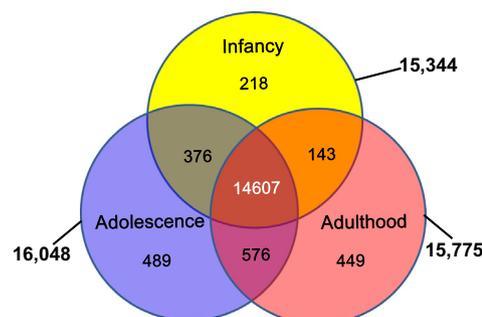


Figure 1 Distribution of expressed genes during the postnatal development of the mouse cerebrum

With the threshold of 5 reads/gene, 15,344, 16,048 and 15,775 expressed genes were identified in the infancy, adolescence and adulthood cerebrum, respectively. Among them, 14,607 genes were universally expressed at all three stages, whereas 218, 489 and 449 genes were expressed uniquely at infancy, adolescence and adulthood, respectively.

and interaction, were also actively expressed (Figure S1B). Combined with previous studies showing that the cell number of the pallium and hippocampus gradually increased but the cells were distributed relatively dispersed from day 7 after birth (P7) [22], the observation may suggest that during infancy and adolescence, synaptogenesis and neuronal migration become frequent.

We then generated a heatmap using R language to cluster all expressed genes at the three stages. Gene expression profiles of the cerebrum were clustered into two subgroups, with adulthood classified as a separate branch and the other two stages together. Gene expression in the cerebrum was highest at adolescence among the three stages. Interestingly, the pattern of gene expression in infancy and adulthood appeared to be opposite to each other, as the highly expressed genes in infancy became inactivated in adulthood, and *vice versa* (Figure 2). These data suggested that difference in gene expression between adolescence and adulthood was higher than that between infancy and adolescence, especially after adolescence, like an “Adult Ceremony” of the cerebrum, some genes trailed off into silence while other genes take their place. On the other hand, although the genes expressed in infancy and adolescence shared some in the same cluster, approximately 38% did change significantly ($P < 0.001$).

To test whether there are possible variations in gene expression during postnatal cerebral development at the chromosomal level, we compared the transcriptional activity of each chromosome at the three stages, which was represented as “the sum RPKM of expressed genes in each chromosome”. We found that chromosomes displayed higher transcriptional

activities at adolescence compared to the other stages. Considering that the numbers of expressed genes were different among chromosomes, the level of transcriptional activity was evaluated using average RPKM of expressed genes in each chromosome (Figure 3). It was shown that the average transcriptional activity levels of chromosomes increased from infancy to adolescence and decreased afterward. It was noteworthy that, heightened transcriptional activity level of Chr18 was readily observed at adolescence and adulthood. Subsequently we scrutinized the expression of genes on Chr18 and found that such elevated transcription was mainly attributed to the extremely high expression of *Mbp* that codes for myelin basic protein (MBP). The expression of *Mbp* was about 26 and 50 times higher in adolescence and adulthood than that in infancy, respectively. Myelin was generated approximately from the postnatal week 2 in the mouse cerebrum. It wrapped neuronal axons and played roles in insulating, protecting axons and improving the conduction of nervous impulse [23]. As the main component of myelin in the central nervous system, the abundance of MBP could reflect the process of myelination, with high expression of MBP suggesting myelin formation or regeneration [24,25]. As a result, our data suggested that the adolescence was a critical stage for initiation of myelin generation and the production of myelin continued into the mice adulthood.

Differential gene expression analysis

By comparing the expression profile between the different stages, we detected 5768 differentially-expressed genes (DEGs) between infancy and adolescence, and 6787 DEGs were found between adolescence and adulthood. Approximately 71% genes (4106 out of 5768 genes) were up-regulated from infancy to adolescence, whereas an almost opposite trend was observed from adolescence to adulthood (Table 1). Among the genes with altered expression during the postnatal development, approximately 74% of the genes followed a similar pattern across all three postnatal stages: expression of 2351 genes was increased during infancy to adolescence and decreased after that; expression of 1536 genes was altered (increased) only during infancy to adolescence; and expression of 2703 genes was altered (reduced) only during adolescence to adulthood (Table 2). These data demonstrated that genes showed altered expression among different stages and the difference between adolescence and adulthood was higher than that between infancy and adolescence. The active genes at adolescence may be closely-related to the cerebral development.

GO analysis revealed that DEGs between any two stages were biased to certain functional classifications. DEGs that are related to auxiliary transport protein, electron carrier, nutrient reservoir and proteasome regulator appeared to be active from infancy to adolescence (Figure 4A) but inactive from adolescence to adulthood (Figure 4B). These genes might have great influence on signal transduction, nutrient transportation and storage. Similar trend was also noticed in genes related to rhythmic process, which was controlled by the spinal cord and pallium in higher animals. Pallium controlling is the characteristic of rhythmic movements in higher animal [26–28]. The overall trend of increasing gene expression in rhythm system suggested that the adolescence mouse cerebrum began to regulate rhythmic movements.

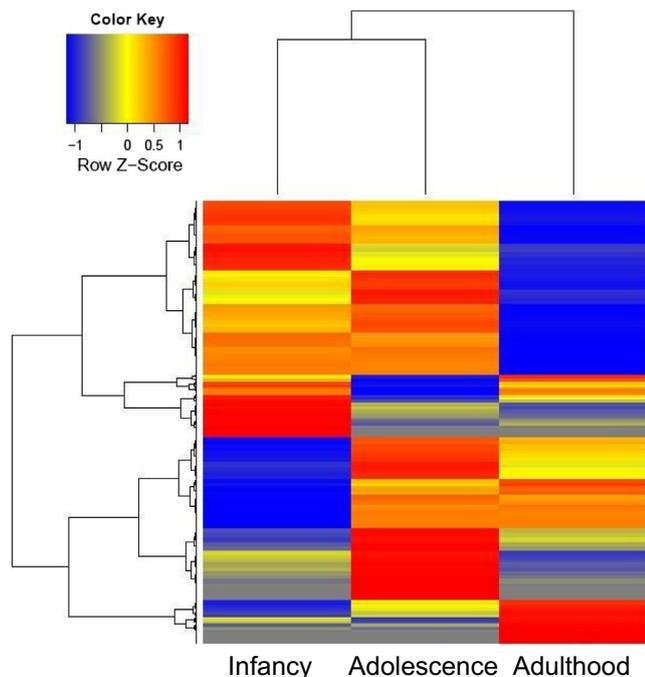


Figure 2 Clustering analysis of mouse gene expression profiles at three developmental stages

RPKM of all expressed genes in infancy, adolescence and adulthood were used to generate the heatmap, for clustering the gene expression profiles of the three stages. The expression intensity was color coded in gradient from blue to red (lack to abundance).

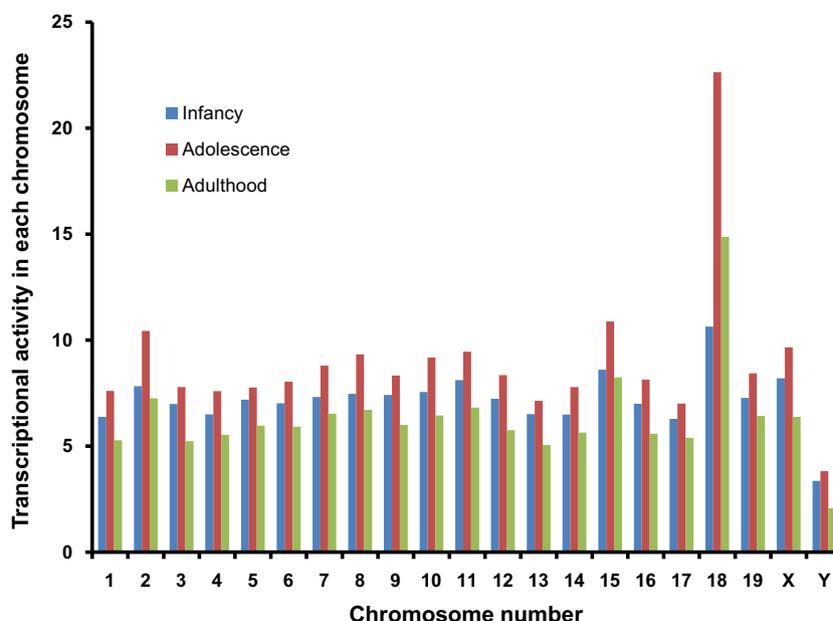


Figure 3 Transcriptional activity in each chromosome

Transcriptional activity in a chromosome was defined as RPKM of expressed genes in a chromosome.

Table 1 DEGs in the mouse cerebrum between different developmental stages

Developmental stages	Number of DEGs		
	Total	Up-regulated	Down-regulated
Infancy–adolescence	5768	4106	1662
Adolescence–adulthood	6787	1164	5623

Note: DEG, differentially-expressed gene.

Table 2 Gene expression profile during postnatal cerebral development

Type	Infancy–adolescence	Adolescence–adulthood	Number of genes
Up-up	↑	↑	219
Up-down	↑	↓	2351
Up-null	↑	●	1536
Down-up	↓	↑	209
Down-down	↓	↓	569
Down-null	↓	●	884
Null-up	●	↑	736
Null-down	●	↓	2703
Null-null	●	●	7651

Note: Up-regulation and down-regulation were indicated with ↑ and ↓, respectively, and ● indicates that expression of genes did not change significantly between the two developmental stages indicated.

KEGG analysis based on DEGs was used to computerize the molecular pathways involved in the postnatal cerebral development. Mitogen-activated protein kinase (MAPK) signaling pathway plays important roles in growth, proliferation, maturity and apoptosis of nerve cells, as well as abnormal development and damage repair of the brain [29–34]. The MAPK signaling pathway was active through infancy to adolescence, especially several genes encoding the key proteins in the pathway, such as BDNF, proto-oncogene Ras and protein kinase C (PKC), as well as important TFs like fetal liver kinase-1 (Flk-1), and activating transcription factor-2 (ATF-2)

(Figure S2A). Meanwhile these genes were inactive from adolescence to adulthood (Figure S2B). Other signaling pathways, such as Wnt pathway, which could cause the intracellular accumulation of beta-catenin, vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), *etc.*, also showed the same trend after birth. We inferred that the signal transduction pathways related to the cerebral development increased continuously from infancy to adolescence.

Neurons migrate accurately during the mammalian cerebrum development to their target location to perform specific physiological functions. This process required the coordination

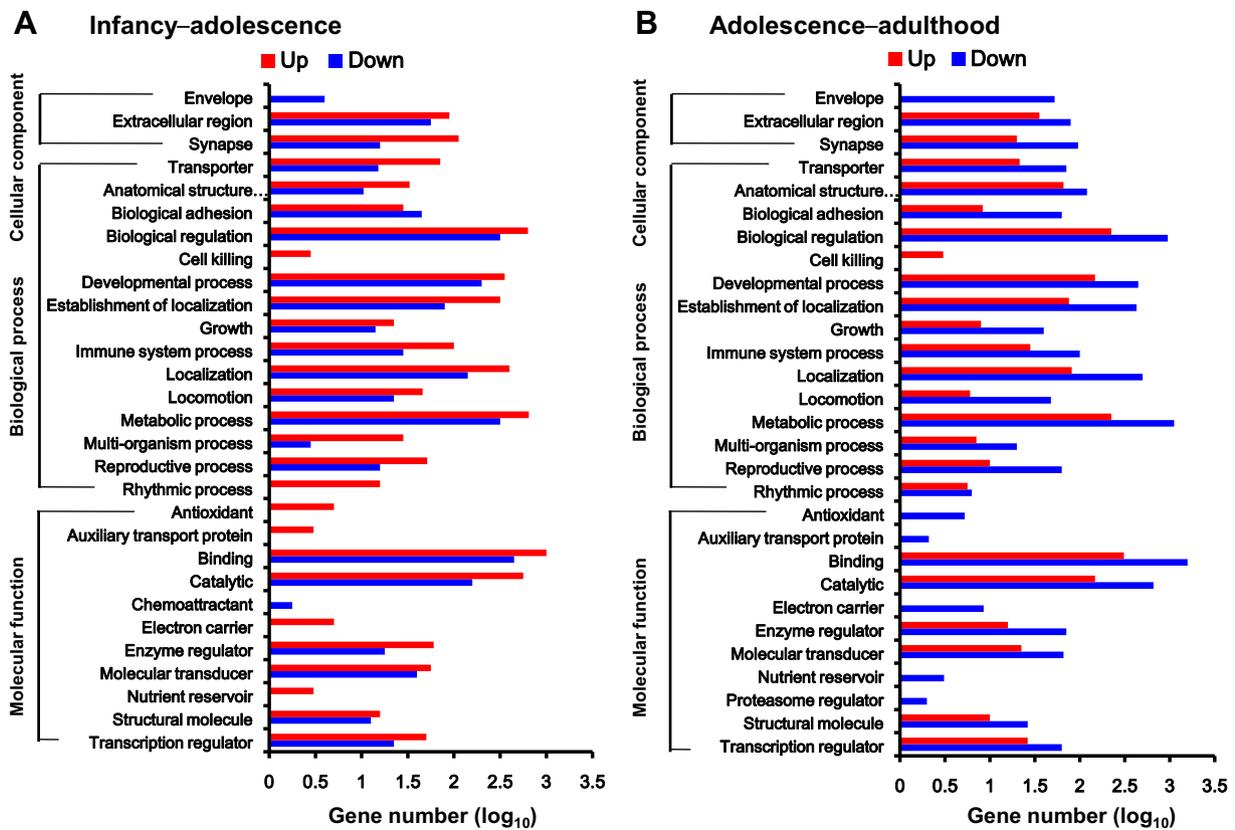


Figure 4 GO analysis of DEGs

DEGs related to auxiliary transport protein, electron carrier, nutrient reservoir and proteasome regulator were active from infancy to adolescence (A), but inactive from adolescence to adulthood (B).

of signal transductions, cytoskeleton proteins, microtubules proteins, and so on [35]. Alex et al reported that migrating interneurons can be found in the marginal zone (MZ) as early as E13.5 [20]. Our data on the involvement of DEGs in the regulation of actin cytoskeleton pathway suggested that neuron migration became more active till adolescence (Figure S3). Moreover, expression of genes in the pathways related to cell migration and adhesion, such as focal adhesion, adherens junction and tight junction, was also increased, suggesting that cell migration was also active from infancy through adolescence.

Axon guidance is the process when axons move to the target sites through complicated paths and the participation of axon guidance cues is required [36]. Genes in the axon guidance pathway were up-regulated during neonatal mice development to adolescence, including neurotransmitter receptors, neuron development related genes and axon growth related genes, such as Rho kinase (ROCK), p21-activated kinase (PAK), nuclear factor of activated T cells (NFAT), calcium-binding protein (CALN), receptor-associated kinase (RAK), extracellular regulated protein kinase (ERK), and so on. During this period, the expression of axon repulsion-related genes was generally up-regulated, such as focal adhesion kinase (FAK), ROCK and PAK. At the same time, the activity of axon attraction-related genes such as Rho GTPase (Rac) and actin binding LIM protein (Ablim) was down-regulated (Figure S4A). From adolescence to adulthood, axon guidance pathway was inactive overall (Figure S4B). To sum up, our data suggest that axon repulsion of the postnatal mouse cerebrum was activated at infancy and

maintained active throughout adolescence, whereas axon attraction decreased during the entire postnatal development, which would lead to more dispersed axon distribution.

Transcription factor analysis

In order to further verify the results above, we examined the expression of TFs reported in an atlas of 1727 mouse TFs [37]. There were 1343, 1393 and 1419 TFs expressed in the infancy, adolescence and adulthood, respectively, whereas 1289 TFs were commonly expressed at all three stages. We defined TFs with RPKM ≥ 10 as highly-expressed TFs and found that highly-expressed TFs were involved in 8, 15 and 7 functional classifications in the infancy, adolescence and adulthood, respectively. These data suggest that TFs in adolescence would involve a wider range of functional activities than those of the other two stages. Moreover, TFs related to signal transduction, neurogenesis, signaling molecules and interaction showed higher expression in adolescence (Table 3), suggesting the cerebral development at this stage was more active than others.

Further analysis indicated that among the highly-expressed TFs, expression of 101 TFs was different between infancy and adolescence, and expression of 103 was different between adolescence and adulthood. This observation is consistent with the result of DEG analysis. Meanwhile, GO analysis showed that TFs, which are related to synapse, transporter, establishment of localization, response to stimulus and rhythmic process, were remarkably up-regulated in the cerebrum during infancy to

Table 3 Functional classification of highly-expressed transcription factors

Functional classification		Developmental stage		
		Infancy	Adolescence	Adulthood
Nucleotide metabolism	Purine metabolism	✓		
	Pyrimidine metabolism	✓		
Amino acid metabolism	Lysine degradation	✓	✓	
Transcription related	RNA polymerase	✓		
	Spliceosome	✓	✓	✓
Signal transduction	Wnt signaling pathway	✓	✓	
	MAPK signaling pathway		✓	✓
	VEGF signaling pathway		✓	
	ErbB signaling pathway		✓	
Nervous system	Neurotrophic protein signaling pathway	✓	✓	✓
Cellular communication	Tight junction	✓	✓	✓
	Focal adhesion		✓	✓
Neurogenesis	Axon guidance		✓	
	Dorso-ventral axis formation		✓	
Protein folding and degradation	Ubiquitin-mediated protein hydrolysis		✓	
Signaling molecules and interaction	Neuroactive ligand-receptor interaction		✓	
Cell growth and death	Apoptosis		✓	✓
Behavior related	Rhythm process		✓	✓

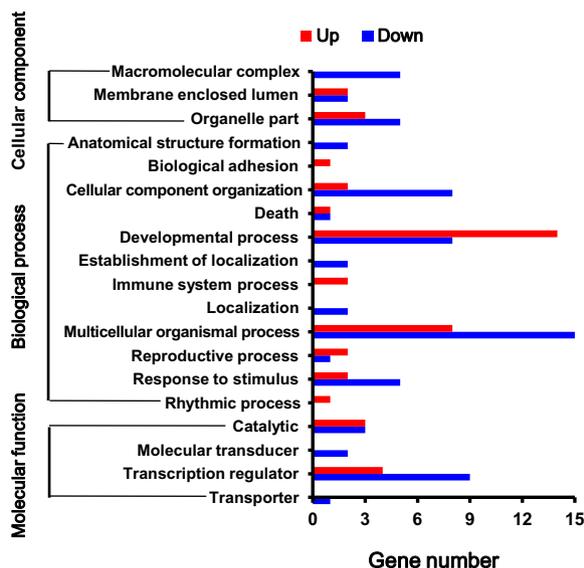
Note: ✓ indicates the presence of genes involved in the functional classification at the respective stages.

adolescence (Figure 5A). The active expression of genes related to these GO terms was also revealed with other analyses. After adolescence, the activities of TFs related to transporter and localization decreased significantly (Figure 5B). KEGG analysis indicated that genes related to both cell migration and axon growth showed increased expression from infancy to adolescence and reduced thereafter.

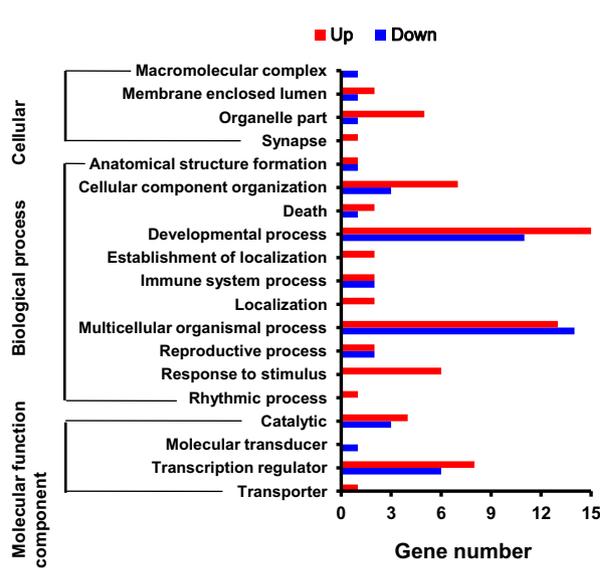
We further surveyed the expression levels of important TFs in detail (Table 4). TFs generally related to neurogenesis differentiation [e.g., neurogenic differentiation 1 (NeuroD1) and

NeuroD2] and oligodendrocyte lineage determination [e.g., YRPW motif protein (HEY1 and HEY2) and oligodendrocyte transcription factor 1 (OLIG1)] [38–41] shared the dynamic pattern of increasing expression before adolescence and decreasing expression afterward. These results suggested that adolescence was the active phase during the postnatal cerebral development. This dynamical pattern was also found in TFs related to circadian rhythm regulation [e.g., circadian locomotor output cycles kaput (CLOCK)] [42], which was consistent with our data of rhythmic process.

A Infancy–adolescence



B Adolescence–adulthood

**Figure 5** GO analysis of differently high expressed TF among libraries

From infancy to adolescence, TFs that were related to synapse, transporter, establishment of localization, response to stimulus and rhythmic process were up-regulated remarkably in the cerebrum (A). After adolescence, the activities of TFs related to transporter and localization decreased obviously (B).

Table 4 Expression of brain development-related transcription factors

Function of TF	Name of TF	Developmental stage		
		Infancy	Adolescence	Adulthood
Neurogenesis differentiation	NeuroD1	5.05	6.34	1.92
	NeuroD2	10.33	12.97	9.88
	HEY1	6.58	16.83	8.48
	HEY2	1.34	2.41	1.46
Oligodendrocyte determination	OLIG1	32.01	54.63	28.42
Circadian rhythm regulation	CLOCK	7.53	21.73	9.51

Note: Expression level was calculated using RPKM. TF, transcription factor; NeuroD1, neurogenic differentiation 1; HEY1, hairy/enhancer-of-split related with YRPW motif protein 1; OLIG1, Oligodendrocyte transcription factor 1; CLOCK, circadian locomotor output cycles kaput.

Conclusion

The cerebral gene expression increased dramatically from infancy to adolescence and decreased through the adolescence to adulthood stage, in terms of both number of involved genes and level of expression. The adolescence was the most active stage and several key pathways were identified in the present study. The difference in gene expression profiles between adolescence and adulthood was more evident than that between infancy and adolescence. Our results also indicated that TFs shared a consistent dynamic pattern, such as the ones related to neurogenesis differentiation, oligodendrocyte lineage determination and circadian rhythm regulation [38–42]. Further analyses indicated that *Mbp* expression appeared to be high in mouse adolescence and adulthood, which may suggest the activation of myelin formation or regeneration in adolescence [24,25]. Additionally, our study also showed that the rhythmic pathway appeared to be activated during infancy to adolescence, but inactivated during adolescence to adulthood. This may suggest the beginning of cerebral involvement in rhythmic movement regulation in mouse. Furthermore, it is of note that from infancy to adolescence, axon repulsion-related genes were generally up-regulated while the activity of axon attraction-related genes was reduced. Accordingly we speculate that during these periods, axon distribution would become more and more dispersed.

Materials and methods

Mouse treatment and sampling

BALB/c mice at three different developmental stages including infancy (1-week old), adolescence (4-week old) and adulthood (10-week old) were bought from Vital River Laboratories (Beijing, China). The animals were sacrificed by cervical dislocation and 6 cerebrum tissue samples were collected from 6 different animals at each stage. To minimize the potential RNA degradation, the cerebrum from each mouse was dissected within 10 min with the ambient temperature below 4 °C. The tissues were then rinsed with 0.01 M phosphate buffer solution (PBS) briefly and homogenized in liquid nitrogen using mortar and pestle.

Library construction for RNA-seq sequencing

Total RNAs were extracted from the cerebrum tissues using Trizol and rRNAs were depleted with the Ribo-minus Eukaryote kit (Invitrogen; Catalog No. 10837-08). We constructed three transcriptomic libraries using SOLiD™ Whole Transcriptome

Analysis Kit for the three stages separately with a starting material of 1 µg rRNA-depleted RNA for each sample. The cDNA library generated was amplified, cleaned using Qiagen MinElute PCR purification Kit (Qiagen; Catalog No. 28004 and 28006) and purified on a native 6% polyacrylamide gel. The cDNA products, which were 140–200 bp in length, were used for sequencing.

Sequencing read mapping

We adopted the method from [43] and used *corona_lite_v0.31R2* as mapping tool to maximally align the SOLiD sequencing reads to mouse genome reference sequence (release mm9, July 2007) from UCSC. To be specific, sequencing reads were first filtered according to the criteria of length ≥ 35 bp, average quality value ≥ 8 and mismatch ≤ 3 bp. Second, the 35-bp reads that were aligned to rRNAs were excluded to avoid confounding effects on analyses and the remaining reads were mapped to the mouse genome. After removal of the tag sequences beyond 30 and 25 bp, the second step was repeated for the 30- and 25-bp truncated reads. The mismatches allowed for 25-, 30- and 35-bp reads were 2, 2 and 3 bp, respectively. The sequencing data can be provided upon request.

Analyses of gene expression

Based on the RefSeq database annotation, we defined the gene whose exons had > 5 uniquely-aligned reads as an expressed gene. We used RPKM, defined as number of exon unique reads $\times 10^9 / (\text{length of exon} \times \text{number of all unique reads})$, to normalize the gene expression abundance [21]. R language was used to draw heatmap for clustering different gene expression profiles. Gene expression difference between any two libraries was determined using IDEG6 software. Audic–Claverie (AC) method was used to calculate the *P* value [44] and DEGs were defined with $P < 0.001$. TFs were identified through searching an atlas of mouse TFs that was reported previously in [37]. GO was used to classify and annotate the defined genes [45], while KEGG was used to predict and display the related pathways [46].

Authors' contributions

JY, PC and SH conceived the project and designed the study. WG collected the samples; YZ and FD carried out the sequencing library construction; CX, QL and WX participated in the data analysis. WX drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared no competing interests.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gpb.2014.04.004>.

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