Chinese Herbal Medicines 11 (2019) 39–44

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

Positive effects of low dose IMPX977 on Rett syndrome related MeCP2 targeted-genes

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ARTICLE INFO

Article history:
Received 28 March 2018
Revised 24 April 2018
Accepted 11 May 2018
Available online 24 October 2018

Keywords:
IMPX977
MeCP2
Rett syndrome
RNA-seq

ABSTRACT

Objective: To investigate the effect of IMPX977 on MeCP2 targeted-genes and the feasibility of IMPX977 acting as a therapeutic candidate drug for Rett syndrome by genomewide transcription profiling.
Methods: Rats' cortex of control group, IMPX977-treated low-dose group (10 mg/kg), and IMPX977-treated high-dose group (30 mg/kg) were collected and RNA was extracted from the tissues. Then, RNA was subjected to RNA-sequencing. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) were used in functional enrichment analysis of differentially expressed genes.
Results: Six MeCP2 targeted-genes were identified in the low/control categories, but not in the high/control categories.
Conclusion: Low-dose treatment of IMPX977 (10 mg/kg) showed a positive effect on MeCP2 targeted-genes and it may serve as a drug candidate for Rett syndrome therapy with proper dosage.

1. Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder with severe neurological regression, which was first reported by Andreas Rett in 1966 (Rett, 1966). The disease mainly occurs in girls. The classical characteristics of RTT are severe mental retardation, learning and cognitive disabilities, repetitive stereotyped hand movements, breathing disturbances and so on. Most cases are primarily caused by mutation of the X-linked gene *Mecp2* (Amir et al., 1999). Methyl-CpG-binding protein 2 (MeCP2), encoded by *Mecp2* gene, can positively or negatively regulate gene expression by binding to 5-methylcytosine of DNA sequence (Chahrour et al., 2008).

MeCP2 is composed of five major domains: N-terminal domain (NTD), methyl binding domain (MBD), inter-domain (ID), transcription repression domain (TRD), and C-terminal domain (CTD). The MBD and TRD are the two crucial regions for binding to methylated DNA (Ghosh, Horowitz-Scherer, Nikitina, Shlyakhtenko, & Woodcock, 2010) and recruiting other protein complexes (Forlani et al., 2010; Nan et al., 1998), respectively. MeCP2 is widely expressed, but with the highest level in brain (Shahbazian et al., 2002), especially in cortex and cerebellum (Zachariah & Rastegar, 2012). Research shows that the *Mecp2* knockout mice have Rett syndrome-like features (Guy, Hendrich, Holmes, Martin, & Bird, 2001; Ito-Ishida, Ure, Chen, Swann, & Zoghbi, 2015; Shahbazian et al., 2002). Consistently, increasing the exogenous or the endogenous MeCP2 protein levels could rescue the phenotype related to *Mecp2* deletion (Foust et al., 2009; Guy, Gan, Selfridge, Cobb, & Bird, 2007). Consequently, elevating the level of MeCP2 may have some advantages for the RTT treatment to some extent. To date, the therapeutic strategies for RTT primarily focus on restoring the expression and function of MeCP2 and the targeted-genes of MeCP2 (Liyanage & Rastegar, 2014).

We previously reported that IMPX977 can alter the MeCP2 expression levels in rat cortex (Hu et al., 2017), but it is unknown whether IMPX977 impacts the expression of MeCP2 targeted-genes and acts as a candidate drug to alleviate RTT. To answer this question, we conducted experiments to explore the influence of

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https://doi.org/10.1016/j.chmed.2018.10.003
1674-6384/

IMPX977 on the rats' cortex, focusing on the transcription levels of MeCP2- targeted genes using RNA sequencing (RNA-seq) technique.

2. Materials and methods

2.1. Animals and drugs

Sprague-Dawley (SD) adult rats were maintained in an SPF room (22 ± 2)°C, with 12 h light/dark cycle and fed with standard rat chow and water. Eighteen male rats were randomly divided into three groups ($n = 6$), including the control group, the low-dose IMPX977 group (10 mg/kg), and the high-dose IMPX977 group (30 mg/kg). All the animals were ig administered every other day for two weeks. All experimental processes in this study were approved by the University Animal Care Committee.

IMPX977, a single component from Malvaceae plants, was dissolved in olive oil which was purchased from Star Fine Foods Borges USA, Inc.

2.2. Tissue collection and RNA isolation

The tissues were immediately dissected from the euthanized animals and snap-frozen in liquid nitrogen. Then they were stored in −80 °C freezer. Total RNA was extracted from rats' cortices with TRIzol reagent (Transgen Biotech). The integrity of RNA was determined by 1.5% agarose gels and RNA Integrity Number (RIN). The RIN was determined by ribosomal 28S/18S ration, with the value between 1 (completely degraded) and 10 (intact). RNA concentration, purity, and RINs were measured with Bioanalyzer RNA 6000 Pico Kit (Agilent 2100).

2.3. Real-time PCR analysis

500 ng RNA, extracted from rat cortices of three groups ($n = 6$), was reverse-transcribed into complementary DNA (cDNA). The expression of mRNA was measured by SYBR Fast qPCR Mix (Takara, Japan) according to the manufacturer protocol in StepOne Real-Time PCR System (Applied Biosystems, USA). The expression of mRNA was normalized to GAPDH. A two-step process was applied: 95 °C for 30 s, and 40 cycles with 95 °C for 5 s, 60 °C for 15 s. The intron-spanning primers are as follows:

BDNF: 5′ATCCACTGAGCAAAGCCGAAC3′ (forward) and 5′CAGCC TTCATGCAACCGAAGTA3′ (reverse)

GAPDH: 5′GGCACAGTCAAGGCTGAGAATG3′ (forward) and 5′A TGGTGGTGAAGACGCCAGTA3′ (reverse)

2.4. RNA sequencing and bioinformatics analysis

The RNA, purified by oligo-dT coated magnetic beads, were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (4368814, Applied Biosystems). The obtained cDNAs were amplified and subjected to sequencing on the Illumina HiSeq 2500. The raw data were processed with filtering, alignment, and feature counting. Processed data were used to analyze the gene expression levels with the HTSeq software (FPKM ≥ 1). Differential gene expression was performed with DESeq2 (Love, Huber, & Anders, 2014) with fold change > 1.5 and $P < 0.05$. Heatmap was analyzed with http://www1.heatmapper.ca/expression/. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis were applied for process and pathway enrichment.

3. Results

3.1. Verification of RNA quality

Extracting high quality of RNA is the key step for qPCR analysis and RNA-seq. The quality of RNA was evaluated before conducting any of the experiments. Three bands were observed in the 1.5% agarose gels, which corresponded to 5S rRNA, 18S rRNA, and 28S rRNA, respectively (Fig. 1). The RIN and RNA concentration were tested with Agilent 2100 bioanalyzer (Table 1). All the RIN from samples were higher than 6, which indicated these RNA with a high quality (http://www.gtexportal.org/home/).

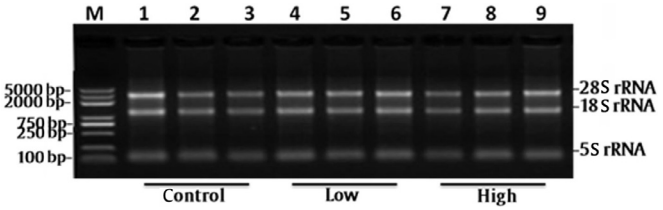

Fig. 1. Electrophoresis of RNA samples.
Lanes 1–3 were control group, lanes 4–6 were low-dose group (10 mg/kg IMPX977), and lanes 7–9 were high-dose group (30 mg/kg IMPX977).

Table 1
RIN and concentration of RNA.

Lanes	RIN	Concentration/(ng·μL^{-1})
1	8.8	138
2	8.3	130
3	8.8	128
4	8.5	147
5	8.7	162
6	8.4	183
7	8.6	134
8	8.7	155
9	8.7	207

3.2. Effects of low dose IMPX977 on MeCP2 targeted-gene transcription

The RNA-seq data were used for analyzing the effect of IMPX977 on rats' cortex gene transcription. First, the correlation across nine samples was evaluated with Pearson method. We observed a close correlation existed in intra-groups (Fig. 2). Then, differentially expressed genes (DEGs) were performed in inter-groups. In comparison with control group, 325 DEGs were identified in low/control sets, including 213 upregulated genes and 112 downregulated genes. However, only 216 DEGs were detected in high/control sets, of which 56 genes were upregulated and 160 genes were downregulated (Fig. 3A). Only 50 DEGs were caused by IMPX977 in both low and high IMPX977 groups. At the same time, different doses of IMPX977 also resulted in different gene alterations. A total of 275 genes were only expressed in low-dose/control sets. However, 165 genes were selectively appeared in high-dose/control sets (Fig. 3B). In contrast with high/control sets, more DEGs were found in low/control sets. To get a global knowledge of the DEGs, all the DEGs screened from the two IMPX977 treated groups were used for clustering analysis, respectively. The heatmap showed that not all the genes of a group had the same change with another group (Fig. 3C and 3D), but they had the similar trend to some extent. In addition, we analyzed the effects of IMPX977 on the MeCP2 targeted-genes. Six MeCP2 targeted-genes, including *Aldh1a1*, *B3gnt5*, *Dlx5*, *Sgk1*, *Bdnf*, and *Oprk1*, were present in low/control DEG list but none was identified in high/control DEGs (Table 2). The fold change of BDNF

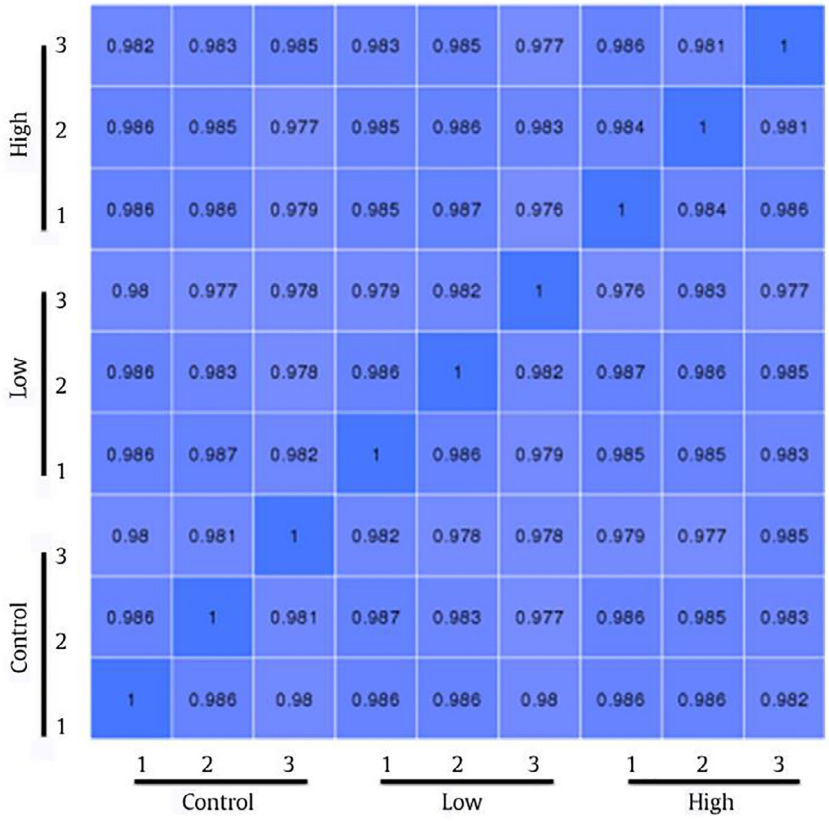

Fig. 2. Correlation analysis of samples.
Correlation analysis was conducted in control, low-dose, and high-dose groups with Person method.

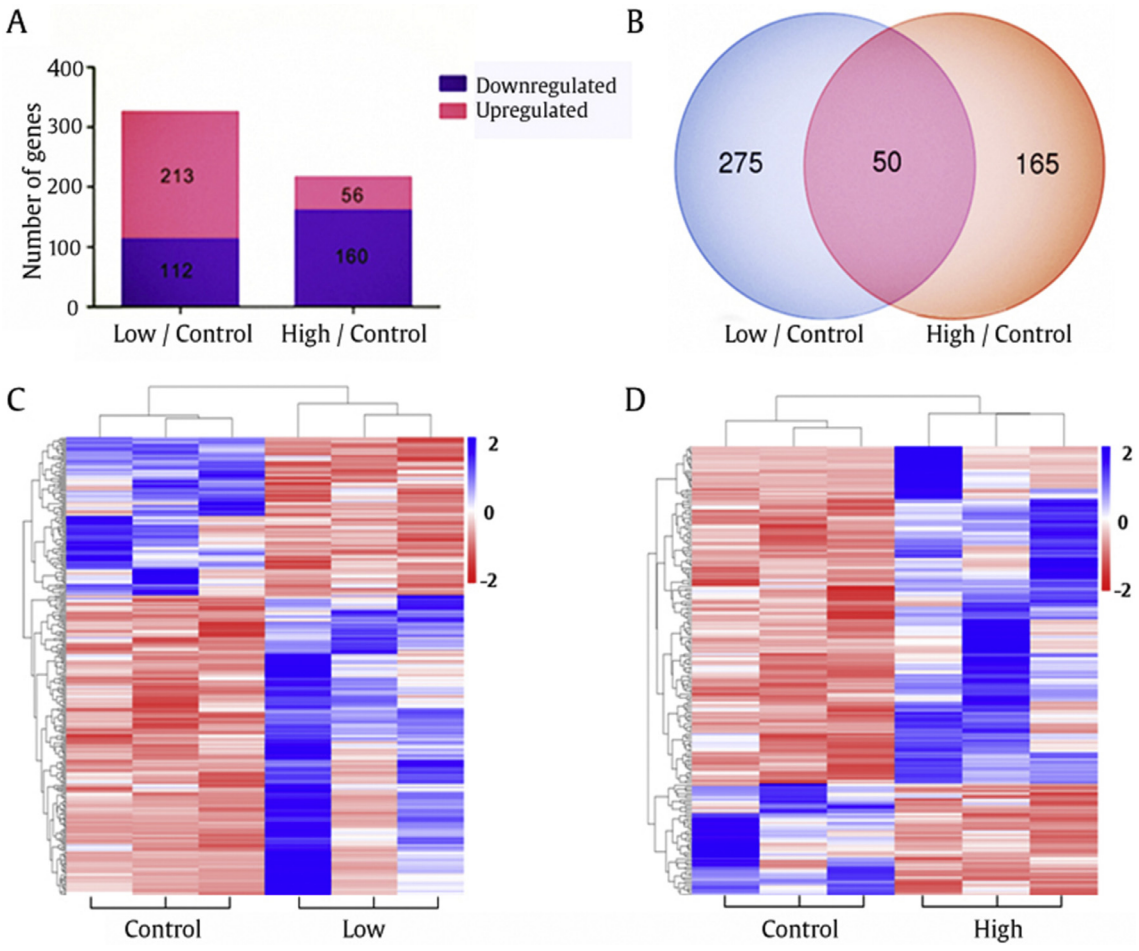

Fig. 3. RNA-seq analysis of rat cortex with different dose of IMPX977.
(A) Upregulated gene and downregulated genes of rat cortex. (B) Venne digram indicated gene number of overlap and difference between low-dose/control categories and high-dose/categories. (C) Heatmap of control group and low-dose group. (D) Heatmap of control group and high-dose group.

Table 2
Differentially expressed genes overlapped with MeCP2 targeted-genes.

Gene names	Description	$Log2^{Fold\ change}$ / direction of expression	
		RTT	Low-dose IMPX977 (10 mg/kg)
Aldh1a1	Retinal dehydrogenase 1A1	−0.67 (Pacheco et al., 2017)	−0.66
B3gnt5	Lactosylceramide 1,3-N-acetyl- beta-D-glucosaminyltransferase	−0.35 (Lin et al., 2016)	2.12
Dlx5	Homeobox protein DLX-5	Decreased (Horike, Cai, Miyano, Cheng, & Kohwi-Shigematsu, 2005)	1.41
Sgk1	Serine/threonine-protein kinase 1	1.37 (Pacheco et al., 2017)	−1.01
Bdnf	Brain-derived neurotrophic factor	Decreased (Chang, Khare, Dani, Nelson, & Jaenisch, 2006)	0.55
Oprk1	Kappa-type opioid receptor 1	Decreased (Chahrour et al., 2008)	Increased

Table 3
Analysis of *Bdnf* expression with RNA-seq and qPCR.

Gene names	RNA-seq		qPCR	
	Fold change	*P* value	Fold change	*P* value
Bdnf	1.47	2.28×10^{-2}	2.99	4.34×10^{-2}

expression was approximate 1.47 (Table 3), which was below the screened criteria of 1.5 fold change. Thus, BDNF expression was further verified with qPCR. Although subtle discrepancy of fold change existed between the RNA-seq and qPCR, the direction of expression was identical (Table 3).

3.3. DEGs function enrichment and pathway analysis

To further understand the biological process and pathway that the DEGs were involved in, GO and KEGG analysis were performed for the upregulated DEGs from low-dose/control categories. Majority of biologic processes were enriched in neuronal functions, calcium ion regulation, and G-protein coupled signaling pathway, such as striatum development, forebrain neuron differentiation, Adenylate cyclase-modulating G-protein coupled receptor signaling pathway, and regulation of cytosolic calcium ion concentration (Fig. 4A and 4B). Only one enrichment pathway, cell-substrate adhesion, was identified in the downregulated DEGs from the low-dose/control categories (Table 4). Upregulated DEGs from the low-dose/control categories were mapped to cocaine addiction, morphine addiction, neuroactive ligand-receptor interaction, dopaminergic synapse, cAMP signaling pathway, and serotonergic synapse with the KEGG method (Fig. 5). In contrast, for the downregulated DEGs, no pathways were enriched. In the high-dose/control sets, less biological processes and pathways, associated with neuron function, were enriched for the upregulated DEGs and downregulated DEGs (Tables 4 and 5).

4. Discussion

Although much is known in the pathology of RTT, there are no proper medicines or therapeutic approaches available in clinic. It

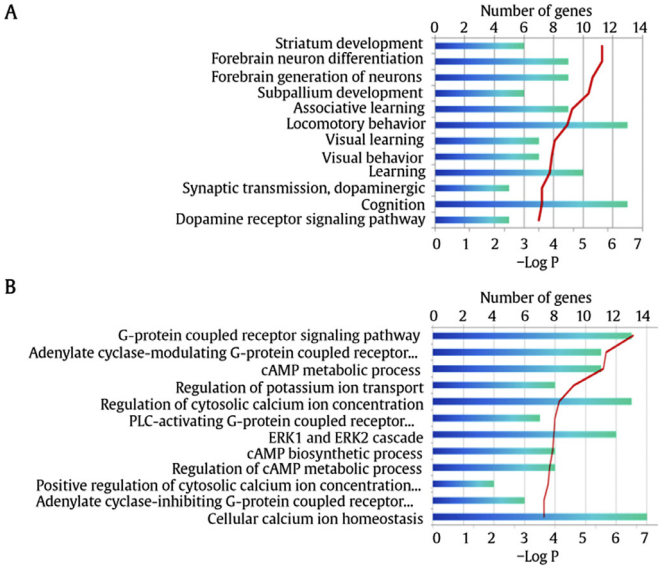

Fig. 4. Function enrichment of upregulated DEGs in low-dose/control categories. Biological processes were enriched with GO analysis. (A) neuron function enrichment with GO analysis. (B) G-protein coupled signaling pathway and biological process associated with calcium ion ($q < 0.05$).

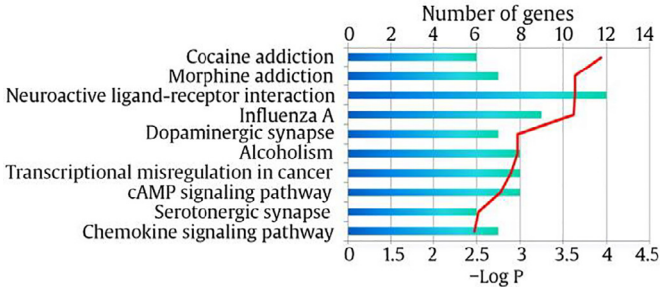

Fig. 5. Pathways enrichment of upregulated DEGs in low-dose/control categories. Pathways were enriched with KEGG analysis ($q < 0.05$).

Table 4
Function enrichment of downregulated DEGs in low-dose/control categories and high-dose/control categories.

No.	Pathway	Term	*q* value	Count
1	GO:0031589	cell-substrate adhesion	2.6×10^{-2}	9
2	GO:0042060	wound healing	7.43×10^{-4}	15
3	GO:0009612	response to mechanical stimulus	6.67×10^{-3}	11

The biological process of No. 1 was enriched by GO analysis in low-dose/control categories ($q < 0.05$), and biological processes of No. 2– No. 3 were determined by GO analysis in high-dose/control categories ($q < 0.01$). No pathways were identified with KEGG ($q < 0.05$) in two categories.

Table 5
Function enrichment of upregulated DEGs in high-dose/control categories.

No.	Pathway	Term	q value	Count
1	GO:0031397	Negative regulation of protein ubiquitination	9.11×10^{-4}	5
2	GO:1903321	Negative regulation of protein modification by small protein conjugation or removal	9.11×10^{-4}	5
3	GO:0007623	Circadian rhythm	9.11×10^{-4}	7
4	GO:0048511	Rhythmic process	2.21×10^{-3}	8
5	GO:0048701	Embryonic cranial skeleton morphogenesis	2.21×10^{-3}	4
6	GO:0042754	Negative regulation of circadian rhythm	2.75×10^{-3}	3
7	GO:0032922	Circadian regulation of gene expression	3.07×10^{-3}	4
8	GO:1904888	Cranial skeletal system development	3.07×10^{-3}	4
9	GO:0007626	Locomotory behavior	4.81×10^{-3}	6
10	GO:0001963	Synaptic transmission, dopaminergic	8.88×10^{-3}	3
11	GO:0035150	Regulation of tube size	9.71×10^{-3}	5
12	rno04710	Circadian rhythm	1.04×10^{-2}	3

Biological process of No. 1–11 were performed with GO analysis ($q < 0.01$), and only one pathway was identified with KEGG pathway enrichment ($q < 0.05$).

is an attractive approach using genomewide transcription profiling to screen drugs or to identify a strategy for RTT therapy. We first employed the RNA-seq technology to analyze the feasibility of IMPX977 being a potential therapeutic target. In our study, we detected that the expression of MeCP2 targeted-genes, including *Bdnf, Sgk1, Oprk1, B3gnt5, Dlx5*, and *Aldh1a1*, were affected by the low-dose treatment of IMPX977. However, these targeted-genes were not observed in DEGs from the high-dose/control sets. In terms of function enrichment, numerous DEGs were enriched in pathways associated with neuronal function, calcium ion regulation, and G-protein coupled signaling pathways in low-dose/control categories rather than the high-dose/control categories. The selectivity of genes regulated by the dosage of IMPX977 may contribute to explain the phenomenon. The influence of low-dose IMPX977 on the MeCP2 targeted-genes expression would be analyzed in the following discussion.

BDNF is widely distributed in the brain and it is crucial for neuronal systems survival, development, and the synaptic functions (Huang & Reichardt, 2001; Maisonpierre et al., 1990; Zagrebelsky & Korte, 2014). Our researches suggested that BDNF expression was suppressed in *Mecp2-null* mice and the RTT patients, but increased BDNF expression would ameliorate Rett syndrome-like phenotype and prolong the lifespan of *Mecp2* deletion animals (Chang, Khare, Dani, Nelson, & Jaenisch, 2006; Kline, Ogier, Kunze, & Katz, 2010; Larimore et al., 2009). Consequently, enhancing BDNF expression may be a preferable therapeutic method for *Mecp2-null* mice and RTT patients. Interestingly, we detected that low-dose IMPX977 upregulated the level of BDNF in the rats' cortex, which can rescue BDNF levels in wildtype and *Mecp2-null* models. Moreover, BDNF activates tyrosine kinase receptor B (TrkB) resulting into three major pathways activation, including the phospholipase C (PLC) pathway, the phosphatidyl-inositol 3-kinase (PI3K) pathway, and the extracellular receptor kinase (ERK) pathway, which plays an important role in neuronal functions (Berridge, 1998). Compared with control, many pathways associated with BDNF-TrkB signaling were identified in the low-dose group, such as, regulation of cytosolic calcium ion concentration, PLC-activating G-protein coupled receptor signaling pathway, and ERK1 and ERK2 cascade pathway. Thus, we suspected the function of IMPX977 acting on the rat cortex might through PLC pathway and ERK pathway with calcium ion influx. First, activation of PLC pathway would lead to glutamate release (Li & Pozzo-Miller, 2014; Zhang, Fan, Ren, Zhou, & Yin, 2013). As an excitatory neurotransmitter, the release of glutamate is crucial for synaptic plasticity and the neurological function that related with learning and memory (Kendell, Krystal, & Sanacora, 2005). In addition, the activation of ERK pathway plays an important role in neural functions including synaptic plasticity and memory (Spencer, 2008).

Serum/glucocorticoid-regulated kinase 1 (SGK1), a target of glucocorticoid (GR), is involved in the intracellular response stress and neuronal functions (Anacker et al., 2013). A research suggested that SGK1 expression was upregulated in *Mecp2* knockout animals with neural function disorder (Nuber et al., 2005), which was further supported by the view that enhancing SGK1 expression would cause morphological abnormalities of oligodendrocytes and reduction of neurogenesis (Anacker et al., 2013; Miyata et al., 2011). Surprisingly, low-dose IMPX977 decreased SGK1 expression in the rats' cortex, which may be ascribed to the MeCP2 regulation. This hypothesis was supported by reports that MeCP2 could bind to promoter-proximal regions of Sgk1 to repress SGK1 expression in the brain (Guy et al., 2001; Nuber et al., 2005). In addition, some studies suggested that SGK1 participated in the downstream signaling transduction of BDNF and the level of SGK1 had a negative correction with BDNF (Anacker et al., 2013; Lang et al., 2006). Therefore, except for direct MeCP2 regulation, we considered that the function of SGK1 may also be impacted by BDNF expression.

DLX5, abundant in forebrain subcortex γ-minobutyricaeid (GABA) neurons and dopaminergic neurons, is involved in osteogenic differentiation and the development of brain. Multiple deficiencies were recognized in the *Dlx5*-knockout mice, including abnormal ears, noses, skull bones, and neurodevelopment (Acampora et al., 1999; Perera et al., 2004). OPRK1 is broadly distributed in brain and it plays an important role in regulating the states of motivation and emotion, like mood disorders and addiction (Aita, Byers, Chavkin, & Xu, 2010). Antidepressant-like phenotype is observed in the mice with *Oprk1* conditional knockout in dopamine neurons (Van't Veer et al., 2013). Lactosylceramide 1, 3-*N*-acetyl-beta- D-glucosaminyltransferase, encoded by *B3gnt5*, is mainly expressed in the spleen and placenta in adult mice (Biellmann, Hulsmeier, Zhou, Cinelli, & Hennet, 2008). It plays an important role in embryonic development and brain morphogenesis. A recent research reported that *B3gnt5* knockout mice demonstrated reproductive defects and abnormal spleen B-cells, but no neurological abnormalities were detected (Kuan et al., 2010). As MeCP2 targeted-genes, the levels of DLX5, OPRK1, and B3gnt5 expression were downregulated in *Mecp2* deficit models (Table 2). Surprisingly, these gene expression levels were elevated by administrating low-dose IMPX977 (Table 2), which build up a good basis for the RTT treatment with IMPX977. Aldehyde dehydrogenase1A1 (Aldh1a1), a maker of non-small cell lung cancer (NSCLC) stem cell (Gao et al., 2015), is widely investigated in lung, colorectal, and breast cancers. Although *Aldh1a1* was identified as a target gene of MeCP2, the role of Aldh1a1 played in neurodevelopment and its specific mechanism implicated in RTT were not clear yet. Consequently, further studies need to be conducted in RTT models or patients.

5. Conclusion

To conclude, low-dose IMPX977 have shown an effect on the target genes of MeCP2 by directly or indirectly regulating transcription, and it may act as a drug candidate to cure RTT animal models and patients. However, it should be noted that this study has examined only in wild type rats. Thus, further studies should be investigated on RTT animal models.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgment

This work was supported by National Natural Science Foundation of China (No. 81271255).

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