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16p11.2 is required for neuronal polarity

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ABSTRACT

Since Autism Spectrum Disorder (ASD) is strongly associated with chromosomal abnormalities of 16p11.2, and Autism has been linked to neuronal polarity defect, our study aimed to explore the role of 16p11.2 genes in regulating neuronal polarity. We performed a neuronal polarity assay in a high throughput manner for candidate genes at 16p11.2. Our most interesting finding was that three 16p11.2 candidate genes, *DOC2a*, *Tbx*-6 *and KIF* 22, affected neuronal polarity. Our research, for the first time, indicates a novel association between 16p11.2 and neuronal polarity. Our results support the hypothesis that 16p11.2 is required for neuronal polarity. Our research provides new important insights into molecular mechanisms underlying the tight association between 16p11.2 and several neural developmental disorders, including autism, epilepsy, mental retardation and schizophrenia.

Keywords: Neuronal Polarity; 16p11.2; Autism; Epilepsy; Neuronal Development

1. INTRODUCTION

Neuronal polarity, defined as neurons which are highly polarized cells, presenting two molecularly and functionally different compartment, single axon and multiple dendrites, is fundamental for neural development and function [1,2]. The formation of axon-dendrite polarity is crucial for neurons to make the proper information flow within the neural system [1]. Neuronal polarity has been studied using dissociated mice embryonic hippocampal neurons, which undergo characteristic growth stages in culture [1,3]. These neurons initially form multiple lamellipodia after plating and generating minor neurites with similar lengths at stage 1 - 2. Then, at stage 3, one of these initially equivalent neurites grows rapidly and becomes the axon, whereas the other neurite subsequently develop into dendrite at stage 4 - 5 [4].

In the past decade, numerous proteins controlling the establishment of neuronal polarity were identified, including microtubule-associated protein [5], kinesin motor proteins [6], N-cadherin [7], glycogen synthase kinase-3 beta (GSK-3β) [8], SAD kinases [9,10], scaffolding proteins [11] and actin cytoskeleton [12].

Autism is a complex childhood neurodevelopmental disorder, which is characterized by impaired social communication and restricted repetitive behavior [13]. Neuronal polarity facilitates the directional flow of information, which is fundamental not only for communication between neurons but also between neurons and effector cells. In light of this point, neuronal polarity defect leads to a group of neuropsychiatric disorders, including autism [14,15], epilepsy [16], mental retardation [17], and schizophrenia [18].

Intriguingly, autism is tightly associated with chromosomal abnormalities of 16p11.2 [14,19,20]. It is confirmed that a region of chromosome 16p11.2 influences susceptibility to autism when either deleted or duplicated [14]. Furthermore, a 600 kb deletion at 16p11.2 leads to a group of neuropsychiatric disorders [21]. 16p11.2 encompasses twenty-five genes, which spans 500 kb and is flanked by 147 kb low copy repeats that are 99% identical [20]. To sum up, 16p11.2 is a novel gene domain which is strongly associated with autism.

Since autism is tightly associated with chromosomal abnormalities of 16p11.2, and autism has been linked to neuronal polarity defect, we hypothesized that 16p11.2 might be associated with neuronal polarity. In light of these, our study aimed to explore the role of 16p11.2 genes in regulating neuronal polarity.

We performed a neuronal polarity assay in a high throughput manner for candidate genes at 16p11.2 which

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contains 25 candidate genes as followings: MAPK3/DOC 2A/MAZ/QPRT/SEZ6L2/HIPIP3/SPN/ALDOA/TaoK2/Tbx-6/ASPHD1/KCTD13/LOC124446/GDPD3/YPEL3/KIF22/CCDC95/FLJ25404/FAM57B/C16orf53/PPP4C/C16orf54/PRRT 2/MVP/CDIPT.

Our most interesting finding is that three 16p11.2 candidate genes, DOC2a, Tbx-6 and KIF 22, affect neuronal polarity. Our research, for the first time, indicates a novel association between 16p11.2 and neuronal polarity. Our results support the hypothesis that 16p11.2 genes involve the neuronal polarity regulation. Our finding that 16p11.2 is required for neuronal polarity lays the foundation for the mechanism underlying the new viewpoint that 16p11.2 is tightly associated with several neural developmental disorders, including autism [14,15], epilepsy [16], mental retardation [17], and schizophrenia [18].

2. METHODS

2.1. Hippocampal Neurons Culture

We constructed a stable hippocampal neurons culture system. Culture of hippocampal neurons prepared from E18 mice embryos was performed as described by Banker G. [22]. Hippocampi were dissected from embryonic 18 (E18) mice, digested with a mixture of proteases at 37˚C for 15 min and dissociated with a fire-polished Pasteur Pipette in plating medium MEM (minimal essential medium), containing 10% fetal bovine serum, 0.5% glucose, 1 mM sodium pyruvate, 25 μM glutamine, and penicillin/streptomycin.

Neurons were then plated onto glass coverslips with poly-D-lysine (Sigma) at a density around 100 - 200 neurons/mm^2. Neuronal culture was incubated at 37˚C with 5% CO_2. After neurons attached to the substrate (normally around 3 - 4 hr after plating), the medium was exchanged to neuronal culture medium in neurobasal medium (Invitrogen) supplemented with B-27 supplement (Invitrogen) and 1 mM glutamine. After plating, hippocampal neurons were electroporated with shRNA by Amaxa® Mouse Neuron Nucleofector® Kit [23].

All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and the study was approved by the Animal Care and Use Committee of Boston Children's Hospital, Harvard Medical School.

2.2. sRNAs Electroporation

We knocked down candidate genes via shRNAs (short hairpin RNAs). The shRNAs which knocked down reciprocal candidate genes were designed by Invitrogen Company. We electroporated neurons with short hairpin RNAs-encoding plasmids by Amaxa® Mouse Neuron Nucleofector® Kit [24]. One Nucleofection® Sample contains 2×10^6 hippocampal neurons, 3 μg short hairpin RNAs-encoding plasmids together with 2 μg pmaxGFP® Vector and 100 μg Nucleofector® Solution.

We prepared coated coverslips in 12-well plates by filling appropriate number of wells with 300 μl culture medium and equilibrated plates in a humidified 37˚C/5% CO_2 incubator, equilibrated additional volume of 500 μl per Nucleofection®, centrifuged 2×10^6 hippocampal neurons at 80 ×g for 5 minutes at room temperature and suspended the cell pellet carefully in 100 μl Nucleofector® Solution per sample. We combined 100 μl of cell suspension with 3 μg short hairpin RNAs-encoding plasmids, 2 μg pmax GFP® Vector, transferred cell/DNA suspension into certified cuvette, selected appropriate Nucleofect® Program, and then inserted the cuvette with suspension into the Nucleofector® Cuvette Holder [25]. After electroporation, we added 500 μl of the pre-equilibrate culture medium to the cuvette and gently transferred hippocampal neurons into the prepared culture dish with the coated coverslip. We incubated hippocampal neurons and replaced fresh culture medium until analysis [26].

2.3. Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde for 10 min and washed with PBS. Fixed neurons were permeablized and blocked with a solution containing 3% goat serum, 3% BSA, and 0.1% Triton X-100 in PBS. Neurons were incubated with anti-Tau-1(5 μg/ml) diluted in PBS overnight at 4˚C. After washing with PBS, cells were stained with secondary antibodies at room temperature for 2 hours.

2.4. Image Acquisition and Quantification

Neuron morphology was analyzed with a fluorescence microscope equipped with Hamamatsu ORCA-ER camera. Neuronal polarity phenotypes were categorized into three groups-no-axon neuron (**Figure 1**), single axon neuron (**Figure 2**) and multiple-axon neuron (**Figure 3**). Neuronal polarity was assessed by determining the percentage of neurons with multiple axons.

2.5. Statistical Analyses

Statistical analysis of neuronal polarity defects in neurons was carried out by analysis of variance (ANOVA), which was performed with GraphPad Prism 5 software (La Jolla, CA).

3. RESULTS

Our most interesting finding is that three 16p11.2 candidate genes, *DOC*2a, *Tbx*-6 *and KIF* 22, affect neuronal polarity.

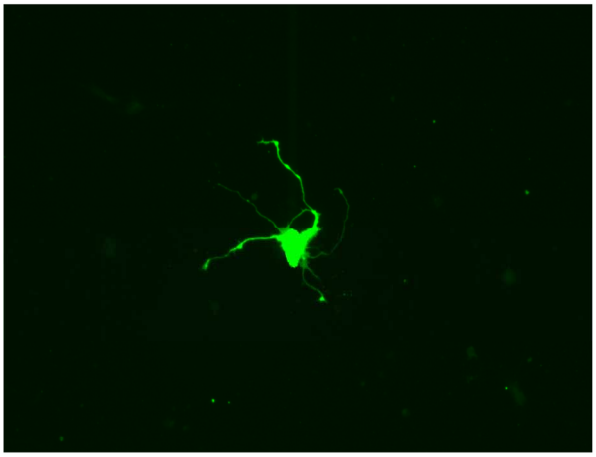


Figure 1. No-axon neuron.

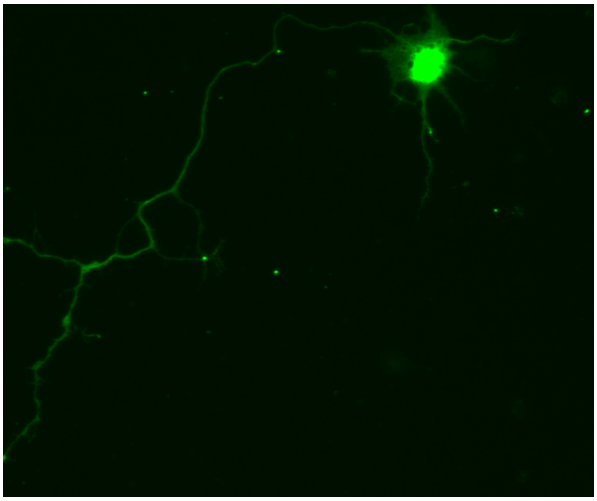


Figure 2. single-axon neuron.

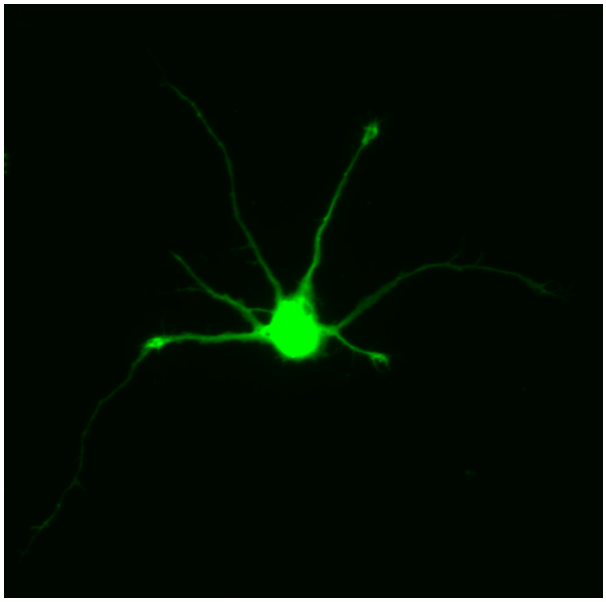


Figure 3. Multiple axon.

3.1. DOC2a Knockdown Affects Neuronal Polarity

Neuronal polarity quantification analysis showed that DOC2a knock-down via shRNA significantly increased the number of multiple-axon neurons from 9.1% to 21.6% and decreased the number of single-axon neurons from 83.1% to 70.3% (**Figure 4**).There was significant difference between control group and DOC2a knock-down group ($p < 0.001$, ANOVA).

3.2. Tbx-6 Knockdown Affects Neuronal Polarity

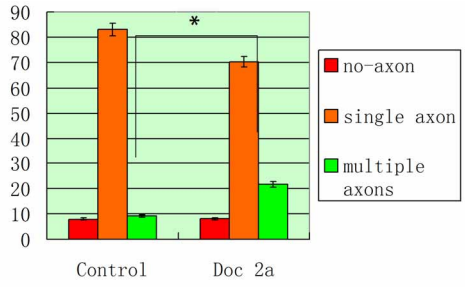
Similarly, Tbx-6 knockdown via shRNA significantly increased the number of multiple-axon neurons from 9.1% to 21.9% and decreased the number of single-axon neurons from 83.1% to 71.5% (**Figure 5**). There was significant difference between control group and Tbx-6 knock-down group ($p < 0.001$, ANOVA).

3.3. KIF 22 Knockdown Affects Neuronal Polarity

Furthermore, KIF 22 knockdown via shRNA signify-cantly increased the number of multiple-axon neurons from 9.1% to 22.1% and decreased the number of sin-gle-axon neurons from 83.1% to 69.6% (**Figure 6**).There was significant difference between control group and KIF 22 knock-down group ($p < 0.001$, ANOVA).

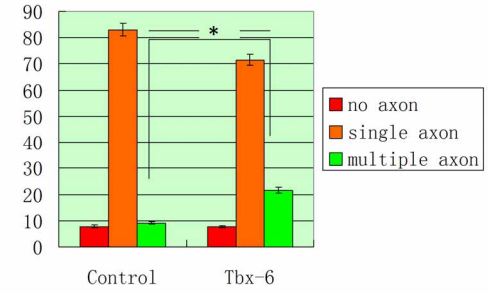
4. DISCUSSION

In this study, we provides evidence that 16p11.2 is re-quired for neuronal polarity. We perform a neuronal po-



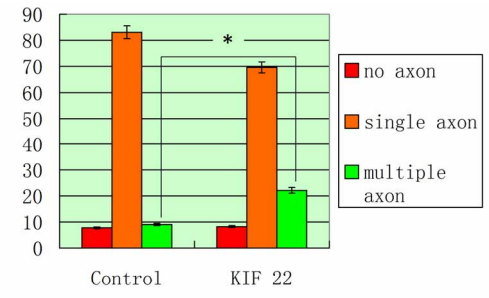
ShDOC2α knock down affects neuronal polarity: Neuronal polarity quantification analysis showed that DOC2a knock-down via shRNA significantly increased the number of multiple-axon neurons from 9.1% to 21.6% and decreased the number of single-axon neurons from 83.1% to 70.3%.There was significant difference between control group and DOC2a knock-down group (*p<0.001, ANOVA).

Figure 4. Results for neuronal polarity assay 16p11.2 genes: DOC2a.



ShTbx6 knock down affects neuronal polarity: Tbx-6 knockdown via shRNA significantly increased the number of multiple-axon neurons from 9.1% to 21.9% and decreased the number of single-axon neurons from 83.1% to 71.5%.There was significant difference between control group and Tbx-6 knock-down group (*p<0.001,ANOVA).

Figure 5. Results for neuronal polarity assay 16p11.2 genes: Tbx-6.



ShKIF22 knock down affects neuronal polarity: KIF 22 knockdown via shRNA significantly increased the number of multiple-axon neurons from 9.1% to 22.1% and decreased the number of single-axon neurons from 83.1% to 69.6%.There was significant difference between control group and KIF 22 knock-down group (*p<0.001,ANOVA).

Figure 6. Results for neuronal polarity assay 16p11.2 genes: *KIF* 22.

larity assay in a high throughput manner for 16p11.2 candidate genes. We find that three candidate genes, *DOC*2*a*, *Tbx*-6 *and KIF* 22, significantly affect neuronal polarity. Our research, for the first time, indicates a novel association between 16p11.2 and neuronal polarity. Our results support the hypothesis that 16p11.2 is required for neuronal polarity. Our research provides new insights into molecular mechanisms underlying the tight association between 16p11.2 and several neural developmental disorders, including autism [14,15], epilepsy [16], mental retardation [17], and schizophrenia [18].

The axons and dendrites of neuronal cells differ from each other in the composition of their proteins and organelles [27]. Axons are typically long and thin, with a uniform width, and they branch at right angles from the cell body. Dendrites are relatively short; as they emerge from the cell body they appear thick, but become thinner with increased distance from the cell body and then undergo Y-shaped branching [28]. Axons contain synaptic vesicles from which they release neurotransmitters at axon terminals in response to electrical signals from the cell body. Dendrites, especially dendritic spines, contain receptors for these neurotransmitters, as well as organelles and signalling systems. These two distinct cellular structures are fundamental for neuronal function and brain development, as they enable neurons to receive and transmit electrical signals. However, the molecular mechanisms that underlie this neuronal polarization were unclear until a decade ago [2].

The polarization of axon and dendrites underlies the ability of neurons to integrate and transmit information in the brain. Many experiments using cultured embryonic hippocampal neurons have revealed that, as they develop, neurons initially generate several equivalent neurites, but then begin to polarize so that one neurite becomes an axon while the remaining neuritis become dendrites. This early asymmetric neurite outgrowth is regulated by various molecules that have established roles in cytoskeletal rearrangements and protein trafficking. A balance of positive and negative signaling regulates these cellular functions, which result in the formation of a single axon [2]. In the past decade, numerous proteins controlling the establishment of neuronal polarity were identified, including microtubule-associated protein [5], kinesin motor proteins [6], N-cadherin [7], glycogen synthase kinase-3 beta (GSK-3β) [8], SAD kinases [9,10], scaffolding proteins [11] and actin cytoskeleton [12].

Nevertheless, to date, the specific gene domain which controls neuronal polarity has never been elucidated.

Intriguingly, it has been demonstrated that 16p11.2 is strongly associated with a group of neural developmental disorders, including autism spectrum disorder 9 [14,15], schizophrenia [18], attention-deficit hyperactivity disorder [29] and abnormal head size [30]. Deletion or duplication of one copy of the 16p11.2 interval is tightly associated with impaired brain function, indicating the importance of 16p11.2 gene domain [31,32].

16p11.2 gene region includes 25 known genes, of which 22 are expressed in the developing human fetal nervous system [19,33]. As yet, the mechanisms leading to neurodevelopmental abnormalities and the broader phenotypes associated with deletion or duplication of 16p11.2 have never been clarified.

In our experiments, we detect three 16p11.2 genes affecting neuronal polarity: *DOC*2*a*, *Tbx*-6 *and KIF*22. *Doc*2*a* is specifically expressed in neuronal cells and

localized on synaptic vesicles [33,34]. *In vitro* experiments indicate that *Doc2a* is kinetically tuned to function as a Ca^{2+} sensor for asynchronous neurotransmitter release [35]. Mice deleted for *Doc2a* show alterations in synaptic transmission and long-term potentiation and exhibit learning and behavioral deficits that include an abnormal passive avoidance task [36].We conclude that *DOC2a* controls neuronal polarity (**structure**) and synaptic transmission (**function**) to facilitate information flow.

The *Tbx* genes, which belong to a family of T-box transcriptional factors, expressed in the paraxial mesoderm, play essential roles during the development of posterior somites [37,38]. It has been confirmed that *Tbx* is required for proper neural crest migration and to stabilize spatial patterns during middle and inner ear development [39]. Furthermore, *Tbx* gene programs a variety of hindbrain motor neurons for migration, independent of directionality, and in facial neurons is a positive regulator of the non-canonical Wnt signaling pathway [40]. We conclude that Tbx controls neuronal polarity to regulate neural crest migration.

KIF 22, one of kinesin superfamily, a microtubule associated motor proteins, has been confirmed to transport vesicles involved in synapses [41]. Different motor proteins of the kinesin superfamily (KIFs) are responsible for selective transport of synaptic vesicle components to the axon and of transmitter receptors to the dendrite. For example, KIF5 is preferentially transported to the axon and accumulated at the axonal tip [42], and thus used for transporting axon-targeting proteins, such as VAMP2, GAP43, apolipoprotein E receptor 2 and amyloid precursor protein [41,42]. Moreover, KIF17 is responsible for transporting dendrite-targeting NR2B [43,45]. We infer that KIF22 is a novel kinesin motor protein which is required for neuronal polarity establishment.

In summary, our analyses, for the first time, indicate a novel association between 16p11.2 and neuronal polarity. Our finding supports the hypothesis that 16p11.2 is required for neuronal polarity. Our research provides new important insights into molecular mechanisms underlying the tight association between 16p11.2 and several neural developmental disorders, including autism, epilepsy, mental retardation, and schizophrenia. Future studies will focus on these three 16p11.2 candidate genes affecting neuronal polarity development in animal model (*in vivo*) to highlight the crucial role of 16p11.2 in neural development and a group of neurodevelopmental diseases.

5. CONCLUSION

Our research, for the first time, indicates a novel association between 16p11.2 and neuronal polarity. Our results support the hypothesis that 16p11.2 is required for neuronal polarity. Our research provides new important insights into molecular mechanisms underlying the tight association between 16p11.2 and several neural developmental disorders, including autism, epilepsy, mental retardation and schizophrenia.

6. ACKNOWLEDGMENTS

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7. AUTHOR CONTRIBUTIONS

X. H. and J. F. designed and managed the experiments. Z. L. performed the experiments. Z. L. wrote the paper. J. F. revised the manuscript.

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