UNIVERSITY OF CALIFORNIA SAN DIEGO

A Possible Role of CASK in Regulating the Function of the Mediator Complex in Neurons

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

In

Biology

By

Junneng Wen

Committee in charge:

Francesca Telese, Chair
James Kadonaga, Co-Chair
Ashley Juavinett

2020

The Thesis of Junneng Wen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2020

DEDICATION

I dedicate this thesis to my parents and my brother for their constant love and support, and to DD for his companionship and comfort.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Telese for her mentorship and support throughout my project. Her patience and guidance have helped me become a better researcher.

I would like to express my sincere gratitude to Dr. James Kadonaga and Dr. Ashley Juavinett for generously serving on my thesis committee and for being awesome professors of some of my favorite classes at UC San Diego.

I would also like to thank all of the current and former Telese lab members, namely, Patricia Montilla-Perez, Ermanno Florio, Attilio Iemono, Yinuo Meng, and Elvis Kim for their supports and friendships.

Data included in this thesis, such as MED1 ChIP, will be part of a paper currently being prepared for submission for publication. Telese, F., Montilla-Perez, P.; Meng, Y. Benner, C., Duttke, SH., and the thesis author are co-authors of this material.

ABSTRACT OF THE THESIS

A Possible Role of CASK in Regulating the Function of the Mediator Complex in Neurons

By

Junneng Wen

Master of Science in Biology

University of California San Diego, 2020

Professor Francesca Telese, Chair

Professor James Kadonaga, Co-Chair

Calcium/ calmodulin-dependent serine protein kinase (CASK) is a protein highly expressed in neuronal cells and associated to the etiology of neurodevelopmental disorders, such as intellectual disability and microcephaly. CASK is known to regulate the trafficking of membrane receptors at pre- and post-synaptic terminals. Accumulating evidence also suggests a role of CASK in transcriptional regulation; however, the

molecular mechanism by which CASK modulate transcription in neurons is currently unknown. To investigate the underlying mechanism, I manipulated the level of CASK by infecting primary cortical neurons with a lentiviral particle carrying short hairpin (sh)RNA targeting CASK. Following CASK knockdown, I showed that the activation of neuronal activity-dependent genes induced by KCl treatment is significantly reduced in the absence of CASK. Based on the analysis of a protein-protein interactions database, I found that CASK can bind to mediator complex, which is required for transcription initiation and long-range chromatin interactions. Thus, I used chromatin immunoprecipitation followed by quantitative PCR to study the binding of mediator complex on CASK targets after the neurons were depolarized and CASK is knocked down. This study showed that the recruitment of the mediator on the regulatory regions of CASK targets is induced by neuronal activity, but it's reduced in the absence of CASK. Such results suggest that CASK may regulate transcriptional initiation or long-range chromatin interactions via its interaction with the mediator complex. My findings provide novel insights into the mechanism by which CASK modulates activity-regulated gene expression in neuronal cells.

INTRODUCTION

Calcium/ calmodulin-dependent serine protein kinase (CASK) is a multidomain scaffolding protein highly expressed in neuronal cells (Y.-P. Hsueh, 2006). CASK was originally discovered in yeast two-hybrid screens as an interacting partner of neurexins, which are neuronal cell surface proteins (Hata et al., 1996). CASK belongs to the family of membrane-associated guanylate kinase (MAGUK) proteins that are abundant at neuronal synapses. Similar to many members of the MAGUK family, CASK regulates synaptic transmembrane protein anchoring and ion channels trafficking (Y. P. Hsueh et al., 2000). What makes CASK unique is its intracellular localization reported not only at synapses but also in the nuclear compartment (Y.-P. Hsueh, 2006).

CASK knock-out (KO) mice have been developed to study its physiological importance (Atasoy et al., 2007). While the complete deletion of CASK gene was lethal for the mice within hours after birth due to a cleft palate syndrome, the electrophysiological analysis of CASK deficient neurons showed that deletion of CASK does not impair core electrical properties of neurons, except for a defect in the frequency of spontaneous release events. In humans, mutations in the CASK gene are associated with a neurodevelopmental disorder, referred to as CASK-related intellectual disability that manifests in two main forms: microcephaly with pontine and cerebellar hypoplasia (MICPCH), and X-linked intellectual disability (XL-ID) with or without nystagmus(Hackett et al., 2010; Hayashi et al., 2012). These observations suggest that CASK exerts an important function for brain development and function. However, we still have a limited understanding of how CASK functions in neuronal cells and how mutations in CASK can

affect the role of this protein in different subcellular compartments. The function of CASK in the nucleus is of particular interest because altered nuclear processes can lead to altered gene expression programs, which are linked to several neurodevelopmental disorders.

In the nucleus, CASK forms a complex with different binding partners, including T-box transcription factor (Tbr1) and a nucleosome assembly protein CASK-interacting nucleosome assembly protein (CINAP) (Wang et al., 2004). It has been reported that Tbr-1-CASK-CINAP complex may regulate the expression of NMDA receptor subunit 2b (NR2b) (Wang et al., 2004). A T724A point mutation of CASK protein in rats disrupts the interaction between CASK, Tbr1 and CINAP, and leads to altered expression of NMDA receptor subunit 2b (NR2b) (Huang & Hsueh, 2009). However, only few genes have been reported to be modulated by CASK, and further studies are needed to explore the involvement of CASK in specific gene expression programs.

Preliminary transcriptomic data in the Telese lab show that CASK knock-down in primary cultured neurons impairs the activation of neuronal activity-dependent genes, which are rapidly activated by KCl-induced membrane depolarization in neuronal cultures, and in response to various stimulation in the brain (Lanahan & Worley, 1998). Activity-dependent transcription is promoted in the presence of synaptic activities and it is considered an essential mechanism of learning and memory formation (Yap & Greenberg, 2018). Moreover, preliminary ChIP-seq data in the lab show that CASK is recruited to the proximal promoter and distal enhancer regions of neuronal activity-dependent genes, suggesting a direct role of CASK in regulating gene expression. In my master thesis, I further investigate the mechanism by which CASK may regulate transcription in neurons.

MATERIALS AND METHODS

Antibodies

The antibodies used include MED1 (cat# A300-793A, RRID: AB_577241) from Bethyl Laboratories; PolII (cat# GTX102535, RRID: AB_1951313) from Gentex; GAPDH (cat# MA1-16757, RRID: AB_568547) from Thermo Fisher Scientific; and YY1 (cat# ab109237, RRID: AB_10890662) from Abcam.

Primary Cortical Neurons and Cell Cultures

Primary cortical cultures were established by dissecting E15.5 mouse embryos. Cultures were maintained for 9 days *in vitro* before treatment. Neuronal cultures were treated with 3x KCL depolarization buffer (170mM KCl, 1mM MgCl2, 2 mM CaCl2, 10mM HEPES pH 7.9) for 1 h before collection. HEK293-T was obtained from ATCC.

Lentivirus Production and Infection

HEK-293T cells are plated in 10 cm tissue culture dish (Genesee Scientific) and maintained in Complete Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% FBS until 90% confluent. 70% of the media were changed 30-60 min before transfection. Lentiviral plasmids pLKO1.0 containing shCASK or shControl (SIGMA) were co-transfected with psPAX2 (Addgene #12260) and pCMV-VSV-G (Addgene #8454) into HEK-293T cells using LipoD293™ In Vitro DNA Transfection Reagent (Signagen). Lentiviruses were harvested in Neurobasal Media (Life Technologies) 72 hours after transfection and used for infection of neurons. Neurons were

infected at DIV3-4 by incubating neurons for 6 hours with viral particles in NB media. After infection, viral particles were removed and neurons were cultured in original culture media. Treatment and collection were performed at least 100 h after infection.

RNA isolation, cDNA synthesis, and RT-qPCR

RNA was extracted by iScript™ RT-qPCR Sample Preparation Reagent (Bio-Rad). RNA was then reverse-transcribed to cDNA by SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher). Real-time quantitative polymerase chain reaction was done using SYBR® Green PCR Master Mix (Thermo Fisher) and carried out in Mx3000P QPCR Systems (Stratagene).

Chromatin Immunoprecipitation

ChIP was performed as previously described with some modifications (Nelson et al., 2006). Cells were crosslinked with 2 mM disuccinimidyl glutarate (DSG) for 45 min and 1% formaldehyde for 10 min at room temperature and the reaction was stopped by adding glycine to final concentration 0.125M for 5 minutes at room temperature. Fixed cells were washed with PBS and resuspended in hypotonic buffer (10 mM HEPES pH 9, 85 mM KCL, 0.5% IGEPAL CA-630 SIGMA I8896). The lysate was resuspended in sonication buffer (1% IGEPAL, 0.5% NaDoc) and sonicated for 18 cycles (30 sec on/ 30 sec off) in Diagenode Bioruptor PICO and centrifuged at 14,000 rpm for 15 min. The sheared chromatin was incubated at 4C overnight with 2 to 3 µg antibodies prebound to 20-ml Dynabeads Protein G (Life Technologies). ChIPed DNA was washed four times with Low Salt Buffer (100 mM Tris-HCL pH 7.5, 250 mM LiCl, 1% IGEPAL, 1% NaDoc),

and eluted in Elution Buffer (0.1 M NaHCO3, 1% SDS, 10 mM Tris-HCL pH 8, 1 mM EDTA). ChIPed DNA was reverse-crosslinked by incubating with RNase at 37C for 30 min, and 300 mM NaCl and Proteinase K at 65°C for 4 h. ChIPed DNA was purified by ChIP DNA purification (Zymo D5201). For ChIP-seq, the DNA libraries were constructed following KAPA HTP/LTP Library Preparation Kits (KK8232) and were sequenced on the Illumina NovaSeq 6000 sequencer at the IGM sequencing core (UCSD).

Western Blotting

Protein lysates were acquired by N-PER Neuronal Protein Extraction Reagent (Thermo Scientific cat#: 87792). Protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific cat#: 23225). Equal amounts of protein were loaded per well with LDS Sample Buffer (Invitrogen) and 100 mM dithiothreitol (DTT). Samples were separated by electrophoresis on 4-12% Bis-Tris polyacrylamide gels at 100V for 90 min (Thermo Fisher). Samples were transferred to PVDF membranes (Millipore) for 60 minutes at 200mA. Membranes were blocked with Casein (Bio-Rad) for 60 minutes. Membranes were then incubated with primary antibody diluted 1:1000 in Casein overnight at 4°C and then washed 3 times with TBS-tween20 (0.25%) for 5 minutes each time on a rocker. Secondary antibody was added 1:10000 into Casein and incubated 60 minutes at room temperature. Membranes then followed the same wash pattern as after primary antibody. After the last wash, membranes were incubated in enhanced chemiluminescence reagent (ECL) (Thermo Fisher) and signal was detected on imager (Bio-Rad). Gel images were analyzed on Image Lab (Bio-Rad).

Statistical Analysis

Results are expressed as means +/- standard error of the mean (SEM). Data were analyzed and statistics were performed using unpaired two-tailed Student's t-test or one-way ANOVA as appropriate using Prism 8 GraphPad (San Diego, CA). $P \leq 0.05$ was defined as statistically significant (*). $P \leq 0.01$ was denoted as (**). $P \leq 0.001$ was denoted as (***). $P \leq 0.0001$ was denoted as (****).

RESULTS

Lentiviral-mediated gene knockdown is a valid approach to study CASK function in primary cortical neurons.

To study the function of CASK in neuronal cells, an experimental model using primary mouse neuronal cultures was established. Brain tissue is isolated from mice at embryonic day 15.5 and cortices are dissected under the microscope. At E15.5 embryonic day, brain cells are highly enriched in neuronal types, with negligible number of astrocytes and oligodendrocytes, resulting in higher purity of neuronal cultures. Moreover, the meninges and tissues are easier to remove at this age (Sciarretta & Minichiello, 2010). After enzymatic homogenization of the tissue, the isolated neurons are plated, and cultures are maintained in a serum-free media for ~9 days to allow neuronal cells to develop projections and establish connections. The morphology of neuronal cultures was checked under the microscope to ensure the neurons are healthy at the time of experiment **(Figure 1A)**.

To manipulate the level of expression of CASK in primary neurons, shRNA against CASK messenger RNA (shCASK) was used to knock down CASK expression. Transient transfection using Lipofectamine 2000 transfection reagent was tested as a potential method to deliver the shRNA plasmids into primary neuronal cultures. An expression vector for expression of GFP was transfected to test the transfection efficiency. Only a small fraction of neurons produces green fluorescence signal when examined at a fluorescent microscope, indicating that the direct transfection of neurons is not efficient **(Figure 1B)**. An alternative way to deliver shCASK plasmid into the neurons is by

transduction of neurons with lentiviral particles, which have the capacity to infect nonproliferating neuronal cells (Ding & Kilpatrick, 2013). The second-generation packaging system was used to generate lentiviral particles. This system contains a transfer plasmid with the viral LTRs (pLK0.1-shRNA), a plasmid encoding the envelop protein (pVSV-G), and a single packaging plasmid encoding the Gag, Pol, Rev, and Tat genes (psPAX2). Human Embryonic Kidney 293T (HEK-293T) cells are co-transfected with the lentiviral, envelope and packaging vectors. The lentiviral particles are collected from the conditioned medium and used to transduce neurons. The efficiency to deliver the plasmids into neurons by lentivirus was much higher (90%) than direct transfection (5%) as a higher fraction of neurons shows green fluorescence signals **(Figure 1C)**. The efficiency of the shRNA was validated by western blot showing that CASK has been knocked down successfully **(Figure 1D).**

The cultures were treated with depolarization buffer (55mM KCl) for subsequent experiments. Elevating the concentration of extracellular KCl causes membrane depolarization and is widely used for studying electrical activity-dependent signaling in neurons, such as neuronal activity-dependent gene expression (Levitan et al., 1995). The western blot shows both non-treated and KCL-treated protein lysates. The knockdown efficiency for non-treated and KCL-treated samples are 91.77% and 90.76% respectively (**Figure 1E**). GAPDH antibody is used as loading control. The cells are then ready for various applications such as Chromatin immunoprecipitation, RT-qPCR, and western blot analysis. The schematic of the model to study CASK is depicted in **Figure 1F**.

CASK is required for the activation of the Neuronal Activity-dependent Genes

Preliminary ChIP-seq data in the Telese lab (data not published) show that CASK is recruited to the proximal promoter and distal enhancer regions of neuronal activity-dependent genes, suggesting a direct role of CASK in regulating gene expression. Neuronal activity-dependent genes respond to various stimulation in the brain and expressed transiently after a stimulus. Their protein products are transcription factors that may induce change in neuron activities (Eggermont, 2013; Lanahan & Worley, 1998; Pfaff & Joëls, 2016). To explore the role of CASK in regulating neuronal activity-regulated gene expression, I selected different targets identified in the ChIP-seq experiments, which are known neuronal activity-dependent genes (Chen et al., 2014; Greenberg & Ziff, 1984; Lin et al., 2008; Lubin et al., 2008; Lyford et al., 1995)

Specifically, I selected c-fos (FOS), Neuronal PAS Domain Protein 4 (NPAS4), nuclear receptor subfamily 4 group A member 1 (NR4A1), brain-derived neurotrophic factor (BDNF), and activity-dependent cytoskeletal protein (ARC) as CASK target candidates whose expression may be affected by CASK. Cannabinoid receptor type 1 (CB1) was selected as a negative region because its expression is not known to be affected by membrane depolarization. Four-hour treatment of 55 mM KCL buffer was used to elevate the concentration of extracellular KCL to study the activity-dependent changes after membrane depolarization (Levitan et al., 1995). I confirmed the validity of these genes as activity-dependent genes using RT-PCR. The expression level in KCL-treated neurons is significantly higher compared to non-treated neurons for FOS, NPAS4, and ARC genes ($p<0.05$). Although no significant difference was observed after KCl

treatment for NR4A1 and BDNF genes, the expression was upregulated **(Figure 2A)**. These results confirm that the genes chosen are neuronal activity-regulated genes.

To investigate the role of CASK in the transcription of neuronal activity-dependent genes, I used the *in vitro* model as shown in **Figure 1** to knockdown the CASK level and measure the expression change in these genes by RT-qPCR. KCl depolarization significantly induced the expression of the neuronal activity-dependent genes in the shControl neurons, confirming previous findings that neurons react to KCl-induced membrane depolarization transcriptionally. In the CASK knockdown neurons, upon KCl treatment, the relative expression of these neuron activity-dependent genes is still upregulated compared to the non-treated groups, meaning that membrane depolarization by KCl took place. However, comparing the shControl treated with KCl and shCASK treated with KCl, I observed that the expression level of each gene is lowered in the CASK knockdown groups **(Figure 2B).** These observations provide evidence that CASK upregulates neuronal activity-dependent genes.

Neuronal activity-regulated gene expression is independent of Tbr1.

To explore the mechanism by which CASK affects the transcription of neuron activity-dependent genes, I identified several binding partners of CASK with whom the interactions may explain the effects on transcription. CASK interacts with the T-BOX, Brain, 1 (Tbr1), a T-box transcription factor which is essential for cerebral cortex development (Y. P. Hsueh et al., 2000). CASK has the capability to enhance the transcriptional activity of Tbr1 when using luciferase assays (Y. P. Hsueh et al., 2000). CASK forms a complex with Tbr1 and a nuclear protein CASK-

interacting nucleosome assembly protein (CINAP) inside the nucleus and affects transcriptions of genes under the control of Tbr1 (Wang et al., 2004).

To explore whether CASK modulates the transcription of neuronal activity-regulated genes via the same mechanism, I used an shRNA against Tbr1. If the CASK/ Tbr1 complex is involved in activity-dependent transcriptional regulation, the knock-down of Tbr1 should impair the activation of KCl-induced genes. Using the experimental *in vitro* model in **Figure 1**, primary cortical neuron cultures were infected with lentiviral particles containing short hairpin RNA targeting Tbr1. The protein level and mRNA level of Tbr1 were checked by western blot and RT-qPCR after the knockdown. The protein level of Tbr1 was reduced by over 97% compared with neurons infected with the shControl **(Figure 3A)**. The mRNA level of Tbr1 was also significantly reduced ($p=0.0088$) **(Figure 3B)**.

I then carried out RT-qPCR using primers targeting neuronal activity-dependent genes in Tbr1 knockdown and Control cells in presence or absence of KCl depolarization buffer for 4 hours. I observed that the knockdown of Tbr1 does not significantly alter the expression level of neuronal activity-dependent genes **(Figure 3C)**. These results suggest that CASK/Tbr1 complex may not be involved in regulating neuronal activity-induced gene expression. This result promotes us to explore another possible mechanism of CASK's transcriptional role.

The Mediator Complex is Recruited to CASK Binding Sites in KCl-dependent Manner

To further investigate the mechanism by which CASK may regulate transcription, I used BioGrid, a repository of protein-protein interactions to identify candidate binding partners of CASK, especially those that are known to be involved in transcriptional mechanisms (Stark, 2006). Among CASK binding partners, I focused on MED1, a subunit of the mediator complex, which is known to play an important role in transcription regulation (Tóth-Petróczy et al., 2008)**.** Interestingly, MED1 has been implicated in long-range chromatin interactions between promoter and distal elements (Chereji et al., 2017). It is generally accepted that DNA looping between promoter and enhancer regions is an important regulatory step of transcription. Therefore, I hypothesized that CASK regulates activity-induced gene expression by interacting with MED1 at specific regulatory DNA elements in proximity of CASK target genes.

To test this hypothesis, I performed ChIP-qPCR using antibodies against MED1 and primers targeting CASK binding regions in primary neuronal cells stimulated with KCl. Compared to the non-treated groups, neurons treated with KCl have a much higher recruitment of MED1 on CASK target regions (**Figure 4A**). The ChIP-ed DNA was used for the library for sequencing to identify the MED1 binding sites within the whole genome. Preliminary ChIP-seq data analysis shows an increased MED1 recruitment near the regulatory elements at selected CASK target genes when induced by KCl (**Figure 4C**). Further analysis is required to evaluate the global overlap of the chromatin occupancy of CASK and MED1. These results show that MED1 and CASK are recruited on the same

regulatory regions of neuronal activity-dependent genes, and that this binding is strongly induced by KCl-induced membrane depolarization.

To test the hypothesis that KCl induces looping between promoter and enhancers occupied by CASK/MED1 complex, I performed ChIP using antibodies against a well-known looping factor, YY1 (Weintraub et al., 2017). I found that YY1 binding at CASK-bound regions is strongly induced by KCl (**Figure 4B**). These results together indicate that CASK regulates neuronal gene expression by forming a complex with MED1 at specific regulatory elements. This binding is signaling dependent and involves looping between enhancers and promoters in proximity of activity-dependent genes.

CASK knock-down impairs the binding of the mediator complex on regulatory regions of activity-dependent genes.

Having confirmed that MED1 is recruited to CASK binding sites, I continued to explore whether CASK is required for the binding of MED1 at its binding sites using lentiviral-mediated shRNA approach. ChIP-qPCR against MED1 shows that the recruitment of MED1 is impaired on several regulatory regions in proximity of KCl-induced genes, including NPAS4, NR4A1 and ARC loci. Even though the recruitment level increased upon KCl treatment in the CASK knockdown group compared to the non-treated group, the fold recruitment of MED1 on these regions is lowered in CASK knockdown group comparing with the control groups. Notably, we did not observe an effect of CASK knock-down on FOS locus **(Figure 5)**. Overall, these results indicate that CASK regulates the KCl-induced MED1 recruitment of target region.

Data included in this thesis, such as MED1 ChIP, will be part of a paper currently being prepared for submission for publication. Telese, F., Montilla-Perez, P.; Meng, Y. Benner, C., Duttke, SH., and the thesis author are co-authors of this material.

DISCUSSION

Our results demonstrate that primary cortical neuronal cultures and lentiviral mediated manipulation of CASK expression is a valid system to study the function of this protein in neuronal activity-regulated gene expression (**Figure 1**). By reducing the expression of CASK, our results demonstrate that CASK is required for the activation of KCl-induced genes (**Figure 2**) and suggests for the first time a role of this protein in mechanisms underlying learning and memory. These results confirm that KCl treatment is useful system to activate activity-dependent genes known to be involved in mechanisms of synaptic plasticity and learning and memory (**Figure 2**). However, it is also known that KCl can induce toxicity in neurons and doesn't represent a physiological condition of neuronal activation (Ramnath et al., 1992). Therefore, it will be important to use different treatments to modulate neuronal activity, such as bicuculline or Reelin (Telese et al., 2015). It is also important to note that, although primary cultures constitute a useful experimental system to model membrane depolarization-induced gene expression, they have several limitations (Sciarretta & Minichiello, 2010). *In vitro* cultures lack the cellular complexity of the brain tissue that include other cell types important for neuronal homeostasis and function, such as astrocytes. Co-cultures with astrocytes or studies *in vivo* could provide additional important information in the context of regulation of neuronal transcription.

Finally, *in vivo* experiments are required to prove that the regulation of activity dependent genes by CASK is linked to specific behavioral outcomes, such as learning and memory. CASK knockout is lethal for the mice, and thus a new animal models of

CASK knockout specifically in the brain needs to be generated to conduct behavioral studies (Atasoy et al., 2007).

Our results highlight a potential mechanism by which CASK regulates transcription by interacting with MED1, a subunit of the mediator complex (**Figure 4**). The mediator complex transduces signals from the transcription activators in the enhancer regions to the transcription machinery, which is assembled at promoters as the PIC to control transcription initiation (Soutourina, 2018). The mediator complex is a large complex with modular organization and is required for transcription by RNA polymerase II (Yin & Wang, 2014). MED1 is only one of the subunits of the mediator. Although it reflects partially how the mediator complex functions as a whole, more experiments on other subunits of the mediator complex are required to understand the overall interaction between CASK and the mediator complex. Moreover, other potential binding partners of CASK might contribute to its transcriptional activity. For example, the BioGrid repository database reports Cyclin-dependent kinase 9 (CDK9) as an interacting partner with CASK (Stark, 2006). CDK9 is one of the subunits of Positive transcription elongation factor (P-TEFb), which interacts with the PIC and allows the elongation phase of transcription to occur (Bacon & D'Orso, 2019; Price, 2000).

It's important to note that the mediator complex is also involved in chromatin looping, which brings together distant regulatory regions such as enhancers and promoters (Soutourina, 2018). The results of the ChIP-qPCR using MED1 and YY1 antibodies suggest that neuronal activity enhancers the recruitment of these factors on regulatory elements and, thus, suggests that DNA looping may underlie mechanisms of transcriptional regulation in neurons. Even though ChIP is a useful technique to study the

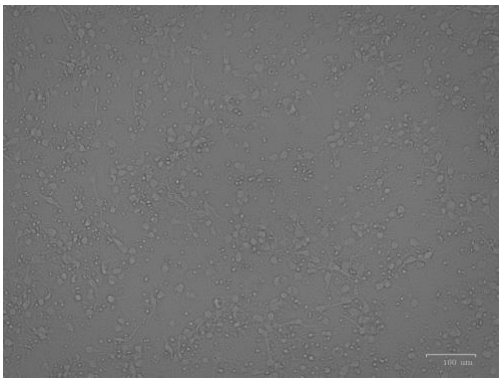
interaction of protein with chromatin, nevertheless, it does not provide information about chromatin architecture dynamics. To answer whether CASK plays a role in chromatin architecture, additional experiments, such as Circularized Chromosome Conformation Capture (4C), are required to study how CASK affects DNA looping (Zhao et al., 2006).

In conclusion, we have established an *in vitro* model to study CASK function in primary neurons. We have also demonstrated that CASK affects the expression of neuronal activity-dependent genes. Furthermore, we have shown that MED1 and YY1 are recruited to the CASK binding sites in signaling-dependent fashion. These results led us to formulate a new hypothesis that CASK might regulate transcription by interacting with the mediator complex. Overall, our current studies shed light on a novel function for CASK in transcription regulation and may contribute to identifying mechanisms underlying neurodevelopmental disorders related to CASK.

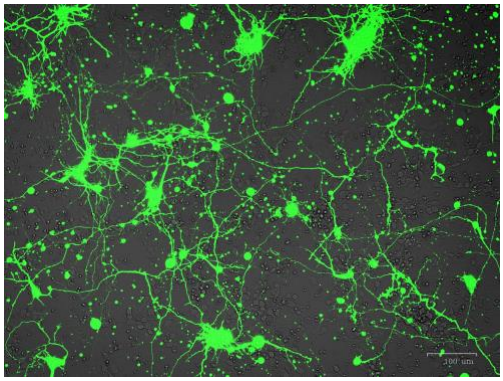
Figure 1. Lentiviral-mediated gene knock-down to study CASK function in primary cortical neurons.

(A) Brightfield image of primary neuronal culture at DIV 9. (B) Image of primary cortical neurons transfected with GFP plasmid using Lipofectamine 2000 transfection reagent. (C) Image of primary cortical neurons infected with lentiviral particles containing GFP plasmid. (D) Western blot analysis was performed using CASK antibody and 20 µg of protein lysate from cortical neuronal cultures treated or not with 55mM KCl (1hr). Cells were infected with lentiviral particles containing shRNA against CASK or scrambled shRNA control (shC). GAPDH was used as a loading control. (E) Relative expression of CASK protein for both non-treated and KCl treated cortical neurons. shControl was used to normalize the signal. (F) A schematic of the *in vitro* model.

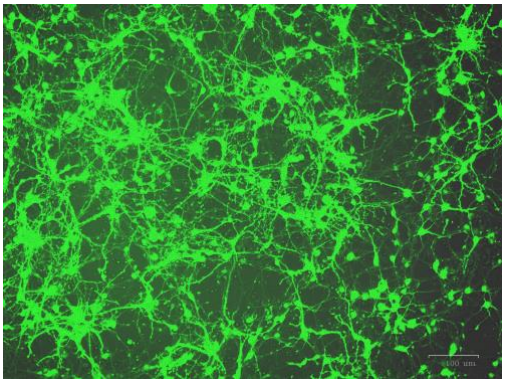
A



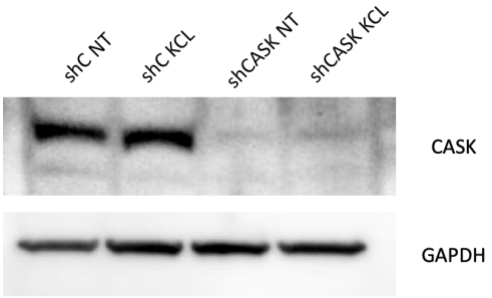
B



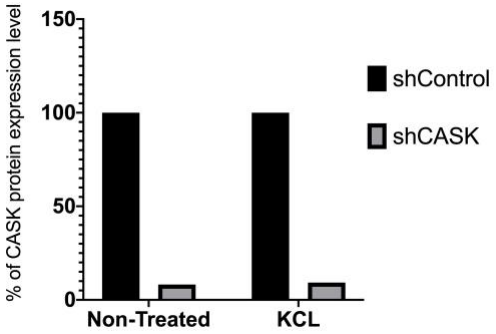
C



D


shC NT
shC KCL
shCASK NT
shCASK KCL
CASK
GAPDH

E


% of CASK protein expression level
150
100
50
0
shControl
shCASK
Non-Treated
KCL

F

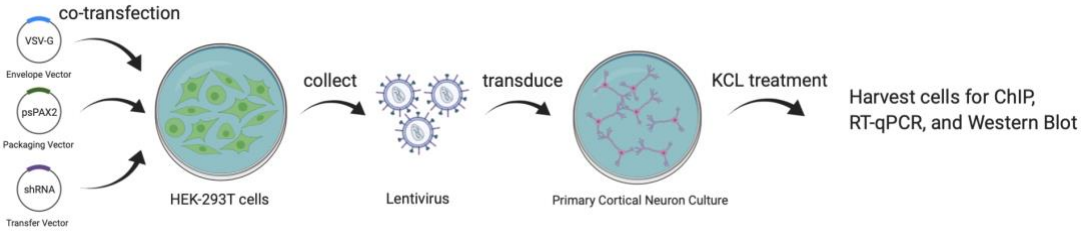

co-transfection
VSV-G
Envelope Vector
psPAX2
Packaging Vector
shRNA
Transfer Vector
HEK-293T cells
collect
Lentivirus
transduce
Primary Cortical Neuron Culture
KCL treatment
Harvest cells for ChIP,
RT-qPCR, and Western Blot

Figure 2. CASK is required for the activation of the Neuronal Activity-dependent Genes.

(A) RT-PCR showing expression levels of neuronal activity-dependent genes in before and after KCl stimulation of primary neuronal cultures for 4 hours. Expression levels of different genes were normalized to housekeeping gene 18s, calculated by the delta-delta Ct method, and represented as relative fold change in mRNA levels over non-treated condition. CB1 was used as control for a KCl-independent gene. qPCR bar graph shows average relative fold change values ± SEM of three independent replicate cDNA samples. (B) Relative expression of neuron activity-dependent genes in CASK knockdown and control cortical neurons before or after KCl treatment (4 hours). Expression levels were normalized to that of 18s, calculated by the delta-delta Ct method and represented as the relative expression in mRNA levels. qPCR data bars indicate average relative expression values ± SEM (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ One-way ANOVA)

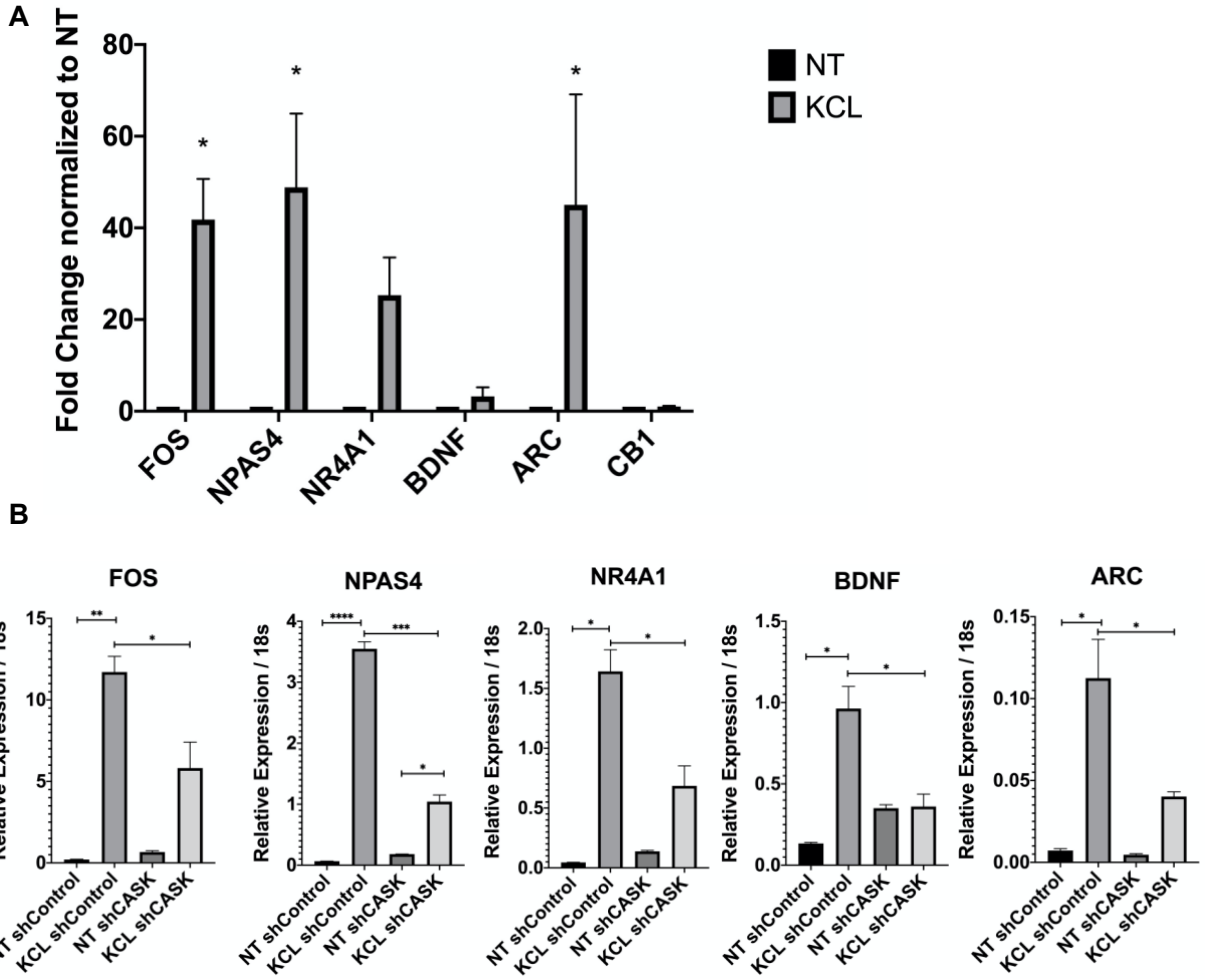

A
Fold Change normalized to NT
80
60
40
20
0
NT
KCL
FOS
NPAS4
NR4A1
BDNF
ARC
CB1
B
FOS
NPAS4
NR4A1
BDNF
ARC
Relative Expression / 18s
NT shControl
KCL shControl
NT shCASK
KCL shCASK

Figure 3. Neuronal activity-regulated gene expression is independent of Tbr1.

(A) Western blot analysis was performed using Tbr1 antibody and 35 µg of protein lysate from cortical neuronal cultures treated or not with 55mM KCl (1hr). Cells were infected with lentiviral particles containing shRNA against Tbr1 or scrambled shRNA control (shC). GAPDH was used as a loading control. (B) Relative expression of Tbr1 in neurons transduced with lentiviral particles carrying scrambled shRNA control or shRNA against Tbr1. The expression levels of Tbr1 were measured using real time PCR, normalized to that of ACTB, calculated by the delta-delta Ct method and represented as the relative expression in mRNA levels. qPCR data bars indicate average relative expression values ± SEM for three independent replicates (**$p \leq 0.01$ Unpaired t-test) (C) Relative expression of neuron activity-dependent genes in KCl-treated and non-treated Tbr1 knockdown and control cortical neurons. CB1 was used as a negative control for a KCl-independent gene. Expression levels were normalized to that of ACTB, calculated by the delta-delta Ct method and represented as the relative expression in mRNA levels. qPCR data bars indicate average relative expression values ± SEM for three independent replicates (ns=not significant, One-way ANOVA).

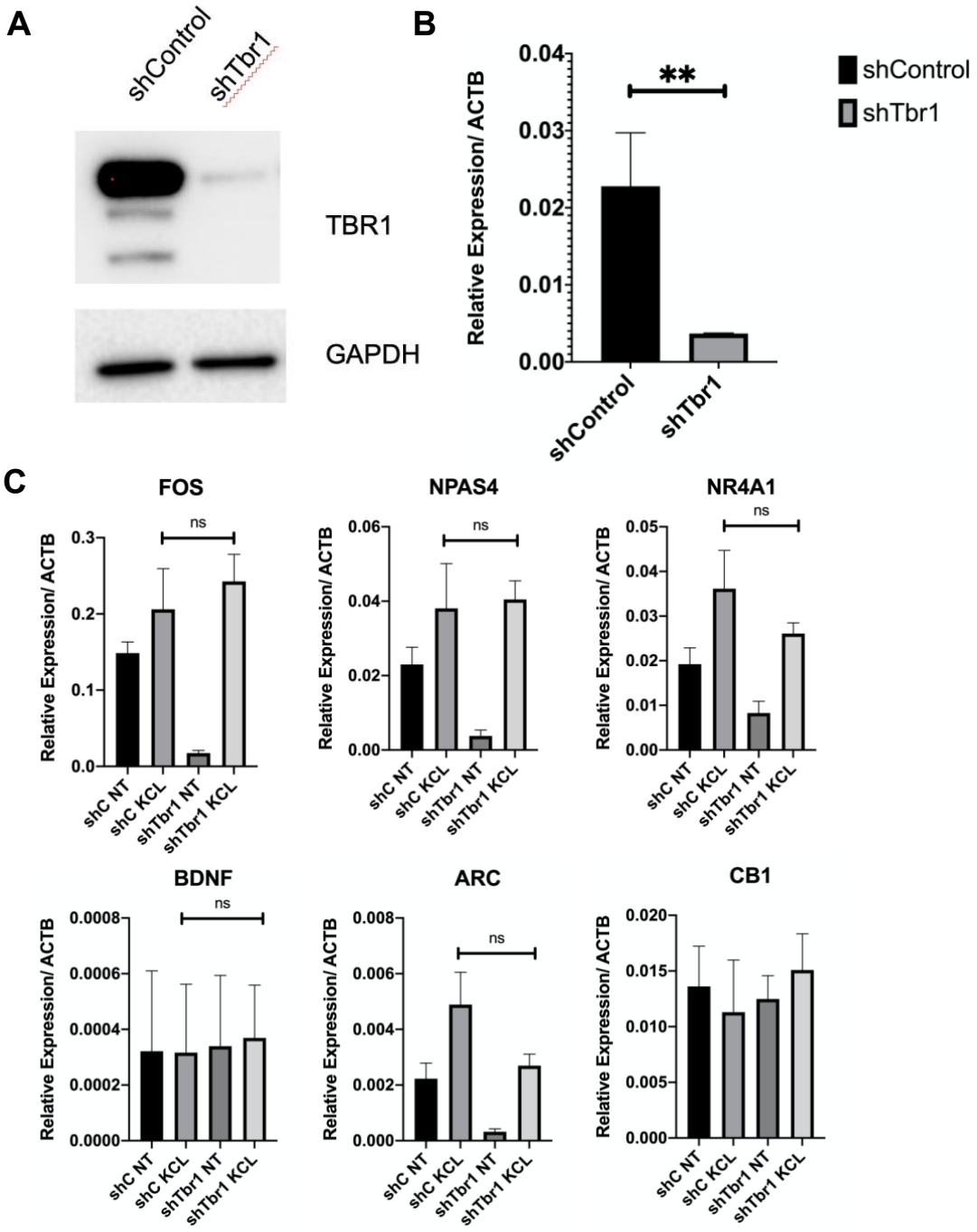
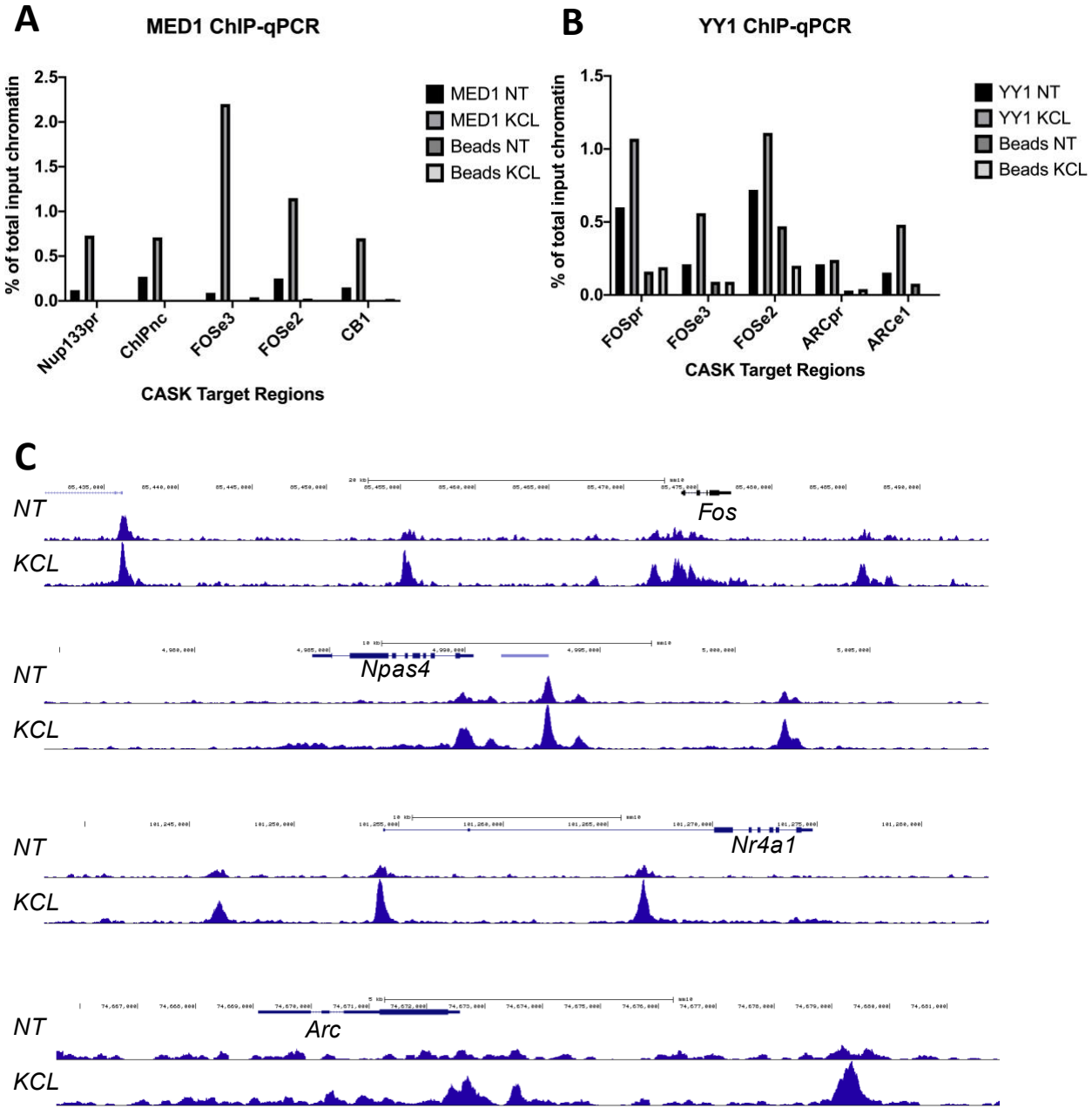

A
shControl
shTbr1
TBR1
GAPDH
B
shControl
shTbr1
**
Relative Expression/ ACTB
0.04
0.03
0.02
0.01
0.00
shControl
shTbr1
C
FOS
NPAS4
NR4A1
BDNF
ARC
CB1
ns
Relative Expression/ ACTB
shC NT
shC KCL
shTbr1 NT
shTbr1 KCL

Figure 4. The Mediator is Recruited to CASK Binding Sites in KCl-dependent Manner

(A) ChIP-qPCR analysis was carried out in cortical neurons with an antibody against MED1 to analyze the occupancy of the MED1 at multiple CASK targets in non-treated and in KCl-treated cells for 1 hour. (B) ChIP-qPCR analysis was carried in cortical neurons with an antibody against YY1 to analyze the occupancy of YY1 at different CASK targets before and after KCl treatment for 1 hour. The ChIP-qPCR signals are expressed as percentage of total chromatin input. (C) The UCSC browser view of the MED1 ChIP-seq signal for non-treated and KCl-treated neurons on selected promoter/ enhancer regions of neuronal activity-regulated genes.


A
MED1 ChIP-qPCR
% of total input chromatin
2.5
2.0
1.5
1.0
0.5
0.0
MED1 NT
MED1 KCL
Beads NT
Beads KCL
Nup133pr
ChIPnc
FOSe3
FOSe2
CB1
CASK Target Regions
B
YY1 ChIP-qPCR
% of total input chromatin
1.5
1.0
0.5
0.0
YY1 NT
YY1 KCL
Beads NT
Beads KCL
FOSpr
FOSe3
FOSe2
ARCpr
ARCe1
CASK Target Regions
C
NT
KCL
Fos
Npas4
Nr4a1
Arc

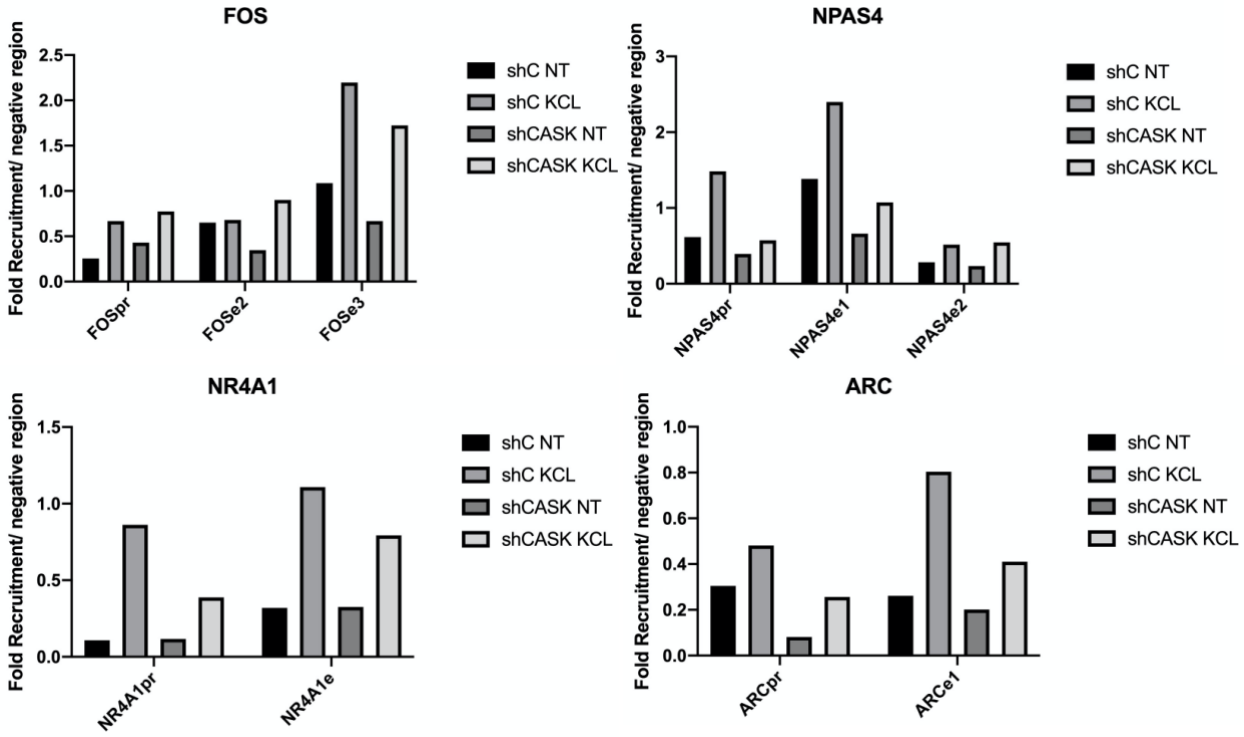


Figure 5. CASK knock-down impairs the binding of the mediator complex on regulatory regions of activity-dependent genes.

ChIP-qPCR analysis was carried out in cortical neurons with an antibody against MED1 to analyze the occupancy of the MED1 at multiple CASK targets in CASK knockdown and control cells, non-treated and KCl-treated. The ChIP-qPCR signals are expressed as fold recruitment over the negative control region where MED1 does not bind.

Table 1. Primer sequences employed in RT-qPCR analysis

Target	Forward (5'-3')	Reverse (5'-3')
18s	CGAACGTCTGCCCTATCAACT	CTGCCTTCCTTGGATGTGGT
ACTB	CCTGGATGGCTACGTACATGGCTG	ACCTTCTACAATGAGCTGCGTGTG
ARC	GAGCTGAAGCCACAAATGCAGCTG	TCATTCTCCTGGCTCTGTAGGCTC
BDNF	TGAGTCTCCAGGACAGCAAA	GACGTTTACTTCTTTCATGGGC
CASK	ATGGGGGTATGATTCACAGG	CTGATGCCATTGATTTCTCG
CB1	AAGTCGATCTTAGACGGCCTT	TCCTAATTTGGATGCCATGTCTC
Comtd1	TACTTTCACGGGCTACTCGG	CACGGCTATGTCGAAGGTTCC
FOS	CAGAGCGCAGAGCATCGGCA	CGCTTGGAGTGTATCTGTCA
Foxp2	AGTGTGCCCAATGTGGGAG	CATGATAGCCTGCCTTATGAGTG
GRIN2B	CCTCCTGTGTGAGAGGAAAGAA	GTCATTCCCAAAGCGTCCCC
NCAM1	ACCACCGTCACCACTAACTCT	TGGGGCAATACTGGAGGTCA
NPAS4	TCTTGAGCAGAGAGAAGCCC	TGCACATCATGAGTCTTGCC
NR4A1	CAATGCTTCGTGTCAGCACT	TGGCGCTTTTCTGTACTGTG
Tbr1	TAAACAGGGAAGGCGCATGT	TGGGATCCGCCAAAATCACA

Table 2. Primer sequences employed in ChIP-qPCR analysis

Target	Name	Forward (5'-3')	Reverse (5'-3')
ARC enhancer	ARCe1	GGCTGGAGACTGGTGACATT	CCATCTGCTTTCTCCTGGAA
ARC promoter	ARCpr	CAGCATAAATAGCCGCTGGT	GAGTGTGGCAGGCTCGTC
CB1 promoter	CB1	GGCAGGACAAAGGCTCATTA	TCTCGCTCCAGTCCCATTTA
FOS enhancer 2	FOSe2	CCAATTCCTGGATTCAGTGC	ATGTACGCCGGCTAGAAGAA
FOS enhancer 3	FOSe3	GCCAAGCTCCTTGCTATCAG	CTCAACACTCTGCCATCAGC
FOS promoter	FOSpr	GAAAGCCTGGGGCGTAGAGT	CCTCAGCTGGCGCCTTTAT
Negative region	NEG	CTGTCACCCTGGCATTTCTT	GAGCGTTAAGGTCGTTCGAG
Non-coding target	ChIPnc	GACCAATCGGGAAGTGTCTG	GAGTCCTCAAGCCAATCACC
NPAS4 enhancer 1	NPAS4e1	AATAGCCTGGCAGCAGCTT	CTGCTTGCTATTTCGGAACC
NPAS4 enhancer 2	NPAS4e2	TGTGCCTCAGTTTCCCTTTC	ACAGAGAGACAACGCCAGGT
NPAS4 promoter	NPAS4pr	GAGGCTTCCTCTTCCTTGCT	GGAGCTATATAAGGCGGATCG
NR4A1 enhancer	NR4A1e	TTCTGCAGCCCTTCTGTTTT	CCTTTTCCGTTAGACCCACA
NR4A1 promoter	NR4A1pr	CAGGATGGATGCACCCTAGT	GTTCAAGACCCCATTGTGCT
Nup133 promoter	Nup133pr	AGGCGCCTAGAGATGACGTA	GGATGTGACGTCAACAAAGC

REFERENCES

Atasoy, D., Schoch, S., Ho, A., Nadasy, K. A., Liu, X., Zhang, W., Mukherjee, K., Nosyreva, E. D., Fernandez-Chacon, R., Missler, M., Kavalali, E. T., & Südhof, T. C. (2007). Deletion of CASK in mice is lethal and impairs synaptic function. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0611003104

Bacon, C. W., & D'Orso, I. (2019). CDK9: a signaling hub for transcriptional control. In *Transcription*. https://doi.org/10.1080/21541264.2018.1523668

Chen, Y., Wang, Y., Ertürk, A., Kallop, D., Jiang, Z., Weimer, R. M., Kaminker, J., & Sheng, M. (2014). Activity-induced Nr4a1 regulates spine density and distribution pattern of excitatory synapses in pyramidal neurons. *Neuron*. https://doi.org/10.1016/j.neuron.2014.05.027

Chereji, R. V., Bharatula, V., Elfving, N., Blomberg, J., Larsson, M., Morozov, A. V., Broach, J. R., & Björklund, S. (2017). Mediator binds to boundaries of chromosomal interaction domains and to proteins involved in DNA looping, RNA metabolism, chromatin remodeling, and actin assembly. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkx491

Ding, B., & Kilpatrick, D. L. (2013). *Lentiviral Vector Production, Titration, and Transduction of Primary Neurons*. https://doi.org/10.1007/978-1-62703-444-9_12

Eggermont, J. J. (2013). Noise and the Brain: Experience Dependent Developmental and Adult Plasticity. In *Noise and the Brain: Experience Dependent Developmental and Adult Plasticity*. https://doi.org/10.1016/C2011-0-06149-5

Greenberg, M. E., & Ziff, E. B. (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature*. https://doi.org/10.1038/311433a0

Hackett, A., Tarpey, P. S., Licata, A., Cox, J., Whibley, A., Boyle, J., Rogers, C., Grigg, J., Partington, M., Stevenson, R. E., Tolmie, J., Yates, J. R., Turner, G., Wilson, M., Futreal, A. P., Corbett, M., Shaw, M., Gecz, J., Raymond, F. L., Stratton, M. R., Schwartz, C. E., & Abidi, F. E. (2010). CASK mutations are frequent in males and cause X-linked nystagmus and variable XLMR phenotypes. *European Journal of Human Genetics*. https://doi.org/10.1038/ejhg.2009.220

Hata, Y., Butz, S., & Südhof, T. C. (1996). CASK: A novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *Journal of Neuroscience*. https://doi.org/10.1523/jneurosci.16-08-02488.1996

Hayashi, S., Okamoto, N., Chinen, Y., Takanashi, J. I., Makita, Y., Hata, A., Imoto, I., & Inazawa, J. (2012). Novel intragenic duplications and mutations of CASK in patients with mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH). *Human Genetics*. https://doi.org/10.1007/s00439-011-1047-0

Hsueh, Y.-P. (2006). The Role of the MAGUK Protein CASK in Neural Development and Synaptic Function. *Current Medicinal Chemistry*.

https://doi.org/10.2174/092986706777585040

Hsueh, Y. P., Wang, T. F., Yang, F. C., & Sheng, M. (2000). Nuclear translocation and transcription regulation by the membrane- associated guanylate kinase CASK/LIN-2. *Nature*. https://doi.org/10.1038/35005118

Huang, T. N., & Hsueh, Y. P. (2009). CASK point mutation regulates protein-protein interactions and NR2b promoter activity. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.03.015

Lanahan, A., & Worley, P. (1998). Immediate-early genes and synaptic function. *Neurobiology of Learning and Memory*. https://doi.org/10.1006/nlme.1998.3836

Levitan, E. S., Gealy, R., Trimmer, J. S., & Takimoto, K. (1995). Membrane depolarization inhibits Kv1.5 voltage-gated K+ channel gene transcription and protein expression in pituitary cells. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.11.6036

Lin, Y., Bloodgood, B. L., Hauser, J. L., Lapan, A. D., Koon, A. C., Kim, T. K., Hu, L. S., Malik, A. N., & Greenberg, M. E. (2008). Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature*. https://doi.org/10.1038/nature07319

Lubin, F. D., Roth, T. L., & Sweatt, J. D. (2008). Epigenetic regulation of bdnf gene transcription in the consolidation of fear memory. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.1786-08.2008

Lyford, G. L., Yamagata, K., Kaufmann, W. E., Barnes, C. A., Sanders, L. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Lanahan, A. A., & Worley, P. F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*. https://doi.org/10.1016/0896-6273(95)90299-6

Pfaff, D. W., & Joëls, M. (2016). Hormones, Brain and Behavior: Third Edition. In *Hormones, Brain and Behavior: Third Edition*.

Price, D. H. (2000). P-TEFb, a Cyclin-Dependent Kinase Controlling Elongation by RNA Polymerase II. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.20.8.2629-2634.2000

Ramnath, R. R., Strange, K., & Rosenberg, P. A. (1992). Neuronal injury evoked by depolarizing agents in rat cortical cultures. *Neuroscience*. https://doi.org/10.1016/0306-4522(92)90530-F

Sciarretta, C., & Minichiello, L. (2010). The preparation of primary cortical neuron cultures and a practical application using immunofluorescent cytochemistry. *Methods in Molecular Biology (Clifton, N.J.)*. https://doi.org/10.1007/978-1-59745-019-5_16

Soutourina, J. (2018). Transcription regulation by the Mediator complex. In *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm.2017.115

Stark, C. (2006). BioGRID: a general repository for interaction datasets. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkj109

Telese, F., Ma, Q., Perez, P. M., Notani, D., Oh, S., Li, W., Comoletti, D., Ohgi, K. A., Taylor, H., & Rosenfeld, M. G. (2015). LRP8-Reelin-Regulated Neuronal Enhancer Signature Underlying Learning and Memory Formation. *Neuron*. https://doi.org/10.1016/j.neuron.2015.03.033

Tóth-Petróczy, Á., Oldfield, C. J., Simon, I., Takagi, Y., Dunker, A. K., Uversky, V. N., & Fuxreiter, M. (2008). Malleable machines in transcription regulation: The Mediator complex. *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1000243

Wang, G. S., Hong, C. J., Yen, T. Y., Huang, H. Y., Ou, Y., Huang, T. N., Jung, W. G., Kuo, T. Y., Sheng, M., Wang, T. F., & Hsueh, Y. P. (2004). Transcriptional modification by a CASK-interacting nucleosome assembly protein. *Neuron*. https://doi.org/10.1016/S0896-6273(04)00139-4

Weintraub, A. S., Li, C. H., Zamudio, A. V., Sigova, A. A., Hannett, N. M., Day, D. S., Abraham, B. J., Cohen, M. A., Nabet, B., Buckley, D. L., Guo, Y. E., Hnisz, D., Jaenisch, R., Bradner, J. E., Gray, N. S., & Young, R. A. (2017). YY1 Is a Structural Regulator of Enhancer-Promoter Loops. *Cell*. https://doi.org/10.1016/j.cell.2017.11.008

Yap, E. L., & Greenberg, M. E. (2018). Activity-Regulated Transcription: Bridging the Gap between Neural Activity and Behavior. In *Neuron*. https://doi.org/10.1016/j.neuron.2018.10.013

Yin, J. W., & Wang, G. (2014). The Mediator complex: A master coordinator of transcription and cell lineage development. *Development (Cambridge)*. https://doi.org/10.1242/dev.098392

Zhao, Z., Tavoosidana, G., Sjölinder, M., Göndör, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Sandhu, K. S., Singh, U., Pant, V., Tiwari, V., Kurukuti, S., & Ohlsson, R. (2006). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nature Genetics*. https://doi.org/10.1038/ng1891