UNIVERSITY OF CALIFORNIA, SAN DIEGO

Investigating the changes in expression of the synaptic regulator neurotrypsin in human Rett syndrome neurons

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

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2013

The thesis of Sonia Nan Kim is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2013

DEDICATION

In recognition of the love and support from my family and friends.

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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Lawrence S.B. Goldstein and Dr. Angels Almenar-Queralt for their encouragement and support during this project and my time in the lab. Their guidance and advice have helped me develop the skills for a strong foundation to pursue scientific research.

I would also like to acknowledge Dr. Alysson Muotri, Dr. Cassiano Carromeu, and Allan Acab for their willingness to collaborate on this project. In addition to providing advice, Dr. Cassiano Carromeu supplied the cell lines and the plasmid components for the lentiviruses, and shared a MeCP2 antibody and a *BDNF* primer to compare the antibodies and primers used in this project. He also provided tissue and RNA samples of the dissected brains from the *Mecp2*-deficient mice (supplementary figure 2). Allan Acab also gave advice and assisted in dissecting mice brains.

I want to thank Grace Woodruff, Dr. Paulina Ordonez, and Dr. Jessica Young for training me to use the BD Biosciences FACS ARIA II system. I would also like to recognize Dr. Jessica Young, Floyd Buen, and Arra Revilla for training me on lentivirus production. In particular, Dr. Jessica Young provided the scramble plasmid for the control lentivirus. Next, I would like to acknowledge Sol Reyna and Lauren Fong for sharing and providing the *Nestin* and *GFAP* primer sequences. I would like to thank the members of the Goldstein lab for their support.

Lastly, I want to thank all my family and friends for their encouragement and support as I was working on this project and my graduate degree.

ABSTRACT OF THE THESIS

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Sonia Nan Kim

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Rett syndrome (RTT) is a X-linked neurodevelopmental disorder caused by mutations in the gene encoding the transcriptional regulator methyl CpG binding protein 2 (MeCP2). It is not fully understood whether the genes regulated by MeCP2 contribute to the neuronal RTT phenotype. Recently, we identified *Neurotrypsin* as a

gene repressed by MeCP2. Neurotrypsin is a serine protease, which may play a synaptic role by processing the synaptic regulator agrin. Altered levels of neurotrypsin's proteolytic activity lead to major synaptic and cognitive defects. Given neurotrypsin's synaptic role, MeCP2's regulatory function on *Neurotrypsin* expression, and MeCP2 dysfunction's involvement in causing RTT, we wondered whether *Neurotrypsin* expression is altered in RTT. To start addressing this question, we used two different clones of human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs) from a male RTT patient carrying a nonsense *MECP2* mutation (RTT-Q83X). RTT-Q83X NSCs showed no detectable MeCP2. Basal levels of *Neurotrypsin* expression were variable between the RTT-Q83X NSC clones; however, both clones exhibited increased *Neurotrypsin* expression, relative to control cells, during differentiation. Additionally, fluorescence-activated cell sorting (FACS)-purified RTT-Q83X neurons also exhibited *Neurotrypsin* upregulation. To mimic the RTT-Q83X neurons, we knocked-down *MeCP2* in FACS-purified control neurons. Surprisingly, *Neurotrypsin* was downregulated, suggesting a more complex regulatory mechanism for *Neurotrypsin* expression than anticipated. In conclusion, we found *Neurotrypsin* upregulation in neurons derived from a RTT patient. This could potentially lead to increased levels of neurotrypsin and neurotrypsin-dependent cleavage of agrin, which might contribute to the synaptic defects observed in RTT-derived neurons and ultimately the disease.

I:

Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder characterized by a regression in developmental skills, particularly in motor and language abilities, after six to thirteen months of normal development (Rett, 1966; Charour and Zoghbi, 2007, Hagberg et al., 1983). The disorder primarily affects females at a rate of one in every 10,000 live female births; however, there are some cases of males with Rett syndrome (Charour and Zoghbi, 2007; Jan et al., 1999). In addition to the regression of motor and speech abilities, Rett syndrome patients also experience apraxia, ataxia, cardiac problems, seizures, and respiratory abnormalities (Charour and Zoghbi, 2007; Williamson and Christodoulou, 2006). The brains of these individuals maintain a normal external appearance and also show no signs of degeneration (Armstrong, 2005; Jellinger et al., 1988; Reiss et al., 1993). The soma size for RTT neurons are smaller, compared to controls (Armstrong, 2005). In addition, there are decreased dendritic spines and arbors for RTT neurons (Armstrong, 2005). Most classical RTT cases are caused by sporadic mutations in the X-linked methyl CpG binding protein 2 (*MECP2*) gene (Amir et al., 1999). The gene encodes for the MeCP2 protein which functions as a transcriptional regulator, controlling repression and activation of many genes during neural development (Amir et al., 1999; Charour et al., 2008).

It is not fully understood whether the genes modulated by MeCP2 contribute to Rett syndrome, particularly to the neuronal phenotypes associated with the disease. Unpublished work in the Goldstein lab found that, using chromatin immunoprecipitation (ChIP) assays, MeCP2 is recruited to the *Neurotrypsin* promoter (Almenar-Queralt et al., submitted). Experiments utilizing siRNAs to knock down

MeCP2 in mouse embryonic fibroblasts (MEFs) showed changes in *Neurotrypsin* expression: a reduction of MeCP2 led to *Neurotrypsin* upregulation, suggesting that MeCP2 may repress *Neurotrypsin* expression (Almenar-Queralt et al., submitted).

Neurotrypsin (*Motopsin*, *PRSS12, BSSP-3*) is a neuronal-secreted serine protease with a role in synaptic and neuronal circuit reorganization required for higher cognitive functions (Molinari et al., 2002). The primary locations of expression are the lateral amygdala, cerebral cortex, and hippocampus, suggesting a potential role for neurotrypsin in learning and memory (Gschwend et al., 1997). Functionally, it may play a role in important synaptic processes through its proteolytic activity on the heparan sulfate proteoglycan agrin, which functions as a regulator of synaptic formation (Reif et al., 2007). Neurotrypsin cleaves agrin at two sites, producing a 90 kDa and a 22 kDa C-terminal fragments (Reif et al., 2007). Studies with *Neurotrypsin*-deficient mice showed that when there was no agrin cleavage, there was reduced formation of hippocampal dendritic filopodia, which is thought to be synaptic precursors (Matsomoto-Miyai et al., 2009; Mitsui et al., 2009). When the 22 kDa agrin fragment was exogenously administered *in vitro*, filopodia formation was restored, suggesting that the 22 kDa agrin fragment is important in the promotion of dendritic filopodia (Matsumoto-Miyai et al., 2009). Additionally, an overexpression of *Neurotrypsin* in motor neurons can also lead to synaptic defects at the neuromuscular junction (Bolliger et al., 2010). In humans, a four base pair deletion in the *Neurotrypsin* gene, predicted to encode the protease domain, leads to mental retardation (Molinari et al., 2002).

Given MeCP2's repressive regulation on *Neurotrypsin* expression and neurotrypsin's involvement in synaptic activities via neurotrypsin-dependent agrin cleavage, the potential contribution of changes in *Neurotrypsin* expression to Rett syndrome becomes a question of interest. The development of induced pluripotent stem cells (iPSCs) from patients' fibroblasts have allowed researchers to create a human neuronal model of Rett syndrome (Marchetto et al., 2010). The neurons from these iPSCs recapitulate the major neuronal defects described for Rett syndrome, such as fewer synapses, reduced dendritic spine densities, among other physiological changes (Marchetto et al., 2010). Thus, this model serves as a tool for us to ask the question about the potential relevance of changes in *Neurotrypsin* expression to Rett syndrome.

The specific RTT line used in this project was derived from iPSCs of a male RTT patient with a nonsense mutation in the *MECP2* gene, referred to as RTT-Q83X. Two different clones from the same patient, termed RTT-Q83X clones 1 and 2, were used in this project to account for cell line variability. The corresponding control cell line was derived from iPSCs of the patient's father, a normal male; these cells would be genetically closer to the patient than that of an unrelated control. Only one clone from this control line was used in this project due to availability issues with obtaining a second clone.

Given neurotrypsin's role in synaptic function, MeCP2's regulatory function on *Neurotrypsin* expression, and MeCP2 dysfunction's involvement in causing Rett syndrome, we examined whether there are changes in *Neurotrypsin* expression in Rett

syndrome neurons. Exploring this relationship of *Neurotrypsin* expression to Rett syndrome neurons, and possibly the associated synaptic defects seen in these cells, will lead to a better understanding of the different MeCP2-influenced genes that may contribute to the disease and its development.

II:

Results

Variable changes in *Neurotrypsin* expression between RTT-Q83X neural stem cells (NSCs) clones

Control and RTT-Q83X neural stem cells (NSCs) were plated and harvested to observe any changes in *Neurotrypsin* expression at this stage in their lineage. Morphology between the control and the RTT-Q83X NSC lines was similar by eye (figure 1A). A western blot using these samples demonstrated the absence of the MeCP2 protein in the RTT-Q83X NSCs as expected, whereas the control NSCs had detectable MeCP2 protein levels at 62 kDa (figure 1B). Results from the RT-qPCR show that the RTT-Q83X clone 2 line has a significant increase in *Neurotrypsin* expression, compared to the control NSCs (figure 1C). However, the other RTT-Q83X line, clone 1, is significantly down-regulated in *Neurotrypsin* expression (figure 1C). The difference in *Neurotrypsin* expression between the RTT-Q83X NSC clones is significant (figure 1C). Cell line variability between the two clones at the NSC stage may be a potential explanation for these different results.

Given agrin's involvement in the neurotrypsin cleavage pathway and a published study showing that peaks in *Agrin* expression temporally coincide with synaptogenesis in rat brains (Hoch et al., 1993), we looked at *Agrin* expression to see if there were any significant changes at the NSC stage. *Agrin* expression was not significantly changed for RTT-Q83X clone 2, meanwhile RTT-Q83X clone 1 showed a significant downregulation in *Agrin* expression (figure 1D).

Studies observing changes in *Neurotrypsin* expression during mouse development show that *Neurotrypsin* expression, and subsequently neurotrypsin-

dependent agrin cleavage, peak during synaptogenesis (Reif et al., 2007). To address the possibility that the *Neurotrypsin* upregulation seen in RTT-Q83X clone 2 was not due to an early differentiation of that NSC line, expression of *MAP2*, a neuronal marker, was measured in the NSCs (figure 1E). There was no significant change in MAP2 expression for the RTT-Q83X NSCs in comparison to the control NSCs, which was expected since these NSCs are non-differentiating (figure 1E).

Results concerning the RTT-Q83X clone 2 line are in agreement with the suggestion that MeCP2 behaves as a repressor of *Neurotrypsin* expression; meanwhile, the *Neurotrypsin* expression differences of the two RTT-Q83X clones may be due to cell line variability at the NSC level. Given the unique neuropathology of Rett syndrome neurons and the peak in *Neurotrypsin* expression during synaptogenesis, we next investigated whether there are changes in *Neurotrypsin* expression in differentiating NSCs.

Control and RTT-Q83X differentiating NSCs have similar morphology

Differentiation of NSCs was initiated by removing FGF from the NPC-base media. Differentiating NSCs were left in culture for up to five weeks and cells were observed and collected at the following time points: weeks 0, 1, 3, and 5. At the respective time points, the control and RTT-Q83X differentiating NSCs exhibited similar morphology (figure 2).

RTT-Q83X overexpress *Neurotrypsin*, but not *Agrin*, during differentiation

Cells collected at the week 0, 1, 3, and 5 time points were analyzed by RT-qPCR to observe any expression changes over time within each cell line. Since NSCs can differentiate into non-neuronal cells, we first examined the possibility of a presence or absence of neurons and other non-neuronal cell types in each differentiating line. Expression levels of *MAP2*, *GFAP*, and *Nestin*, which are markers of neurons, astrocytes, and NSCs respectively, were used to determine the overall presence of these cell types in the mixed cell population. An increase in *MAP2* expression is seen as the NSCs differentiate, indicating that the NSC population is differentiating into neurons (figure 3A). There is an upregulation or increasing trend in *GFAP* expression by week 5, suggesting that there is an increased astrocytic presence during NSC differentiation (figure 3B). *Nestin* levels decreased overall as expected, indicating that the population of NSCs is decreasing as each cell line becomes more differentiated (figure 3C). *BDNF* expression was examined, since published studies reported that BDNF levels were reduced in *Mecp2*-mutants in RTT mouse models (Chang et al., 2006; Li et al., 2012). *BDNF* expression was upregulated in each cell line as they differentiated over time (figure 4A). These *GFAP*, *Nestin*, and *MAP2* expression results suggest that the control and RTT-Q83X cell lines are differentiating similarly within their respective lines, whereas the *BDNF* expression results indicate that similar expression behavior is exhibited within each cell line.

All differentiating NSCs showed a significant increase in *Neurotrypsin* expression over time (figure 4B). This fits with published work which suggests that

Neurotrypsin expression peaks during synaptogenesis (Reif et al., 2007). *Agrin* expression also had an overall significant increase as the NSCs became more differentiated (figure 4C). This also fits with a published finding which suggests that the peak of *Agrin* expression parallels with synapse formation (Hoch et al., 1993).

Given that these results show an increase in *Neurotrypsin* and *Agrin* expression in all differentiating lines, we compared *Neurotrypsin* and *Agrin* expression changes between the control and RTT-Q83X differentiated NSCs at each time point. At the non-differentiation stage (week 0) and at the beginning of differentiation (week 1), the two RTT-Q83X clones show similar results: the clone 1 line shows a significant *Neurotrypsin* downregulation and the clone 2 line shows a significant *Neurotrypsin* upregulation (figure 5A). This could be due to variability between the two RTT-Q83X clone lines at the NSC stage. This speculation is further strengthened by evidence that there is still a large presence of a NSC population, suggested by the *Nestin* expression, at the week 1 time point in comparison to the week 3 time point in each of the lines (figure 3C). The possibility of RNA sample degradation for RTT-Q83X-clone 1 in weeks 0 and 1 was ruled out, as RNA integrity tests showed minimal RNA degradation and similar RNA quality for all samples collected from the three lines at weeks 0 and 1 (supplementary figure 1).

Later in differentiation (weeks 3 and 5), RTT-Q83X clone 1 had a significant upregulation in *Neurotrypsin*; the RTT-Q83X clone 2 experienced a significant *Neurotrypsin* increase only at week 3 (figure 5A). This upregulation in both clones at week 3 may be associated with the increased presence of a mature neuronal

population, as evidenced by the significant *MAP2* upregulation in each of the lines by week 3 (figure 3A). The non-significant result for *Neurotrypsin* expression seen in RTT-Q83X clone 2 at week 5 may be due to an increased presence of other non-neuronal cells, such as astrocytes. This speculation is strengthened by evidence showing an upregulation or increased trend in *GFAP* expression for all three differentiating lines at week 5 (figure 3B).

Although *Agrin* expression was upregulated for each differentiating cell line, it did not have a similar increasing trend that is seen with *Neurotrypsin* expression in the RTT-Q83X cells (figure 5B). *BDNF* expression analysis shows that there is no change in *BDNF* expression for RTT-Q83X cells at weeks 0, 1, and 3, with the exception of RTT-Q83X clone 2 experiencing a significant *BDNF* upregulation at week 1 (figure 5C). By week 5, only the RTT-Q83X clone 1 achieved a statistically significant *BDNF* downregulation (figure 5C). There are several different isoforms of *BDNF*, due to the several promoters which produce the isoforms of the *BDNF* gene (Liu et al., 2006), so it is possible that the specific *BDNF* isoform used for this RT-qPCR could result in this behavior.

MAP2 expression was analyzed to see if there were any differences in neuronal differentiation between the control and RTT-Q83X differentiated NSCs at each time interval. As previously seen in the non-differentiating NSCs, the two RTT-Q83X clones did not have a significant *MAP2* difference relative to the control NSCs (figure 5D, week 0). At the beginning of the differentiation stage (week 1), there is a significant increase in *MAP2* expression for both RTT-Q83X lines (figure 5D). By

weeks 3 and 5, the two RTT-Q83X differentiating NSCs showed no significant change in *MAP2* expression compared to differentiating control NSCs, suggesting that all three lines do not have significant neuronal population differences at these two differentiation time points (figure 5D).

Since a general increase in *Neurotrypsin* expression is seen with the RTT-Q83X differentiating NSCs along with an increase in *GFAP* expression for all differentiating NSCs, the non-homogenous cell population in these differentiating cultures becomes an issue; it could be that a non-neuronal cell type is responsible for the *Neurotrypsin* upregulation, instead of an increased neuronal population. In fact, preliminary experiments suggest that *Neurotrypsin* is expressed in iPSC-derived astrocytes (personal communication by Angels Almenar-Queralt).

Significant *Neurotrypsin* upregulation seen in FACS-purified RTT-Q83X neurons

To address the issue of a non-homogenous cell population, fluorescence-activated cell sorting (FACS) was used to isolate a more purified population of neurons from differentiating NSCs (Figure 6). NSCs were differentiated for three weeks and FACS-purified according to protocols established in the Goldstein lab (Yuan et al., 2011). Isolated neurons were then in culture for two weeks. For the first week in culture, neurons were incubated with glial-conditioned media (GCM) with the factors (BDNF, GDNF, and dbcAMP) to ensure neuronal survival after the FACS-procedure. After one week under these conditions, the neurons were switched to either glial-conditioned media with the three factors or glial-conditioned media alone.

These two conditions were kept separate due to the possibility that the presence of these factors may affect gene expression, such as correcting for expression changes in RTT-Q83X neurons. Additionally, these two culture conditions were done in parallel, in case that the FACS-purified neurons could not survive without the presence of these factors in the media. Cells were collected and examined for any expression changes in *Neurotrypsin* and *Agrin*. In addition to isolating a more homogenous neuronal population, the FACS protocol also reduces the likelihood of working with various differentiation states in a mixed population.

Comparing *Neurotrypsin* and *Agrin* expression between cells from the two different post-FACS culture conditions, there is a significant increase in *Neurotrypsin* expression in the FACS-purified control and RTT-Q83X clones which had BDNF, GDNF, and dbcAMP in the second culture week (figure 8A). This suggests that either these factors are influencing gene expression or that an extended presence of these factors may encourage overall neuronal survival after FACS purification. Expression of *MAP2*, a neuronal marker, was analyzed to compare general differences between the neuronal populations in the two culture conditions. Overall, no significant change was seen for the control and RTT-Q83X lines in the two culture conditions (figure 8C). *Agrin* expression changes in the control and the RTT-Q83X clones did not correlate with the *Neurotrypsin* expression changes seen for the cells in the corresponding condition; there was no significant upregulation in *Agrin* expression (figure 8B).

FACS-purified control and RTT-Q83X neurons without factors during the second week of culture still survived and also showed similar morphology after the purification procedure (figure 7). Given the *Neurotrypsin* expression differences between the two post-FACS culture conditions and the confirmation that FACS-purified neurons can survive without the factors in the second week, we focused on analyzing expression changes in *Neurotrypsin* and *Agrin* from cells without factors during the second culture week.

Comparing *Neurotrypsin* expression changes between FACS-purified control and RTT-Q83X neurons, there is a significant *Neurotrypsin* upregulation in RTT-Q83X neurons (figure 9A). Considering that the differences in differentiation states and mixed cell populations are reduced in this cell population, the resulting *Neurotrypsin* upregulation is significant and distinct, fitting with the current hypothesis that an absence of MeCP2 in RTT-Q83X cells leads to *Neurotrypsin* upregulation. Regarding changes in *Agrin* expression, the RTT-Q83X clone 1 neurons had a statistically significant upregulation, whereas the RTT-Q83X clone 2 neurons showed no significant change relative to the control neurons (figure 9B).

We also analyzed *MAP2* and *GFAP* expression differences between the FACS-purified control and RTT-Q83X neurons to verify that the resulting cell populations were a majority homogenous neuronal population. The FACS-purified RTT-Q83X neurons showed no significant *MAP2* expression change when compared to the FACS-purified control neurons (figure 9C). *GFAP* expression analysis of the lines suggests that there may have been non-neuronal cell contaminants present in culture (figure

9D). If astrocytes were present in the culture, as suggested by the *GFAP* expression, then the astrocytes could provide an additional support system for the FACS-purified neurons, perhaps increasing survival of these neurons after the sorting procedure. However, the presence of a non-neuronal cell type, such as astrocytes, throws into question whether the associated *Neurotrypsin* upregulation for these cells is due to a more homogenous neuronal population. When examining the plated FACS-purified neurons, the non-neuronal cell types were seen on occasion, which may correlate with the *GFAP* expression changes. However, it was visually confirmed that there were not many contaminant cells seen and that the FACS-purified neurons was the dominant cell type seen in these cultures (figure 7). For these reasons, the presence of a non-neuronal cell type in these cultures does not significantly affect the conclusions regarding *Neurotrypsin* upregulation. Overall, these results indicate that the FACS purification procedure does not completely isolate a neuronal population, but that the sorting procedure isolates for a more homogenous neuronal population. Additionally, it reduces differences in various differentiation states, as seen with the differentiated NSC population, making the FACS purification procedure still a useful tool.

MeCP2 knock down in FACS-purified control neurons leads to *Neurotrypsin* downregulation

The potential for variability between cell lines derived from the same individual is a concern, especially since this project did not use a large number of cell lines derived from the same patient. To address this, we cloned a MeCP2 shRNA

(shMeCP2) into a pLentilox3.7 lentiviral vector to infect FACS-purified control neurons one week after FACS purification. Cells were allowed to recover for a week in culture after the infection. If the lentiviral infection worked, then we would expect to see the FACS-purified control neurons infected with shMeCP2 lentivirus to have similar gene expression changes to that of the RTT-Q83X neurons.

The efficiency rate of the shMeCP2 infection was not as high compared to the infection efficiency rate of the control virus, as evidenced by the florescent differences of the infected neurons (figure 10). The neurons infected with the shMeCP2 lentivirus showed a significant knock down of *MeCP2* (figure 11A). However, there was a unexpected significant downregulation of *Neurotrypsin* expression in shMeCP2-infected neurons compared to the neurons infected with the control-scramble (pSICOR) lentivirus (figure 11B). *MAP2* expression was not significantly different between the control- and shMeCP2-infected neurons, suggesting that the results seen in figures 11A and 11B are not due to a large-scale cell death resulting from potential toxicity from using the shMeCP2 lentivirus (figure 11C). *Agrin* expression did not significantly change in response to the *MeCP2* knock-down (figure 11D). These experiments were repeated in two different control lines; one control line was an unrelated control. The results of these experiments were recapitulated and have been compiled in the figures shown.

A second set of FACS-purified control neurons were infected with twice the concentration of shMeCP2 lentivirus to observe if *MeCP2* knock-down would be more efficient and to confirm if the expression changes of *Neurotrypsin* and *Agrin* behaved

in the same fashion in a more severe *MeCP2* knock-down condition. Expression changes for *MeCP2* and *Neurotrypsin* were similar to the prior infection experiments; however, there was a slight upregulation seen for *MAP2* expression and a downregulation for *Agrin* expression (figure 11E).

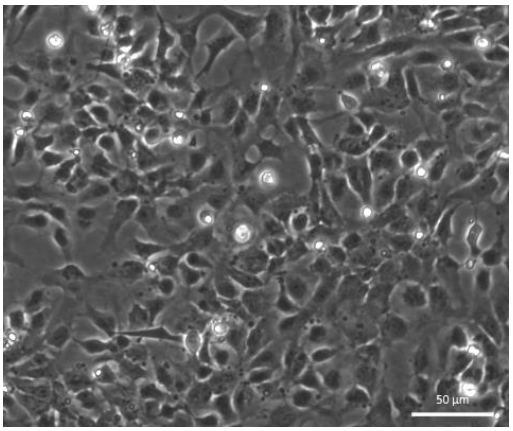
These lentiviral infection experiments show unexpected results for *Neurotrypsin* expression changes because the shMeCP2-infected control neurons did not behave similarly to the RTT-Q83X FACS-purified neurons. This result suggests several things including the idea that there may be more complex mechanisms regulating *Neurotrypsin* expression in these cells.

Figure 1: Variable changes in *Neurotrypsin* expression between RTT-Q83X neural stem cells (NSCs) clones

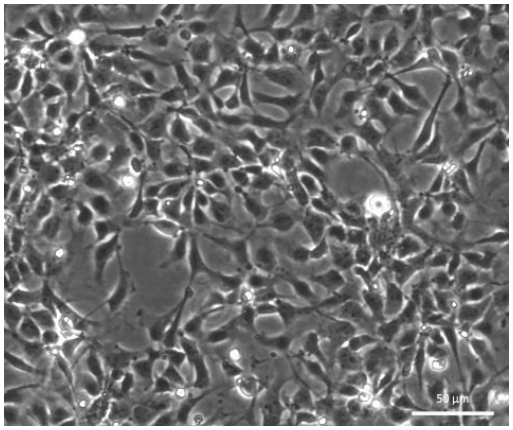
A. Representative images depicting similar morphology between control and RTT-Q83X NSC clones. The scale is set to 50 µm.
B. MeCP2 protein is detected by Western blot only for the control and not for the RTT-Q83X clones. 10 µg of protein was loaded on each lane. Membranes were probed with an antibody against MeCP2 and were reprobed with an antibody against actin as a loading control.
C-E. RT-qPCR shows variable expression changes for *Neurotrypsin* (C) and *Agrin* (D) between RTT-Q83X NSC clones; meanwhile, there is no significant change for *MAP2* expression between the control and RTT-Q83X cells (E). For *Neurotrypsin* and *Agrin* expressions: control, n = 5; RTT-Q83X clone 1, n = 2; RTT-Q83X clone 2, n = 5. For *MAP2* expression: control, n = 3; RTT-Q83X clone 1, n = 2; RTT-Q83X clone 2, n = 3.

A

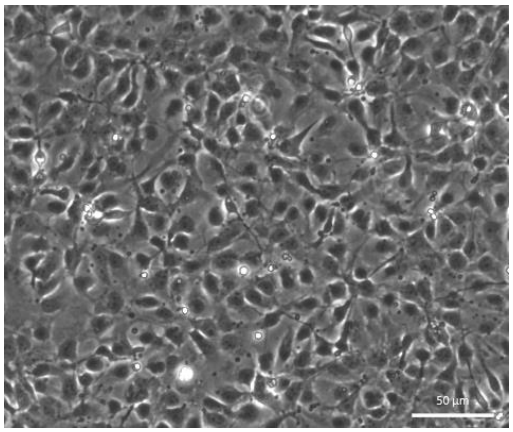
Control



RTT-Q83X #1



RTT-Q83X #2



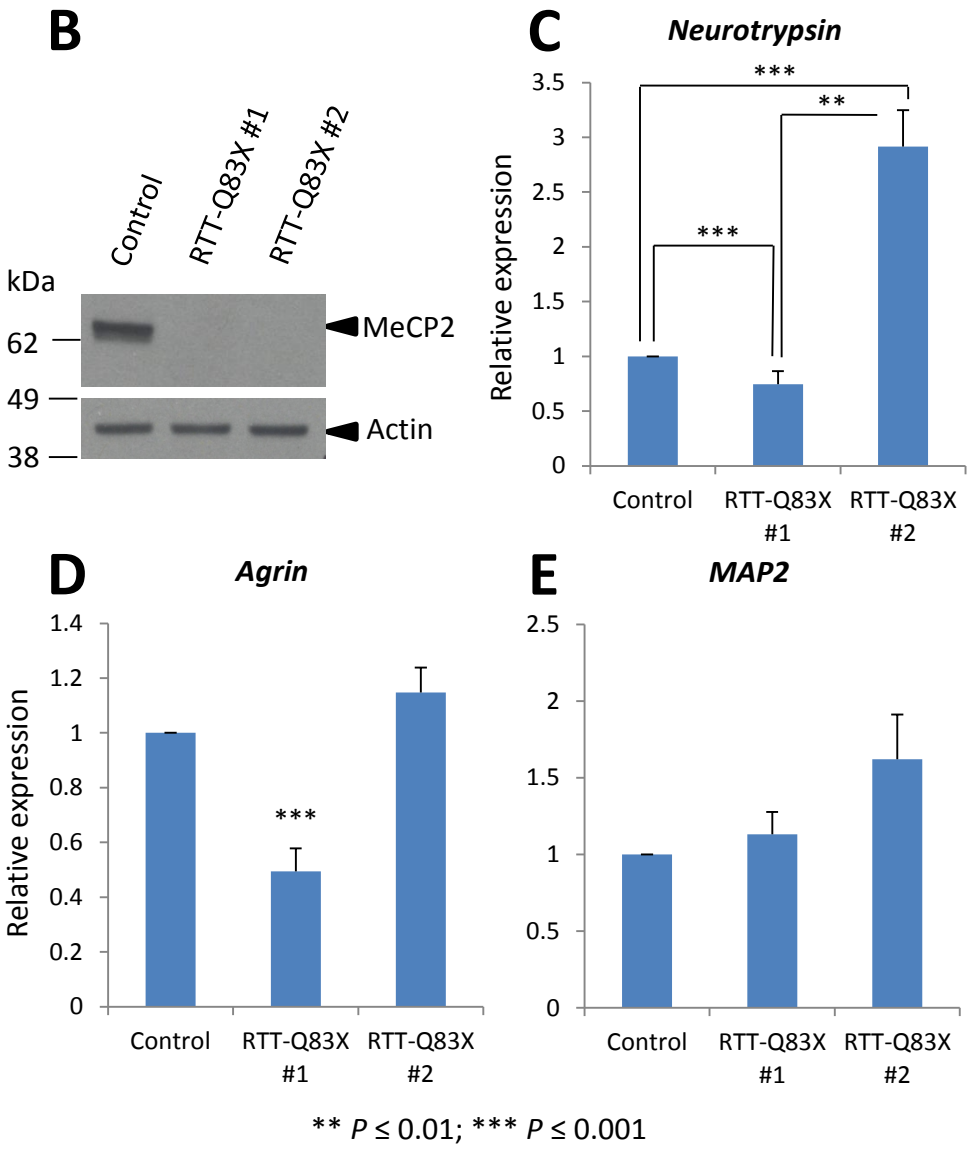


Figure 1, continued.

Figure 2: Control and RTT-Q83X differentiating NSCs have similar morphology

Representative images for control and RTT-Q83X differentiating NSCs show similar morphology at various differentiation time points (weeks 0, 1, 3, and 5). The red arrows indicate axonal projections. Although only one clone is shown, RTT-Q83X clones 1 and 2 showed similar morphology at each time point. The scale is set to 100 µm.

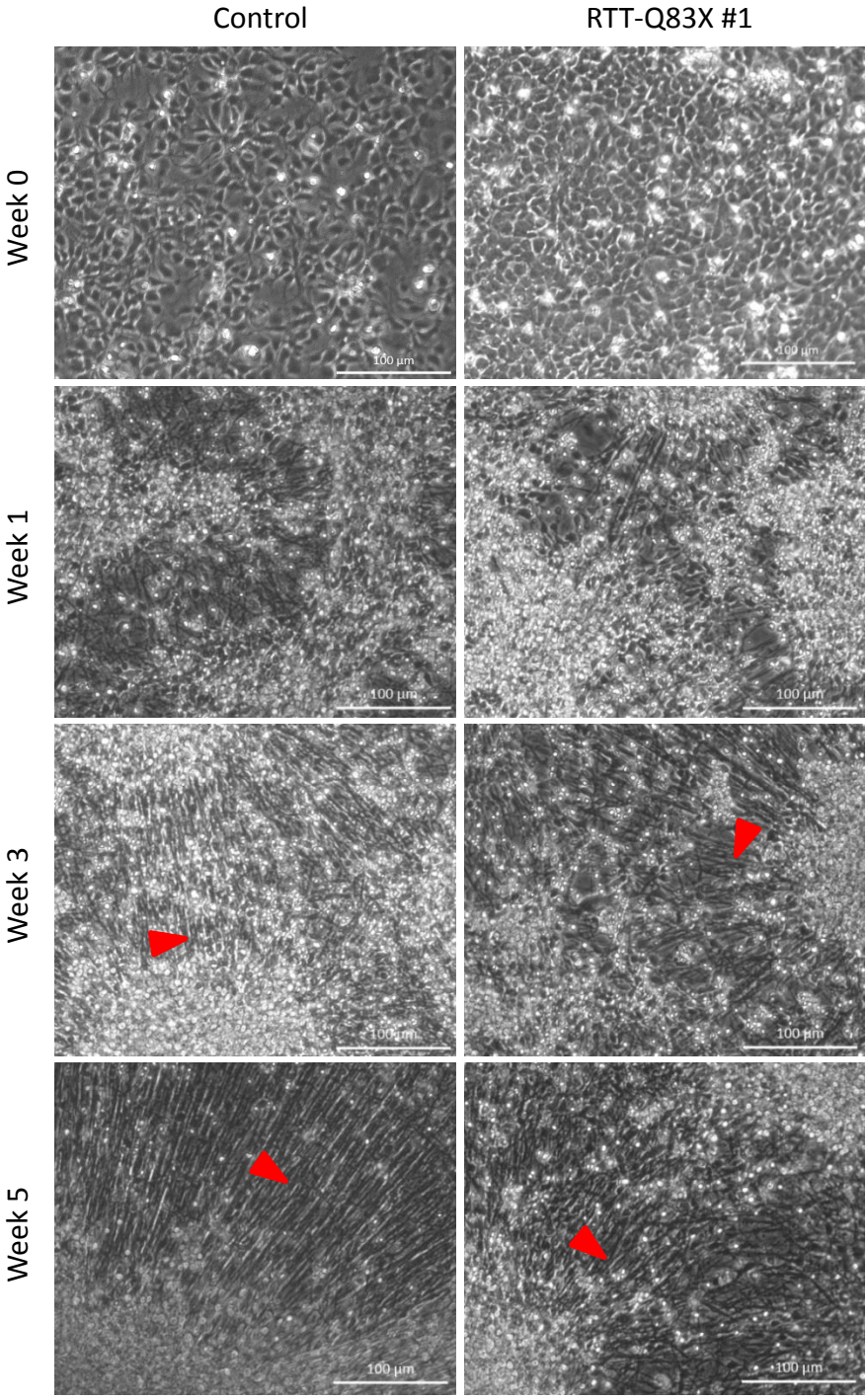
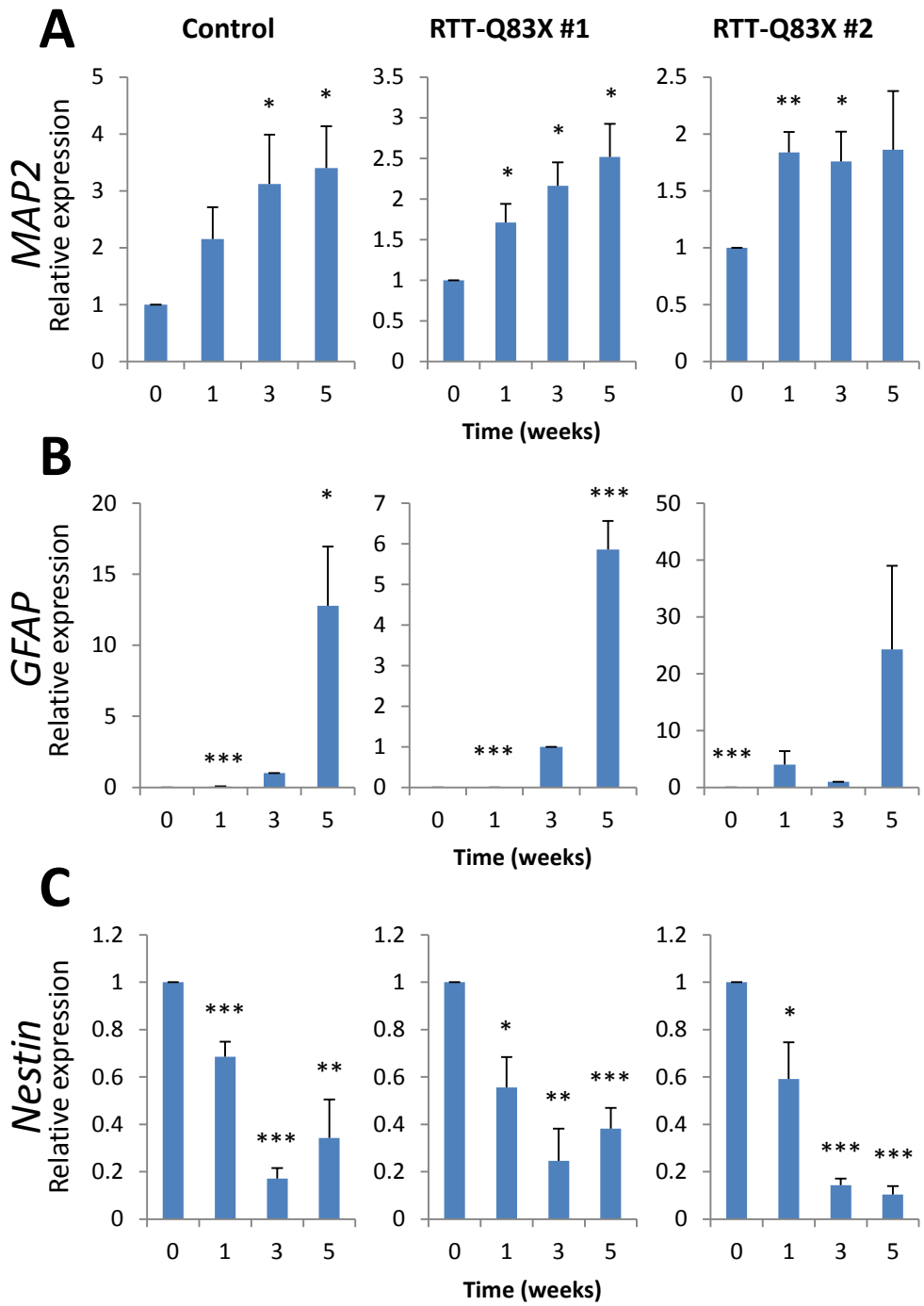

Control
RTT-Q83X #1
Week 0
Week 1
Week 3
Week 5
100 µm

Figure 3: Control and RTT-Q83X have similar differentiation

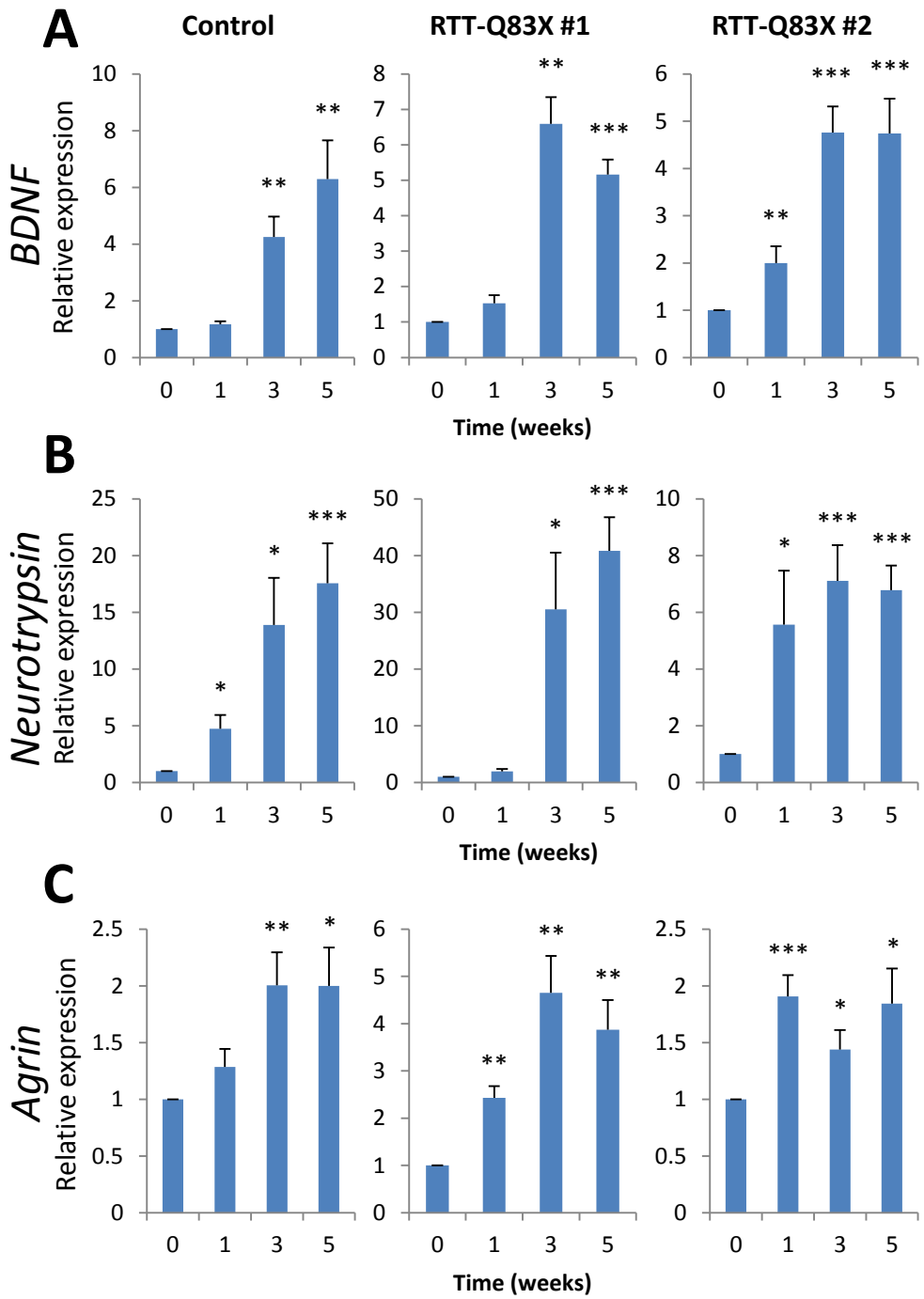
A-C. Expression changes for *MAP2* (A), *GFAP* (B), and *Nestin* (C) indicate that all cell lines are differentiating. Gene expressions were detected by RT-qPCR. Control, n = 3; RTT-Q83X clone 1, n = 2; RTT-Q83X clone 2, n = 3.



* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Figure 4: Differentiating NSCs have *Neurotrypsin* and *Agrin* upregulation

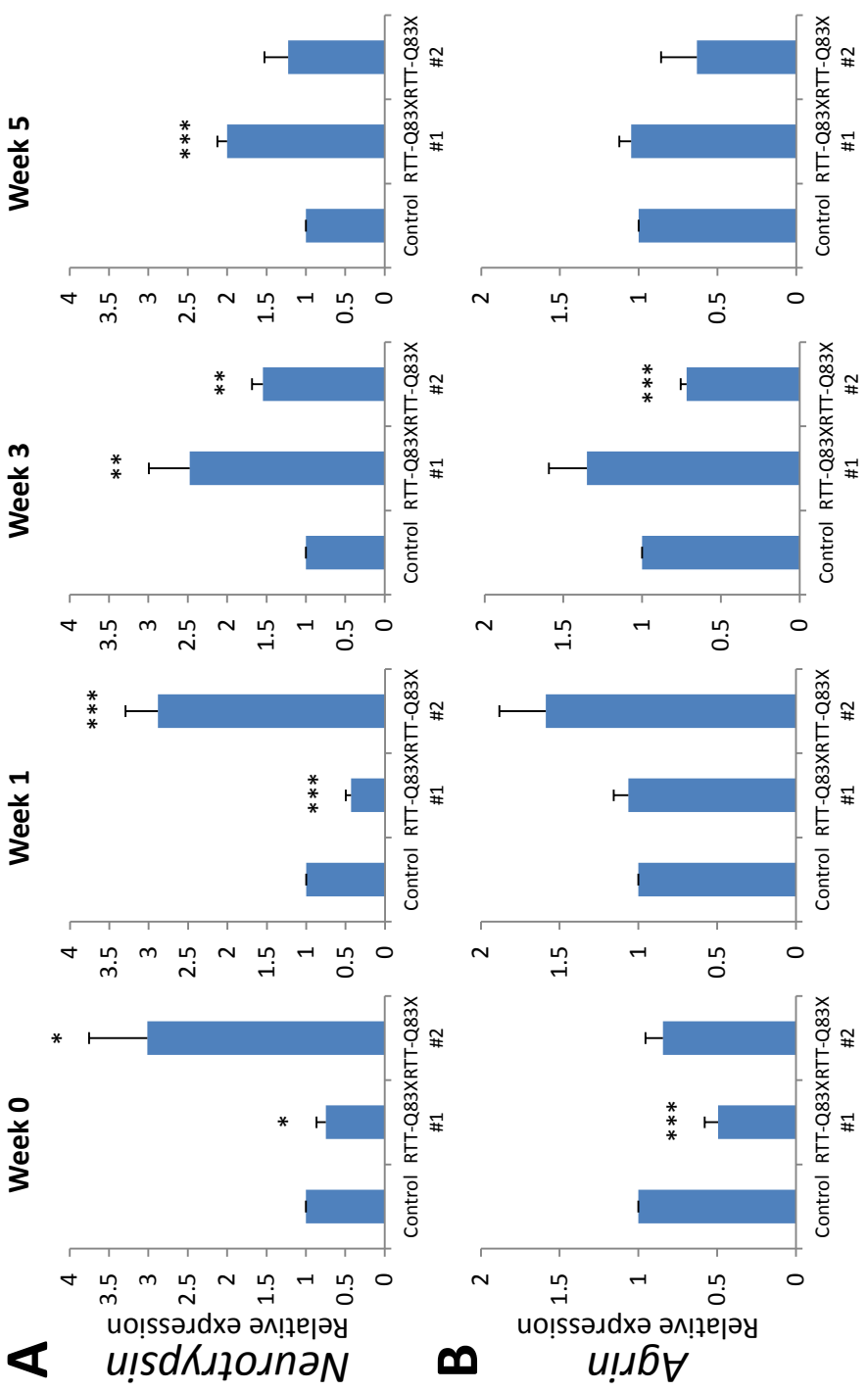
A-C. The control and RTT-Q83X differentiating NSCs exhibit similar behavior with an overall upregulation for *BDNF* (A), *Neurotrypsin* (B), and *Agrin* (C). Control, n = 3; RTT-Q83X clone 1, n = 2; RTT-Q83X clone 2, n = 3.

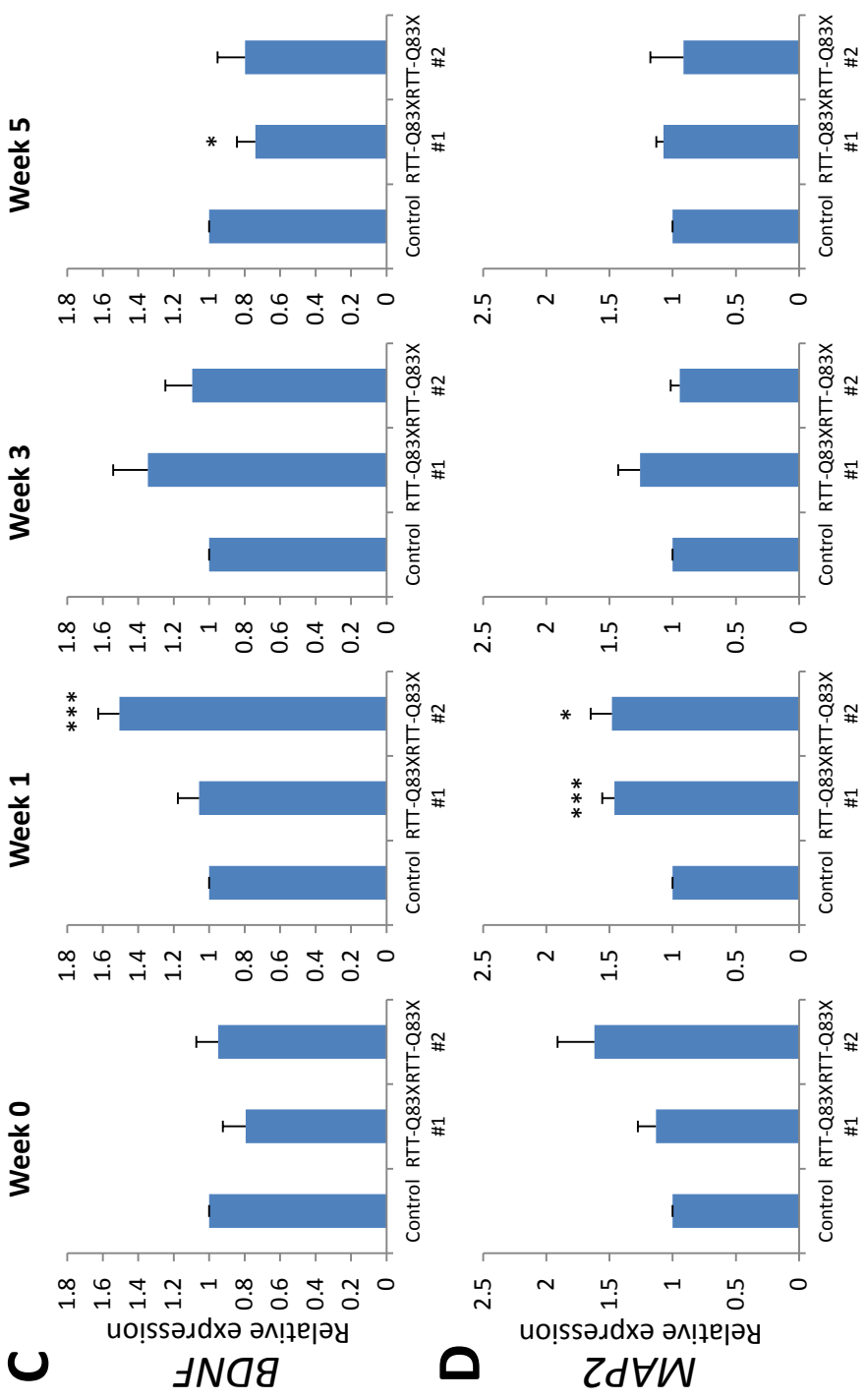


* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Figure 5: RTT-Q83X overexpress *Neurotrypsin*, but not *Agrin*, during differentiation

A – D. *Neurotrypsin* upregulation is seen for RTT-Q83X differentiating NSCs at a later differentiation stage (A); meanwhile *Agrin* expression changes do not show a similar increasing trend over time (B). Overall, there is no significant change for *BDNF* (C) and *MAP2* (D) expressions. Control, n = 3; RTT-Q83X clone 1, n = 2; RTT-Q83X clone 2, n = 3.


A
Neurotrypsin
Relative expression
Week 0
Week 1
Week 3
Week 5
Control
RTT-Q83XRTT-Q83X #1
RTT-Q83XRTT-Q83X #2
B
Agrin
Relative expression
* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001



* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Figure 5, continued.

Figure 6: Overview of obtaining FACS-purified neurons and culture conditions

Unsorted NSCs are differentiated for 3 weeks by removing FGF from the NPC base media. Cells are then sorted, according to protocols and the cell signatures established in the Goldstein laboratory (Yuan et al., 2011). FACS-purified neurons are plated for one week in glial conditioned media with factors (BDNF, GDNF, and dbcAMP). Afterwards, cultures are separated into two conditions for another one week culture: glial conditioned media with or without the factors.

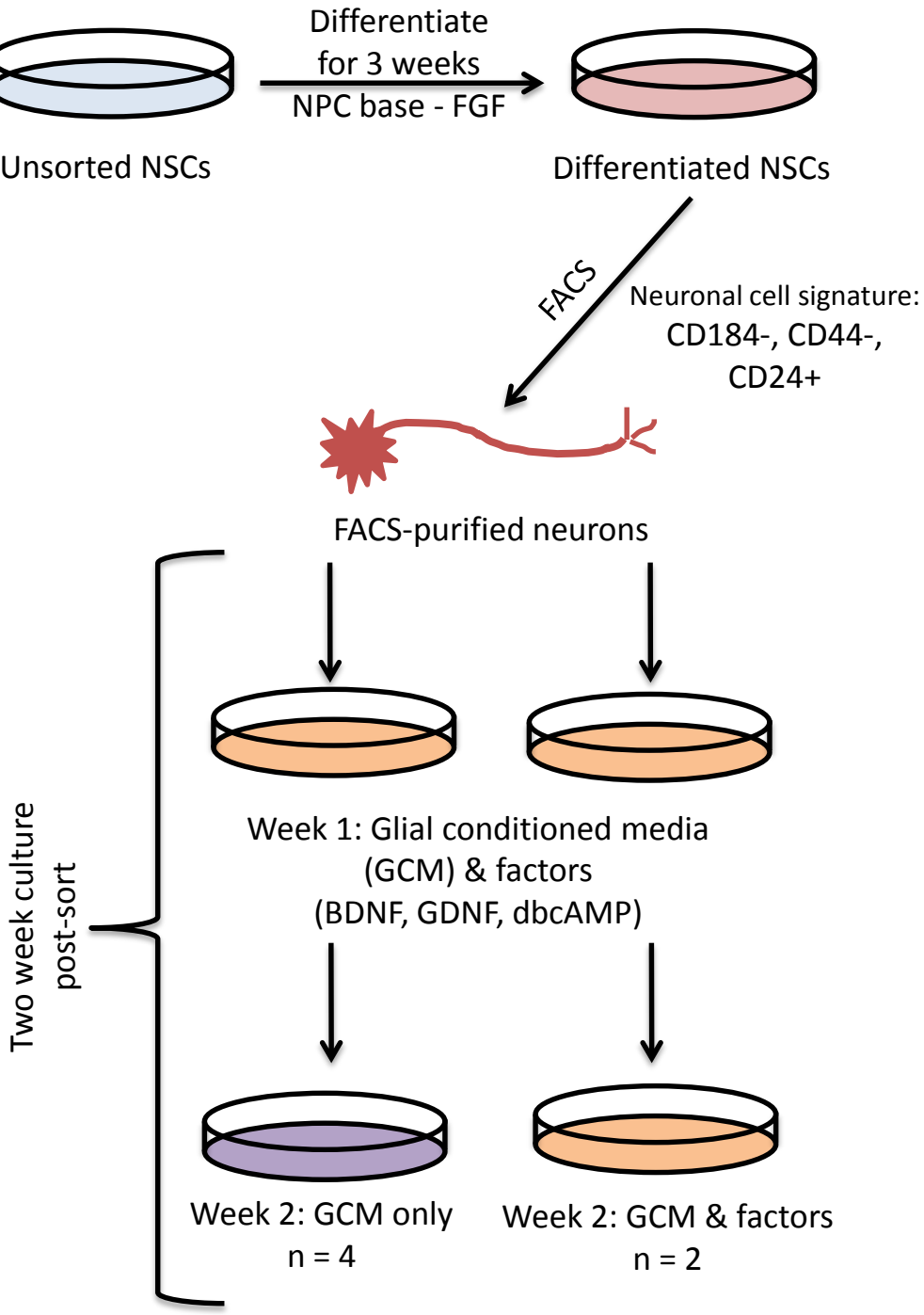
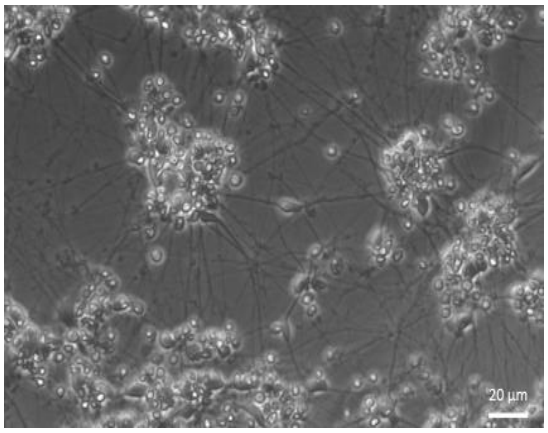

Differentiate
for 3 weeks
NPC base - FGF
Unsorted NSCs
Differentiated NSCs
FACS
Neuronal cell signature:
CD184-, CD44-,
CD24+
FACS-purified neurons
Two week culture
post-sort
Week 1: Glial conditioned media
(GCM) & factors
(BDNF, GDNF, dbcAMP)
Week 2: GCM only
n = 4
Week 2: GCM & factors
n = 2

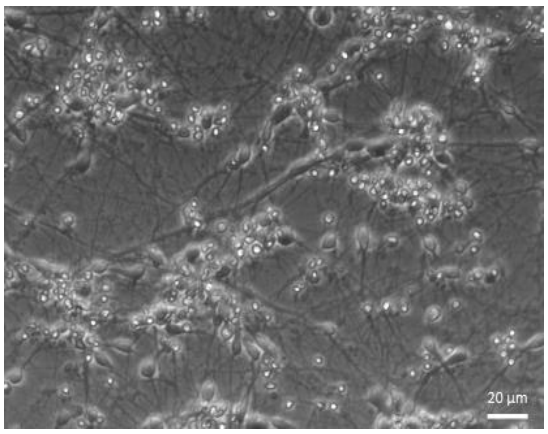
Figure 7: FACS-purified control and RTT-Q83X neurons exhibit similar morphology

The neurons in these images were in the following post-FACS culture conditions: the first week had factors (BDNF, GDNF, and dbcAMP) in the glial-conditioned media (GCM), and the second week had no factors in the GCM. Neurons incubated with the GCM and factors for the second week showed similar morphology to the neurons with the GCM alone for the second week. The scale is set to 20 μm.

Control



RTT-Q83X #1



RTT-Q83X #2

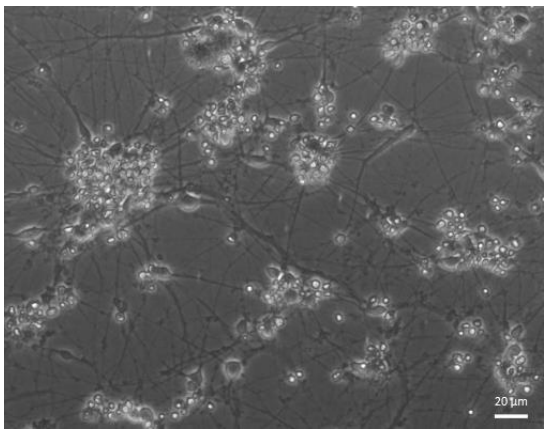


Figure 8: Presence of BDNF, GDNF, and dbcAMP for second culture week increases *Neurotrypsin* expression in FACS-purified neurons

A-C. *Neurotrypsin* upregulation is seen in each cell line with the factors (BDNF, GDNF, and dbcAMP) in the glial-conditioned media (GCM) for the second culture week (A). This is relative to the cell lines without the factors in the GCM for the second culture week. There was no significant change in *Agrin* (B) and *MAP2* (C) expressions for each cell line in the two different culture conditions. Control and RTT-Q83X #1, n = 2; RTT-Q83X #2 (for *Neurotrypsin* expression), n = 2; RTT-Q83X #2 (for *Agrin* and *MAP2* expressions), n = 3.

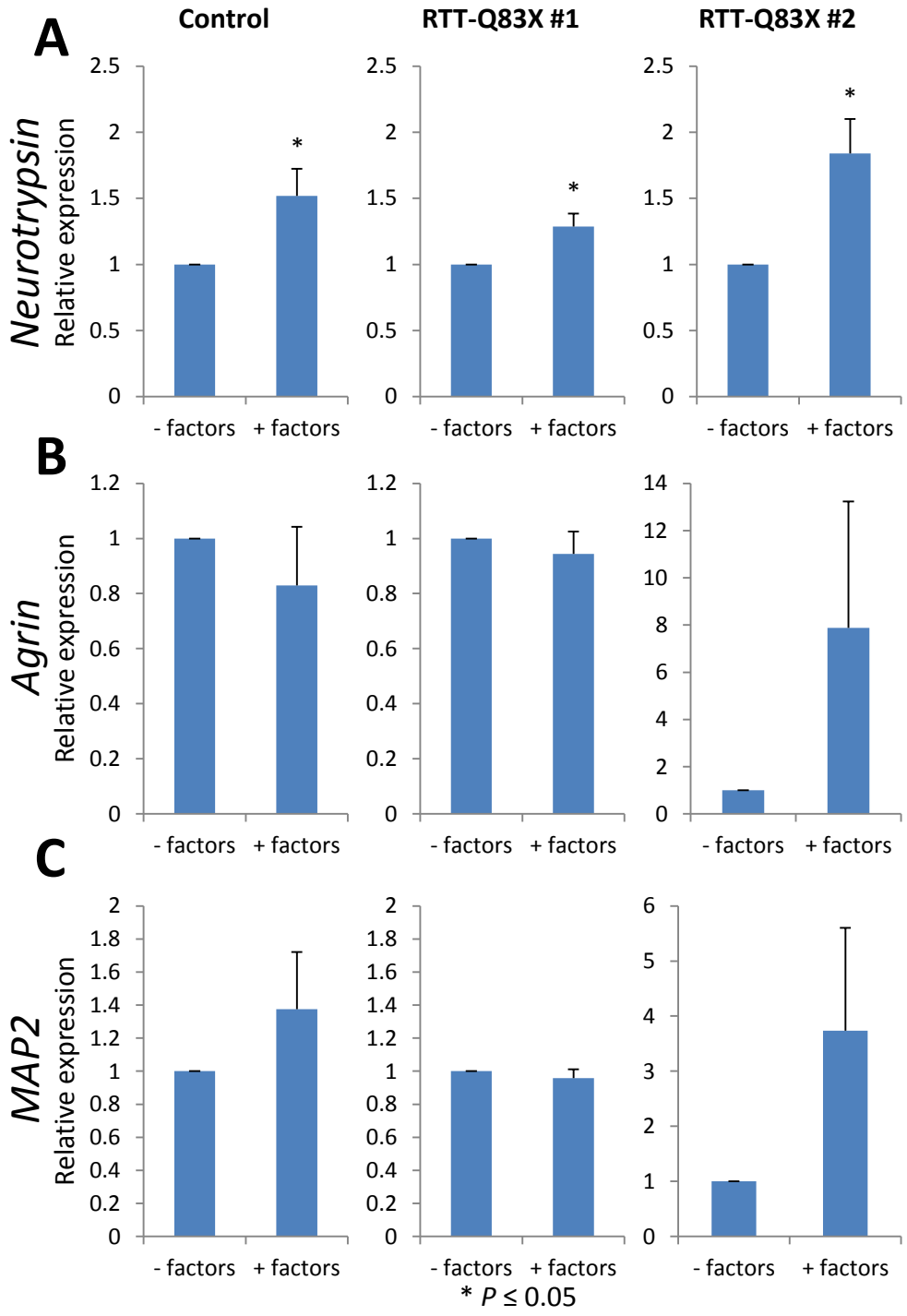
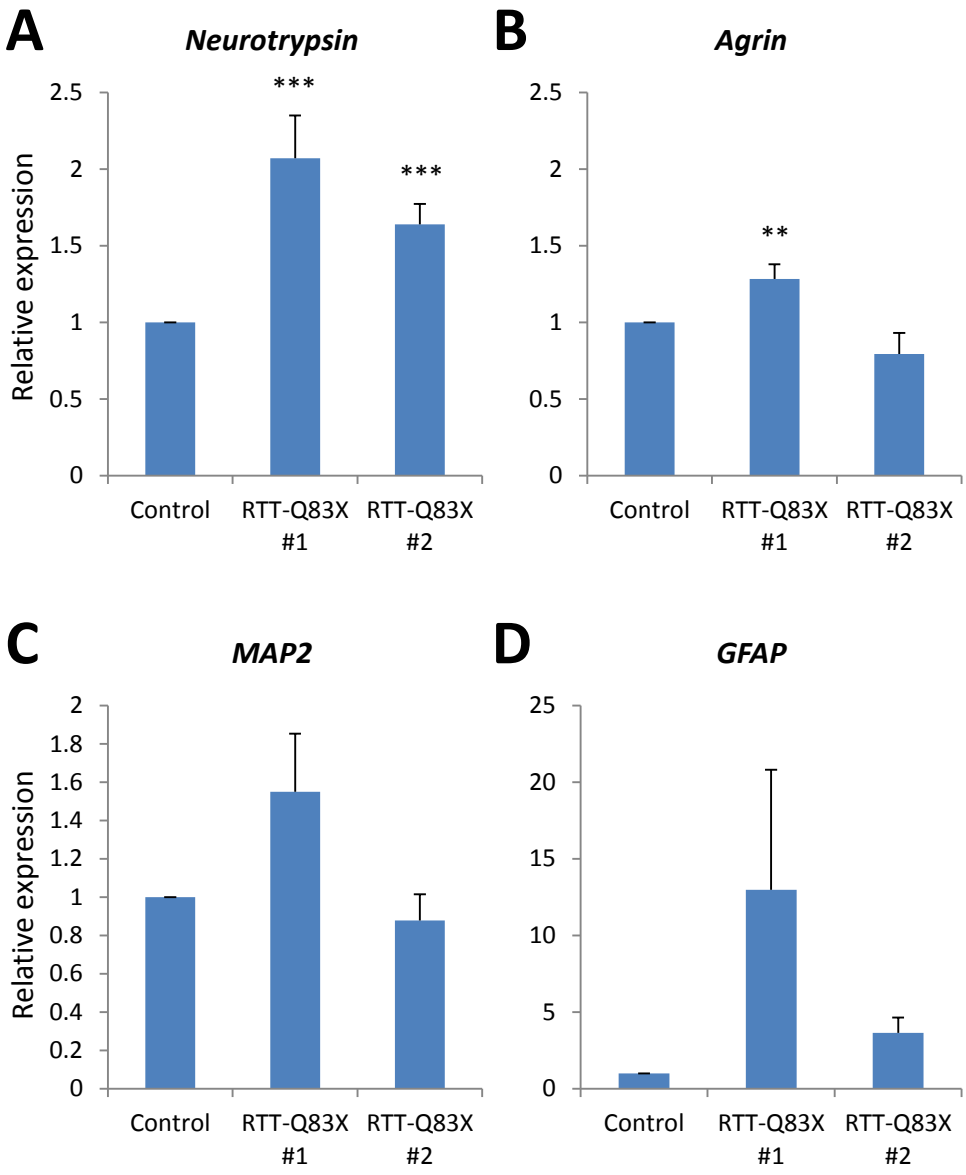

A
Control
RTT-Q83X #1
RTT-Q83X #2
Neurotrypsin
Relative expression
B
Agrin
Relative expression
C
MAP2
Relative expression
- factors
+ factors
* P ≤ 0.05

Figure 9: Significant *Neurotrypsin* upregulation seen in FACS-purified RTT-Q83X neurons

A-D. *Neurotrypsin* upregulation is seen in FACS-purified RTT-Q83X neurons in glial-conditioned media (GCM) without the factors (BDNF, GDNF, and dbcAMP) in the second week of post-FACS culture (A). Only the RTT-Q83X clone 1 line showed a statistically significant *Agrin* upregulation (B). There was no significant *MAP2* expression change for the RTT-Q83X cells (C). *GFAP* expression suggests the presence of a non-neuronal cell type in culture (D). A: Control and RTT-Q83X #1, n = 4; RTT-Q83X #2, n = 3. B, C, D: n = 4.



** $P \leq 0.01$; *** $P \leq 0.001$

Figure 10: The infection efficiency rate of shMeCP2 lentivirus is low compared to the control lentivirus

Representative bright-field (left) and immunofluorescent (right) images of FACS-purified control neurons infected with a control-scramble virus (top row) or a shMeCP2 virus (bottom row). Cells infected with the control virus expressed the *GFP* gene, whereas cells infected with the shMeCP2 virus expressed the *dsRed* gene.

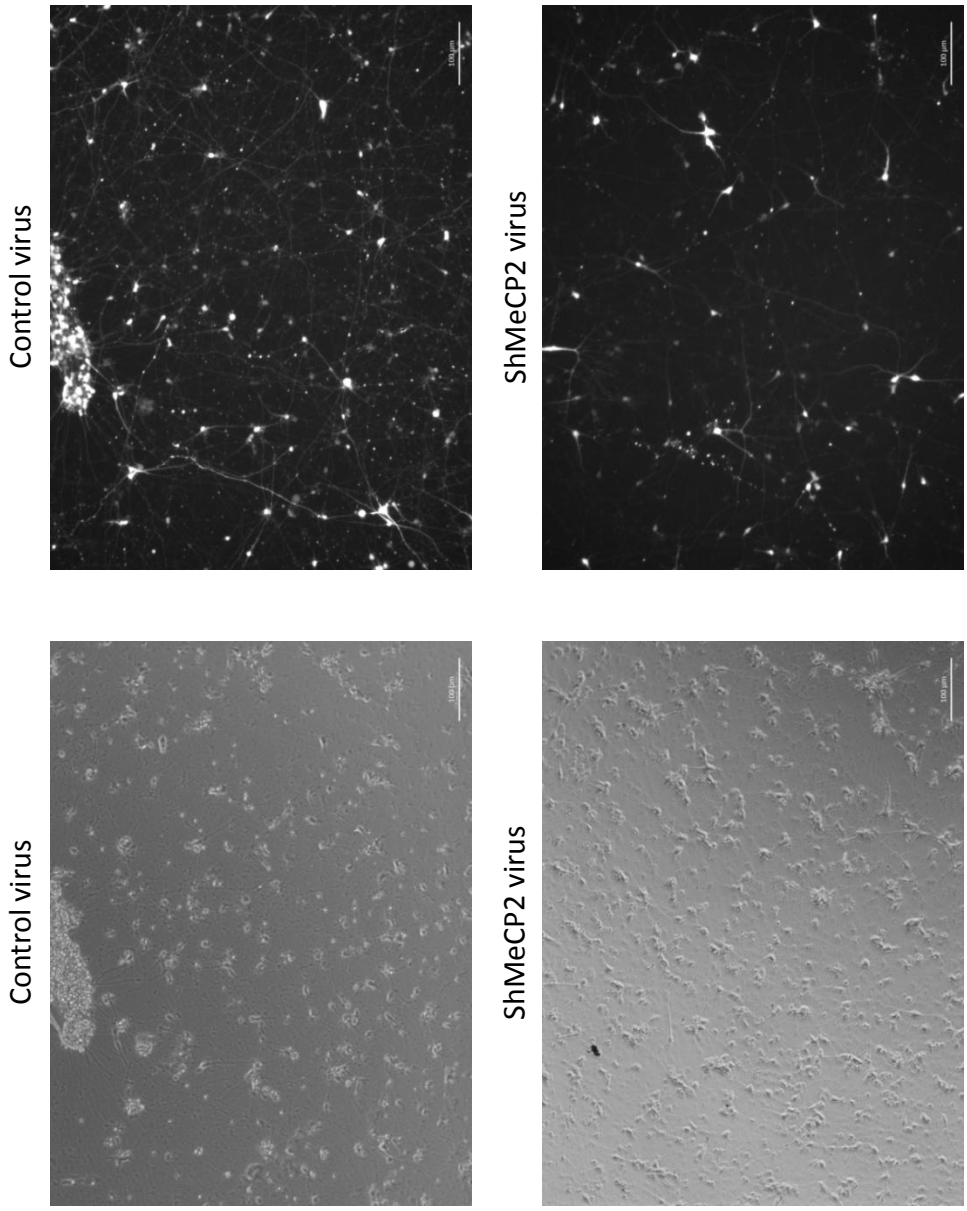
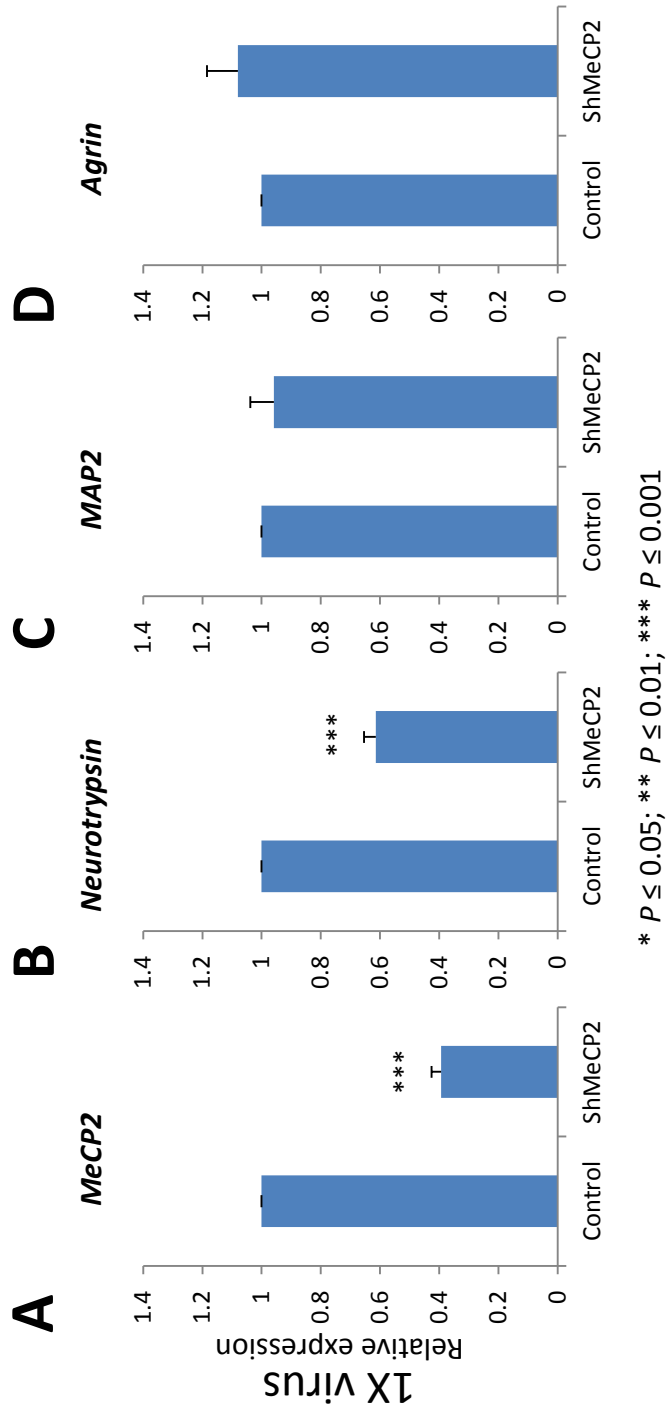
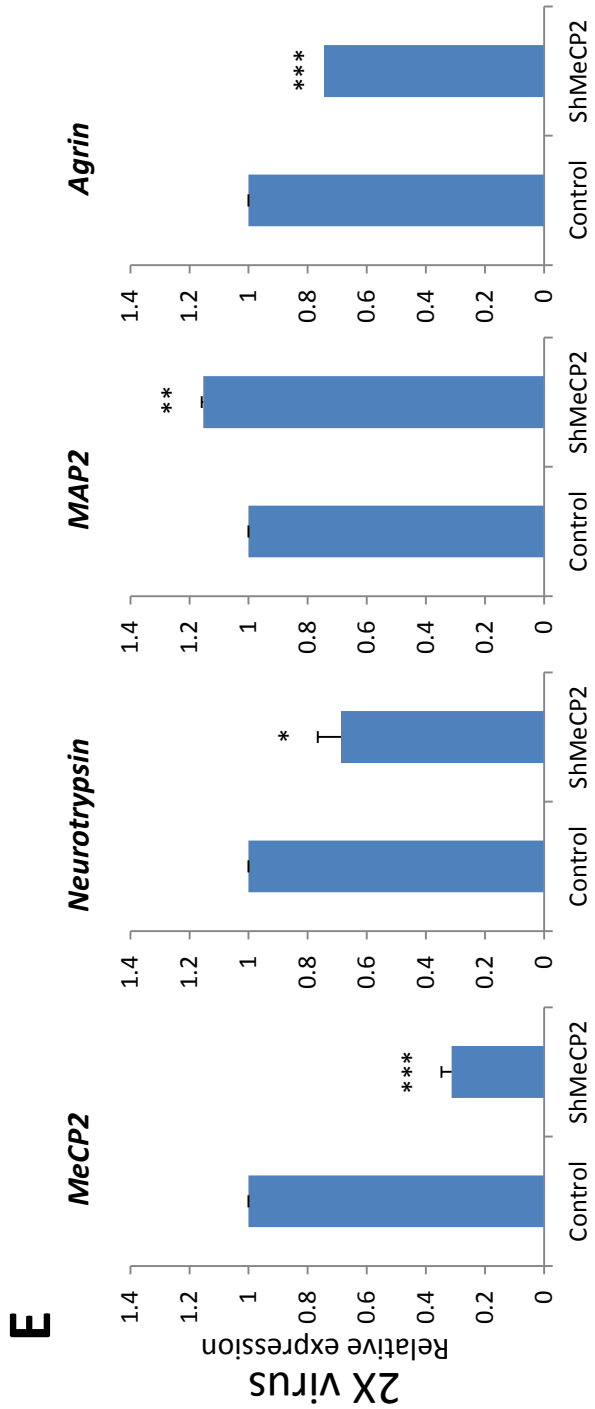

Control virus
ShMeCP2 virus
Control virus
ShMeCP2 virus

Figure 11: *MeCP2* knock-down leads to *Neurotrypsin* downregulation

A-D. *MeCP2* knockdown was successful (A). However, there was an unexpected *Neurotrypsin* downregulation (B). There were no significant expression changes for *MAP2* (C) and *Agrin* (D). N = 3.
E. The experiment was performed with twice the concentration of virus (F), yielding similar results for *Neurotrypsin* and *MeCP2* expressions but not for *MAP2* and *Agrin* expressions. N = 1.


A
MeCP2
B
Neurotrypsin
C
MAP2
D
Agrin
1X virus
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
ShMeCP2
***
* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001



* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Figure 11, continued.

III:

Discussion

We have analyzed *Neurotrypsin* expression changes in control and RTT-Q83X cells at different time points of the neural lineage and found *Neurotrypsin* expression is significantly upregulated in FACS-purified RTT-Q83X neurons. Through a series of RT-qPCRs, we also found that RTT-Q83X differentiating NSCs experience a general increase in *Neurotrypsin* expression in later differentiation. However, a *MeCP2* knock-down experiment in FACS-purified control neurons showed an unexpected downregulation of *Neurotrypsin* expression, leading to some ideas to possibly explain this event.

There are some speculations that may explain the downregulation of *Neurotrypsin* expression in the lentiviral infection experiments. Firstly, the viral backbone of the control lentivirus (pSICOR) is not the same backbone used for the shMeCP2 lentivirus (pLentiLox3.7). Given this difference, it calls into question whether it is appropriate to compare the expression results between the two viruses. However, if the results were hypothetically the same even with a more appropriate control, this suggests that the prior model, *Neurotrypsin* expression is repressed by MeCP2, is more complicated. A possibility is that other factors are recruited to the *Neurotrypsin* promoter in these control neurons now that *MeCP2* is knocked-down. Previous studies show that, using the chromatin immunoprecipitation (ChIP) assay, that CREB, CBP, and SP1 are also recruited to the *Neurotrypsin* promoter in mouse embryonic fibroblasts (Almenar-Queralt et al., submitted). Although a ChIP assay has not been done to confirm what is recruited to the promoter in this system, the possibility of other factors being recruited and altering *Neurotrypsin* expression cannot

be dismissed. There is also the speculation that the downregulation of *MeCP2* may result in other effects, such as reduced neuronal activity which could indirectly regulate *Neurotrypsin* expression.

Referring back to the initial model of *Neurotrypsin* expression repression by MeCP2, it was suggested that there is an upregulation of *Neurotrypsin* for RTT patients who have a *MECP2* loss-of-function mutation. This upregulation of *Neurotrypsin* transcript would suggest that there is more neurotrypsin protein present, which may produce more agrin fragments, such as a 22 kDa agrin fragment reported to rescue synaptic defects in an *in vitro* model (Matsumoto-Miyai et al., 2009). Given this information along with published studies reporting that either an excess or deficiency in neurotrypsin-dependent agrin cleavage leads to important synaptic defects, it would be interesting to expand this work to study the subsequent effects on synapses in RTT neurons as a result of a change in neurotrypsin-dependent cleavage of agrin. This work regarding *Neurotrypsin* expression changes in RTT cells can also be branched out to other non-neuronal cell types by studying the *Neurotrypsin* expression changes in those cells: emerging results suggest a role for glia in the RTT phenotype, making it a relevant cell to study for this disorder.

Studying changes in *Neurotrypsin* expression in an *in vivo* model may also be promising. Preliminary data using *Mecp2*-deficient mice from a RTT mouse model shows that *Neurotrypsin* expression is upregulated in the cerebral cortex and hippocampus but not the cerebellum (supplementary figure 2). This fits with published data which suggests that *Neurotrypsin* expression is primarily expressed in neurons of

the cerebral cortex, hippocampus, and lateral amygdala (Gschwend et al., 1997). Additionally, there have been reports suggesting that the cerebral cortex is the most affected meanwhile the cerebellum is the least affected in regards to localized weight changes in Rett syndrome brains (Armstrong, 2005).

In summary, we found an upregulation in *Neurotrypsin* expression in neurons derived from a RTT patient. This suggests that there may be altered levels of neurotrypsin, and subsequently neurotrypsin-dependent cleavage of agrin, indicating a potential relevance between *Neurotrypsin* expression and synaptic defects seen in Rett syndrome neurons.

IV:

Materials and Methods

Cell culture and differentiation

Neural stem cells (NSCs) were kept on 20 µg/mL poly-L-ornithine (Sigma) and 5 µg/mL laminin (Sigma) (POL) coated plates and incubated at 37°C. Media, NPC base with FGF, was changed every two days. NPC base media consisted of 1X DMEM: F12 + Glutamax (Gibco), 0.5X B27 (Gibco), 0.5X N2 (Gibco), and penicillin/streptomycin (Gibco). NSCs were split with Accutase (Innovative Cell Technologies). NSCs were differentiated by replacing the existing media with NPC base media. Rock inhibitor (0.001X, Ascent Scientific) was present in the NPC-base media for the first three to four days of differentiation and was subsequently replaced with NPC base media alone. At this point, media was changed every three to four days. FACS-purified neurons were plated on POL coated plates after sorting. For the first week after sorting, cells were plated with glial-conditioned media with BDNF (20 ng/mL; Peprotech), GDNF (20 ng/mL; Peprotech), and dbcAMP (1000X; Sigma). The media for the second week of culture was either glial-conditioned media alone or with BDNF (20 ng/mL; Peprotech), GDNF (20 ng/mL; Peprotech), and dbcAMP (1000X; Sigma). The media was half-changed at the 0.5- and 1.5- week time points; meanwhile a full media change was done at the one week time point. Glial-conditioned media was produced by collecting NPC-base media that incubated for 24 hours on Lonza human astrocytes. FACS-purified neurons for virus infection were also plated on POL coated plates. Cells were incubated with glial-conditioned media with BDNF (20 ng/mL; Peprotech), GDNF (20 ng/mL; Peprotech), and dbcAMP (1000X; Sigma) for the first week, and GCM alone for the second week. All reprogramming work was completed

by Dr. Cassiano Carromeu (Muotri lab). According to the Muotri lab's naming scheme, the RTT-Q83X #1 line is the RTT-Q83X clone 3 NSC line; the RTT-Q83X #2 line is the RTT-Q83X clone 13 NSC line; and the control line is the WT83 clone 7 NSC line.

FACS-purification: Isolating neurons from differentiated NSCs

NSCs were differentiated by adding NPC base media with rock inhibitor for three to four days. This media was replaced by NPC base media; media was changed every three to four days for three weeks. FACS isolation of neurons was completed by using the BD Biosciences FACS ARIA II system to sort for cells with the neuronal cell surface signature (CD184-, CD44-, CD24+) established by the Goldstein lab (Yuan et al., 2011). FACS-purified neurons were cultured in the conditions described in "cell culture and differentiation."

Lentiviral infection

The *MeCP2* shRNA (Cassiano Carromeu, Muotri lab) was cloned into a pLentiLox3.7 lentiviral vector. The corresponding control, a scramble plasmid (Jessica Young, Goldstein lab), was cloned into a pSICOR lentiviral vector. FACS-purified control neurons were infected with both viruses after a one-week culture in glial-conditioned media with the BDNF (20 ng/mL; Peprotech), GDNF (20 ng/mL; Peprotech), and dbcAMP (1000X; Sigma). The media on the neuronal cultures was reduced before adding the virus, to increase infection efficiency. DMEM media (1X;

Gibco) was used with the scramble virus to equate the total volume between the wells with the scramble virus and the wells with the shMeCP2 virus. Neurons were infected approximately 24 hours before replacing the existing virus-infected media with glial-conditioned media without BDNF (20 ng/mL; Peprotech), GDNF (20 ng/mL; Peprotech), and dbcAMP (1000X; Sigma). Neurons recovered for a week in culture after changing the media.

RNA extraction and RT-qPCR

Cellular RNA from NSCs and differentiated NSCs were collected using the PARIS kit (Ambion), according to manufacturer's instructions. These samples underwent a DNAse treatment by using the TURBO DNA-free kit (Ambion), following manufacturer's instructions. FACS-purified neurons were collected for cellular RNA using the RNeasy kit (Qiagen), according to manufacturer's instructions. Reverse transcription was completed on all samples by using SuperScript III First-Stand Synthesis System RT-qPCR (Invitrogen). The FastStart Universal SYBR Green Master (ROX) mix (Roche) was used for RT-qPCR. All results from the human cell lines were normalized to human *TBP*, human *RPL-27*, human *RPS-13*, and human *RPL-13A*. In addition to those primers, the human *NONO* primer was also used to normalize *GFAP* expression for the differentiating NSCs. Results were then compiled together based on the experimental primer. The human *MeCP2* primer used for the virus-infected control neurons is a primer that recognizes *MeCP2* isoforms 1 and 2.

Protein isolation and Western blot analysis

Protein was isolated from NSCs using the PARIS kit (Ambion). Ten micrograms of protein was loaded on a 4-12% bis-tris SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with primary antibodies overnight at 4°C. An antibody against MeCP2 (1:1,000; Sigma) was followed by a horseradish-peroxidase secondary antibody (1:5,000; Invitrogen). ECL was used to visualize results, using the ECL Western blotting substrate (Pierce). Membranes were re-probed with an antibody against actin clone C4 (1:10,000; Millipore) as a control. Protein was not isolated from FACS-purified neurons and virus-infected FACS-purified neurons.

Statistical analysis

RT-qPCR results are given as mean ± S.E.M. Two-tailed t-tests were used to determine statistical differences. $P \leq 0.05$ was considered statistically significant.

References

Almenar-Queralt, A., Kim, S.N., Herrera, C., Kang, D., Brenner. C., Garcia-Bassets, I., and Goldstein, L.S.B. Presenilins Modulate Neurotrypsin Expression and Neurotrypsin-Dependent Agrin Cleavage. Submitted.

Amir, R.E., Van den Veyver, I.B., Wan, M., Tran, C.Q., Francke, U., and Zoghbi, H.Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. 23: 127 – 128.

Armstrong, D.D. (2005). Neuropathology of Rett syndrome. J Child Neurol. 20:747.

Bolliger, M.F., Zurlinden, A., Lüscher, D., Bütikofer, L., Shakhova, O., Francolini, M., Kozlov, S.V., Cinelli, P., Stephan, A., Kistler, A.D., Rülicke, T., Pelczar, P., Ledermann, B., Fumagalli, G., Gloor, S.M., Kunz, B., Sonderegger, P. (2010). Specific proteolytic cleavage of agrin regulates maturation of the neuromuscular junction. J Cell Sci. 123: 3944-3955.

Chahrour, M., Jung S.Y., Shaw, C., Zhou, X., Wong, S.T., Qin, J., Zoghbi, H.Y. (2008). MeCP2, a key contributor to neurological disease, activates and represses transcription. Science. 320: 1224-1229.

Charour, M., Zoghbi, H.Y. (2007). The story of Rett syndrome: from clinic to neurobiology. Neuron. 56: 422-437.

Chang, Q., Khare, G., Dani, V., Nelson, S., Jaenisch, R. (2006). The disease progression of *Mecp2* mutant mice is affected by the level of BDNF expression. Neuron. 49: 341-348.

Chen, R.Z., Akbarian, S., Tudor, M., Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genet. 27: 327-331.

Falzone, T.L., Stokin, G.B., Lillo, C., Rodrigues, E.M., Westerman, E.L., Williams, D.S., Goldstein, L.S. (2009). Axonal stress kinase activation and tau misbehavior induced by kinesin-1 transport defects. J Neurosci. 29: 5758-5767.

Gonzales, M., and LaSalle, J. (2010). The role of MeCP2 in brain development and neurodevelopmental disorders. Curr Psychiatry 12: 127-134.

Gschwend, T.P., Krueger, S.R., Kozlov, S.V., Wolfer, D.P., and Sonderegger, P. (1997). Neurotrypsin, a novel multidomain serine protease expressed in the nervous system. Mol Cell Neurosci 9: 207-219.

Hagberg, B., Aicardi, J., Dias, K., Ramos, O. (1983). A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. Ann Neurol. 14: 471-479.

Hoch, W., Ferris, M., Campanelli, J.T., Hall, Z.W., Scheller, R.H. (1993). Developmental regulation of highly active alternatively spliced forms of agrin. Neuron. 3: 479-490.

Israel, M.A., Yuan, S.H., Bardy, C., Reyna, S.M., Mu, Y., Herrera, C., Hefferan, M.P., Van Gorp, S., Nazor, K.L., Boscolo, F.S., Carson, C.T., Laurent, L.C., Marsala, M., Gage, F.H., Remes, A.M., Koo, E.H., Goldstein, L.S. (2012). Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature. 482: 216-220.

Jan, M.M., Dooley, J.M., Gordon, K.E. (1999). Male Rett syndrome variant: application of diagnostic criteria. Pediatr Neurol. 20(3): 238-240.

Jellinger, K., Armstrong, D., Zoghbi, H.Y., Percy, A.K. (1988). Neuropathology of Rett syndrome. Acta Neuropathol. 76 (2): 142-158.

Killian, R.L., Flippin, J.D., Herrera, C.M., Almenar-Queralt, A., Goldstein, L.S. (2012). Kinesin light chain 1 suppression impairs human embryonic stem cell neural differentiation and amyloid precursor protein metabolism. PLoS One. 7: e29755.

Li, W., Calfa, G., Larimore, J., Pozzo-Miller, L. (2012). Activity-dependent BDNF release and TRPC signaling is impaired in hippocampal neurons of *Mecp2* mutant mice. Proc Natl Acad Sci USA. 109 (42): 17087-17092.

Liu, Q.R., Walther, D., Drgon, T., Polesskaya, O., Lesnick, T.G., Strain, K.J., de Andrade, M., Bower, J.H., Maraganore, D.M., Uhl, G.R. (2005). Human Brain Derived Neurotrophic Factor (BDNF) genes, splicing patterns, and assessments of associations with substance abuse and Parkinson's disease. American Journal of Medical Genetics Part B (Neuropsychiatric Genetics). 134B: 93–103.

Marchetto, M., Carromeu, C., Acab, A., Yu, D., Yeo. G., Mu, Y., Chen, G., Gage, F., and Muotri, A. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell. 143: 527-539.

Matsumoto-Miyai, K., Sokolowska, E., Zurlinden, A., Gee, C.E., Lüscher, D., Hettwer, S., Wölfel, J., Ladner, A.P., Ster, J., Gerber, U., Rülicke, T., Kunz, B., and Sonderegger, P. (2009). Coincident pre- and postsynaptic activation induces dendritic filopodia via neurotrypsin-dependent agrin cleavage. Cell. 136: 1161-1171.

Meins, M., Lehmann, J., Gerresheim, F., Herchenbach, J., Hagedorn, M., Hameister, K., Epplen, J.T. (2005). Submicroscopic duplication in Xq28 causes increased expression of the MECP2 gene in a boy with severe mental retardation and features of Rett syndrome. J Med Genet. 42: e12.

Mitsui, S., Osako, Y., Yokoi, F., Dang, M.T., Yuri, K., Li, Y., Yamaguchi, N.(2009). A mental retardation gene, motopsin/neurotrypsin/prss12, modulates hippocampal function and social interaction. Eur J Neurosci. 30: 2368-2378.

Molinari, F., Rio, M., Meskenaite, V., Encha-Razavi, F., Augé, J., Bacq, D., Briault, S., Vekemans, M., Munnich, A., Attié-Bitach, T., Sonderegger, P., and Colleaux, L. (2002).Truncating neurotrypsin mutation in autosomal recessive nonsyndromic mental retardation. Science. 298: 1779-1781.

Muotri, A., Marchetto, M., Coufal, N., Oefner, R., Yeo, G., Nakashima, K., and Gage, F. (2010). L1 retrotransposition in neurons is modulated by MeCP2. Nature. 468: 443-446.

Reif, R., Sales, S., Hettwer, S., Dreier, B., Gisler, C., Wolfel, J., Luscher, D., Zurlinden, A., Stephan, A., Ahmed, S., et al. (2007). Specific cleavage of agrin by neurotrypsin, a synaptic protease linked to mental retardation. FASEB J. 21: 3468–3478.

Reiss, A.L., Farugue, F., Naidu, S., Abrams, M. Beaty, T., Bryan, R.N., Moser, H. (1993). Neuroanatomy of Rett syndrome: a volumetric imaging study. Ann Neurol. 34 (2): 227-234.

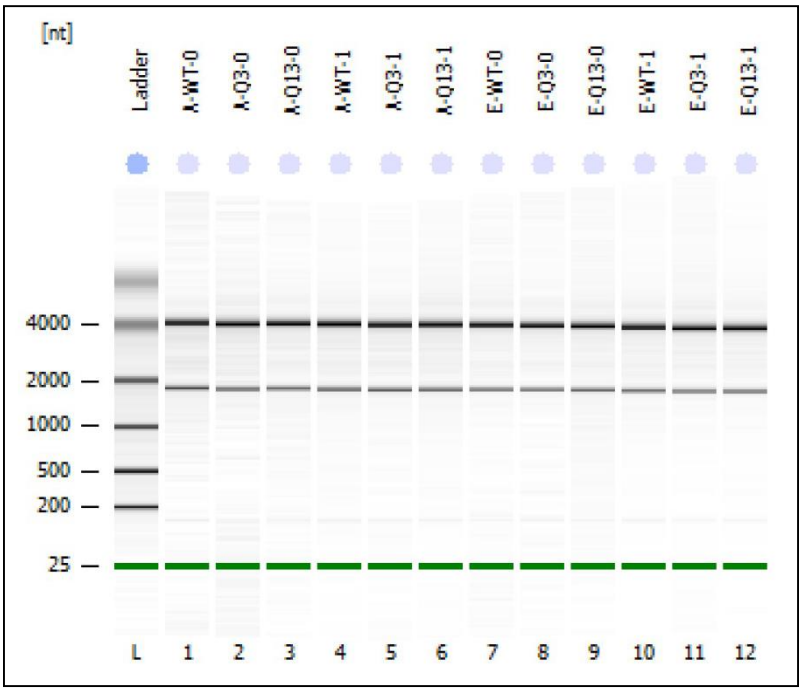
Rett, A. (1966). On a unusual brain atrophy syndrome in hyperammonemia in childhood. Wien Med Wochenschr. 116: 723-726.

Yuan, S.H., Martin, J., Elia, J., Flippin, J., Paramban, R.I., Hefferan, M.P., Vidal, J.G., Mu, Y., Killian, R.L., Israel, M.A., Emre, N., Marsala, S., Marsala, M., Gage, F.H., Goldstein, L.S., Carson, C.T. (2011). Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. PLoS One. 6(3):e17540.

Williamson, S. L., Christodoulou, J. (2006). Rett syndrome: new clinical and molecular insights. Eur J Hum Genet. 14: 896 – 903.

Supplementary figure 1: Minimal RNA degradation and similar RNA quality for all lines collected at weeks 0 and 1

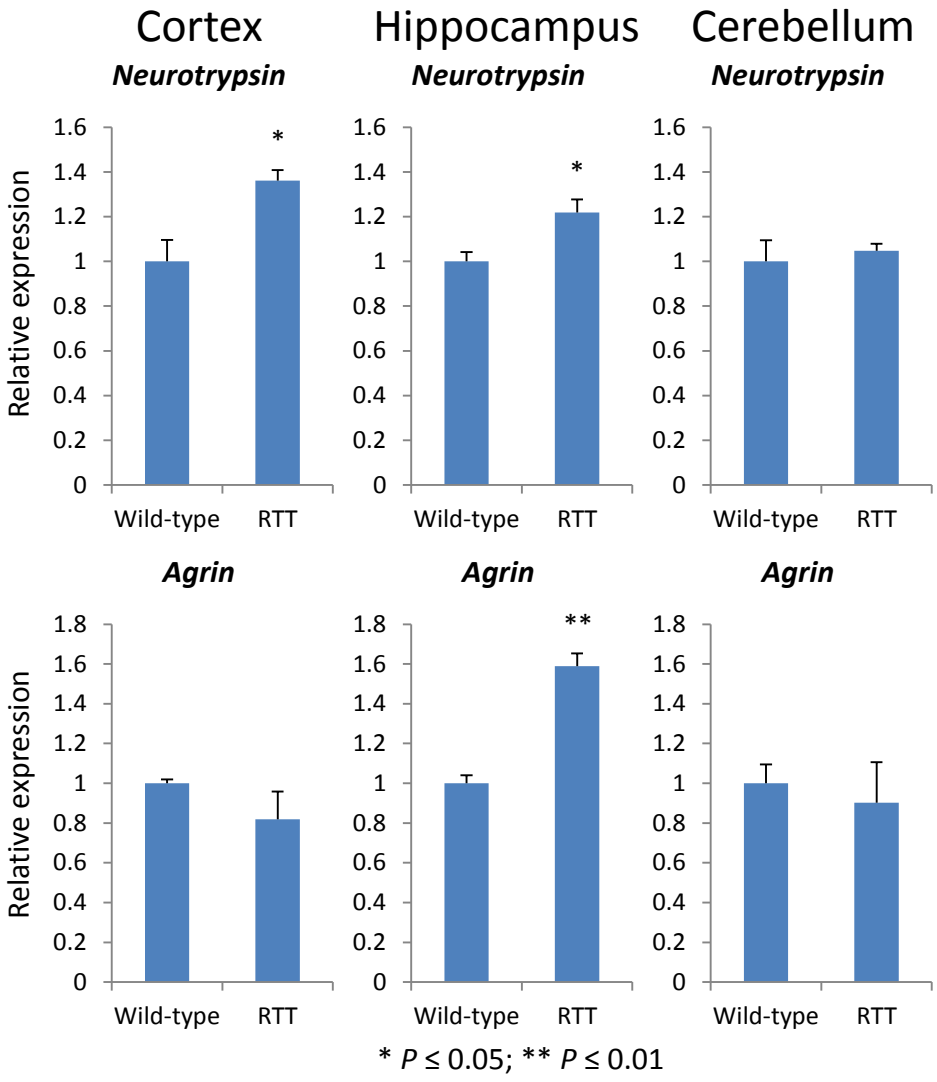
RNA integrity tests were completed using the Agilent Bioanalyzer. RIN values range from 1 (degradation) to 10 (intact RNA). For this test, the RNA samples are named to indicate: “cell line – week number,” with “WT” indicating “control,” “Q3” indicating “RTT-Q83X #1,” and “Q13” indicating “RTT-Q83X #2.” For instance, “A-Q3-1” indicates “RTT-Q83X #1 differentiated NSCs at week 1.” An image of the electrophoresis gel (top) and RIN values (bottom) are shown.



Sample Name	Sample Comment	Statu s	Result Label
A-WT-0		✔	RIN:9
A-Q3-0		✔	RIN: 9.60
A-Q13-0		✔	RIN: 9.20
A-WT-1		✔	RIN: 9.50
A-Q3-1		✔	RIN: 9.80
A-Q13-1		✔	RIN: 9.80
E-WT-0		✔	RIN:10
E-Q3-0		✔	RIN: 9.90
E-Q13-0		✔	RIN:9
E-WT-1		✔	RIN: 9.60
E-Q3-1		✔	RIN:10
E-Q13-1		✔	RIN: 9.80
Ladder		✔	All Other Samples

Supplementary figure 2: The cerebral cortex and hippocampus of *Mecp2*-deficient mice have *Neurotrypsin* upregulation

Neurotrypsin upregulation is seen in isolated cerebral cortex and hippocampus of *Mecp2*-deficient mice from a RTT mouse model. There was no *Neurotrypsin* upregulation seen in the cerebellum of these animals. *Agrin* expression was also analyzed: there is *Agrin* upregulation in the hippocampus of these mice. N = 1.


Cortex
Hippocampus
Cerebellum
Neurotrypsin
Agrin
Relative expression
Wild-type
RTT
*
**
* P ≤ 0.05; ** P ≤ 0.01

Supplementary table 1: List of primers used to determine human gene expression by RT-qPCR

Target mRNA	Forward	Reverse
Neurotrypsin	ACATAACAGGATGGGGTGACA	CGTTTGTGTTCATGGAGGTTT
Agrin	CCTGCAACTGAGCTACAACCT	AACCTTCCCTCTGCTCCCTAT
MAP2	GCCCAAGCTAAAGTTGGTTCT	CTTCCAGACGAGGAGACATTG
GFAP	GTACCAGGACCTGCTCAATG	TGTGCTCCTGCTTGGACTCCTT
Nestin	GAAACTCAAGCACCAC	TTTTAAACTCCAGCCATCC
BDNF	TGGAGCCTCCTCTTCTCTTTC	CTTTTTGTCTGCTGCCGTTAC
MeCP2 isoforms 1 & 2	ACGGAAGCTTAAGCAAAGGAA	CCCAGTTACCGTGAAGTCAAA
RPL27	AAACCGCAGTTTCTGGAAGA	TGGATATCCCCTTGGACAAA
TBP	GAACCACGGCACTGATTTTC	CCCCACCATGTTCTGAATCT
RPS13	ACCAGGGCAGAGGCTGTAG	AATAGAGAGCCGGATTCACC
RPL13A	GGACCTCTGTGTATTTGTCAATTTT	GCTGGAAGTACCAGGCAGTG
NONO	GATGGAACTTTGGGATTGACC	TAGTATCGGCGACGTTTGTTT

Supplementary table 2: List of primers used to determine mouse gene expression by RT-qPCR

Target mRNA	Forward	Reverse
Neurotrypsin	TAACAGGATGGGGAGACACAG	ACGGTTGTCTTCTTGGAGGTT
Agrin	CACACAATTGGACACAGATGG	GCAGGGTCTTAGCTCTGGTTT
Nono	ATAAACAATAGAGGCGCGATG	ATTGCTCCAATTCCTTCCATT