Genes that regulate synaptic and intrinsic properties of neocortical interneurons after perturbations of DNA methylation or network activity

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by
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ABSTRACT
Genes that regulate synaptic and intrinsic properties of neocortical interneurons after perturbations of DNA methylation or network activity

A thesis presented to the Department of Biochemistry
Brandeis University
Waltham, MA
By Liliya Leybova

Brain networks rely on complex circuitry and neurotransmitter communication between neurons to relay information. Because of their criticality, even when disruptions arise, these networks have to readjust themselves in order to maintain proper function. Networks can be disrupted by changes in gene expression due to alterations in methylation patterns after excisions in DNA methyltransferase genes DNMT1 and DNMT3a or prolonged activity deprivation. Interneurons with decreased methylation have a decrease in excitatory synaptic input while neuronal cultures subjected to prolonged activity deprivation show both altered synaptic changes and are more excitable. I investigated what genes might be responsible for the observed synaptic and intrinsic excitability changes in both conditions. RT-qPCR and immunohistochemistry show that in the DNMT1/3 double mutant there is a difference in the gene expression and a decrease in the protein levels of ErbB4, a receptor involved in regulating synapse size and number. Western blot analysis of activity deprived organotypic cultures shows non-significant decreases in ErbB4 and Twik1, a potassium channel, levels and a large but non-significant increase in cortisol releasing factor binding protein (CRFBP). These results provide a set of molecular pathways that are changed in response to alterations in activity and DNA methylation and can provide a mechanism to explain the observed physiological changes.
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LIST OF ABBREVIATIONS

ACTH – adrenocorticotropic hormone
Chmp1a – chromatin modifying protein
CNS – central nervous system
CRF-BP – corticotropin-releasing factor binding protein
CRH – corticotropin releasing hormone
DNMT – DNA methyl transferase
EPSC – excitatory post-synaptic current
Esco1 – establishment of sister chromatid cohesion N-acetyltransferase 1
Fgf1 – Fibroblast growth factor
FPKM – fragment per kilobase of exon mapped
GABA - γ-aminobutyric acid
GIRK – G-protein activated K⁺
IPSC – inhibitory post-synaptic current
MHC – major histocompatibility complex
OPRL-1 – opiate receptor-like 1
PBS – phosphate-buffered saline
PC2 – prohormone convertase 2
PFA – paraformaldehyde
PNOC – prepronociceptin
PNS – peripheral nervous system
PPF – paired-pulse facilitation
PSD-95 – post-synaptic density protein 95
PV⁺ – parvalbumin-positive
PVDF – polyvinylidene difluoride
SAM – S-adenosyl-methionine
Scg5 – secretogranin V
TTX – tetrodotoxin
β2m - β2-microglobulin
Introduction

Brain networks consist of neurons communicating with one another by sending chemical signals at synapses via neurotransmitters. When these networks are disrupted, it often leads to changes in communication which contribute to many neurological diseases including schizophrenia and epilepsy. Disruptions can occur in multiple ways both on a global scale and more locally. Both DNA methylation, which is able to activate or repress specific genes, and activity deprivation, which alters synapses and changes the inhibition/excitation balance, are able to globally change neuronal circuits by mechanisms which are currently unknown.

Neurons in the central nervous system can be categorized broadly as excitatory or inhibitory neurons, depending on the type of the neurotransmitters released. Excitatory neurons often make long range projections and act through type I synapses, which contain synaptic vesicles, a high electron density region at the active zone, and an even higher electron density region in the post-synaptic membrane (Harris & Weinberg 2012). On the other hand, inhibitory neurons, commonly referred to as interneurons due to their local circuitry in most forebrain structures, usually release γ-aminobutyric acid (GABA) and act on type II synapses, which contain less visible synaptic densities than the previously described type I synapses (Harris & Weinberg 2012). The interaction between excitatory and inhibitory neurons is important for the formation and regulation of brain circuits.
**Fast-Spiking Cells**

Inhibitory neurons can be divided into subcategories depending on their different morphological, electrophysiological and neurochemical features (Ascoli et al 2008). One important type of GABAergic interneuron is the parvalbumin-positive (PV⁺) fast-spiking cell consisting of two subtypes: the chandelier and basket cells. PV⁺ cells express parvalbumin, a calcium-binding protein suggested to affect inhibitory post-synaptic currents (IPSCs) as well as gamma oscillations (Neddens & Buonanno 2010). Basket cells are regulated by GABA_A receptors containing α1 subunits, which are able to produce IPSCs with a much faster decay time as well as enabling them to fire synchronously (Hefft & Jonas 2005).

PV⁺ cells play an important role in neocortical circuitry and are the single largest source of synaptic inhibition in the adult cortex (Okaty et al 2009). However, PV⁺ cells do not fully mature until late in post-natal development between P25 and P30 (Miller et al 2011). Physiological properties are refined through the course of maturation until they eventually achieve their phenotypic characteristics of high firing rates, narrow spikes relative to other neocortical cell types, and little to no spike-frequency adaptation (McCormick et al 1985). At this point, they exhibit an increased amplitude and frequency of both mEPSCs and mIPSCs. These currents reach their peaks at slightly different times; mEPSCs peak at P15 for both amplitude and frequency, whereas mIPSCs have a frequency peak at P15 and an amplitude peak at P25 (Okaty et al 2009).

It is believed that these firing properties result from voltage-gated potassium channels. The Kv3 subfamily, encoded by Knc1 and Kcn2 genes, has been found important in PV⁺ cell maturation. Traits that are unique to PV⁺ cells, like short action potential half-width and ability
to follow repetitive high frequency excitatory inputs, are absent in immature PV+ cells and seem to be driven by developmentally regulated expression of potassium channel subunits Kv3.1 and Kv3.2 (Goldberg 2011). From P10 to P18, the protein and mRNA levels of Kv3.1 and Kv3.2 are dramatically up-regulated and follow similar developmental patterns as the maturation of PV+ cells themselves suggesting that the potassium channels play a role in their maturation (Goldberg 2011, Okaty et al 2009). In comparing PV+ and somatostatin-positive interneurons, Kv3.1b was present in about 80% of PV+ interneurons but absent from somatostatin interneurons and its developmental onset was identical to the parvalbumin (Du et al 1996).

PV+ cells play an important role in maintaining neuronal circuitry but there is limited information about how their interactions with other neurons are altered after network perturbations. Changing activity levels can lead to network rearrangement that would “fine tune” connections and promote excitability (Nelson & Turrigiano 2008, Turrigiano & Nelson 2004) allowing cells to respond appropriately to stimuli even when signal strengths are altered. These changes can be observed by measuring mEPSC amplitude and frequency. Changes to mEPSC amplitude are often linked to a change in receptor number or conductance while frequency changes are often due to changes in the probability of transmitter release (Turrigiano & Nelson 2004).

There are many different ways to disrupt network activity including changes in DNA methylation and activity deprivation. However, little is known about the mechanisms governing these changes and whether there are genes responsible for network changes that are independent of how the disruption came out. In my project, I investigated gene changes that are related to synaptic and intrinsic properties of neurons after changes in DNA methylation and after activity deprivation.
DNA Methylation

One way in which the maturation of PV neurons can potentially be altered is through DNA methylation, commonly associated with gene regulation through transcriptional silencing (Kadriu et al 2012). DNA methylation is catalyzed by DNA methyl transferases (DNMTs), which bind a methyl group derived from a methyl donor S-adenosyl-methioine (SAM) to the 5’ carbon of cytosine and by doing so prevents gene expression (Kadriu et al 2012). By directly affecting the expression of specific genes in the central nervous system, DNA methylation could have an important role in regulating nervous system functions (Nguyen et al 2007). Neurons in the adult brain have a particularly high expression of DNMTs and they are thought to have a continued functional role in post-mitotic neurons (Nelson et al 2008, Nguyen et al 2007).

Conditional double knockouts of DNMT1 and DNMT3a in excitatory forebrain neurons late in development show abnormal long-term plasticity and deficits in learning and memory (Day & Sweatt 2011, Kadriu et al 2012). Comparisons of single knockout mice with the double knockouts, shows the two isoforms have overlapping, but not interchangeable, roles in post-mitotic neurons. Both DNMT1 and DNMT3a appear to have important roles in the epigenetic regulation of GABAergic gene expression (Kadriu et al 2012). In order to study the effect of DNMTs, conditional knockouts in specific regions need to be created through the Cre-loxP recombination system (Nguyen et al 2007) as complete knockouts result in lethality (Li et al., 1992; Okano et al., 1998, 1999).

Inhibiting DNA methylation in hippocampus reveals that they play a role in long-term memory and synaptic plasticity (Miller & Sweatt 2007). In hippocampal neurons, DNMT inhibition results in activity dependent demethylation of genomic DNA and there is a decrease in
the frequency of mEPSCs, impacting the neuronal excitability and network action. This is the same pattern observed after enhancing excitatory activity in the absence of DNMT inhibitors, suggesting that DNA methylation may control homeostatic synaptic plasticity (Nelson et al 2008). In these hippocampal cultures, DNMT inhibition affects excitatory but not inhibitory synaptic activity. The observed changes in mEPSCs were shown to occur from a decrease in their amplitude, rather than from a decrease in the number of excitatory inputs (Nelson et al 2008).

The decrease in the spontaneous firing rates of neurons caused by DNMT inhibition does not change the intrinsic properties of the neurons indicating that demethylation leads to changes in overall network activity. It was also found that the number of excitatory synapses was unaffected after DNA methylation; however there was a change in the spontaneous vesicle release from the presynaptic terminals but only during synaptic activity (Nelson et al 2008).

**Activity Deprivation**

Another way to alter the brain network and cell firing properties is with drugs that directly block firing by blocking sodium channels. Prolonged application of the sodium channel blocker TTX causes seizures if applied beginning around post-natal day 10 and has therefore been used as a model for epilepsy (Galvan et al 2000, Galvan et al 2003, Lee et al 2008).

TTX treatment prevents axon remodeling causing them to remain in an immature state with an over abundance of connectivity leading to hyper-excitability (Lee et al 2008). Analyzing homeostatic synaptic plasticity after TTX application shows differences in both mEPSCs and mIPSCs in pyramidal and PV+ cells. Inhibitory input onto excitatory cells is decreased, as is
excitatory input onto inhibitory cells. At the same time, there is an increase in the excitatory input onto excitatory cells and inhibitory input onto inhibitory cells (Escobedo-Lozoya, unpublished results). These observed synaptic changes all work together to increase the overall excitability of the cell following TTX activity deprivation. However it is unclear as to the exact mechanism by which these changes occur. In my experiments, I looked at several targets that are known to be involved in mediating synaptic plasticity in other conditions.

Both human and animal studies have implicated corticotropin releasing hormone (CRH) in causing infantile spasms (Brunson et al 2001) and this affect may be mediated by adrenocorticotropic hormone (ACTH) (Hrachovy et al 1983, Stafstrom et al 2006). It is believed that increased stress levels during infantile spasms causes an increase in CRH levels and leads to increased neuronal excitability and ultimately seizures. ACTH functions in a negative feedback loop to decrease CRH expression and this may explain why application of ACTH decreases spasms (Brunson et al 2001).

Aside from the interaction with ACTH, little is known about the underlying mechanism leading to infantile spasms. I wanted to probe this question by comparing protein expression levels of known neuromodulators after TTX application. Significant changes would provide potential explanations for the mechanisms underlying the observed hyperactivity after activity withdrawal and could lead to drug therapies.

**ErbB4/Neuregulin Interactions**

Neuregulin 1 is a neurotrophic growth factor that activates ErbB tyrosine kinase receptors. Of these, the interaction with ErbB4 is particularly interesting because of its potential involvement in schizophrenia and epilepsy (Li et al 2012, Mei & Xiong 2008, Stefansson et al
ErbB4 is also the only neuregulin 1 receptor highly expressed in the central nervous system in a subpopulation of interneurons that migrate tangentially toward the cortex. Loss of ErbB4 disturbs interneuron migration decreasing the number of GABAergic interneurons in the post-natal cortex (Flames et al 2004, Mei & Xiong 2008).

Through interactions with ErbB4, neuregulin increases both the number and size of excitatory synapses in GABAergic, but not pyramidal, neurons and strengthens existing synapses through the stabilization of post-synaptic density protein 95 (PSD-95). PSD-95 plays a role in interactions between NMDA and AMPA receptors, as well as potassium channels (Ting et al 2011). Research done on brain tissue from schizophrenia patients shows that ErbB4 association with both PSD-95 and NMDA receptors is enhanced in these patients without altering the number of either molecule but rather by enhanced ErbB4 phosphorylation levels (Hahn et al 2006).

Neuregulin is also thought to increase the probability of GABA release in response to depolarization as it increases evoked EPSC in a dose dependent manner while not having any effect on IPSC or basal GABA release (Woo et al 2007). Specifically in GABAergic interneurons, neuregulin decreases both the frequency and amplitude of mEPSCs (Ting et al 2011). Deletion of ErbB4 specifically in PV-FS cells also leads to a decrease in the number of inhibitory synapses from pyramidal cells and an increase in the frequency of sEPSC (Del Pino et al 2013).

Recordings done on DNMT knockout animals and after TTX application both show a decrease in the amplitude of mEPSC in PV+ cells and could involve the neuregulin/ErbB4 pathway. In addition, post-mortem studies on schizophrenia patients show a decreased level of
both DNMT and ErbB4 and it is thought that these two may act in either parallel or additive pathways (Stefansson et al 2002).

**Nociceptin/Orphanin FQ**

Another set of genes that could regulate synaptic plasticity after activity perturbations are neuropeptides. Advances in visualization of neuropeptides and their receptors has linked their function to regulating cell to cell communication as neuromodulators (Merighi et al 2011). One of these is nociceptin/Orphanin FQ, a neuropeptide that acts on a G-protein coupled receptor opiate receptor-like 1 (OPRL-1) and reduces neuronal excitability and inhibits neurotransmitter release (Schlicker & Morari 2000) by stimulating K+ conductance and inhibiting high-voltage activated N-type Ca^{2+} channels (Mallimo & Kusnecov 2013). Nociceptin and its receptor, OPRL-1, is expressed in both the central nervous system (CNS) and peripheral nervous system (PNS) but primarily in the cortical areas of the brain, hippocampus, and amygdala (Tariq et al 2013). Its expression patterns and targets lead it to be a prime candidate in regulating synapse activity. Furthermore, past work in the lab has identified the inactive form of nociceptin to be differentially expressed at the mRNA level after activity deprivation (O’Toole et. al, unpublished results).

Secretogranin V (scg5), a neuroendocrine protein, plays an important role in the processing of nociceptin from its inactive form of prepronociceptin (PNOC). Seg5 is involved in prohormone convertase 2 (PC2) maturation and PC2 converts PNOC into nociceptin (Apletalina et al 2000). Seg5 is found primarily in neuronal or endocrinal tissues and has high sequence alignment among mammals (Mbikay 2001). Additionally, while scg5 null mice are able to create mature PC2 peptides, they are completely nonfunctional even if exogenous scg5 is added in vitro (Hwang et al 2000, Westphal 1999). Deletion of, or inactive, PC2 leads to a dramatic reduction
in the levels of nociceptin but small levels of PC2 are still sufficient to convert a small fraction of nociceptin (Allen 2001). Together, this suggests that scg5, and its role in PC2 maturation, plays an important role in nociceptin production and its function is not easily compensated for.

Experiments done on rat cortical neurons demonstrated nociceptin’s role in mediating NMDA and GABA responses and cell excitability. Nociceptin inhibits the electrically evoked released from both GABA and NMDA receptors in a concentration dependent manner and when cultures are pre-treated with TTX the inhibition was no longer present suggesting nociceptin acts selectively through sodium channels (Bianchi et al 2004).

Other studies done in the rat lateral amygdala also measured the effect of nociceptin on IPSCs and EPSCs and found that nociceptin was able to decrease the amplitude of EPSCs in a concentration dependent manner up to 10 nM amounts (Meis & Pape 2001, Yu 1997). The concentration dependence corresponded to the previously found nociceptin concentration dependence of post-synaptic potassium conductance (Meis & Pape 1998). Additionally, they determined through synthetic antagonists that this mediation occurs through nociceptin binding to the OPRL-1 receptor.

Studies also found that addition of nociceptin decreased the mean frequency in both mEPSCs and mIPSCs as compared to control but had no affect on amplitude in either case (Meis & Pape 2001, Roberto & Siggins 2006). This is similar to the decreased mean frequency of mEPSCs seen when ErbB4 is removed and unable to interact with neuregulin and elicits the question whether these act on parallel pathways and whether their actions are additive.

While data supports nociceptin’s role in synaptic regulation, the direct mechanism for how nociceptin works is still unclear. Most evidence points to action occurring at presynaptic
terminals and acts by decreasing presynaptic release of excitatory neurotransmitter (Yu 1997). When TTX is applied concurrently with nociceptin, the frequency but not the amplitude of miniature postsynaptic currents are reduced suggesting that the receptors are located presynaptically and not post-synaptically (Meis & Pape 2001). The combination of decreased mIPSC frequency and decreased paired-pulse facilitation (PPF) ratio of IPSC due to nociceptin suggest that nociceptin decreases GABA release through pre-synaptic terminals (Roberto & Siggins 2006).

Nociceptin is also able to mediate neuronal excitability through G-protein activated K⁺ (GIRK) channels (Ikeda et al 1997). Through functional coupling of OPRL-1 to the GIRK channel, nociceptin was able to induce an outward current in CA3 pyramidal neurons (Ikeda et al 1997). Additionally, nociceptin-induced hyperpolarization showed a near steady state current that had an inward rectification reversal near the potassium equilibrium potential as commonly seen with inwardly rectifying channels. When a GIRK channel blocker was applied, nociceptin-induced outward current was significantly reduced (Parsons & Hirasawa 2011).

Past work in the lab, as well as on-going projects, look at the role of global activity changes to the brain network, in addition to local circuitry. My project focuses on two different types of changes – DNA methylation and activity deprivation – and investigates changes that occur at the gene level to identify the mechanism as to how intrinsic and synaptic changes occur. I was specifically interested in seeing if there was an overlap between the DNMT knockout conditions and TTX treatments as these two conditions serve as models for different neurological diseases – decreased levels of DNMTs are seen in schizophrenic patients while TTX application early in mouse development mimics infantile spasms. In both of these cases, as well as other neurological diseases where brain circuitry is disrupted, the molecular pathways that bring on the
pathological activity are still unknown. Since both the synaptic and neuronal activity changes and the molecular players, such as neuropeptides, that I am specifically looking at have been implicated in different human diseases such as schizophrenia and epilepsy, being better able to understand how these proteins change the network would allow a more targeted approach in developing potential treatments.
Materials and Methods

1. RT-qPCR

RT-qPCR was performed on a Roto-Gene 3000 (Corbett Research) to validate differential expression of candidates that were previously identified in RNA Sequencing experiments. Primers were selected using Primer3 to specifically amplify the region of interest for Fgf1, β2m, Esco1, Scg5, Chmp1a, and Mgat5b using the following primers:

**Fgf1**
Forward: ggaccttggtgatgcaaagtt  
Reverse: aattgtgcggtctctgtgtagg

**β2m**
Forward: atgggaagccgaacatactg  
Reverse: cagtctcagtgggggtgaat

**Esco1**
Forward: cttcaccgcagatcacaag  
Reverse: atctctcactgcgcaaaagacc

**Scg5**
Forward: gggggatttttttctgtggga  
Reverse: cccaaacaccaacaccaaaa

**Chmp1a**
Forward: tctgcttgatggactcactc  
Reverse: tgaagcagacgctttttgc

**Mgat5b**
Forward: ttcaggacagcagccttcg  
Reverse: tttgctctggattctgctg

PCR programs were as follows: 98°C for 2 minutes, followed by an annealing at 53°C for 10 seconds, followed by a melt at 60°C rising by 1 degree each step. β-actin (Forward: ttcagcagcttcgctttc; Reverse: aatccagggcaacagc) was used as an endogenous internal control. To create a standard curve used to determine relative concentrations, we pooled aliquots
containing 2µL from each library and then created a serial dilution. All reactions were run in triplicate and results were analyzed with ΔΔCT method (Livak & Schmittgen 2001). Relative expression of genes was calculated by the equation $2^{-\Delta\Delta CT}$.

2. Immunohistochemistry

Mice were deeply anesthetized with isoflurane and then injected with a cocktail consisting of 100mg/mL ketamine, 100mg/mL xylazine, and 10mg/mL acepromazine. Then they were perfused intracardially with 1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). 50µm coronal sections were cut on a Vibratome after post-fixation in PFA for at least 24 hours. Sections were then blocked and permeabilized in PBS with 10% normal goat serum and 0.3% Triton X for one hour. After blocking, they were incubated overnight at 4°C with primary antibody in PBS, 0.1% Triton and 1% goat serum. After 3 fifteen minute washes with PBS/0.1% Triton, secondary antibody incubation was done for 2 hours at room temperature in PBS, 0.1% Triton, and 1% goat serum. After another set of washes, sections were mounted using Fluoromount. The antibodies were used as follows: polyclonal anti-rabbit ErbB4 1:300 (Bethyl Laboratories), polyclonal anti-mouse parvalbumin 1:1000 (Swant), anti-mouse Alexa 488 1:250 (Invitrogen), anti-rabbit 594 1:250 (Invitrogen). Confocal images were taken on a Leica SP5 spectral confocal microscope using a 63x oil-immersion lens. Image J/FIJI was used for quantification. A two-tailed student’s t-test was used to determine significance, using a cutoff of $p < 0.05$.

3. Western Blot

Organotypic slices were suspended in sample buffer (60mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol, 0.1% bromophenol blue, H$_2$O) and boiled for 10 minutes. Protein concentration of each sample was determined using Nanodrop (Thermo Scientific).
Equivalent protein concentrations were loaded on SDS-PAGE with 10% gel under reducing conditions, then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked overnight with 5% dry milk and then incubated with primary antibody in BSA/PBST overnight. Primary antibodies used include polyclonal anti-mouse ErbB4 antibody 1:500 (Bethyl Laboratories, A300-667A), polyclonal anti-goat CRF-BP antibody 1:500 (Santa Cruz, sc-20315), polyclonal anti-rabbit twik1 antibody 1:500 (Alamone Labs, APC-110), polyclonal anti-mouse alpha tubulin 1:500 (Santa Cruz, sc-8035), and polyclonal anti-mouse actin (Thermo Scientific). After 3 15-minute washes with PBS/0.1% Tween, a horseradish peroxidase-conjugated secondary anti-rabbit, mouse or goat antibody (Promega W4011, Cell Signaling 7076S, Santa Cruz sc-2020) depending on the primary used was incubated for 1 hour at room temperature. Blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and then developed on film. Film was scanned in and analyzed using FIJI/Image J to compare mean intensities.

4. Genotyping

Tails from animals that were used for immunohistochemistry were clipped to confirm genotype. Samples were first digested with 25µL extraction buffer, 6µL tissue prep followed by 25µL neutralization solution. After digestion, the DNA was tested for PvCre, DNMT1, and DNMT3a using the following primers:

Test for presence of PvCre allele (has two sets of primers; PV-Cre\textsuperscript{++} 727bp, PV-Cre\textsuperscript{+-} 450-500 bp, heterozygotes contain both alleles and express PV-Cre)

\[ \text{5'}-\text{TCCACTCTGGTGCTGAAAGCTAA-3'} \]

\[ \text{5'}-\text{ATCTCCTTGTGGGAAAGGTGCAGA-3'} \]

\[ \text{5'}-\text{GCCAGCTAAACATGCTTCATC-3'} \]
5’-ATTGCCCTGTTTCTACTATCC-3’

Test for presence of DNMT1 PV-Cre driven lox sites (DNMT1+/+ 370bp, DNMT1-/- 335bp, lox sites will only be excised if PV-Cre present)

5’ - GGG CCA GTT GTG TGA CTT GG-3’

5’ – CTT GGG CCT GGA TCT TGG GGA- 3’

Test for presence of DNMT3a PV-Cre driven lox sites (DNMT3a+/+ 250 bp, DNMT3a-/- 150bp, lox sites will only be excised if PV-Cre present)

5’-CCTCTGGGGAGTTAAGACTCTTGGCCAGCCC-3’

5’-CCTGTGTGCAGCAGACACTTCTTTGCGTC-3’

For both DNMT1 and DNMT3a amplification reactions were carried out with 2µL dH2O, 1µL primer mix, 5µL PCR master mix, and 2µL DNA template while PvCre amplification used 1µL dH2O, 2µL total primer mix, 5µL PCR master mix, and 2µL DNA template. Reactions ran for 30 cycles with an initial 5 minute denaturation at 95°C, 45 second denaturation at 94°C, 45 seconds at the annealing temperature of 62°C, 45 second extension at 72°C. Following the amplification cycles, a final extension at 72°C for 10 minutes. Amplification results were detected by electrophoresis of 2% agarose gel.
Results

Differentially expressed genes in DNMT1 knockout mice

Past research in the lab has found that when DNMT1 is conditionally knocked out in PV+ FS cells, it leads to a decrease in the excitatory input to these inhibitory cells and I was interested in investigating whether there was an epigenetic basis to this change. RNA Sequencing was done on a manually sorted PV+ inhibitory cells from the motor cortex and the results showed that there were differentially expressed genes in animals that lacked the DNMT1 allele in comparison to those that had it (Fig 1).

Figure 1. Differentially expressed genes in DNMT knockout animals obtained from RNA Sequencing data.
Mgat (A), Scg5 (B), Esco1 (C), and Fgf1 (D) are up-regulated in mutant animals while B2m (E) and Chmp1a (F) are down-regulated. *** p < 0.001; ** p < 0.01, significant unpaired student’s t-test (RNA Sequencing was done by Yasuyuki Shima)
I wanted to validate these genes using real-time qPCR to test whether there was a difference in mRNA levels between the two groups. I compared mRNA levels of 6 different genes (Fgf1, Scg5, Esco1, Chmp1a, B2m, and Mgat5b) in animals with the intact DNMT1 allele and those lacking it. Of the 6 that I tested all but one, Mgat5b, matched the RNA Sequencing data (Fig 2).

Data collection was done in triplicate and standardized to β-actin. All trials were combined and qPCR fold change was calculated as $2^{-\Delta\Delta ct_{(test
gene)}-\Delta\Delta ct_{(ref.
gene)}}$. The genes found were associated with neuronal function or linked to DNA replication mechanisms (Table 1). Of these, the most interesting target was scg5, which I wanted to investigate further though immunohistochemistry to look at expression at the protein level.

Figure 2. Changes in mRNA levels in response to decreased DNMT levels. Fold change of various genes using both qPCR, shown in green, and RNA Sequencing results, shown in blue, between animals lacking DNMT1 compared to those with the DNMT1 allele. Actg was used to normalize data because of its constant expression. Mgat was not validated by qPCR. qPCR done in triplicate and shown as mean ± s.e.m. (RNA Sequencing was done by Yasuyuki Shima)
Table 1. Function of gene candidates found in RNA Sequencing and validated by qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Change in DNMT cKO</th>
<th>Known Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fgf1</strong> Fiberblast growth factor</td>
<td>Increase</td>
<td>Inhibits protein kinase B and regulates NF-κB signaling neurons</td>
</tr>
<tr>
<td><strong>Sgc5</strong> Secretogranin V</td>
<td>Increase</td>
<td>Regulates prohormone convertase responsible for producing family of active polypeptides</td>
</tr>
<tr>
<td><strong>Esco1</strong> establishment of sister chromatid cohesion N-acetyltransferase 1</td>
<td>Increase</td>
<td>Important for DNA replication</td>
</tr>
<tr>
<td><strong>Chmp1a</strong> Chromatin modifying protein</td>
<td>Decrease</td>
<td>Regulates proliferation of CNS progenitor cells</td>
</tr>
<tr>
<td><strong>B2m</strong> β2-microglobulin</td>
<td>Decrease</td>
<td>Component of MHC; neuronal MHC important in synaptic formation, remodeling and plasticity</td>
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**Down-regulation of ErbB4 protein in PV⁺ cells of DNMT knockout mice**

Due to its association with schizophrenia (Stefansson et al 2002), and its previously discovered role in regulating synaptic plasticity (Del Pino et al 2013, Ting et al 2011, Woo et al 2007), I was interested to see whether ErbB4 expression was regulated by DNMTs. Because DNMT1 or DNMT3 were selectively deleted in PV⁺ cells, I co-stained for ErbB4 and PV in control and DNMT knockout animals (Fig 3A) and then selectively compared the levels of ErbB4 only in PV⁺ cells. My results show that ErbB4 expression is decreased in the knockout animals (normalized control: 1 ± 0.186, n=95; normalized mutant: 0.762 ± 0.145, n=76; p < 0.001) (Fig
suggested that DNA methyltransferases regulate ErbB4 expression either by directly acting on ErbB4 or indirectly through decreased DNA methylation of a repressor of ErbB4. I have also compared the levels of ErbB4 expression in PV⁺ and PV⁻ cells and found that the decrease in ErbB4 staining is not limited to PV⁺ cells although DNMTs were only excised in PV⁺ cells (control average ErbB4 mean intensity day 1 = 15.49±4.34 control average ErbB4 mean intensity day 2 16.95±3.06 mutant average ErbB4 mean intensity day 1 = 11.29±2.44; mutant average ErbB4 mean intensity day 2 12.35±2.20).

Figure 3. Immunohistochemistry show decreased ErbB4 staining in PV⁺ cells.  
(A) Top row, Representative control mice cortical slices co-stained for parvalbumin (magenta) and ErbB4 (green).  
Bottom row, Representative mutant mice cortical slices co-stained for parvalbumin (magenta) and ErbB4 (green).  
Scale Bars, 5 μm. Total 2 mutant and 2 control mice used, 2 sections per animal stained  
(B) Quantified data show 25% decrease in ErbB4 expression in PV⁺ cells where DNMT 1/3a is knocked out. Data shown as mean ± s.t.d, n refers to number of cells, *** p < 0.001, significant unpaired student’s t-test
This suggests that the difference in expression levels is not localized to PV+ cells but rather in the entire cell network. Despite the observed change however, my staining contradicts previously published results which identify ErbB4 as a selective marker for inhibitory cells (Vullhorst et al 2009). Instead, I have found that ErbB4 is expressed throughout the brain and appears too widespread to be restricted only to inhibitory neurons and due to time constraints have not confirmed the specificity of this antibody. All animals used for comparisons were genotyped for PV-Cre expression (Fig 4A) as well as the flox sites for DNMT1 and DNMT3a (Fig 4B). Control animals both lacked PV-Cre and therefore the genes coding for DNMTs were not excised and expressed as normal.

**Figure 4. DNMT1/3a and PV-Cre genotyping on all animals used for immunohistochemistry experiments.**

(A) Wild type and litter mate control both lacked PV-Cre and therefore expressed DNMT alleles as normal. (PV-Cre +/+ 727 bp; PV-Cre −/− 400-500 bp; Heterozygotes contain both bands and express PV-Cre)

(B) Double knockouts contained lox site alleles for both DNMTs (DNMT1 +/+ 370 bp; DNMT1 −/− 335 bp; DNMT 3a +/+ 250 bp; DNMT3a −/− 150 bp)
TTX Treatment leads to changes in protein expression

Other on-going work in the lab looks at changes to synaptic and intrinsic properties of neurons as a result of TTX treatment effectively blocking sodium channels and causing activity deprivation. Their work has shown an increased intrinsic cellular excitability as well as increased excitatory input onto excitatory cells and decreased excitatory input onto inhibitory cells. ErbB4 has been associated with altering the size and number of excitatory synapses in inhibitory cells and I was interested in whether the decreased mEPSCs in inhibitory cells seen after TTX treatment could be due to ErbB4. This time because I was not interested specifically in PV⁺ cells, I was able to run a Western Blot on whole cell samples obtained from slice culture to compare total ErbB4 expression as compared to tubulin loading control between the two conditions (Fig 5A, B). Preliminary results show a decrease in ErbB4 expression in TTX conditions, which does not yet reach significance (normalized control 1; normalized TTX 0.617 ± 0.080, p > 0.05, n=2) however the experiment needs to be repeated to increase the number of samples in order to draw conclusions.

I was also interested in investigating TTX treated samples to see if other neuropeptides were also affected. Similar to DNMT knockouts, RNA Sequencing was previously done on TTX treated samples, however this was performed on PV⁺ cells, which make up only a small fraction of neurons. I was curious whether the observed changes were restricted only to PV⁺ interneurons, or were present more broadly. Prior results (O’Toole et al. unpublished results) had shown that corticotropin-releasing factor binding protein (CRF-BP) is down-regulated following TTX treatment both by RNA sequencing as well as by immunohistochemistry. However, analysis by Western Blot showed a strong, but not statistically significant, increase in CRF-BP protein levels in the TTX condition (normalized control 1, n=3; normalized TTX 5.90 ± 3.20, n=3) (Fig 5C,
D). This result suggests that CRF-BP proteins are increased after activity withdrawal but is contradictory to previous sequencing results.

In addition to synaptic changes, there is a strong increase in intrinsic excitability of both inhibitory and excitatory neurons after TTX treatment. I was interested in testing whether potassium leak channels are involved in the increased intrinsic excitability observed after TTX treatment. Like with ErbB4, I saw a downward trend in TTX conditions (normalized control 1, n=1; normalized TTX 0.683, n=1) (Fig 5E, F) however since I only tested one set of samples, more experiments need to be done to see if there is in fact a decrease and the change is consistent or if twik1 is highly variable between different samples. Given the increased excitability seen in TTX recordings, it is reasonable that leak potassium channels would be decreased causing the cells to be more depolarized, therefore requiring less input to fire.

Figure 5. Protein changes in mouse neocortex after TTX application
(A, C, E) Comparison of ErbB4, CRF-BP, and Twik1 protein levels, respectively, in control brain slices and after TTX application as compared to tubulin or actin loading controls.
(B, D, F) Quantitative representation of protein levels using unpaired student’s t-test. ErbB4 (n=2, p > 0.05) and Twik1 (n=1) show a decrease in protein levels after TTX. CRF-BP (n=3, p > 0.05) shows a decrease in protein levels after TTX. Data shown as mean ± s.t.d
Discussion

My experiments investigated the mechanism underlying changes to intrinsic and synaptic plasticity due to DNA methyltransferase and TTX treatment to gain a better understanding of brain networks. Preliminary results suggest ErbB4’s involvement in both indicating that it is an interesting target to investigate further. Additionally, preliminary results suggest that different mechanisms may be at play to regulate intrinsic cell properties and synaptic plasticity.

mRNA changes due to DNA methyltransferase mutations

Using qPCR, we were able to demonstrate the presence of differentially expressed genes at the mRNA level. It is known that in early development DNMTs add or remove methyl groups and thereby change expression patterns in cells. My results, which were obtained from animals where DNMT1 was conditionally knocked out around P15, suggest DNMTs continue to function past development and can change methylation patterns even in adult animals. Other labs have had similar findings in recent years relating DNMT function to long-term plasticity and memory (Miller et al 2008, Morris et al 2014, Rahn et al 2013)

RNA Sequencing results provided a list of genes that may be differentially expressed when DNMTs are knocked out by looking at the difference in fragment per kilobase of exon mapped (FPKM) in each animal sequenced. However, the data was largely variable per animal and therefore the top hits were validated with qPCR experiments. I began with analyzing the same samples primarily to ensure that the qPCR was working. For the genes that replicated the results, the next step would be to validate the genes in a new population of sorted cells.
Fibroblast growth factor (Fgf1), establishment of sister chromatid cohesion N-acetyltransferase 1 (Esco1), and Secretogranin V (Scg5) were all up-regulated more than two-fold in both RNA Sequencing and qPCR data. Esco1 plays an important role in DNA replication as it is essential for the establishment of sister chromatid cohesion in mammalian cells (Price et al 2014). This up-regulation most likely results from the decrease in DNMTs and consequential increase in DNA expression and does not seem to be a likely candidate to explain the synaptic properties that result with this mutation. Likely acting with Esco1 is chromatin modifying protein (Chmp1a), which was down-regulated in DNMT knockouts, and responsible for regulating chromatin levels. Chmp1a would act in opposition to Esco1 and therefore should have inverse correlation patterns as is seen.

Upregulation of fgf1 is also consistent with decreased DNA methylation due to its involvement in neuronal differentiation and survival. Fgf1 has been shown to interact with SH2B1β-STAT3 complexes - SH2B1β is an adaptor/scaffold protein and STAT3 is a signal transducer and activator of transcription (Chang et al 2014). Other evidence suggests that this interaction can then lead to an increase in expression changes, especially during neuronal differentiation, as SH2B1β has been shown to participate in signaling pathways for different tyrosine receptor kinases and regulates a subset of neuron growth factor genes. This provides yet another mechanism by which DNMT knockouts show altered gene expression. (Chang et al 2014, Loeffler et al 2005).

However, neither of these changes have been implicated with intrinsic or synaptic changes. β2-microglobulin (β2m), which is down-regulated in RNA sequencing qPCR data, is a component of the major histocompatibility complex (MHC) and has been shown to play an important role in synaptic formation, remodeling and plasticity (Elmer & McAllister 2012).
Although β2m was shown to play a role in regulating synapses, the observed changes do not go in the same directions as our data shows. Past work found that β2m<sup>−/−</sup> mice had increased synapse density and increased frequency and amplitude of mEPSCs (Glynn et al 2011). Work in our lab saw a decrease in the frequency and amplitude of mEPSC in DNMT knockout animals and therefore it seems unlikely that β2m would explain the changes seen and is most likely down-regulated for a different reason.

I was particularly interested in scg5 as a potential target for explaining the physiological change seen in the DNMT cKO. As a chaperone protein, scg5 is responsible for regulating the conversion of PNOC into the active form of nociceptin, which leads to decreases in the frequency of mEPSC with no change in amplitude (Tallent et al 2001). While the mRNA levels of scg5 only increased about 3-fold, it is possible that, through translational means, the protein levels of nociceptin could be significantly higher than that. While I attempted to investigate nociceptin protein levels, I was unable to find a nociceptin specific antibody and therefore am unable to say in either direction whether scg5, and nociceptin, are involved in regulating the synaptic properties of the DNMT1 animals and are responsible for the decreased excitatory synaptic drive in PV-FS cells.

I was also interested in looking at ErbB4 and Neuregulin at the protein level despite them not being differentially expressed at the mRNA level because mutations in these genes lead to reduced excitatory synaptic drive (Fazzari et al 2010) and make them more excitable (Li et al 2012) – the same phenotype that is seen in the DNMT knockouts. Additionally, ErbB4 and neuregulin have been identified as schizophrenia candidate genes while DNMT levels were up-regulated in PV<sup>+</sup> cells of schizophrenic patients (Zhubi et al 2009) suggesting that these two networks may act in either parallel pathways or additive pathways.
ErbB4 protein changes due to DNA methyltransferase regulation

ErbB4 and its ligand neuregulin are both associated with regulation of excitatory synapses onto GABAergic neurons (Ting et al 2011, Woo et al 2007) and therefore could explain why knockouts of DNMTs exhibit reduced excitatory drive from inhibitory cells. On the other hand, if ErbB4 levels were not changed then this would strongly suggest that the ErbB4 and neuregulin act in a parallel pathway to DNMTs but do not interact with each other.

The immunohistochemistry done on DNMT knockouts shows a decrease in ErbB4 levels in mutants compared to control, however the high expression in non-interneurons evokes skepticism as to its validity. While I have not tested for the specificity of the antibody in immunohistochemistry, when I used it in a western blot it migrated at the correct molecular weight suggesting that at least in western blots it was recognizing ErbB4. In addition to the band at 184kDa, there were several strong non-specific bands also seen. Despite the non-specific bands, it is possible the change seen in PV\(^+\) cells is due to specific ErbB4 staining and the strong expression seen outside of the PV\(^+\) cells is due to the additional non-specific bands. I have not done a western blot analysis on the DNMT mutants since our mutation is specific to PV\(^+\) cells and they make up only a small portion of neurons in the brain. As a result, any difference that I may see could not be attributed to decreased levels of DNMTs.

If the staining is specific, and ErbB4 levels are decreased in DNMT mutants, this suggests that the two act in a parallel pathway where DNMT regulates the expression of ErbB4 receptors. If this is true, it would be interesting to see if up-regulation of ErbB4 in DNMT knockouts would be sufficient to revert the changes observed. While this is highly simplistic and therefore unlikely, it is still a plausible mechanism due to the high similarity in their expressed phenotypes.
I also wanted to look at the levels of neuregulin in control and mutant animals to see if there was a change in only the ErbB4 receptor or in both the ligand and the receptor. I was particularly interested in this because, unlike ErbB4, neuregulin is expressed predominantly in pyramidal cells where in our experimental set-up the DNMTs were completely intact. Therefore, if we were to see any changes in neuregulin levels it would be a consequence of a feedback loop due to a change in the ErbB4 levels. While I attempted this experiment, the antibody tested was non-specific and did not yield any results. Another difficulty with looking at gene changes due to decreased methylation is the experimental set-up does not allow analysis at the level of individual cell types with western blots as DNMTs are conditionally knocked out in PV⁺ cells which comprise a small portion of the cortex. As a result, to be able to detect changes we need to perform immunohistochemistry co-staining for parvalbumin and our protein of interest. In looking at activity deprivation conditions, western blot analysis can be done on whole cells and looks at gene changes from all cell types.

**Twik1 protein changes due to TTX treatment**

TTX treated slices have an overall increased excitability seen from the increased intrinsic plasticity of both inhibitory and excitatory cells. One possibility is that changes in ion channel expression could increase the excitability of neurons and RNA sequencing results show that some potassium channels part of the voltage gated potassium channel complex were down-regulated.

Twik1 is a leak potassium channel part of this complex that may be involved in regulating intrinsic neuronal properties. While the data has not been done on enough samples to begin drawing conclusions preliminary results suggest that it may be down-regulated after TTX treatment. This is consistent with the RNA sequencing data we have collected and other data
which has found potassium channels to be down-regulated in epilepsy conditions (Li et al 2012). By decreasing leak potassium channels, cells are more depolarized and therefore require less current to reach threshold and fire an action potential (Goldstein et al 2001). This is consistent with our findings that TTX application leads to increased intrinsic excitability (Escobedo-Lozoya, unpublished data).

It is still too early to tell what, if any, effects potassium channels play in TTX conditions but more samples should be tested for changes at the protein level. We could also test other potassium channels in this complex to see if this decrease is found in different potassium channels within a family. If it is found that twik1 is down-regulated in TTX, cell type specific analysis could be done to see if twik1 and other potassium channels have the same role in both inhibitory and excitatory cells.

**ErbB4 protein changes due to TTX treatment**

Past data has implicated ErbB4 and neuregulin to play a role in epilepsy and therefore it would be expected that they are involved in our in-vitro model of epilepsy as well. Preliminary results show that expression is decreased in TTX conditions, however due to the small sample size there is not enough data to determine whether these results are statistically significant.

Neuregulin was found to affect number of excitatory synapses onto inhibitory interneurons and it is believed to act through ErbB4 receptors (Ting et al 2011). Decreased levels of ErbB4 may lead to a lower number of synapses and less excitatory input onto inhibitory cells. My preliminary data shows that ErbB4 appears down-regulated in TTX conditions and is consistent with other findings based on human epileptogenic tissue (Li et al 2012). This experiment should be repeated to increase the sample size and determine if the observed decrease is statistically significant. If ErbB4 levels differ between conditions, it would be useful to
compare the number of synapses onto inhibitory cells to further determine if the hyper-excitability phenotype is directly affected by neuregulin/ErBb4 signaling to decrease excitatory synapse number.

**CRF-BP protein changes due to TTX treatment**

Due to the existing literature on the importance of CRH in epilepsy and past findings in the lab, I wanted to compare the expression of corticotropin releasing factor binding protein (CRF-BP), which binds to CRH and prevents its activation of CRH receptors. In doing so, bound CRF-BP is able to decrease seizure activity. From this, we would expect CRF-BP levels to be decreased in TTX conditions and this is what is seen in immunohistochemistry on cortical slices and RNA sequencing from PV⁺ cells. However, whole slice western blots show a significant increase of CRF-BP after TTX treatment (Fig 5C, D).

One possible explanation for the difference is that the antibody is recognizing different forms of the protein in immunohistochemistry and western blots. Because during western blot preparation, the samples are lysed and reduced, results refer to total concentration of proteins while in immunohistochemistry these proteins are largely in their native conformations and therefore may not be as accessible for binding to the antibody. Taking this into consideration, it suggests that while total protein concentration of CRF-BP is increased in TTX conditions, it has a decreased binding affinity to CRH and this is what we are seeing in the immunohistochemistry. The protein structure of CRF-BP has not yet been crystallized and to my knowledge it is not known whether there are conformational events that occur upon binding which may explain a difference in antibody binding affinity between conditions (Huising et al 2008).

Further experiments should be done to determine the specificity of the CRF-BP antibody in both immunohistochemistry and western blots. However, if only bound protein is being
recognized in immunohistochemistry it may be difficult to test with the current state of research. We should also do a pre-incubation experiment to test for the specificity of the antibody. Since the staining conditions are different between immunohistochemistry and western blots, it is possible that only in one of the conditions the antibody is recognizing CRF-BP and in the other condition it is non-specific.

Taken together, our results strongly suggest a role for ErBb4 in regulating synaptic plasticity both in DNA methylation induced changes as well as activity deprivation changes after TTX treatment. In addition to increasing the sample size for both conditions, it would be interesting to see whether neuregulin 1, a ligand of ErbB4, is also differentially regulated. Although it appears that in both of our conditions ErbB4 levels are decreased, we do not know if this leads to the observed decrease in mEPSC amplitude and frequency. Testing whether increasing ErbB4 levels is able to rescue this phenotype is a critical next step and would get at whether decreased ErbB4 is responsible for decreasing excitatory input onto inhibitory cells or if this is only a by-product of a greater change.

Nociceptin, through its interaction with scg5, may also be involved in regulating synaptic plasticity but not the intrinsic properties. Due to antibody limitations, I was unable to test nociceptin changes in DNMT knockout animals however RNA sequencing on TTX treated slices showed a decrease in the inactive form of nociceptin. Future experiments can investigate whether changes in scg5 and inactive nociceptin levels lead to downstream changes in the expression of nociceptin.

Finally, I began looking into a role for potassium leak channels to regulate plasticity after activity deprivation due to TTX blockage of sodium channels. This appears to be a mechanism by which a neuron is able to adapt its intrinsic properties and still be able to fire appropriately
even when less stimuli is presented. Future experiments can investigate twik1 expression further, as well as other potassium channels that are part of the voltage gated complex and those in other gene families.
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