Deaf1 and MeCP2 interact to coordinately regulate 5-HT_{1A} receptor gene expression

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Abstract

Deaf1 regulates 5-HT1A receptor gene (HTR1A) transcription by binding a 26-bp palindromic DNA sequence containing the rs6295 C(-1019) HTR1A polymorphism. However, Deaf1 cannot bind the G(-1019) HTR1A allele, which is associated with major depression. MeCP2 is implicated in serotonergic regulation and because the C(-1019) is a methylation site, I addressed whether MeCP2 alters Deaf1 regulation of HTR1A. MeCP2 and Deaf1 co-precipitated and bound the putative Deaf1 sites of the mouse HTR1A promoter *in vivo*. Binding of MeCP2 to the Deaf1 site was dependent on Deaf1 and MeCP2 increased Deaf1 recruitment to its site independently of methylation. MeCP2, Deaf1 and their combination modulated transcription of human and mouse HTR1A luciferase reporter constructs, with Deaf1 having a dominant effect, Deaf1 actions were disrupted by mutation of the Deaf1 site(s). Deaf1 KO or MeCP2 cKO in mice increased functional 5-HT1A autoreceptor-mediated hypothermia response. Overall, MeCP2 and Deaf1 coordinately regulate the HTR1A promoter.

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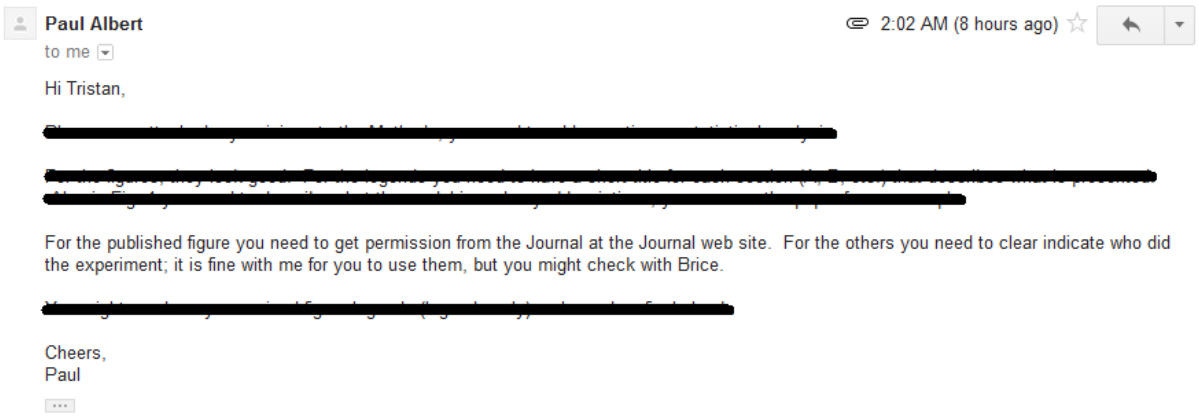

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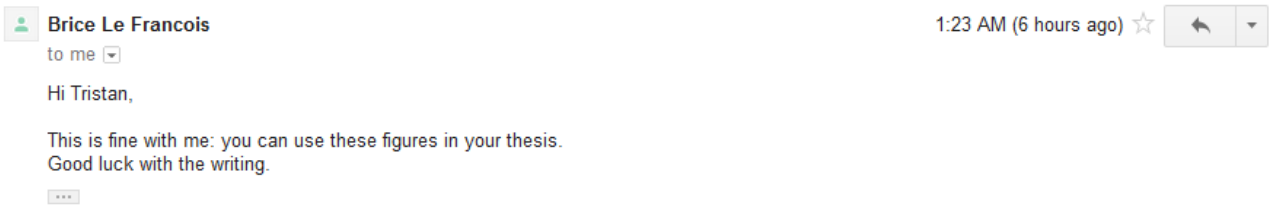

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Abbreviations (Legends)

-/- : Knockout Genotype

+/+ : Wild Type Genotype

5-HIAA : 5-HydroxyIndoleAcetic Acid

5-HT : 5-hydroxy tryptamine (Serotonin)

5-HT1A : 5-hydroxy tryptamine (Serotonin) 1A receptor

5-HTP : 5-HydroxyTryptophan

5-HTT : Serotonin Transporter

8-OH-DPAT : 8-hydroxy-N,N-DiPropyl-2-AminoTetralin

ACTH : AdrenoCorticoTropic Hormone

ANOVA : ANalysis Of VAriance

AVP : Arginine VasoPressin

BDNF : Brain-Derived Neurotrophic Factor

bp : Base Pair

Ca2+ : Calcium ion

cAMP : cyclic Adenosine MonoPhosphate

CpG : Cytosine-guanosine dinucleotides

CRH : Corticotropin-Releasing Hormone

Deaf1 -/- (Deaf1 KO) : Deformed Epidermal Autoregulatory Factor-1 Knockout

Deaf1 +/+ (Deaf1 WT) : Deformed Epidermal Autoregulatory Factor-1 Wild Type

Deaf1 : Deformed Epidermal Autoregulatory Factor-1

DNMT-3B : DNA Methyl Transferase-3B subtype

DNA PK : DNA Protein Kinase

DRN : Dorsal Raphé Nucleus

EPM : Elevated Plus Maze

fl/fl : two copies of the floxed gene

fl/Y : one copy of the floxed gene and no other copy on the Y chromosome

flox; fl; flx : flanked by loxP sites

Freud1 : Five-prime Repressor Element Under Dual repression 1

FS : Forced Swim test

G protein : Guanosine nucleotide-binding protein

GABAA receptor : γ-aminobutyric acid A subtype receptor

Gi : G protein Inhibitory

Go : G protein Other

GR : Glucocorticoid Receptor

GRE : Glucocorticoid Response Element

Gs : G protein Stimulatory

HDAC : Histone DeACetylase

HES1 or 5 : Hairy and Enhancer of Split 1 or 5

HET : HETerozygous

Hippo : Hippocampus

HPA : Hypothalamic Pituitary Adrenal

HVA : HomoVanillic Acid

IP : IntraPeritoneally

K+ : Potassium ion

KO : KnockOut

LD : Light Dark paradigm

MAOIs : MonoAmine Oxydase Inhibitors

MBD : Methyl Binding Domain

MDD : Major Depressive Disorder

MeCP2 cKO : Methyl CpG binding Protein 2 conditional KnockOut

MeCP2 : Methyl CpG binding Protein 2

mRNA : messenger RiboNucleic Acid

MYND : MYeloid Nervy Deaf1

NE : NorepinEphrine

NGFI-A : Nerve Growth Factor Inducible A

ns : Not Significant

NUDR : NUclear Deaf1 Related

OF : Open Field test

PET : Positron Emission Tomography

PFC : Prefrontal cortex

pMeCP2 : phosphorylated MeCP2

REST : Repressor Element 1-Silencing Transcription factor

S.E.M. : Standard Error of the Mean

SAND : Sp100 AIRE-1 NucP41/75 Deaf1

SD : Standard Deviation

SE : Standard Error

SERT : SERotonin Transporter

siRNA : Small Interfering RiboNucleic Acid

SLC6A4 : Serotonin transporter gene

SSRI : Selective Serotonin Reuptake Inhibitor

TCAs : TriCyclic Aantidepressants

TPH : TryptoPhan Hydroxylase

TS : Tail Suspension test

TSS : Transcriptional Start Site

UCMS : Unconditional Chronic Mild Stress

WT: Wild Type

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Depression and serotonin

Major depressive disorder (MDD) is a complex and burdensome mental health disorder that affects 16-20% of the population (Kessler and Bromet, 2013), in which women have twice the risk as men (Dorris et al., 1999; Fava and Kendler, 2000; Albert and Lemonde, 2004). MDD is linked to increased suicide risk (15%), anxiety, obsessive compulsive disorder, PTSD and other mood disorders (Albert and Lemonde 2004; Pineyro and Blier, 1999). While there are many types of treatment for MDD, pharmaceuticals have been shown to work initially in only 60-70% of patients and in only 30% of patients after 6 months (Souery et al., 2006; Trivedi et al., 2006). It remains unclear why only 60-70% of patients respond to treatment and why many patients stop responding to treatment.

Serotonin (5-HT) is thought to play a crucial role in mood and resulting behaviour (Albert and Lemonde, 2004). While there are several theories regarding the mechanism, cause, and treatment of depression, several lines of evidence support the serotonin theory of depression. Depressed patients were shown to have decreased 5-HT activity (Albert and Lemonde 2004; Blier and de Montigny, 1999; Cowen et al., 1989; Lesch and Heils, 2000; Veenstra-Vander Weele et al., 2000) and to be susceptible to tryptophan depletion (Delgado et al., 1999) As a result, specific serotonin reuptake inhibitors (SSRI)s which inhibit the serotonin transporter (SERT) are the first line of treatment for depression and suicide, followed by tricyclic antidepressants (TCAs) which increase the levels of 5-HT and norepinephrine (NE). Despite several aversive effects, monoamine oxidase inhibitors (MAOIs) are used to treat SSRI-resistant forms of MDD and act by inhibiting the degradation of monoamine neurotransmitters (e. g. 5-HT, NE, dopamine (DA)) following their release at the synapse (Blier and de Montigny 1999; Pineyro and Blier, 1999; Albert and Lemonde, 2004). More recently serotonin-norepinephrine reuptake in-

hibitors (SNRIs) or multimodal antidepressants have been introduced. For example, vortioxetine, which acts on 5-HT1A receptors, other receptors and SERT is considered a multimodal agent (Hamon and Blier, 2013). Scientists have been working to understand how these various pharmaceuticals work for several decades since SSRIs were first discovered. While not all of their effects are clear, they underline the importance of 5-HT as well as other monoamine neurotransmitters (NE and DA) in the treatment of depression (Albert et al., 2011; Artigas et al., 1996; Cryan et al., 2005; Pineyro and Blier 1999; Porsolt et al., 1977).

Additionally, suicide attempters and MDD patients have significantly lower 5-HT metabolites in their cerebrospinal fluid (Brown et al., 1982; Lidberg et al., 2000). Since the reduction of 5-HT was suggested to lead to MDD, Delgado and colleagues put depressed patients in remission on a diet where tryptophan (5-HT precursor) was significantly reduced. A reduction of tryptophan in the brain led them to relapse into a depressive episode (Delgado et al., 1999). When patients returned to a normal diet, they also returned to remission (Leyton et al., 1997). As will be discussed further, imaging studies have shown lower 5-HT levels and higher 5-HT1A autoreceptor binding in depressed patients (Jans et al., 2007). Nevertheless, MDD is a complex disorder where several neurological and physiological systems are affected. Overall, this makes it difficult to determine how the underlying mechanisms work together to influence the development of MDD and major behavioural effects (Albert et al., 2011; Celada et al., 2013). Furthermore, a combination of genetic and environmental factors (in particular stress) are thought to predispose to MDD via these mechanisms (Caspi et al., 2003).

Role of the 5-HT1A receptor

Serotonin is recognized by several G protein-coupled serotonin receptors, which are organized into 7 classes (5-HT1 to 5-HT7; Barnes and Sharp, 1999). Of these, the 5-HT1A recep-

tor is present on the cell body and dendrites of the serotonergic "pre-synaptic" neurons where it acts as an inhibitory autoreceptor in the raphé nuclei (Adell et al., 2002; Albert and Lemonde 2004; Riad et al., 2000; Sotelo et al., 1990). The 5-HT1A autoreceptor (aka: pre-synaptic 5-HT1A receptor) negatively regulates the spontaneous activity of the serotonergic raphé neurons on the neurons themselves. These serotonergic raphé neurons release serotonin to "post-synaptic" targets in the brain (e. g. hippocampus, PFC), some of which have 5-HT1A receptors (aka: post-synaptic 5-HT1A receptors), amongst several other kinds of 5-HT receptors. Some post-synaptic targets in the brain also send feedback to the raphé nuclei to regulate the levels of serotonin (Albert and Lemonde, 2004; Albert, 2012; Aznar et al., 2003; Hajos et al., 1999; Liu et al., 2005; Riad et al., 2000; Sotelo et al., 1990). For a graphical representation see Albert and Lemonde (2004). The pre- and post-synaptic 5-HT1A receptor couple to Gi/Go proteins to inhibit cAMP formation which inactivates Ca^{2+} channels and activates potassium channels, ultimately causing the cell to momentarily hyperpolarize and reduce its firing (Barnes and Sharp, 1999).

PET imaging studies have been used to study the levels of 5-HT1A receptors in the living brains of MDD patients and healthy controls. Most but not all of these studies have shown higher 5-HT1A binding in the raphé of MDD patients with and without exposure to antidepressants compared to controls (Drevets et al., 1999; Drevets et al., 2007; Hesselgrave and Parsey, 2013; Parsey et al., 2006a; Parsey et al., 2006b; Parsey et al., 2010; Sargent et al., 2000; Savitz & Drevets, 2009). Others have also shown higher 5-HT1A binding in the raphé of MDD patients during a depressive episode, which correlated with HAMD scores (Parsey et al., 2006a; Parsey et al., 2006b; Parsey et al., 2010), even showing lower 5-HT1A binding following treatment and during remission (Lanzenberger et al., 2013; Meltzer et al., 2004; Martinez et al., 2001). However, increased 5-HT1A binding in the raphé is correlated with antidepressant resistance (Parsey et al.,

2006b; Moses Kolko et al., 2007). In support of these PET imaging results the brains of MDD patients and suicide victims were also shown to have increased levels of the 5-HT1A autoreceptor in post mortem analyses (Albert & Lemonde, 2004; Boldrini et al., 2008; Goswami et al., 2010; Stockmeier et al., 1998). Additionally, several other PET studies have shown decreased 5-HT1A receptor density in the PFC of MDD patients (Drevets et al., 2000; Neumeister et al., 2004; Sargent et al., 2000). These PET and post-mortem studies underline the role of 5-HT1A receptor de-regulation in human depression.

Several studies in mouse have shown that increased 5-HT1A autoreceptors and reduced post-synaptic 5-HT1A receptors are associated with anxiety and depression, and response to antidepressant treatment. Effective SSRI treatment, but not TCA treatment, appears to be dependent on both the auto- and hetero- 5-HT1A receptors since SSRI treatment did not elicit any behavioral response in 5-HT1A KO mice (Albert, 2012; Santarelli et al., 2003). Furthermore, reductions in post-synaptic 5-HT1A heteroreceptors have been associated with anxiety disorders and depression (Albert, 2012; Fisher et al, 2006). As extensively reviewed by Albert and colleagues (2014), altering 5-HT levels or 5-HT1A receptor expression (pre- and post-synaptically), by altering 5-HTT or TPH2, or by knocking out, repressing, or increasing levels of the 5-HT1A receptor all led to complex mood disorders (Albert et al., 2014; Gross et al., 2002; Heisler et al., 1998; Lo Iacono and Gross, 2008; Ramboz et al., 1998; Richardson-Jones et al., 2011; Parks et al., 1998). Overall, these findings lend support for the serotonin hypothesis of depression and the major role the 5-HT1A receptor plays in mood disorders.

As mentioned previously, most antidepressants (SSRIs, MAOIs, and TCAs) increase 5-HT levels (Albert and Lemonde, 2004) but require three weeks before they have an appreciable effect on mood (Pineyro and Blier, 1999). This delay is thought to be caused in part by 5-HT1A

autoreceptors attempting to reduce the now higher 5-HT levels to "normal" levels by feedback inhibition of raphé firing (Albert et al., 1996; Pineyro and Blier, 1999). Higher levels of 5-HT1A autoreceptor would lead to greater inhibition of raphé neuronal firing and reduced 5-HT release (Albert, 2012; Blier and de Montigny, 1987; Czachura and Rasmussen, 2000; Descarries and Riad, 2012; Dong et al., 1997; Rigdon and Wang, 1991). Finally, the 5-HT1A autoreceptors (but not the post-synpatic 5-HT1A heteroreceptors) slowly to desensitize to the higher 5-HT levels and the patient remits after a few weeks (Albert and Lemonde, 2004; Descarries and Riad, 2012; Kennet et al., 1987; Pineyro and Blier 1999). Desensitization can occur at the level of receptor binding, trafficking of the receptor, or genetic expression of the receptor. From these, the levels of 5-HT1A autoreceptor mRNA and binding sites are reduced in the raphé nuclei of rats, particularly in rat depression models (Albert, 2012; Casanovas et al., 1999; Le Poul et al., 2000; Yau et al., 1999). This underlines the importance of 5-HT1A autoreceptor regulation and desensitization at the genetic level as will be discussed further.

However, some patients are more resistant to antidepressant treatment suggesting that they are less able to adapt and change the regulation of their 5-HT1A autoreceptors. Therefore, Lemonde and colleagues (2003) analysed a functional gene polymorphism, the C/G(-1019) HTR1A promoter polymorphism, in depressed patients and normal controls. They found that depressed patients were twice as likely, and suicide victims four times as likely to be homozygous for the 5-HT1A G(-1019) allele compared to controls. Furthermore, a reduced antidepressant response was significantly associated with the G(-1019) allelic variant (Lemonde et al., 2004; Albert and Lemonde, 2004). The G(-1019) allele was associated with depression and suicide in several other studies across several human populations (Anttila et al., 2007; Choi et al., 2010, Dannlowski et al., 2007; Domschke et al., 2006; Drago et al., 2007, Kishi et al., 2009; Kishi et al.,

2011; Kraus et al., 2007; Le Francois et al., 2008; Lemonde et al., 2003; Lenze et al., 2008; Molina et al., 2011; Neff et al., 2009; Straube et al., 2014; Strobel et al., 2003; Wu et al., 2008; Videtic et al., 2009; Zetzsche et al., 2008; Zhang et al., 2009), but not all studies (Hettema et al., 2008; Wasserman et al., 2006). This association was also seen in meta-analyses for depression and bipolar depression (Kishi et al., 2009; Kishi et al., 2011; Kishi et al., 2013) PET studies showed that the G/G(-1019) phenotype in depressed patients was associated with 5-HT1A levels in the raphé (Parsey et al., 2006b; Sullivan et al., 2009; Parsey et al., 2010). Furthermore, Villafuerte and colleagues showed that the G(-1019) allelic variant was associated with more severe depression and reduced response to SSRIs (Villafuerte et al., 2009). By analogy with the 5-HTT long polymorphic repeat s/s genotype (Caspi et al., 2003), Kim and colleagues found that the G(-1019) allele was also associated with depression after a stressful life event, suggesting an interplay between genetics and environment (Kim et al., 2011). Overall these results suggest that the G(-1019) allele deregulates 5-HT1A receptor expression, leading to higher levels presynaptically, which predisposes someone to depression especially after stressful life events. Due to the de-regulation of 5-HT1A receptor expression, antidepressants are not as effective in reducing 5-HT1A autoreceptor levels and increasing 5-HT levels and so the patient is resistant to treatment (Blier and de Montigny, 1999).

Deaf1 regulation of the 5-HT1A receptor

Further study of the C/G(-1019) polymorphism showed that Deaf1 (Deformed autoregulatory factor-1; aka: NUDR) and HES5 bind in this region and regulate gene transcription. Specifically, Deaf1 binds to a 26-bp imperfect palindrome containing the TTCG motif, which includes the C/G(-1019) polymorphism (Lemonde et al., 2003). Deaf1 is a transcription factor present in the human, mouse, rat, and several other species that has been shown to enhance or re-

press gene expression (Albert et al., 2011; Huggenvik et al., 1998; Lemonde et al., 2003; Michelson et al., 1999). Deaf1 contains a conserved KDWK motif in the SAND domain that mediates its ability to bind to the TTCG motif (Bottomley et al., 2001; Jensik et al., 2014). Nevertheless, Deaf1 still binds to single or imperfect TCGs but with lower affinity (Lemonde et al., 2003; Jensik et al., 2014).

Deaf1's SAND domain has also been shown to mediate Deaf1's interaction with other proteins such as DNA protein kinase (DNA PK; Jensik et al., 2012). Mutations of the SAND domain have been shown to prevent protein-protein interaction in association with mental retardation (Jensik et al., 2012; Faqeih et al., 2014; Vulto-van Silfhout et al., 2014). Deaf1 has also been shown to interact with other proteins through other domains (Joseph et al., 2014; Ordureau et al., 2013; Pilot-Storck et al., 2010). Deaf1 contains a zinc finger region known as the MYND domain (Huggenvik et al., 1998), which may mediate DNA interaction (Jensik et al., 2012) but is also important for interactions with other proteins (Liu et al., 2011). Deaf1 can also be phosphorylated at a minimum of three identified sites (Jensik et al., 2012); while the functional relevance of phosphorylation is unknown.

Furthermore, two isoforms of Deaf1 have been identified in mice and humans (Yip et al., 2009). The full length Deaf1 (simply Deaf1) isoform has a nuclear localisation signal and nuclear export signal, which enables Deaf1 to be present in both the nucleus and cytoplasm (Jensik et al., 2004; Yip et al., 2009). While the shorter variant (Deaf1Var1) lacks part of the nuclear localization signal, the nuclear export signal, and the MYND domain, and is primarily located in the cytoplasm (Yip et al., 2009). Higher Deaf1Var1 levels in the pancreas due to inflammation and hyperglycemia were associated with type 1 diabetes in human (Yip et al., 2009; Yip et al., 2015). Yip and colleagues observed that Deaf1Var1 reduced Deaf1's effects on gene transcrip-

tion, possibly by interacting with and subsequently sequestering Deaf1 to the cytoplasm (Yip et al., 2009). Deaf1's interactions with other proteins may break this Deaf1-Deaf1Var1 interaction enabling Deaf1 to enter the nucleus. This process may allow the cell to quickly react to stimuli with changes in gene transcription. In summary, Deaf1 binds to the human HTR1A promoter, is localized in the nucleus and cytoplasm, and interacts with other proteins.

In addition to Hes5, the closely related homologue Hes1 was shown to bind and repress the human HTR1A gene, and knockout of Hes1 in mice lead to de-regulation of 5-HT1A autoreceptors in development (Jacobsen et al., 2008). HES1/5 binds slightly further upstream to an N box (CACNAG). While Deaf1 and HES1/5 can bind to the C(-1019) allele, they are unable to bind to the G(-1019) allelic variant (Czesak et al., 2006; Lemonde et al., 2003; Lemonde et al., 2004; Le Francois et al., 2008; Jacobsen et al., 2008). Considering however, that HES1/5 are expressed primarily during embryonic development (Kageyama and Ohtsuka 1999; Le Francois et al., 2008), our focus has shifted to Deaf1, which is expressed throughout development to adulthood. Furthermore Deaf1 and 5-HT1A receptors are co-localized in the raphé nuclei and 5-HT1A expressing post-synaptic neurons (Lemonde et al., 2003).

Luciferase assays indicated that Deaf1 enhances or represses human C(-1019) 5-HT1A transcriptional activity in different cell types (Repress: HEK-293, RN46A; Enhance: SN48, NG-108, SKN-SH; Albert et al., 2011; Czesak et al., 2006; Lemonde et al., 2003). Knocking out Deaf1 in a mouse model led to an increase in 5-HT1A receptors on serotonergic raphé neurons but a decrease in 5-HT1A receptor RNA in the PFC (Czesak et al., 2012). These results mimic PET and post mortem results in depressed humans compared to healthy controls, which show decreased 5-HT1A levels in the PFC and increased 5-HT1A levels in the raphé (Drevets et al., 2000; Parsey et al., 2010; Stockmeier et al., 1998). Overall these results suggest that the loss of

Deaf1 binding to the G(-1019) allele or by KO in the mouse model, prevents it from differentially regulating the 5-HT1A gene (HTR1A) promoter leading to higher 5-HT1A autoreceptor levels in the raphé and lower 5-HT1A heteroreceptor levels post-synaptically (Le Francois et al., 2008; Albert et al., 2012). As explained above, higher 5-HT1A autoreceptor levels in the raphé would inhibit the neuronal firing rate and reduce 5-HT release, which could increase the likelihood of depression (Fig. 1; Albert et al., 2012). Before the start of this project causational evidence for Deaf1's functional effect on the 5-HT1A receptor and the resultant behavioural effects in mice had not been demonstrated. In addition, the C/G allelic variation that exists in humans and prevents Deaf1 binding does not exist in rodent models. Therefore in order to address Deaf1 function on 5-HT1A receptors *in vivo*, it was important to examine Deaf1 binding and regulation of the mouse HTR1A promoter.

Effects of stress on gene methylation

The role of C/G HTR1A genotype in depression appears to be a modulatory one that can be enhanced by stressful life events. For example, normal subjects with the G/G genotype were not significantly different on depression or anxiety scales than those with the C/G or C/C genotype (Chipman et al., 2010), underlining the concept that the G/G genotype only predisposes someone to depression. Instead significant G/G genotype and environment interactions were found on depression and other mood disorders (Blaya et al., 2011; Caspi et al., 2003; Chipman et al., 2010; Kim et al., 2014). However, the mechanism between this genetic and environmental interaction remains elusive, but several studies have shown that stress results in changes in gene expression via changes in gene methylation or histone modifications (Boku et al., 2014; Chahrour et al., 2008; Labonte et al., 2012; Le Francois et al., 2015; McGowan et al., 2009;

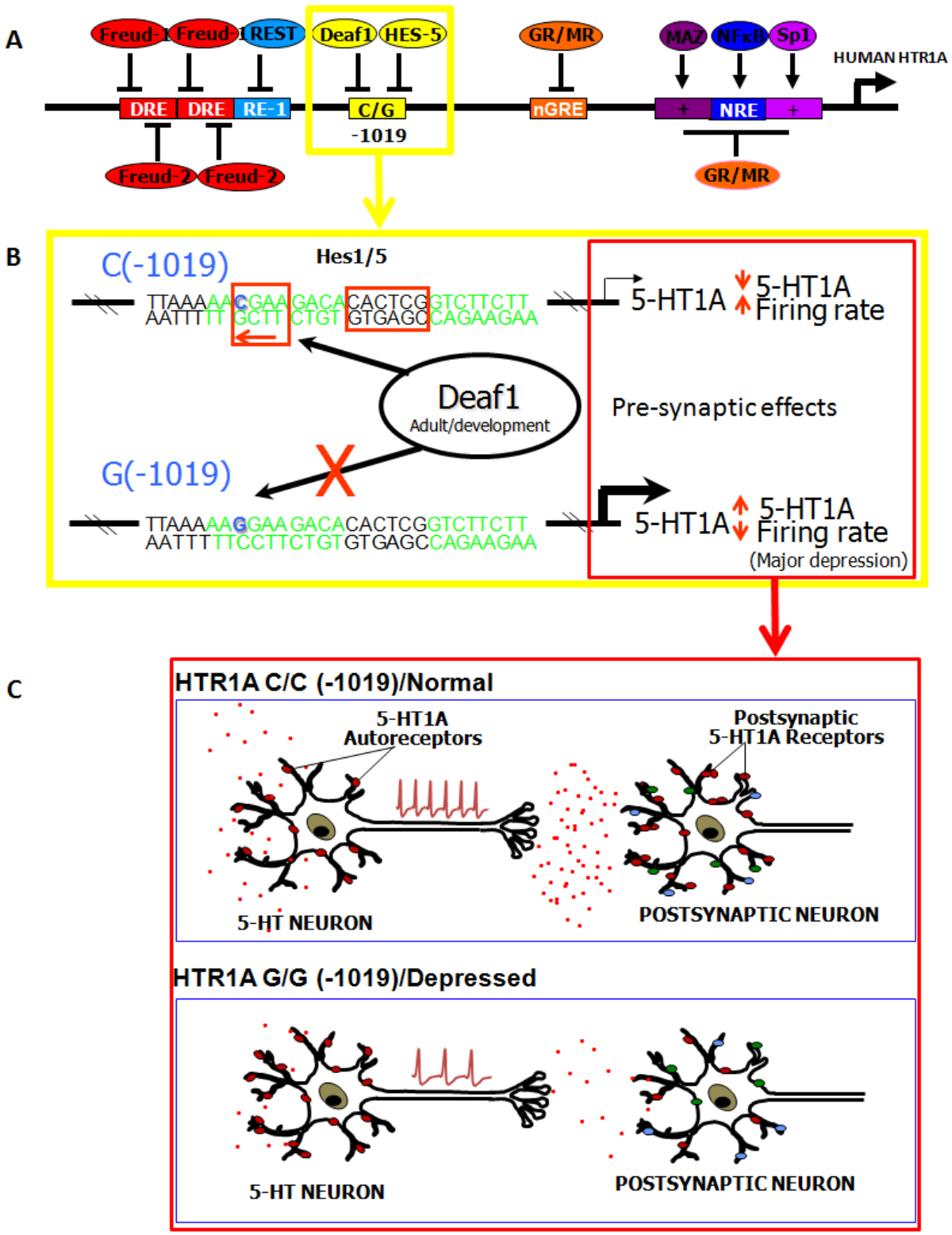


Figure 1. Model of 5-HT1A C(-1019)G polymorphism and presynaptic dysregulation resulting from Deaf1 dysregulation. (A) Human HTR1A Promoter. A model of the human HTR1A promoter, with characterized DNA elements located upstream of the translational start site (bolded arrow) are shown and the C(-1019)G polymorphism boxed in yellow. **(B) Deaf1 26-**

bp site. The DNA sequence of the 26-bp palindrome, and the Deaf1 inverted TTCG site as well as the Hes1/5 site (N-box) is shown. Deaf1 is unable to bind and regulate its site at the the G(-1019) allelic variant. Note that the C(-1019)G polymorphism is a CpG methylation site (red *). **(C) Model of HTR1A genotype effect on 5-HT.** The 5-HT1A autoreceptor (yellow ovals) on 5-HT neurons negatively regulates neuronal firing to maintain normal 5-HT neuron firing rate. While normal subjects have the HTR1A C-allele that can be repressed by Deaf1 and Hes1/5 to normalize 5-HT1A receptor levels (yellow oval), depressed subjects homozygous for the HTR1A G(-1019) allele have lost Deaf1 and Hes1/5 regulation of HTR1A transcription and do not have such a mechanism. Subjects homozygous for the C(-1019) genotype have normal amounts of 5-HT1A receptors (yellow oval) normal 5-HT neuron firing (red action potentials), and normal 5-HT release (red dots), which can activate post synaptic receptors (coloured ovals). In depressed subjects, this loss of Deaf1 regulation results in greater HTR1A gene transcription and increased expression of 5-HT1A autoreceptors pre-synaptically, and therefore a greater inhibitory response to serotonin leading to a lower firing rate and less serotonin release (red dots). In addition, lack of Deaf1 enhancer activity at the HTR1A G-allele in post-synaptic regions leads to decreased HTR1A transcription and decreased levels of 5-HT1A receptors post-synaptically, thus leading to a decreased response to already decreased 5-HT release. Together, these changes greatly reduce 5-HT neurotransmission, predisposing these subjects to depression and reduced response to antidepressants. Borrowed from Paul R. Albert, and Albert et al., 2011.

Meaney and Szyf, 2005; Meaney and Ferguson-Smith, 2010; Murgatroyd et al., 2009; Nestler, 2014; Philibert et al., 2007; Philibert et al., 2008; Oberlander et al., 2008; Weaver et al., 2004). Changes in gene methylation can prevent or alter the binding of transcriptional regulators. DNA methylation is also recognized by methyl-CpG binding proteins (e. g., MeCP2). Methyl-CpG binding proteins recruit other repressive complexes to change the structure of chromatin by post-transcriptional histone modifications, such as histone deacetylation (Klose and Bird, 2006). These modifications in turn change how histones interact with DNA allowing or blocking the interaction of DNA with transcription factors and polymerases (Meaney and Szyf, 2005; Meaney and Ferguson-Smith, 2010).

The link between early life stress and specific changes in DNA methylation at transcription factor sites is becoming clearer (Meaney and Szyf, 2005). For example, high or low maternal licking and grooming in mice was shown to result in changes in DNA methylation of the NGFI-A binding site in the promoter for the glucocorticoid receptor (GR) gene (NR3C1) and histone acetylation in the hippocampus (Weaver et al., 2004). Similarly, human suicide completers with a history of childhood abuse or neglect had decreased methylation of the NR3C1 promoter and showed lower amounts of GR_{1F} protein and mRNA in the hippocampus (McGowan et al., 2009). Furthermore, the same group found that suicide completers who suffered childhood abuse had site specific methylation changes of GR_{1B} and $_{1C}$ which were negatively correlated with GR mRNA and GR_{1B} and $_{1C}$ expression (Labonte et al., 2012). Pertaining more to the development of anxiety and depression, newborns exposed to stress prenatally had increased methylation of specific CpG islands in the NGFI-A binding site in the NR3C1 promoter region. These changes in DNA methylation were then correlated with a higher cortisol response to a stress challenge during adulthood (Oberlander et al., 2008). These studies also show the importance of

DNA methylation in the regulation of NGFI-A binding to NR3C1 and GR levels in response to stress.

Furthermore, mice subjected to early life stress had reduced methylation and MeCP2 binding to the arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) promoters that correlated with higher levels of gene transcription, leading to greater HPA axis activation in response to stress in adulthood (Murgatroyd et al., 2009).

However, the stress-epigenetic relationship is not exclusive to glucocorticoid and HPA axis related genes. Childhood abuse and depression were also associated with higher methylation at specific CpG sites in the serotonin transporter gene (SLC6A4) (Kang et al., 2013) and decreased levels of SLC6A4 mRNA (Philibert et al., 2007; Philibert et al., 2008). Interestingly higher SLC6A4 methylation was also associated with resilience to developing PTSD and lower SLC6A4 methylation with a greater risk of developing schizophrenia (Koenen et al., 2011).

In addition, human suicide completers had an upregulation in the PFC of DNMT-3B, an important DNA methyltransferase. This up-regulation was correlated with hypermethylation at key CpG islands in the $GABA_A$ receptor α1 subunit genomic DNA (Poulter et al., 2008), and with lower levels of the $GABA_A$ receptor α1 subunit in the PFC of suicide completers compared to controls (Merali et al., 2004). These results suggest that the upregulation of DNMTs led to changes in DNA methylation, which may result in significant behavioural effects (Merali et al., 2004; Poulter et al., 2008).

In relation to the HTR1A promoter, increased methylation of CpG 13 (in the HES1/5 binding site) was positively associated with the negative symptoms of schizophrenia (Tang et al., 2013). Recently our lab found that unconditional chronic mild stress (UCMS) in mice resulted in lower nesting and coat state scores (indicators of depression-like behaviour) and increased meth-

ylation of the -681 CpG site of the mouse HTR1A gene, increased levels of 5-HT1A mRNA and protein levels in the PFC and 5-HT neurons of the raphé (Le Francois et al., 2015). However, methylation state was analyzed on the proximal (up to -750bp) region of the HTR1A promoter and did not include the CpG site in the Deaf1 binding sites (Le Francois et al., 2015). However, the effects of stress are not limited to gene expression. Acute stress and glucocorticoid injections led to a desensitization of the 5-HT1A autoreceptor in the raphé without any changes in 5-HT1A mRNA levels (Fairchild et al., 2003; Laaris et al., 1999; Neumaier et al., 2000). Nonetheless, acute corticosterone led to specific changes in 5-HT1A mRNA levels in the hippocampus (Neumaier et al., 2000). Lastly, chronic corticotropin releasing factor in the raphé increased 5-HT1A mRNA levels and anxiety-like behaviour (Clark et al., 2007). Indeed the relationship between stress and depression (via the 5-HT1A receptor) is complicated by the fact that the 5-HT1A promoter region contains a GR/MR site and sites for several other transcription factors (Albert et al., 2011). In addition, the regulatory effects of Deaf1 and HES1/5 are also affected by the C/G(-1019) allelic variation, which can associate with depression (Parsey et al., 2010; Kim et al., 2014).

Role of MeCP2 in gene regulation

Methyl CpG binding protein 2 (MeCP2) is one of the major proteins in the brain that binds to methylated CpG sites and is mutated in Rett syndrome (Amir et al., 1999; Khrapunov et al., 2014). MeCP2 was shown to bind to methylated CpGs and interact directly with the co-repressor mSin3A, which recruits HDACs to deacetylate histones, leading to a contraction of the chromatin making it inaccessible for transcription factor binding (Kass et al., 1997; Lewis and Bird, 1991; Nan et al., 1998). However, MeCP2's role solely as a methyl-DNA binding transcriptional repressor has been challenged since it also enhances the transcription of certain genes, a

dual role that could be explained by its interaction with other transcription factors (Chahrour et al., 2008). MeCP2 is highly expressed in the brain and several mutations thereof were found to result in Rett Syndrome (Amir et al., 1999; Bellini et al., 2014). While most mutations are in the methyl binding domain (MBD), certain mutations that result in Rett syndrome have recently been shown to disrupt MeCP2 phosphorylation and dynamic interaction with the NCoR co-repressor complex (Ebert et al., 2013; Jorgensen and Bird, 2002; Lyst et al., 2013). These mutants highlight both the importance of phosphorylation (one of many post translational modifications) and MeCP2's interaction with other transcriptional regulators.

Role of methylation and MeCP2 in anxiety and depression

Phosphorylation of MeCP2, via the activation of DRD1 receptors and all 5HT receptor subtypes, was also found to be involved in antidepressant action (Deng et al., 2010; Hutchinson et al., 2012a; Hutchinson et al., 2012b), suggesting that changes in MeCP2 function and perhaps interaction with other transcription factors can affect mood disorders. Considering however, the many effects and interacting partners MeCP2 may have, more studies are needed to determine how MeCP2 can affect each of these components.

For example phosphorylation of MeCP2 can affect its interaction with other transcription factors and change its role as an activator or a repressor of the BDNF gene. While MeCP2 was found to be a repressor of BDNF expression in cortical neurons, over-expressing MeCP2 paradoxically promotes BDNF expression. In addition, transgenic mice over-expressing MeCP2 had higher BDNF expression and MeCP2 KO mice had lower BDNF levels (Chahrour et al., 2008; Cheng and Qui, 2014). Furthermore, Ca^{2+}-mediated phosphorylation of MeCP2 was found to affect activity-dependent BDNF transcription in growing neurons (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006; Cheng and Qui, 2014). Considering that low BDNF and neuro-

plasticity have been identified as important factors in the molecular symptomatology of depression (Groves, 2007), this underlines MeCP2's role not only in learning and memory, but also in depression.

In addition, MeCP2 gene duplication in males resulted in anxiety and several symptoms of mental retardation and autism (Ramocki et al., 2009). By contrast, in females, MeCP2 gene duplication increased the prevalence of anxiety, depression, and compulsions (Ramocki et al., 2009). Rett syndrome patients also show reduced levels of DA and 5-HT metabolites (HVA and 5-HIAA, respectively; Temudo et al., 2009; Samaco et al., 2009). Similarly, MeCP2 null mice have lower levels of biogenic amine neurotransmitters (NE, DA, 5-HT) in early post-natal life, due to reduced expression of rate limiting enzymes in neurotransmitter synthesis (Ide et al., 2005; Samaco et al., 2009). Behaviourally, male knock out MeCP2 mice and heterozygous female mice showed increased anxiety in the open field and light dark test, which was partially rescued by reactivating functional MeCP2 (Lang et al., 2014). Contrarily, other studies have found that MeCP2 KO males showed less or no anxiety and increased aggression compared to WT (Samaco et al., 2009; Samaco et al., 2013). However, this full knock out had EEG deficits, tendency towards seizures, lower life span, locomotor deficits, social deficits, memory and learning deficits, and other impairments making it difficult to study anxiety and depression (Lang et al., 2014). While it is difficult to determine all of the effects knocking out MeCP2, these results suggest that changes in MeCP2 expression may result in anxiety and depression, warranting further study.

Deaf1 and MeCP2 have both been found to interact with other transcription factors (Jensik et al., 2012; Ebert et al., 2013; Lyst et al., 2013) and could interact together to influence gene expression. Thus the importance of MeCP2 in stress-mediated changes in gene expression

prompted us to study the potential role of MeCP2 in regulating the 5-HT1A promoter and its potential interaction with Deaf1. This led to the hypothesis that MeCP2 could regulate the HTR1A gene, and below are presented preliminary studies, done before the current thesis, which suggest support this hypothesis.

Potential role of MeCP2 in 5-HT1A regulation

MeCP2 increases HTR1A promoter activity

To address whether HTR1A is regulated by MeCP2, we used mouse and human HTR1A-promoter luciferase vectors to examine the potential role of MeCP2. Luciferase vectors consisted of either: the human HTR1A gene from -1128 bp to the translation start site (TSS, 0 bp), to include the Deaf1 binding site (Lemonde et al., 2003); or the mouse HTR1A gene promoter from -984 bp to the TSS, to include the two putative Deaf1 binding sites (Czesak et al., 2012). These promoters were ligated to the luciferase reporter gene in the pGL3B expression vector. The empty pFLAG vector or pFLAG-MeCP2 fusion vector was co-transfected with the reporter vectors into the human embryonic kidney 293 cells (HEK-293). Co-transfection of MeCP2 with both the human and mouse reporter vectors led to an increase in transcriptional activity, as detected by increased luciferase activity (Fig. 2). These data suggest that MeCP2 can affect HTR1A promoter activity *in vitro*. Nonetheless, it is unclear how MeCP2 regulates the HTR1A promoter; whether it requires the promoter to be methylated or whether Deaf1 is involved in this effect.

(ii) Yeast-one hybrid methylation assay

MeCP2 can regulate transcription either directly by binding to DNA or indirectly through binding to other transcription factors. To test for direct or indirect effects of MeCP2 at the

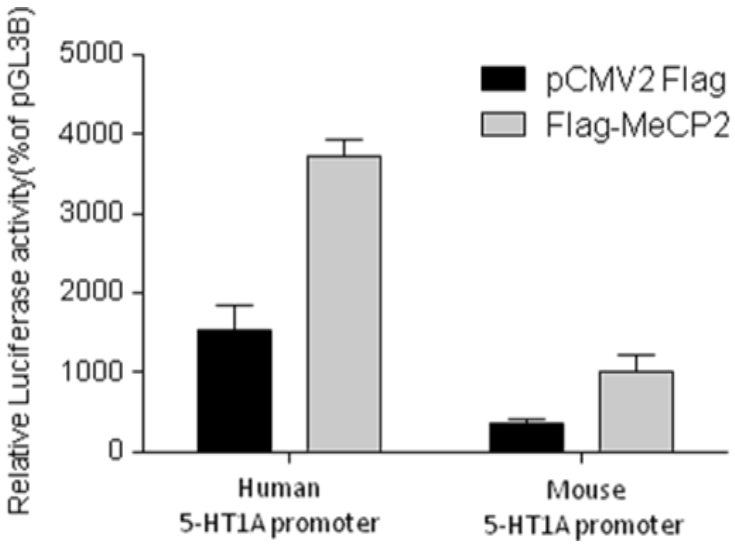


Figure 2. MeCP2 in HEK-293 cells increases 5-HT1A promoter activity. For each well in a 6 well plate 1µg of pFlag-MeCP2 or pCMV2Flag (negative control) were co-transfected with 1µg Human pGL3B-1128 or mouse -984 5-HT1A promoter luciferase constructs and 1µg pCMV-βGal and luciferase activity normalized to β-galactosidase was determined. Both 5-HT1A constructs contain Deaf1 sites. Data shown as mean±S.E., n = 3 independent experiments. Performed by Brice Le Francois.

HTR1A Deaf1 site we used a yeast one-hybrid transactivation assay. Since MeCP2 binds to methylated DNA we examined whether changes in DNA methylation affect Deaf1 or MeCP2 binding to the Deaf1 site of the human 5-HT1A promoter. Since yeast lack DNA methylase enzyme, we transfected with the DNA methylase MSssI. Three copies of the C (p8op-3RC) or G-allele (p8op-3RG) of the 26-bp human HTR1A Deaf1 site fused to β-Gal were used as a target. Deaf1 or MeCP2 fused to Gal4 (pACT2-Deaf1 or MeCP2) or vector Gal4 (pACT2) (Fig. A1) were transduced in the yeast. β- galactosidase activity was measured and used as a read-out for the recruitment of Deaf1 and MeCP2 to the DNA. An increase in β-galactosidase was observed upon transduction of Deaf1, independently of DNA methylation, but was enhanced in the presence of MeCP2 (Fig. 3A). In contrast, MeCP2 did not transactivate at the Deaf1 site unless it was methylated. MeCP2 also showed significantly more transactivation at the C-allele than G-allele, presumably because the G-allele has only one CpG site (at the Hes site) compared to the C-allele, which has two sites. This result suggests that MeCP2 can enhance Deaf1 recruitment to its site, and in the absence of Deaf1, MeCP2 is recruited to Deaf1 the site only upon methylation of the site. However, the nature of MeCP2's interaction with Deaf1 and the resulting functional effects are unknown.

(iii) Methylation affects HTR1A promoter activity

Having observed an effect of DNA methylation on MeCP2 regulation of the HTR1A promoter at the Deaf1 site, we addressed whether methylation of the Deaf1 or adjacent Hes5 CpG sites affect HTR1A promoter activity. A 26-bp fragment of the human HTR1A promoter including the Deaf1 and Hes5 binding sites, were methylated at these sites and ligated into the pGL3P luciferase expression vector to determine the effects of DNA methylation on Deaf1 activity. Deaf1 fused to pCDNA3 (or empty pCDNA3 vector) was co-transfected with the HTR1A-

luciferase constructs in the human SKN-SH neuroblastoma cells, a human model of neuronal cells that express endogenous 5-HT1A receptors (Czesak et al., 2006). Deaf1 was shown to significantly repress C(-1019) human HTR1A promoter activity, an effect which was lost in the G allelic variant to which Deaf1 is unable to bind (Lemonde et al., 2003). Methylation of the HES site (denoted on the figure as me-HES), which overlaps with the area of the Deaf1 site in human HTR1A, or both the C(-1019) (Deaf1 site) and HES sites (on the figure as: Me-HES Me-C(-1019)) significantly increased promoter activity as detected by luciferase (Fig. 4). Despite methylation of the sites, Deaf1 was still able to repress the transcription of the methylated C(-1019) site (on the figure as: me-C(-1019)), HES site, or both the C(-1019) and HES sites. This result suggests that gene methylation is not only important in the repression of gene expression but also in increasing activity. Similarly, MeCP2 increased HTR1A promoter activity, suggesting that the methylation induced increase in HTR1A promoter activity could involve MeCP2 (Fig. 2). The maintained repression by Deaf1 suggests that recruitment of MeCP2 does not prevent Deaf1 activity, and may actually enhance it.

Hypothesis

Based on the above findings, we hypothesized that Deaf1 is an important regulator of the 5-HT1A receptor and that MeCP2 interacts with Deaf1 to modulate Deaf1's effects on HTR1A promoter activity. To address whether Deaf1 and MeCP2 interact and bind in the region of the putative Deaf1 binding site of the HTR1A promoter in mice immunoprecipitation and chromatin immunoprecipitation experiments were performed. To examine Deaf1 and MeCP2's regulatory effects, luciferase reporter constructs with the WT HTR1A promoter region and mutations of the Deaf1 binding sites (in both human and mouse) were co-transfected in several selected cell lines with Deaf1, MeCP2 or Deaf1 and MeCP2. Finally to explore the physiological and behavioural

effects of HTR1A regulation by Deaf1 and MeCP2, Deaf1 was fully knocked out and MeCP2 conditionally knocked out only in TPH2 containing raphé neurons at adulthood.

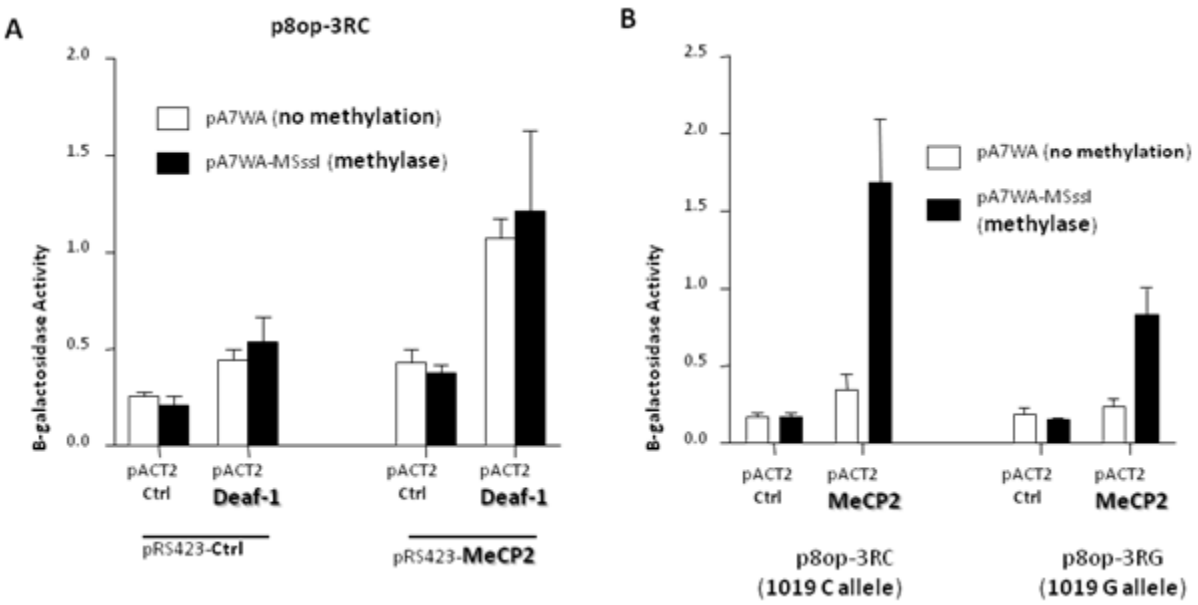


Figure 3. MeCP2 increases Deaf1 recruitment particularly to the C(-1019) independently of promoter methylation based on beta-galactosidase activity in yeast 1 hybrid assay.
(A) Deaf1-GAL4 transactivation. Yeast were transduced with Deaf1-GAL4AD or GAL4AD vector (pACT2), MSssI or vector, and MeCP2 or vector. To determine their effects on β-galactosidase activity resulting from the β-Gal gene fused to three copies of the 5-HT1A 26bp Deaf1 site with either the C (p8op-3RC) or G-allele ((p8op-3RG). **(B) MeCP2-GAL4 transactivation.** Yeast were transduced with MeCP2-GAL4AD or GAL4AD vector (pACT2), MSssI or vector, and the indicated 5-HT1A element (C or G-allele) as in (A). Data shown as mean±S.E., n = 3 independent experiments. Performed by Brice Le Francois.

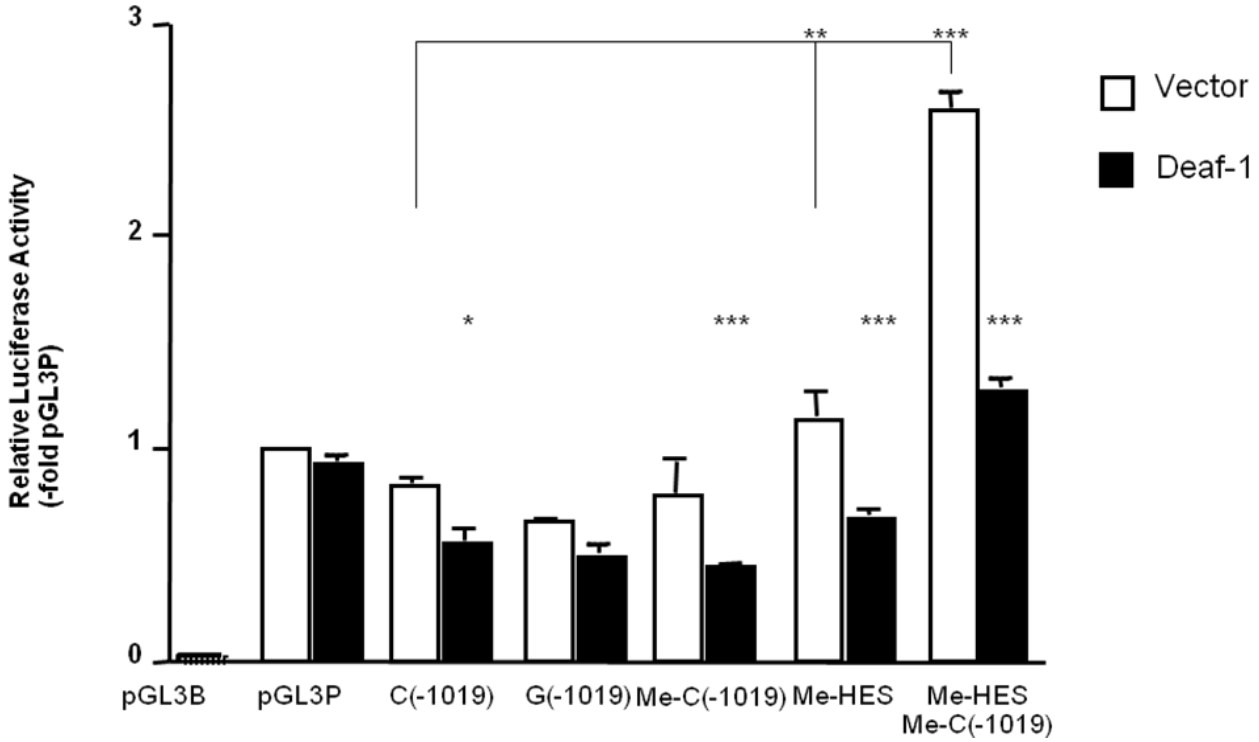


Figure 4. Methylation of Deaf1 and HES sites modulates human HTR1A promoter activity. SKN-SH were transfected using Lipofectamine 2000 (Ou et al., 2000) with either pCDNA3 or Deaf1 and either of the human 5-HT1A 26-bp element pGL3P reporter construct including the Deaf-1 site with the C(-1019), G(-1019) or methylated (me-) C(-1019), methylated (me-) HES site, or both sites methylated me -C(-1019) -HES. Data are shown as mean ± SE of triplicate samples. A two tailed ttest was used for vector vs. Deaf1 comparisons and ANOVA with Sydak corrected post-hoc when comparing the C(-1019), Me-HES, and Me-HES Me-C(-1019) expression vectors. Performed by Jin Lu.

Methods

Yeast one-hybrid

Three copies of the 26bp Deaf1 element of the C or G allele were fused to the β-galactosidase reporter plasmid p8op-SN-LacZ. Deaf1 was then fused into Gal4AD pACT2 plasmids (Clontech). MeCP2 was also fused into the pRS423 plasmids (Clontech); as described in Lemonde et al., 2003. Empty p8op-SN-LacZ, pACT2 and pRS423 plasmids were used as control vectors as indicated. These were transformed into the PJ29-2A yeast strain as indicated in Fig. 2. The effects of gene methylation were analyzed by co-transforming M.SssI as described by Feng et al., 2004. ortho-Nitrophenyl-β-galactoside substrate was used to measure β-galactosidase activity.

Extraction and immortalization of mouse embryonic fibroblasts (MEFs)

Deaf1 HET females were bred to a HET male and checked daily for plugs. Pregnant females were euthanized with CO_2 and cervical dislocation. Under aseptic technique fetuses (E13.5 to E15.5) were removed from the mother's womb and washed with 1X PBS. Embryos were separated, washed, and dissected: dark red tissues (heart and liver) and the developing brain were removed and kept for genotyping. All other tissues (mostly fibroblasts) were minced by razor blade and trypsinized (1 mL) for 10 min at 37°C, with occasional mixing. Tissue was homogenized into a single cell suspension using a 1mL pipette tip, with a 1-2mm opening. DMEM-015, 10% v/v heat-inactivated FBS, and 1% Penicillin/Streptomycin (8 mL; Wisent) was added to the homogenized tissue, mixed 5 times, and allowed to sit for 5 min. The supernatant (i. e., single cell suspension of mouse embryonic fibroblasts (MEFs)) was recovered, plated, grown at 37°C in 5% CO_2 and split as required (Heffernan, 2010; Xu, 2005).

MEFs were immortalized by Brice Le Francois using the pWP TS A58 lentiviral vector.

Cell lines

Human SKN-SH, mouse Deaf1 +/+ and -/- MEF, human HEK-293, mouse NIH-3T3 were grown in DMEM-015 (Wisent) with 10% v/v heat-inactivated FBS (Wisent) at 37°C and 5% CO_2. LTK were grown in EMEM (Wisent) with 5% v/v heat-inactivated FBS (Wisent) at 37°C in 5% CO_2. Rat RN46A cells were grown in Neurocell (Wisent) with 10% heat-inactivated FBS (Wisent) at 33°C and 5% CO_2.

Luciferase assay

Constructs

Reporter constructs, 984, 1128C, 1128G, 26bpC, 26bpG have been described previously (Ou et al., 2000; Lemonde et al., 2003; Czesak et al., 2006, Czesak et al., 2012). Briefly, 984 consists of 984-bp upstream of the WT mouse 5HT1A translation start site; 1128C or 1128G consist of 1128bp upstream of the human 5HT1A translation start site with either the C(-1019) or G(-1019) allele; and 26bpC or 26bpG consist of three copies of human HTR1A 26bp palindrome sequence that includes the Deaf1 binding site with either the C(-1019) or G(-1019) allele. In consultation with us, Zoe Donaldson designed and made reporter constructs WT1A, m1, full, and m1&m2, which consist of 1438 bp upstream of the mouse 5HT1A translational start site and were mutated (or not: WT1A) at specific Deaf1 sites as represented in Fig. A4. Purchased methylated or un-methylated 26bpC or 26bpG double stranded oligonucleotides were ligated at 16°C O/N into the PGL3P vector (as described: Le Francois et al., 2015).

Transcription vectors consisted of empty vector pcDNA3 or pcDNA3-His-Deaf1 (denoted as Deaf1) and empty vector pFLAG or pFLAG-MeCP2 (denoted as MeCP2) (Lemonde et al., 2003).

Transfection

SKN-SH cells were transfected with Lipofectamine 2000 (Life Tech.) as described previously (Le Francois et al., 2015). For Fig. A5, MEFs were transfected using 1 μg of 984 reporter plasmid, where applicable 1 μg of pcDNA3 or Deaf1 plasmid, and 0.5 μg of β-Galactosidase in a 2 μL:1 μg ratio (Lipid:DNA) using Fastfect (Feldan) at 90% confluence in a 6-well plate (Corning) according to product protocol. For Fig. 10 and 11, Deaf1-/- MEF were transfected with 1.5 μg of either reporter plasmid, 1.5 μg of each transcription factor or controls, and 0.5 μg of β-Galactosidase in a 2 μL:1 μg ratio (Lipid:DNA) using Fastfect (Feldan) at 90% confluence in a 5-cm dish (Corning) according to product protocol. HEK-293 cells were transfected with 2.5 μg of either plasmid (as indicated in figure 10 and 11) except for 0.5 μg of β-galactosidase using calcium-phosphate as described previously (Ou et al., 2000) at 70% confluence in a 5-cm plate. Lipofectamine 2000 (Life Tech.) at 1.5 μL:1 μg ratio (lipid:DNA) was used to transfect 2.5 μg of each plasmid other than 1 μg of β-galactosidase in 70% confluent LTK cells on a 5-cm plate. RN46A cells were grown to 60% confluence in a Primaria (Falcon) 10-cm dish prior to transfection with 3 μg of each vector except for 0.5 μg of β-galactosidase using Lipofectamine 3000 (Life Tech.) in a 2 μL:1 μg (lipid:DNA) ratio and 5 μL of Plus reagent (Life Tech.). 5-cm or 10-cm dishes were split into three separate wells of a 6-well plate to create triplicates and treated independently from this point.

Assay

After maintaining transfected cells for 48 hrs, they were lysed with reporter lysis buffer (Promega; described in: Ou et al., 2000). A Victor V3 apparatus (Perkin Elmer), or for Fig. 10 and 11 only, a GloMax-96 microplate luminometer (Promega), was used to measure luciferase luminescence, which was corrected to β-galactosidase activity (measured using a LS50 spectro-

photometer Perkin-Elmer). Corrected luciferase activity was normalized to appropriate PGL3B empty vector control and expressed as a percentage thereof.

Antibody purification and validation

Purification

Frozen serum of the Deaf1 (Lemonde et al., 2003) and 5-HT1A (Czesak et al., 2012) homemade polyclonal rabbit antibodies were thawed on ice and pre-cleared using the Steriflip Filter Unit (Millipore). The IgG was then purified using the Montage antibody purification kit and spin columns with PROSEP-G media (Millipore) and finally desalted and concentrated using the Amicon ultra-15 centrifugal filter devices (Millipore).

Validating the Deaf1 and 5-HT1A antibody

Deaf1 +/+ and -/- MEFs or 5-HT1A expressing LTK cells were grown to 80% confluence on cover slips. Cells were fixed with 4% PFA in PBS, rinsed 3x with PBS and then blocked (1X PBS, 5% normal goat serum, 0.3% Triton X-100) for 1 hr. Deaf1 and 5-HT1A antibody were each diluted (1:10, 1:25, 1:50 v/v) in antibody dilution buffer (1X PBS, 1% BSA, 0.3% Triton X-100), applied to the Deaf1 +/+ and -/- MEF or 5-HT1A expressing LTK cover slips (respectively), and incubated in a humid chamber O/N at 4ºC. They were then rinsed 3x with PBS, before a 2 hr incubation at RT with a CY3 fluorochrome-conjugated anti-rabbit secondary antibody (Jackson Immuno Research; 1:1000) for the Deaf1 +/+ and -/- MEFs and an AF-488 fluorochrome-conjugated anti-rabbit secondary antibody (Jackson Immuno Research; 1:1000) for the 5-HT1A expressing LTK cells. Cover-slips were then rinsed 2x with PBS and 3x with ddH2O, before 1X DAPI (Sigma) in PBS was applied for 10 min. Finally the cover-slips were rinsed twice with ddH2O, washed with ethanol and mounted onto slides.

Slides were viewed using the Axio Observer.D1 (Zeiss). DAPI was used to identify the nuclei of the cell. A representative Deaf1 1:25 antibody dilution is shown (Fig. A2), with little non-specific binding in the Deaf1 -/- MEFs, but significantly stronger detection in the Deaf1 +/+ that is primarily concentrated in the nucleus. The use of the Deaf1 antibody in other applications was optimized based on these results. A representative 5-HT1A 1:50 antibody dilution is shown (Fig. A3) and the use of the 5-HT1A antibody in immunostaining is based on these results.

Western blots

Whole cells (in Fig. 5B only) were lysed using FLAG buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, at pH 7.0) and protease inhibitors (1 mM PMSF, 1 μM aprotinin, 10 μM leupeptin). Proteins in the lysate were quantified using the BCA protein assay kit (Pierce) and absorbance was measured on a LS50 spectrophotometer (Perkin-Elmer) according to manufacturer protocol (Pierce), with bovine serum albumin as standard. Laemmli loading buffer was added to 30 μg protein samples to a factor of 1x (4% w/v SDS, 120 mM Tris-HCl (pH 6.8), 0.01% w/v bromophenol blue, 0.01% v/v β-mercapto-ethanol). Whole cell and brain extract (see below) afterwards (Fig. 5A, 6) were lysed using 1x Laemmli loading buffer and equally loaded based on β-actin levels. Proteins were resolved on 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was stained using Ponceau, washed with TBS and 0.5% Tween (TBST) three times, and blocked with 5% dry milk in TBST for 2 hrs. The Deaf1 (homemade) rabbit antibody (1:500), MeCP2 rabbit antibody (Millipore CAT. # 07-013;1:1000), β-actin mouse antibody (1:4000) were diluted in 5% dry milk in TBST, applied to the membranes, and incubated with rotation at 4°C overnight. The membranes were washed four times with TBST, followed by a 2-hr incubation with HRP-conjugated anti-rabbit (Sigma; 1:4000) or anti-mouse (1:4000) where appropriate. The membranes were washed another 4 times before

HRP substrate (Millipore) was applied and detected using Kodak film (Fig. 5B, 7) or Image Quant LAS4010 (GE) (Fig. 5A, 6, 8).

Co-immunoprecipitation

SKN-SH cells were grown and transfected with pcDNA3-HIS-Deaf1 and pFLAG-MeCP2 or their respective controls as described under "Transfection". For co-immunoprecipitation of endogenous proteins, cells were not transfected; instead 90-100% confluent cells were washed twice with 1X PBS. Brain extracts (see below) were minced with a razor blade and pre-homogenized with a 1-mL pipette with a 1-2 mm opening. All samples were lysed with FLAG buffer (with protease inhibitors) and homogenized in a chilled 7 mL glass Dounce homogenizer with 25 strokes of the large clearance and small clearance pestle (respectively). Lysates were centrifuged at 15000 rpm for 15 min at 4°C and quantified. Lysates of transfected SKN-SH (500µg/500µL) were rotated overnight (16 h) with 100 µL of anti-FLAG beads (Sigma) at 4°C or for 1 hr with 100 µL of cobalt beads (Thermo Fisher; binds the HIS tag) at 4°C. Endogenous lysates from cells and brains (1500 µg/1 mL) were incubated O/N with pre-immune IgG (1:50 v/v), anti-Deaf1 homemade rabbit antibody (1:50 v/v), or anti-MeCP2 rabbit antibody (Millipore CAT.# 07-013;1:100 v/v) and then incubated for 2 hr with 200 µL of protein A-Sepharose bead slurry (Sigma) per 1 mL of lysate. Beads were then washed five times with 500 µL of lysis buffer and protease inhibitors at 4°C. 1X Laemmli loading buffer (60uL) was added to the beads, boiled for 5 min, and centrifuged at 14000 rpm for 15 min to remove the beads. Entire samples were loaded on western blot as described in "Western blots". Anti-FLAG tag rabbit antibody (1:2000; Millipore) was used to detect the FLAG tag; anti-Deaf1 rabbit polyclonal antibody against homemade recombinant full-length human Deaf1 (1:500 v/v) was used to detect transfected Deaf1; monoclonal anti-Deaf1 mouse antibody (Sigma CAT.# WH0010522M4; 1:1000 v/v) was

used to detect Deaf1 in the endogenous lysates; anti-MeCP2 chicken antibody (Millipore CAT.# ABE171; 1:1000 v/v) was used to detect MeCP2 in the endogenous lysates; β-actin mouse antibody was used to detect immunoreactive species. Followed by HRP-conjugated anti-rabbit (Sigma; 1:4000), anti-mouse (1:4000), or anti-chicken (1:4000) where appropriate. HRP substrate (Millipore) was applied and chemically detected using Kodak film (Fig. 5,8) or Image Quant LAS4010 (GE) (Fig. 6, 7, 9).

Chromatin immunoprecipitation

Brain tissue preparation

Brain extracts (see below) were minced with a razor blade and cross-linked in 1.67% formaldehyde in PBS and rotated for 20 min at RT. Glycine (0.125M) was used to halt crosslinking for 5 min at RT. Tissues were washed twice with 1 mL of PBS at 4°C and homogenized with 25 strokes of the small and large pestle (respectively) using the pre-chilled Dounce homogenizer. The tissue was pelleted at 1600 rpm at 4°C for 5 min, resuspended in 100 µL of FLAG lysis buffer with protease inhibitors for every 25 µL of tissue, and incubated on ice for 10 min.

Cell preparation

Deaf1 +/+ or -/- MEF cells were grown to 90-100% confluence before being treated with 1.67% formaldehyde for 10 min at RT. Crosslinking was halted with 0.125M glycine for 5 min at RT. Cells were rinsed three times with 1X PBS and lifted from the plate with hypo-osmotic swelling buffer (10 mM Tris pH 8.0, 85 mM KCl and 0.5% NP-40) and protease inhibitors. Cells were pelleted at 2000 rpm at 4°C and lysed with FLAG lysis buffer and protease inhibitors (100 µL lysis buffer:15 µL of cell pellet).

Chromatin immunoprecipitation and PCR

ChIP grade glass beads (Sigma; 0.1 g per 1 mL of lysate) were used with a 70W, 40KHz Ultra-

sonic water bath (1.9L) MH series, model 15-337-401 (Fisher Scientific, Ottawa ON) for 30 min at 4°C to break up DNA into median lengths of 500 bp (verified by electrophoresis on a 2% agarose gel). Samples were centrifuged at 14000 rpm for 15 min at 4°C. The supernatants (1500 µg per condition) were incubated with either pre-immune rabbit IgG (1:50), Deaf1 rabbit homemade antibody (1:50), MeCP2 rabbit antibody (1:100; Millipore CAT.. # 07-013), or Histone H1 (1:200, Abcam CAT.# AB31972) or no antibody (input) at 4°C overnight with constant rotation. Samples (1 mL) were then incubated for 2 hrs with 200 µL of Protein A-Sepharose beads (Sigma) in PBS, 250 µg/mL BSA and 100 µg/mL sonicated salmon sperm. Samples were washed once with 1 mL of lysis buffer, four times with 1 mL of LiCl buffer (100 mM Tris HCl, 500 mM LiCl, 1% NP-40, 1% deoxycholate, pH 7.5) and once with 1 mL of TE. Bead-protein-DNA complexes were eluted with 200 µL of fresh SDS elution buffer (0.1 M $NaHCO_3$, 1% SDS) at 65°C for 2 hr with vortexing every 10 min. Beads were discarded and the supernatant was de-crosslinked at 65°C overnight (16 hr). Phenol/chloroform extraction was performed to extract the DNA. DNA was precipitated with 0.3 M NaCl, 20 µg of linear acrylamide and 2 volumes of EtOH at -20°C overnight and again with 75% EtOH overnight, after which ethanol was evaporated and the DNA was brought to a final volume of 50 µL in nuclease free water (based on Czesak et al., 2012; Le Francois et al., 2015). PCR was performed with mouse HTR1A promoter primers that encompass both Deaf1 binding sites as follows forward 5'-ATGAAAAGGTC ACAGGCACC and reverse 5'-TCACAGTGTGGCTATCAGGC from -1112/-932 bp from the translation start of the mouse HTR1A promoter (180-bp PCR product), which encompasses both Deaf1 sites. The GAPDH promoter was used as a control as follows 5'-GTCCAAAGAGAGGG AGGAGG forward and 5'-AGGGCTGCAGTCCGTATTTA reverse (179-bp PCR product). A total reaction mixture (50 µL) comprised of 1X Thermobuffer, 220 µM dNTP, 1 µM of each

primer, 2.5 U of Taq polymerase (NEB), and 5 µL of each sample. Cycling was as follows: 1 min at 95°C and 40 cycles of 95°C for 30 sec, 59°C (for HTR1A)/56°C (for GAPDH) for 45 sec, 72°C for 15 sec, followed by a 5 min final extension at 72°C.

mRNA extraction and analysis

Cells (90-100% confluence) were washed with 1X PBS and lysed with TRIzol (Ambion). RNA was extracted with pure chloroform, purified with isopropanol (0.5 volumes) and linear acrylamide (20 µg) at -20°C O/N and precipitated with 75% -20°C ethanol. Samples were treated with DNase (Ambion) and quantified. cDNA synthesis was performed with 3 µg of sample RNA, 0.5 mM dNTPs, 12.5 ng of random primer, 1X mMuLV buffer, and mMuLV (NEB; based on Le Francois et al., 2015).

PCR for MeCP2 mRNA was performed using the following primers: 5'-CAGGCAAAGCAGAA ACATCA forward and 5'-AGGAGGTGTCTCCCACCTT reverse (273-bp PCR product). The final reaction mixture (50 µL) was 1X Thermobuffer, 220 µM dNTP, 1 µM of each primer, 2.5 U of Taq polymerase (NEB), and 5 µL or 2.5 µL of each sample. Cycling was as follows: 1 min at 95°C and 30 cycles (or 20 cycles) of 95°C for 30 sec, 56°C for 45 sec, 72°C for 15 sec, followed by a 5 min final extension at 72°C.

Mice housing, breeding and genotyping

Mice studies were performed in accordance with the University of Ottawa Animal Care Committee guidelines under protocol NSI-65 and NSI-67. Animals were housed with food and water ad libitum, on a 12-hour light/dark cycle (lights on at 6:00 AM), at 21°C.

<u>Deaf1 mice</u>

The Deaf1 mice were rederived from Deaf1 +/- embryos obtained from Dr. Jane Visvader and bred on a C57BL/6-BALB/c background. Mice were genotyped and tagged at 3 weeks of age

using an ear tissue sample from which DNA was extracted using the REDExtract-N-Amp Tissue PCR kit (Sigma). PCR was performed with three primers as follows: 5'-GGGCTTCCGGGTCA TTCTGT, 5'-ACTAAGAGGGTCACACAAAAGAACAAA, and 5'-TGCACCCACCACCAAG ATAAGAA. A total reaction mixture of 25 μL comprised of 1X Phusion buffer HF, 200 μM dNTPs, 1 μM of each primer, 0.5U Phusion HF Taq (NEB), and 2.5 μL of DNA sample. Cycling conditions follow: 30 sec at 98°C, 34 cycles of 98°C for 10 sec, 62°C for 30 sec, 72°C for 20 sec, 84°C for 10 sec and a 10 min extension at 72°C. This amplification yielded a 267-bp product representing WT, and a 421-bp product representing KO allele, and the presence of both represented HET genotype. Mice were re-genotyped from separate tissue samples taken at sacrifice.

MeCP2-TPH2$^{cre\text{-}ERT2}$ mice

MeCP2 and TPH2$^{cre\text{-}ERT2}$ mice were obtained from Jackson labs and bred together on a C57Bl/6 background (Fig. A7). At 3 weeks of age mice were genotyped and tagged, DNA was prepared using the REDExtract-N-Amp Tissue PCR kit (Sigma). PCR for MeCP2 was performed with the two following primers: 5'-GCCACATGACAAGACAAAAACA forward and 5'-GTTACACCG CTGAAATCTCTTG reverse. PCR was done using 1X Taq DNA Polymerase Master Mix Red (Ampliqon), 1 μM of each primer, and 1 μL of DNA from extraction. Genotyping was done using the following conditions: 4 min at 94°C, and 34 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, with a 10 min extension at 72°C. A 238-bp product represented WT while a 313-bp product represented flx MeCP2. PCR for TPH2$^{Cre\text{-}ERT2}$ was performed with two sets of primers to identify the TPH2^{CreERT2} transgene 11679 (5'-GCTGAGAAAGAAAATTACATCG) and 12523 (5'-TGGCTTGCAGGTACA GGAGG) and internal positive controls OIMR8744 (5'-CAAATGTTGCTTGTCTGGTG) OIM-R8744 (5'-GCTAGTCGAGTGCAC AGTTT). PCR was done using 1X Taq polymerase buffer, 160 mM dNTP, 0.2 μL of each primer, 0.25 U of Taq pol

(NEB), and 1 uL of template DNA under the following cycling conditions: 2 min at 94°C, 10 cycles of 94°C for 20 sec, 65°C for 15 sec -0.5°C/cycle, 68°C for 30 sec, 28 cycles of 94°C for 15 sec, 60°C for 15 sec, 72°C for 30 sec and a final extension of 72°C for 10 min. The presence of the $TPH2^{CreERT2}$ yields a product of 300-bp and the internal positive control yields a product of 200-bp. Mice were genotyped again after euthanasia.

Tamoxifen injections

Stock tamoxifen (Sigma CAT # T5648-5G; 30 mg/mL) was dissolved in 10% ethanol mixed by vortexing for 30 sec. and 90% sunflower seed oil (Sigma) mixed vortexing for 30 sec. and heated water bath sonication (10min). 180 mg/kg/day tamoxifen was injected IP at 8 weeks of age for four consecutive days (based on a procedure from Dr. Diane Lagace). All following experiments took place after 10 weeks of age.

Brain extracts

Ten-week old mice were euthanized with CO_2, quickly decapitated, the brain was extracted and put on ice. A 1-mm coronal section (approximately, Bregma -4.0 to -5.0) was made, from which an upright triangle (▲) encompassing the raphé was cut underneath the cerebral aqueduct. The hippocampi were dissected and identified as horseshoe shaped structures underneath the cortex but above the ventral structures. A 1-mm coronal section (approximately, Bregma 3 to 2) was made from which the rostral structures were kept as PFC. All extracts were immediately frozen on dry ice and kept at -80°C prior to use.

8-OH DPAT induced hypothermia

The experiment was performed using 10-week old mice, between 1 pm and 4 pm. The body temperature of mice was measured using a rectal thermometer lubricated with KY jelly. Four baseline measurements were taken at 10 min intervals prior to an IP injection of 0.9% NaCl or 0.5

mg/kg of 8-OH DPAT dissolved in 0.9% NaCl (Sigma CAT. # 87394-87-4; 0.25 mg/kg and 0.75 mg/kg were also attempted in separate Deaf1 mice) after which measurements were resumed at 10 min intervals for 60 min. The first baseline measurement was discarded; the remaining three baseline values were averaged together. The post-injection temperature was subtracted from the averaged baseline temperatures to provide a temperature change.

Immunostaining

Euthanyl (0.01 mL/g) was used to anesthetize mice prior to cardiac infusion of 30 mL of PBS followed by 30 mL of 4% paraformaldehyde. Brains were dissected, post-fixed in 4% paraformaldehyde solution overnight (16 h), then in cryo-protectant (20% sucrose and 0.02% sodium azide), changed daily for five days before finally being frozen at -80°C and stored. Brains were sliced and stained with a rabbit 5-HT1A polyclonal antibody to the 5-HT1A second intracellular loop peptide palmitolyl-CYWAITDPIDYVNKRTPRRAAA (Cedarlane, Hornby, ON), as described previously (Czesak et al., 2012). Sheep anti-TPH (1:100), recognized by CY3 fluorochrome conjugated donkey anti-sheep (1:200), was used to identify serotonergic neurons.

Behavioural testing

Sucrose preference test

Mice were handled once at 12:00 PM daily at 9 weeks of age and singly housed for 1 week. All cages were changed the day before testing, mice were habituated to two drinking bottles, measured daily, with two days where both bottles contained water and two days where both bottle had sucrose. No preference for either bottle was observed during the habituation period. Cages were then changed. During the choice phase, mice were given pre-weighed water and 1% sucrose water bottles, weighed and exchanged daily for four days. Refilling the bottles was not necessary. The daily decrease in the weight of each bottle was calculated. The daily weight of the water was

divided by the daily weight of the sucrose to provide a ratio expressing each mouse's preference (based on Katz et al., 1981; Krishnan et al., 2007; Weiss, 1997).

Statistical analyses

Where a graph is presented, data are presented as averages and standard error (SE) calculated; both were presented graphically using Microsoft Excel. Statistical calculations (i. e. ANOVA and post-hoc test, and t-test) were performed using Graphpad Prism 6 as described in the figure legends and Tables. Minimum significance was set at $p<0.05$.

Results

Endogenous Deaf1 and MeCP2 expression

To determine which cell lines express Deaf1 and MeCP2 for later study of the Deaf1-MeCP2 interaction, Western blot analysis was performed with lysate from several cell lines (Fig. 5) and brain extracts from Deaf1 WT and KO mice (Fig. 6). The lysate contains total cell extract, as nuclear or cytoplasmic fractionation was not done. At first, samples were lysed using FLAG buffer and protein levels were quantified, however this resulted in greater variability in the levels of β-actin (Fig. 5B). From this approach, we detected significant amounts of MeCP2 in Deaf1 +/+ and -/- MEFs, SKN-SH, NIH-3T3, and RN46A. However, to reduce variability in protein levels between each cell type we lysed an equal number of cells with SDS loading buffer, mechanically disrupted cells with the MagNA lyser, and equalized loading amounts based on β-actin levels. These samples showed little detectable MeCP2 (data not shown). Since MeCP2 binds to DNA it is possible that it precipitated out of solution with the DNA it was bound to when the cells were lysed, preventing us from detecting it.

None of the commercial antibodies tested in the lab were able to detect Deaf1. The Deaf1 antibody used here is a "homemade" rabbit IgG raised to purified recombinant human Deaf1 that has been shown previously to detect Deaf1 (Czesak et al. 2012), but also to bind to non-specific proteins (Fig. A2). In analysing Deaf1 protein levels in cells and brain samples we included Deaf1 -/- MEFs and Deaf1 KO brain samples, where the Deaf1 protein has been truncated and rendered non-functional (Czesak et al. 2012) and use this to establish background non-specific staining. Deaf1 was detected in the HEK-293, SKN-SH, and Deaf1 +/+ MEFs with negligible amounts in the Deaf1 -/- MEFs, and a very weak signal in RN46A, NIH-3T3 and SN-48 cells (Fig. 5).

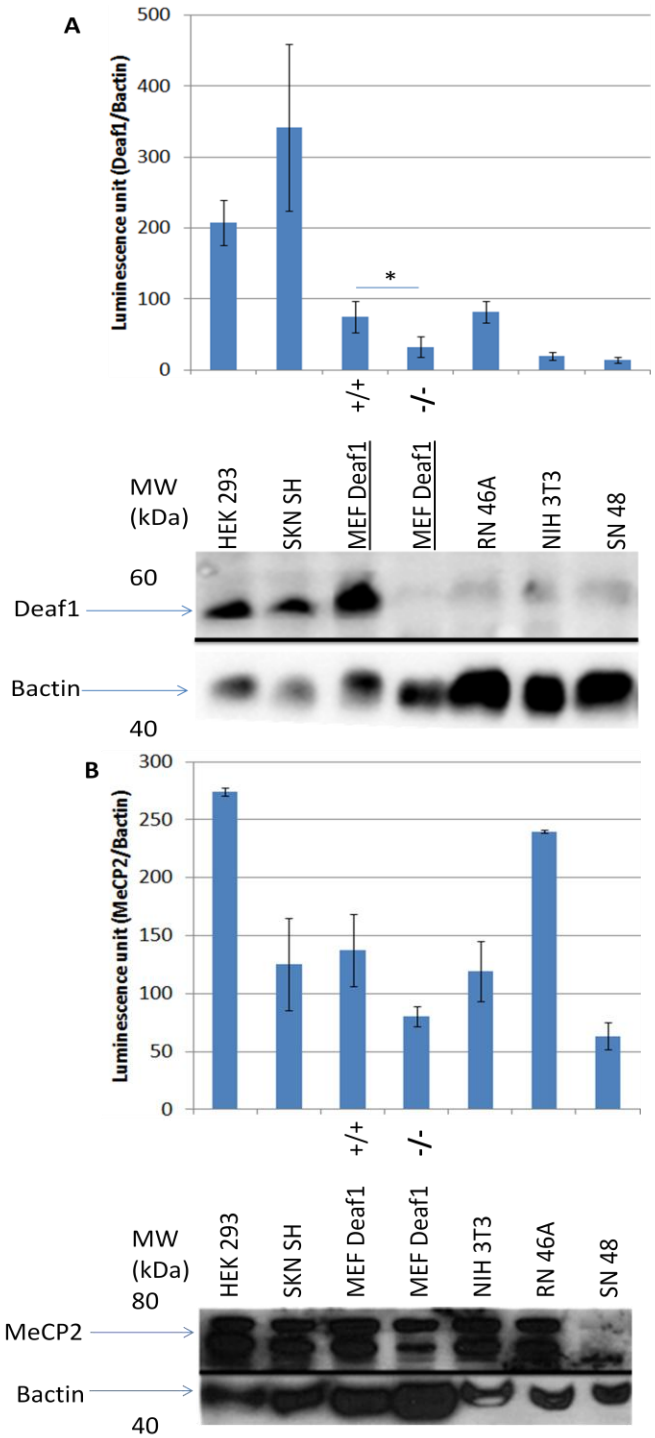


Figure 5. Deaf1 and MeCP2 levels in cell lines. Cell extracts (30 ug per well) from the indicated cell lines were analyzed for Deaf1 and MeCP2 by Western blot, using mouse β-actin (1:4000) as loading control. Representative blots and averaged data are shown. Staining was visualized using HRP-conjugated anti-mouse secondary 1:4000 (Sigma CAT.# A0545) and ECL kit. Molecular weight ladder sizes were 80 kDa, 60 kDa, and 40 kDa as labeled. **(A) Deaf1** was identified using 1:500 of rabbit Deaf1 (homemade). **(B) MeCP2** was identified using 1:1000 of rabbit MeCP2 (Millipore CAT.# 07-013). Samples were quantified using ImageJ software, nor-

malized to β-actin, averaged (A n=5, B n=3 experiments), and SE was calculated and plotted as shown. A one tailed t-test was performed to compare normalized protein levels in Deaf1 +/+ and -/- MEFs. *p<.05

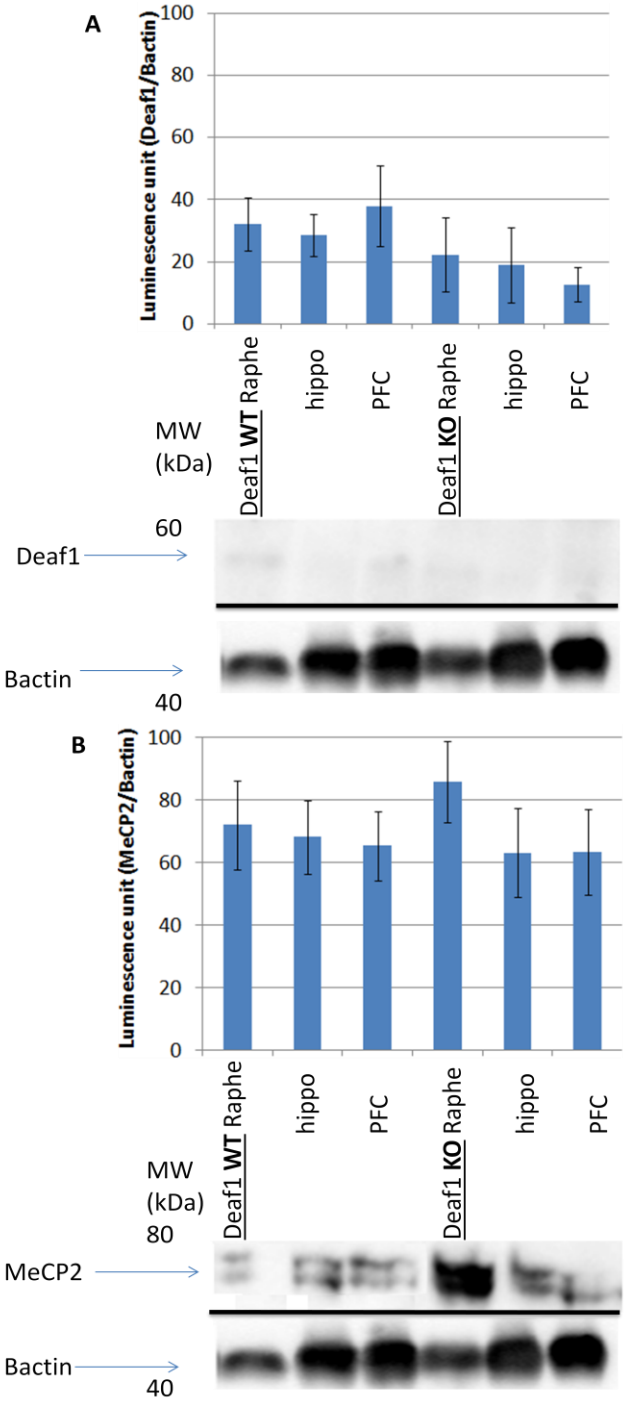


Figure 6. Deaf1 and MeCP2 protein levels in brain extracts from Deaf1 KO and WT mice. Raphe, hippocampus (hippo), and PFC extracts (~30ug/sample) were subjected to Western blot analysis, using β-actin (1:4000) to normalize protein levels across samples. **(A) Deaf1** (1:500) and **(B) MeCP2** (Millipore CAT.# 07-013; 1:1000) rabbit antibodies were identified with HRP-conjugated rabbit secondary (Sigma CAT.# A0454; 1:4000) as described in Fig. 5. ImageJ was used to quantify gels before normalization to β-actin, and plotted as mean ± SE, as shown (A n=5, B n=6 independent experiments).

Deaf1 was very weakly detected in the wild-type compared to Deaf1 knockout brain samples, despite a strong detection of β-actin. MeCP2 was clearly detected as a doublet in both wild-type and Deaf1 knockout tissues in all regions examined, including the raphe nuclei, hippocampus, and PFC (Fig. 6).

Deaf1 and MeCP2 co-precipitate

Transfected SKN-SH

To determine whether MeCP2 interacts with Deaf1, we co-transfected Flag-MeCP2 and His-Deaf1 in a human model of 5-HT1A-positive neuronal cells (Czesak et al., 2006), SKN-SH cells, and pulled down for either MeCP2 (Flag) or Deaf1 (His). Deaf1 was not detected when pulling down the flag tag upon co-transfection with an empty pFlag vector. His-tagged Deaf1 was detected when transfected in SKN-SH cells and pulled down with either the His tag conjugated with Deaf1 or the flag tag conjugated to MeCP2 (Fig. 7, in the red squares of the Deaf1 blot). Correspondingly, MeCP2 was not detected when pulling down the His tag upon co-transfection with an empty pcDNA vector. MeCP2 was detected when pulling down with either the His tag conjugated with Deaf1 or the flag tag conjugated to MeCP2 (Fig. 7, in the red squares of the MeCP2 blot). Overall, these results indicate that MeCP2 was pulled down with Deaf1 and vice versa, which suggests that MeCP2 and Deaf1 physically interact.

Endogenous Co-IPs

To substantiate our findings, we performed Co-IPs for endogenous Deaf1 and MeCP2. From the western blot results for transfected Deaf1 and MeCP2, a co-IP for endogenous Deaf1 and MeCP2 was performed in the SKN-SH, Deaf1 +/+ and -/- MEFs, and in Deaf1 WT and KO hippocampal extract and analyzed by Western blot for Deaf1 and MeCP2. The Deaf1 -/- MEFs and KO hippocampal extracts express a non-functional truncated Deaf1, and these serve as

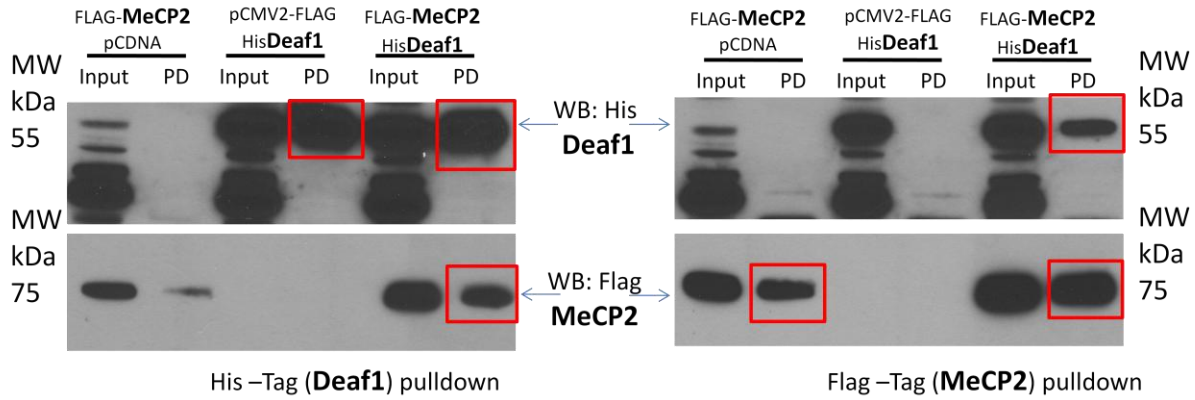


Figure 7. Deaf1 and MeCP2 co-precipitate in transfected human SKN-SH cells. SKN-SH cells were cotransfected with His-Deaf1 and Flag-MeCP2 constructs and proteins extracted. His-Deaf1 was pulled down using cobalt-His tag method (left panel), while Flag-MeCP2 was pulled down using FLAG beads-FLAG tag method (right panel), before being run for Western blot analysis and stained with 1:2000 Flag-tag antibody (MeCP2) and 1:1000 rabbit Deaf1 (homemade) antibody. These were visualized with 1:4000 of the appropriate HRP-conjugated secondary antibody and ECL kit. Pull-down of targeted and interacting proteins are highlighted (red boxes). Molecular weight ladder sizes were 75 kDa and 55 kDa as labeled. Performed by Brice Le Francois.

negative controls for Deaf1 expression. β-actin in the input was used to demonstrate that similar amounts of protein were used from each sample (Fig. 8, β-actin strip). There was a significant enrichment (compared to pre-immune IgG) of MeCP2 when pulling down for Deaf1 or MeCP2 (Fig. 8A, MeCP2 strip). Furthermore, there was a significant enrichment of MeCP2 (compared to IgG) when pulling down for MeCP2 in both the Deaf1 +/+ and -/- MEFs, however there was a detectable enrichment of MeCP2 in the Deaf1 +/+ but not -/- MEFs when pulling down for Deaf1 (Fig. 8B, MeCP2 strip). Similarly, a significant enrichment of MeCP2 (compared to IgG) was observed when pulling down for MeCP2 in both the Deaf1 WT and KO hippo (hippocampal) extracts. There was also an enrichment of MeCP2 when pulling down for Deaf1 in the Deaf1 WT, but not the Deaf1 KO hippocampal extracts (Fig. 8C, MeCP2 strip). This result substantiates and extends our previous findings, suggesting that endogenous MeCP2 and Deaf1 interact.

However, when staining for Deaf1 using a new antibody, Deaf1 in the input of WT MEF and hippocampal tissue was detected but no clear signal could be observed in the other lanes (Fig. 8 Deaf1 strip). This would suggest that Deaf1 was not pulled down, notwithstanding enriched MeCP2 when pulling down for Deaf1, indicating that Deaf1 was indeed pulled down (Fig. 8). After using our homemade rabbit anti-Deaf1 antibody to pull down for Deaf1, we obtained high background due to IgG heavy-chain cross-reactivity that obscured Deaf1 detection on the Western blot. We therefore had to use a different species (mouse) antibody to Deaf1 and MeCP2 for Western blot. The lack of Deaf1 signal in the input of knockout compared to wild-type cells and tissue suggests that the antibody is specific to Deaf1. It is possible that Deaf1 underwent post-transcriptional modifications of the antibody epitope to be able to bind to the mouse monoclonal Deaf1 antibody used in this assay to detect Deaf1.

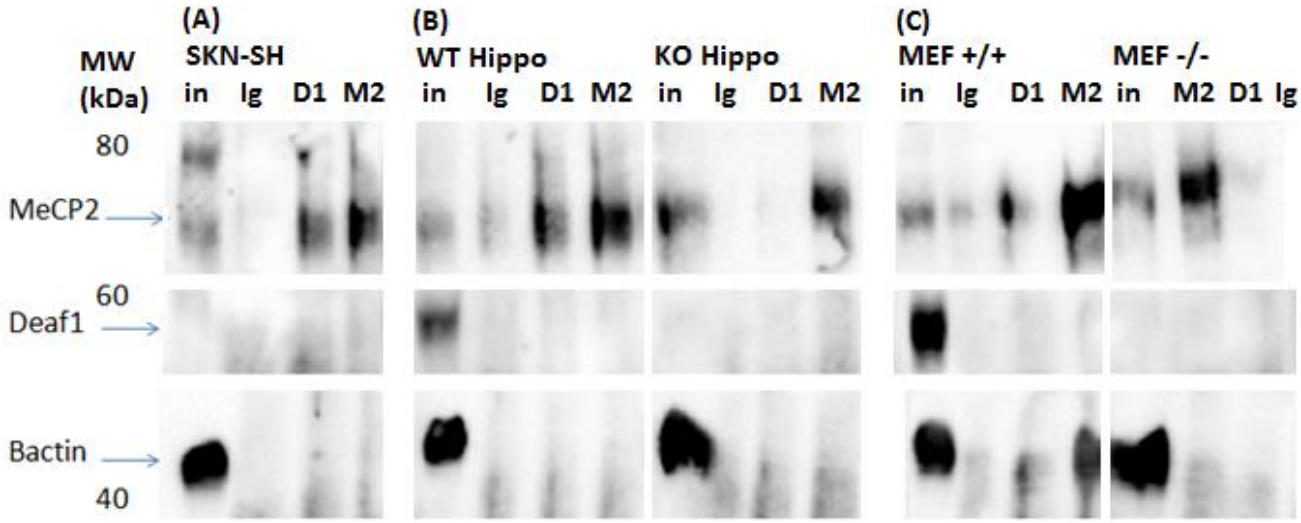


Figure 8. Endogeneous MeCP2 co-precipitates Deaf1, only when Deaf1 is present. Co-IP and IP was performed using rabbit antibodies against Deaf1 (D1) and MeCP2 (M2), respectively; a pre-immune IgG as background control (Ig), and approximately 3 ug of total protein as input (in) and analyzed by western blot. B-actin is used as a loading control to demonstrate that similar amounts of input protein were used. The MeCP2 western was stained with 1:1000 chicken IgY against MeCP2 (Millipore CAT.# ABE171) and then with 1:4000 of a HRP-conjugated anti-chicken secondary antibody. The Deaf1 western was stained with 1:1000 mouse monoclonal IgG against Deaf1 (Sigma CAT.# WH0010522M4) and then with 1:4000 of a HRP-conjugated anti-mouse secondary antibody. The experiment was performed twice loading approximately 1 ug/uL of whole **(A) SKN-SH cell extract, (B) Deaf1 +/+ and -/- MEF cell extract**. **(C) Homogenized hippocampal** (hippo) tissue extract (1µg/µL) from several 10 week old Deaf1 WT or KO mice.

Deaf1 and MeCP2 interact with the HTR1A promoter in a Deaf1 dependent fashion (ChIP)

To examine Deaf1 interaction with the HTR1A promoter region in the mouse and to determine whether MeCP2 also is present, chromatin immunoprecipitation was performed in Deaf1 +/+ and -/- mouse embryonic fibroblasts (MEFs) derived from the Deaf1 mouse line. There was a significant enrichment of the HTR1A promoter region containing the two Deaf1 sites when pulling down for Deaf1, which was not observed in the Deaf1 KO MEFs (Fig. 9A, quantified: Table 1), thus showing a specific interaction of Deaf1 with the HTR1A promoter. To better understand the interaction of Deaf1 and MeCP2 the same experiment was performed pulling down for MeCP2 and showed a significant enrichment of the HTR1A promoter region. Interestingly this enrichment was no longer observed in the Deaf1 KO MEFs (Fig. 9A). Since this could be due to changes in MeCP2 levels, we examined MeCP2 mRNA levels in the Deaf1 WT and KO MEFs and found no differences (Fig. A4). As a negative control, no significant pull-down with Deaf1, MeCP2 or IgG was found when analyzing a portion of the GAPDH gene, while pulling down for histone H1, a ubiquitous component of chromatin, did lead to an enrichment of this region (Fig. 9B).

To substantiate our findings that Deaf1 and MeCP2 bind to the HTR1A promoter in a Deaf1 dependent fashion we performed similar experiments combining several brain extracts from 10 week-old genotyped Deaf1 WT and KO mice from each region of interest. Pulling down for Deaf1 and MeCP2 led to a significant enrichment (compared to the IgG) of the same HTR1A promoter region in dissected raphé nuclei (Fig. 10A), hippocampus (Fig. 10B), and PFC (Fig. 10C) from Deaf1 WT, but not KO mice. These results suggest that Deaf1 is required to recruit MeCP2 to the Deaf1 site of the 5-HT1A promoter *in vivo*. This led us to explore the functional

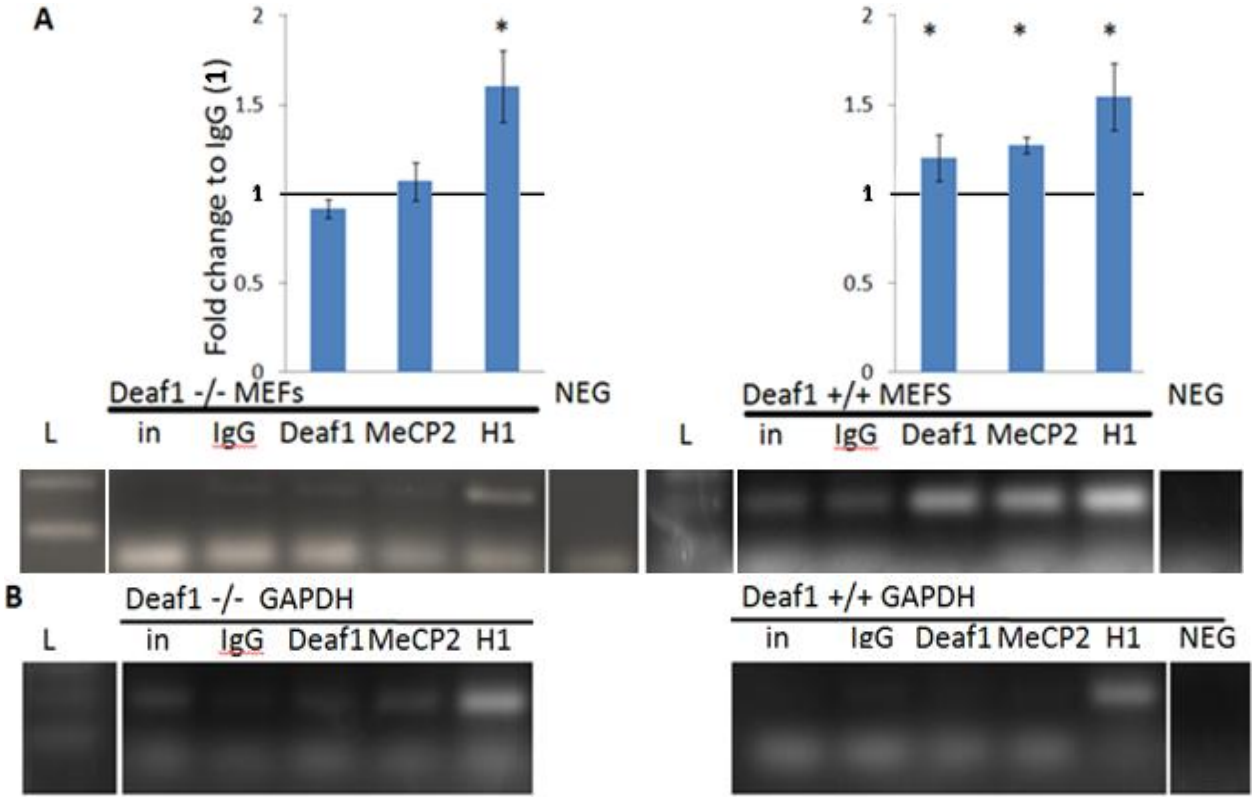


Figure 9. Deaf1 and MeCP2 binding to HTR1A promoter region. Cell extracts from Deaf1 +/+ and -/- MEFs were analyzed by ChIP pulling down with pre-immune rabbit IgG, n=8; rabbit IgG immunized to Deaf1, n=8; anti-MeCP2 (Millipore CAT.# 07-013), n=4; or anti-histone H1, n=4. Shown are molecular size ladder with 200 bp and 100 bp bands (L) and negative (buffer) control for PCR amplification (NEG). **(A) HTR1A promoter.** Shown is a representative gel for PCR of the HTR1A promoter. PCRs for the HTR1A promoter region encompassing the Deaf1 sites were quantified and plotted as Mean ± SE, *$p<0.05$ (Table 1). Total luminosity of each independent PCR was read inside a square of identical size and normalized to that of the pre-immune IgG for either pull downs in the Deaf1 +/+ or Deaf1 -/- MEFs. **(B) GAPDH promoter.** Shown is a representative PCR for GAPDH promoter following pull down in either Deaf1 -/- or +/+ MEFs. No consistent or significant enrichment was observed except using Histone H1.

	Enrichment of HTR1A promoter region		
	Deaf1	MeCP2	H1
F	6.32	6.132	7.202
df	2	2	2
p	**0.0071**	**0.0133**	**0.0078**

Post hoc						
Cell line	Deaf1 +/+ MEFs			Deaf1 -/- MEFs		
Pull down	Deaf1	MeCP2	H1	Deaf1	MeCP2	H1
p	**0.0224**	**0.0074**	**0.0212**	0.7874	0.568	**0.0074**

Table 1. Statistical Analysis for Deaf1 and MeCP2 binding to HTR1A promoter region. Total luminosity of each independent PCR was read inside a square of identical size and normalized to that of the pre-immune IgG for either pull downs in the Deaf1 +/+ or Deaf1 -/- MEFs. Pre-immune IgG pull downs for each of the MEFs rarely differed significantly. Statistical results from one way analysis of variance with Sydak corrected post hoc comparing pull downs with rabbit IgG immunized to Deaf1, MeCP2, or H1 (for both MEF Deaf1 +/+ and -/-) to pre-immune rabbit IgG. Significant values are bolded.

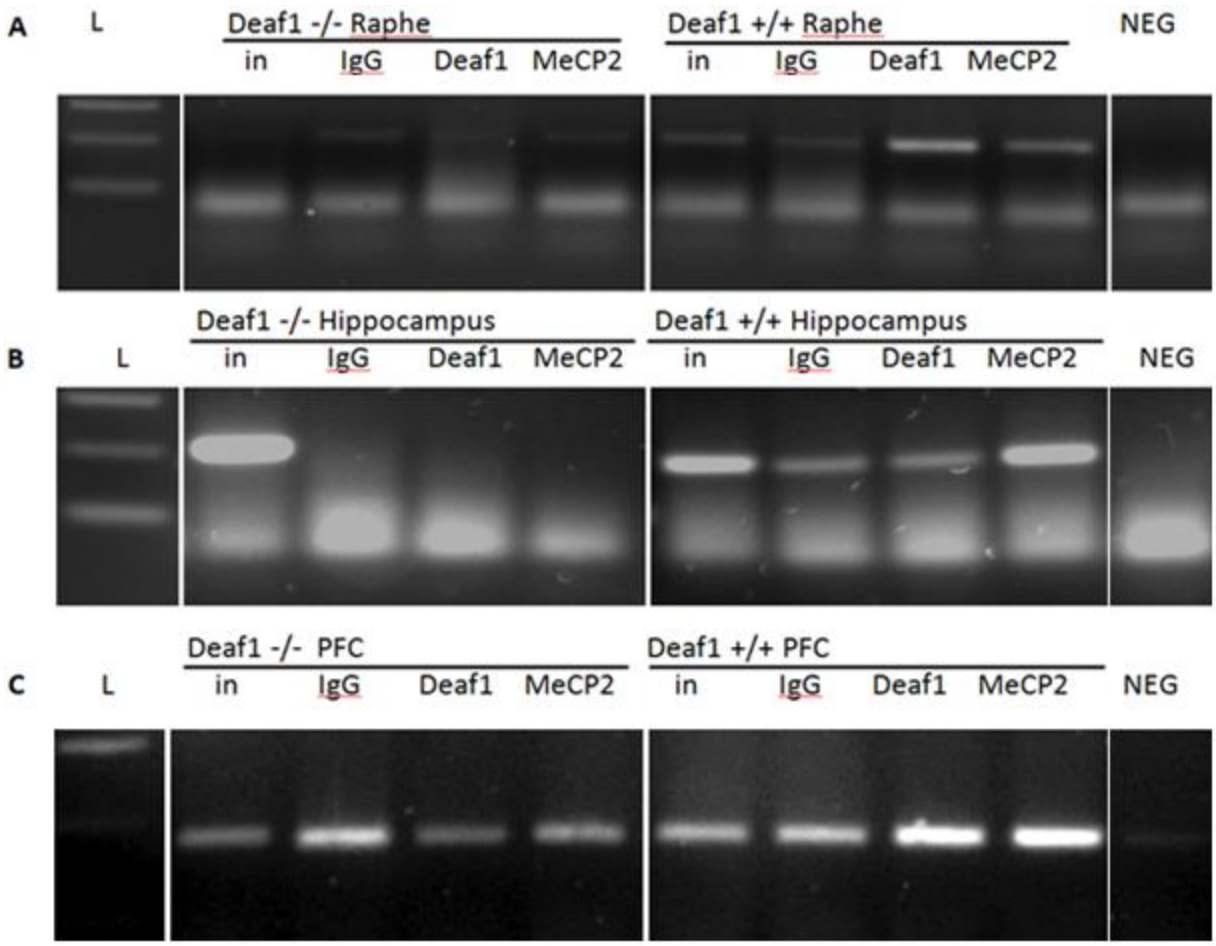


Figure 10. Deaf1 and MeCP2 binding to HTR1A promoter in brain tissues. (A) Raphe, (B) hippocampus, or (C) PFC were dissected from seven 10 week old Deaf1 WT or KO mice, subjected to ChIP analysis (Methods) and pulled down with pre-immune rabbit IgG, rabbit IgG immunized for Deaf1 or MeCP2 (Millipore CAT.# 07-013). Shown are molecular size ladder with 300 bp, 200 bp and 100 bp bands (L) and negative (buffer) control for PCR amplification (NEG).

effects of the Deaf1-MeCP2 interaction and either Deaf1 or MeCP2 on 5-HT1A gene expression.

Deaf1 and MeCP2 modulate HTR1A expression, which is significantly affected by mutations of the Deaf1 binding sites

Previous research has shown that Deaf1 can act both as a repressor and enhancer of gene expression, and for regulation of the HTR1A gene this effect localised in the pre-synaptic serotonergic raphé neurons (repressor activity) and post-synaptic neurons (enhancer activity), respectively (Czesak et al., 2012). We therefore explored the effects of Deaf1, MeCP2 and their combination on HTR1A transcriptional activity in cell types (HEK293, Deaf1 -/- MEF, LTK, and RN46As) where Deaf1 acts either as an enhancer or repressor of gene activity. We first analysed the effects of both transcription factors on the human 5-HT1A promoter region with either the C or G allelic variant of the single Deaf1 site (Fig. 10A; as described in Czesak et al., 2012). The expression vectors consist of a luciferase reporter construct in which the promoter region of interest is added upstream to drive the expression of the luciferase reporter gene in the PGL3B vector. One can infer that the luminescence observed is an indication of promoter activity, once this is subtracted from the basal activity of the vector without any promoter region.

<u>HEK-293</u>

The HEK 293 cells provide efficiently transfected human cell model to study Deaf1 transcriptional activities. In the HEK 293, significant main effects of MeCP2, Deaf1, or their combination on promoter activity of the 1128C HTR1A construct, but not the 1128G construct, assayed by normalized luciferase activity were found (Table 2). Post-hoc analysis using Dunnett's multiple comparisons test showed that Deaf1 had significant repressor activity on the 1128C human promoter activity with or without MeCP2. MeCP2 had no significant effects on gene expression with or without Deaf1. The 1128G variant (M=224.2, SD=146.4) showed a significant

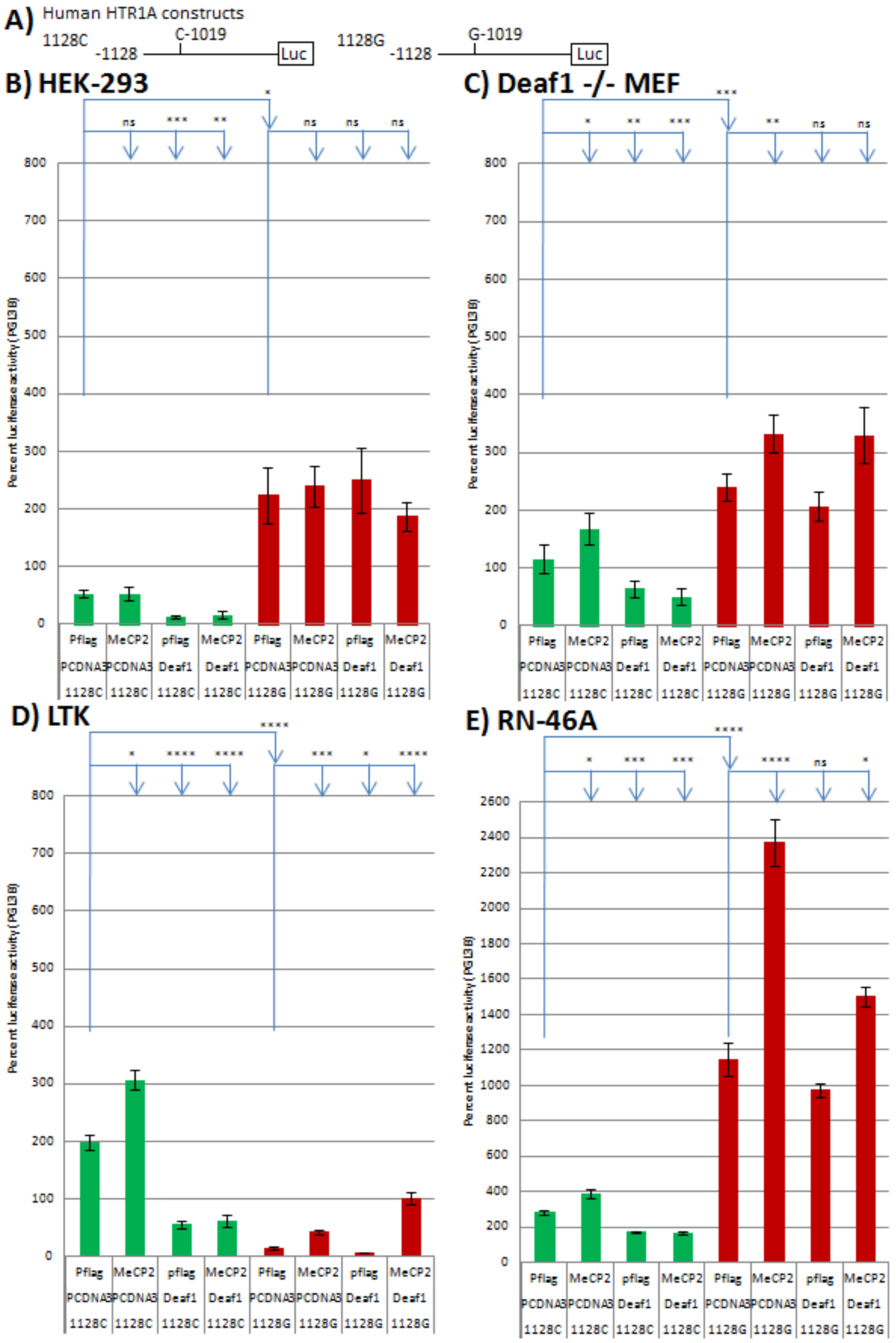

A) Human HTR1A constructs
1128C
C-1019
-1128
Luc
1128G
G-1019
-1128
Luc
B) HEK-293
C) Deaf1 -/- MEF
D) LTK
E) RN-46A
Percent luciferase activity (PGL3B)
Pflag PCDNA3 1128C
MeCP2 PCDNA3 1128C
pflag Deaf1 1128C
MeCP2 Deaf1 1128C
Pflag PCDNA3 1128G
MeCP2 PCDNA3 1128G
pflag Deaf1 1128G
MeCP2 Deaf1 1128G

Figure 11. Differential activity of Deaf1 and MeCP2 at the C or G(-1019) allele of the human HTR1A promoter.

The indicated cell lines were co-transfected with the human 1128C or 1128G HTR1A promoters (described in Czesak et al., 2012; figuratively shown in **(A)** and constructs for Deaf1, MeCP2, or both Deaf1 and MeCP2 with their respective empty vectors and β-Gal plasmid. Cell extracts were used for luciferase and β-galactosidase assays, and all luciferase values were normalized to β-Gal and then normalized to the empty PGL3B vector co-transfected with empty MeCP2 (pflag) and Deaf1 (PCDNA3) vectors and expressed as a percentage of activity. Co-transfection of the empty PGL3B vector with MeCP2 and PCDNA3, pflag and Deaf1, or MeCP2 and Deaf1 yielded no significant differences (data not shown). **Shown are results from (B) HEK-293 cells** (n=12), **(C) Deaf1 -/- MEF cells** (n=9), **(D) LTK cells** (n=9), and **(E) RN46A cells** (n=9)**.** Data are plotted as Mean ± SE and statistics shown in Table 2. ns = not significant; *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001

(A) Main effect of transcription factor(s) in HEK-239

	1128-C expression vector	1128-G expression vector
F	7.94	1.27
df	3	3
p	**0.009**	**0.3081**

(B) Post hoc in HEK-293

Vector x Transcription factor	1128-C expression vector	1128-G expression vector
MeCP2	0.9994	0.9111
Deaf1	**0.0008**	0.812
Deaf1-MeCP2	**0.0063**	0.5924

(C) Main effect of transcription factor(s) in Deaf1 -/- MEF

	1128-C expression vector	1128-G expression vector
F	26.15	8.218
df	3	3
p	**<0.0001**	**0.0042**

(D) Post hoc in Deaf1 -/- MEF

	1128-C expression vector	1128-G expression vector
MeCP2	**0.0129**	**0.0081**
Deaf1	**0.0059**	0.2442
Deaf1-MeCP2	**0.0001**	0.0914

(E) Main effect of transcription factor(s) in LTK

	1128-C expression vector	1128-G expression vector
F	78.37	78.37
df	3	3
p	**<0.0001**	**<0.0001**

(F) Post hoc in LTK

	1128-C expression	1128-G expression

	vector	vector
MeCP2	**0.0109**	**0.0002**
Deaf1	**<0.0001**	**0.0246**
Deaf1-MeCP2	**<0.0001**	**<0.0001**

(G) Main effect of transcription factor(s) in RN46A

	1128-C expression vector	1128-G expression vector
F	49.69	77.54
df	3	3
p	**<0.0001**	**<0.0001**

(H) Post hoc in RN46A

	1128-C expression vector	1128-G expression vector
MeCP2	**0.0145**	**<0.0001**
Deaf1	**0.0002**	0.0831
Deaf1-MeCP2	**0.0004**	**0.0388**

Table 2. Statistical analysis of luciferase activity assay for the human 1128C and 1128G HTR1A promoter constructs. Statistical analyses using a repeated measures one-way ANOVA analysing the effects of transcription factors on each expression vector individually of the other. Cell lines analyzed and tests are as indicated. Post hoc analyses were performed when the main effect of the RM one way ANOVA reached <0.05 significance. Bold indicates statistical significance.

de-repression compared to the 1128C variant (M=53.4, SD=53.4); t(8)=3.423, p=0.009 and no significant effects of transcription factors on vector expression (Fig. 11B, Table 2). These results suggest that the G-allele prevents repression by transfected Deaf1, but also by endogenous Deaf1 or other transcription factors (Hes1/5). However, according to western blot (Fig. 5), HEK cells express a great amount of Deaf1 endogenously, which can affect basal HTR1A promoter activity.

Deaf1 KO MEF

The Deaf1 KO MEFs enable us to examine promoter activity without any endogenous Deaf1 protein present. Previous characterization showed that the loss of Deaf1 in Deaf1 KO MEFs led to a significant increase in -984 −WT mouse HTR1A promoter construct that includes both Deaf1 binding sites− promoter activity compared to WT MEFs, which was returned to baseline upon co-transfection with Deaf1 (Fig. A6; Table A1). Since Deaf1 is inhibitory in these cells, the Deaf1 MEFs model the repressor function of Deaf1 on 5-HT1A promoter activity. Significant effects of transfected Deaf1 and MeCP2 on mouse 5-HT1A promoter activity were found in the Deaf1 KO MEFs (Table 2). Post-hoc analysis using Dunnett's post-test revealed that transfected MeCP2 significantly enhanced human 1128C 5-HT1A promoter activity while Deaf1 inhibited promoter activity, which was dominant when both MeCP2 and Deaf1 were cotransfected. For the 1128G construct, the enhancer activity of MeCP2 appeared to remain, while all repressor activity of Deaf1 was lost. This is consistent with a loss of Deaf1 action in the G-allele, and suggests that MeCP2 may bind to other sites in the promoter to enhance 5-HT1A transcription in the Deaf1 -/- MEF background. Furthermore, the 1128G construct (M=239.6, SD=71.1) was significantly de-repressed compared to the 1128C construct (M=115, SD=71.5); t(22)=4.247, p=0.0003 (Fig. 11B), although not to the same degree as observed in HEK-293 cells. This de-repression may result from the effects of other transcription factors such as Hes1 or Hes5 that are also in-

hibited by the C-G change (Lemonde et al. 2003; Jacobsen et al. 2008).

LTK

The LTK- cells are a mouse derived fibroblast cell line in which Deaf1 represses 5-HT1A gene activity. In these cells a significant effect of Deaf1, MeCP2, or their combination on vector activity was observed (Table 2). Post-hoc in the 1129-C using Dunnett's multiple comparisons test revealed that MeCP2 enhanced gene activity, while Deaf1 significantly repressed gene activity. There was a stark loss of expression in the 1128G (M=197.9, SD=38.5) compared to the 1128C (M=13.7, SD=6.63); $t(8)=13.75$, $p<0.0001$. MeCP2 nevertheless significantly enhanced gene activity, while any effects of Deaf1 were lost or greatly attenuated (Fig. 11D).

RN46A

The RN46A cells are immortalized rat raphe nuclei cells that express endogenous 5-HT and 5-HT1A receptors (unlike the other cell lines tested) and so provide a better representation of Deaf1 and MeCP2's interaction in raphe neurons. Deaf1 has inhibitory effects on the transcription of the HTR1A gene in the RN46A cells (Czesak et al., 2006). Transcription factors had a significant main effect on 1128C and 1128G HTR1A promoter activity (Table 2). Post-hoc analysis with Dunnett's test showed that the activity of the human 1128-C construct was significantly enhanced upon co-transfection with MeCP2, and this effect was even greater in the 1128-G construct. However, Deaf1 had significant repressor activity in the 1128-C but not 1128-G construct. Mutating the C (M=282.2, SD=31.6) to a G (M=1147.5, SD=274.0) led to a stark disinhibition of promoter activity; $t(8)=10.27$, $p<0.0001$. Nevertheless, MeCP2 still had enhancer activity on the 1128-G construct (Table 2, Fig. 11E).

Summary

As summarized in Table 3, Deaf1 had significant repressor activity on 1128-C promoter

		5-HT1A promoter Construct					
	Expression vector	1128-C			1128-G		
Cell	Transcription factor	M	D	MD	M	D	MD
HEK 293		1	**3.8-**	**3.3-**	1	1	1
MEF Deaf1 -/-		**1.5+**	**1.8-**	**2.3-**	**1.5+**	1	**1.5+†**
RN46A		**1.4+**	**1.6-**	**1.7-**	**2.1+**	1	**1.3+**

Table 3. Summary of –fold effect of Deaf1, MeCP2 or both on 1128C and 1128G HTR1A promoter activity. The indicated cell lines were cotransfected with Deaf1 (D), MeCP2 (M), or both Deaf1 and MeCP2 (MD) and their respective empty vectors and significant changes analyzed (Table 2). Significant increases (+) and decreases (-) are shown as -fold changes (bold) normalized to each respective expression vector without Deaf1 or MeCP2 (1x). †Trend, $p<0.10$. Note that data from LTK cells were not included due to low transfection efficiency.

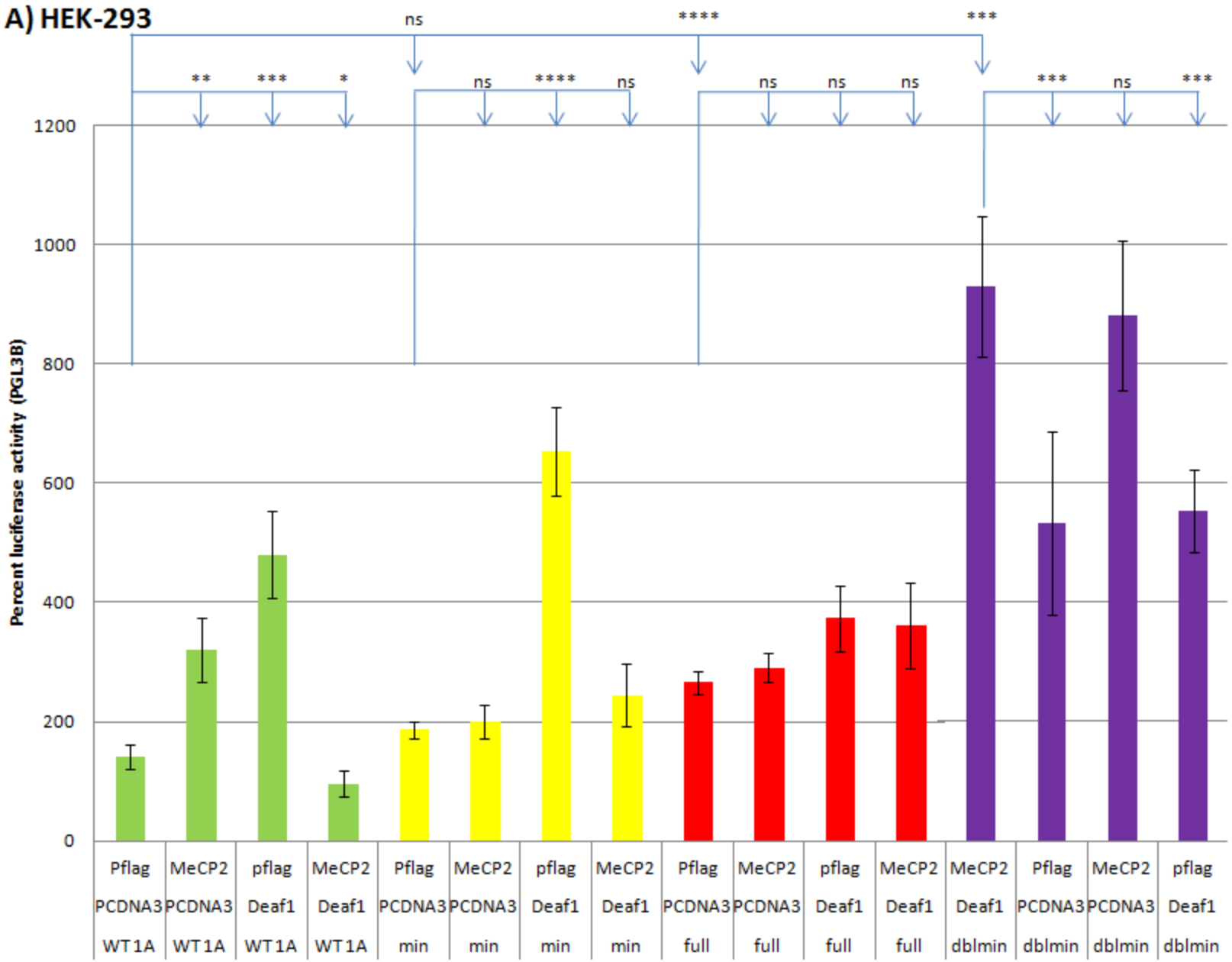
activity in all cell types. MeCP2 had enhancer activity on the 1128-C vector in the MEFs and RN46As, but not the HEK-293. Their combination also had significant repressor activity on the 1128-C vector, in all cell types. Mutating the 1128-C to a G (as in 1128-G) led to a significant increase in promoter activity and loss of Deaf1 effects, consistent with previous studies. However, MeCP2's enhancer activity in the MEFs and RN46A cells remained.

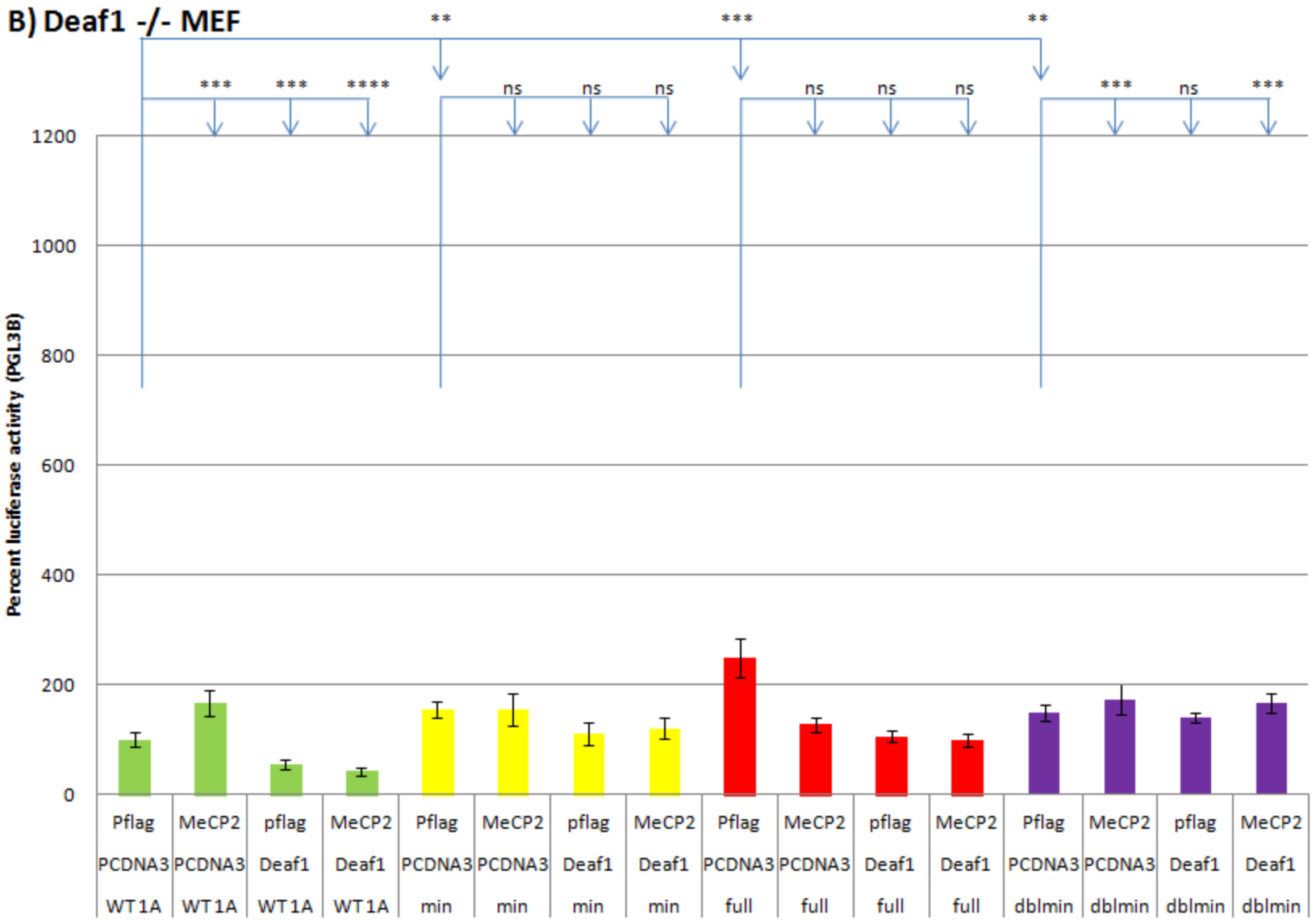
Deaf1 and MeCP2 modulate mouse HTR1A expression which is significantly affected by mutations of the mouse Deaf1 sites

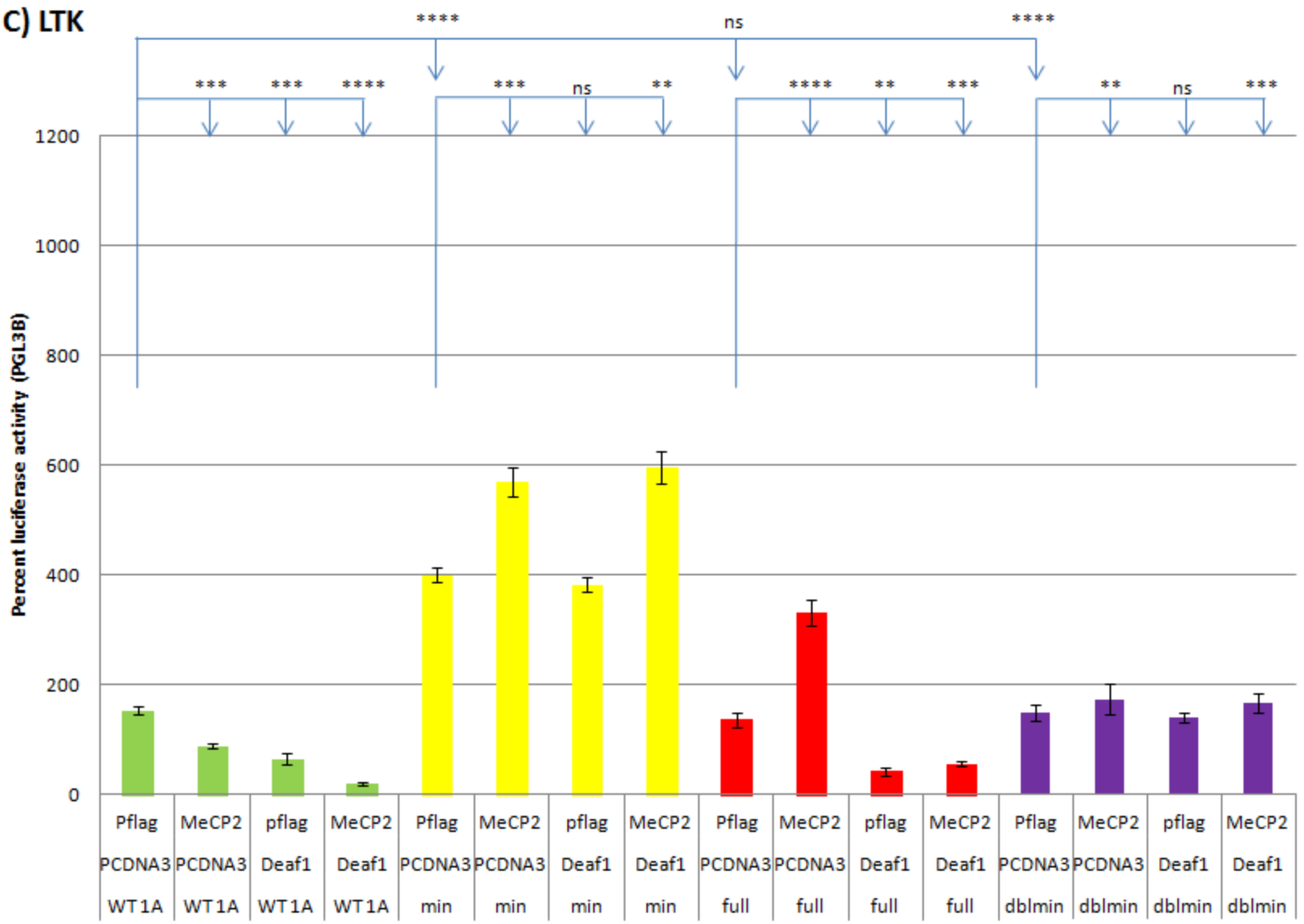
Unlike the human HTR1A promoter, the mouse HTR1A promoter contains two potential Deaf1 sites (Czesak et al., 2012). Using the luciferase reporter approach, we explored the effects of Deaf1, MeCP2, and their interaction on HTR1A promoter activity in PGL3B expression vectors containing mouse HTR1A promoter constructs, including: wild-type mouse HTR1A promoter region (WT), minimal mutation of the first mouse Deaf1 site (m1), full deletion of DNA enclosed by the Deaf1 sites (full), and minimal mutation of both Deaf1 sites (m1&m2) (Fig. A5)

HEK-293

Significant main effects of MeCP2, Deaf1, or their combination were found on mouse HTR1A promoter activity (Table 4), except in the full mutant vector. Post-hoc analysis using Dunnett's multiple comparisons test showed that in HEK293 cells MeCP2 and Deaf1 significantly enhanced WT HTR1A promoter activity compared to control, while their combination significantly repressed gene activity. In the m1 mutant, the enhancer activity of MeCP2 but not Deaf1 was lost, and the enhancer activity of the Deaf1-MeCP2 combination was also lost. No significant interaction of transcription factors and the full mutant was found, so no post hoc analyses were performed. In the m1&m2 mutant all activity of Deaf1 was lost, but a weak


A) HEK-293
Percent luciferase activity (PGL3B)
0
200
400
600
800
1000
1200
ns
****
***
**
***
*
ns
****
ns
ns
ns
ns
***
ns
***
Pflag PCDNA3 WT1A
MeCP2 PCDNA3 WT1A
pflag Deaf1 WT1A
MeCP2 Deaf1 WT1A
Pflag PCDNA3 min
MeCP2 PCDNA3 min
pflag Deaf1 min
MeCP2 Deaf1 min
Pflag PCDNA3 full
MeCP2 PCDNA3 full
pflag Deaf1 full
MeCP2 Deaf1 full
MeCP2 Deaf1 dblmin
Pflag PCDNA3 dblmin
MeCP2 PCDNA3 dblmin
pflag Deaf1 dblmin


B) Deaf1 -/- MEF
Percent luciferase activity (PGL3B)
0
200
400
600
800
1000
1200
**
***
**
***
***
****
ns
ns
ns
ns
ns
ns
***
ns
***
Pflag PCDNA3 WT1A
MeCP2 PCDNA3 WT1A
pflag Deaf1 WT1A
MeCP2 Deaf1 WT1A
Pflag PCDNA3 min
MeCP2 PCDNA3 min
pflag Deaf1 min
MeCP2 Deaf1 min
Pflag PCDNA3 full
MeCP2 PCDNA3 full
pflag Deaf1 full
MeCP2 Deaf1 full
Pflag PCDNA3 dblmin
MeCP2 PCDNA3 dblmin
pflag Deaf1 dblmin
MeCP2 Deaf1 dblmin


C) LTK
Percent luciferase activity (PGL3B)
0
200
400
600
800
1000
1200
****
ns
****
***
***
****
***
ns
**
****
**
***
**
ns
***
Pflag PCDNA3 WT1A
MeCP2 PCDNA3 WT1A
pflag Deaf1 WT1A
MeCP2 Deaf1 WT1A
Pflag PCDNA3 min
MeCP2 PCDNA3 min
pflag Deaf1 min
MeCP2 Deaf1 min
Pflag PCDNA3 full
MeCP2 PCDNA3 full
pflag Deaf1 full
MeCP2 Deaf1 full
Pflag PCDNA3 dblmin
MeCP2 PCDNA3 dblmin
pflag Deaf1 dblmin
MeCP2 Deaf1 dblmin

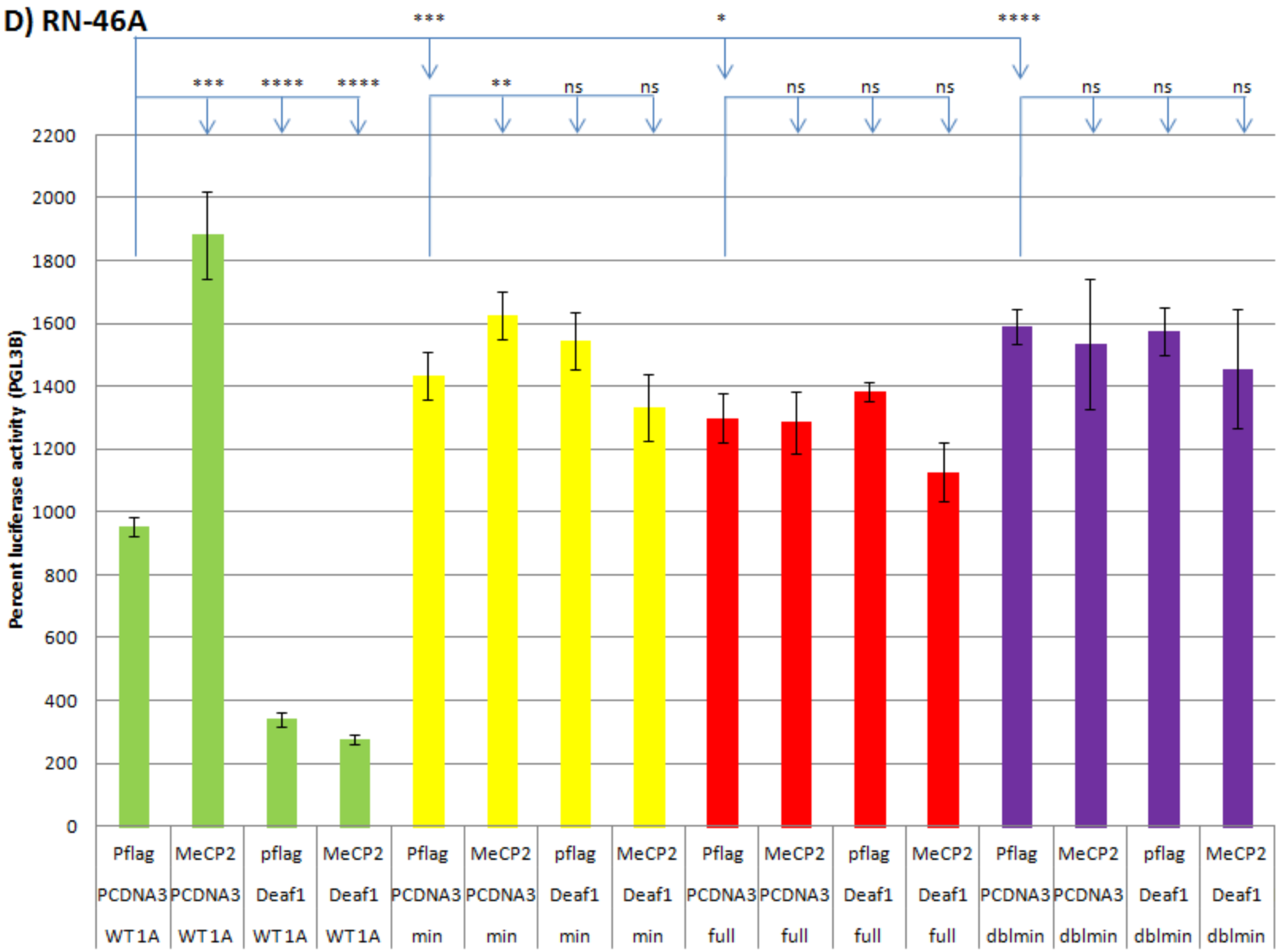

D) RN-46A
Percent luciferase activity (PGL3B)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
***
*
****
***
****
****
**
ns
ns
ns
ns
ns
ns
ns
ns
ns
Pflag PCDNA3 WT1A
MeCP2 PCDNA3 WT1A
pflag Deaf1 WT1A
MeCP2 Deaf1 WT1A
Pflag PCDNA3 min
MeCP2 PCDNA3 min
pflag Deaf1 min
MeCP2 Deaf1 min
Pflag PCDNA3 full
MeCP2 PCDNA3 full
pflag Deaf1 full
MeCP2 Deaf1 full
Pflag PCDNA3 dblmin
MeCP2 PCDNA3 dblmin
pflag Deaf1 dblmin
MeCP2 Deaf1 dblmin

Figure 12. Differential activity of Deaf1 and MeCP2 at mouse HTR1A promoter Deaf1 site mutants. The indicated cell lines were co-transfected with the mouse HTR1A promoter constructs (wild-type WT; Deaf1 site 1 mutant, m1; full mutant, full; Deaf1-site1&2 mutant, m1&m2; figuratively shown below) and constructs for Deaf1, MeCP2, or both Deaf1 and MeCP2 with their respective empty vectors and β-Gal plasmid. Cell extracts were used for luciferase and β-galactosidase assays, and all luciferase values were normalized to β-gal and then normalized to the empty PGL3B vector co-transfected with empty MeCP2 (pflag) and Deaf1 (PCDNA3) vectors and expressed as a percentage of activity. Co-transfection of the empty PGL3B vector with MeCP2 and PCDNA3, pflag and Deaf1, or MeCP2 and Deaf1 yielded no significant differences (data not shown). **Shown are results from (A) HEK-293 cells** (n=12)**, (B) Deaf1 -/- MEF cells** (n=9), **(C) LTK cells** (n=9), and **(D) RN46A cells** (n=9)**.** Data are plotted as Mean ± SE and statistics shown in Table 4. ns = not significant; *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001

(A) Main effect of transcription factor(s) in HEK-239

	PGL3B expression vector	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
F	0.8975	27.46	31.04	15.3	2.079
df	3	3	3	3	3
p	0.4341	**<0.0001**	**<0.0001**	**0.0002**	0.1665

(B) Post hoc in HEK-293

Vector x Transcription factor	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
MeCP2	**0.0014**	0.8957	**0.0002**	
Deaf1	**0.0006**	**<0.0001**	>0.9999	
Deaf1-MeCP2	**0.0254**	0.5586	**0.0008**	

(C) Main effect of transcription factor(s) in Deaf1 -/- MEF

	PGL3B expression vector	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
F	0.6151	47.41	5.38	5.649	22.35
df	3	3	3	3	3
p	0.528	**<0.0001**	**0.0125**	**0.0184**	**<0.0001**

(D) Post hoc in Deaf1 -/- MEF

	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
MeCP2	**0.0004**	>0.9999	0.1333	**0.004**
Deaf1	**0.0002**	**0.0201**	0.0632	**0.0006**
Deaf1-MeCP2	**<0.0001**	0.0501	0.1097	**0.0003**

(E) Main effect of transcription factor(s) in LTK

	PGL3B expression vector	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
F	1.656	82.59	23.48	27.8	101.6
df	3	3	3	3	3
p	0.2239	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

(F) Post hoc in LTK

	WT1A expression	m1 expression	m1&m2 expression	full expression

	vector	vector	vector	vector
MeCP2	**0.0002**	**0.0005**	**0.0011**	**<0.0001**
Deaf1	**0.0004**	0.4296	0.8055	**0.001**
Deaf1-MeCP2	**<0.0001**	**0.0039**	**0.0003**	**0.0005**

(G) Main effect of transcription factor(s) in RN46A

	PGL3B expression vector	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
F	4.086	108.8	7.696	0.3973	2.391
df	3	3	3	3	3
p	**0.0476**	**<0.0001**	**0.0058**	0.5813	0.1387

(H) Post hoc in RN46A

	WT1A expression vector	m1 expression vector	m1&m2 expression vector	full expression vector
MeCP2	**0.0002**	**0.0046**		
Deaf1	**<0.0001**	0.1274		
Deaf1-MeCP2	**<0.0001**	0.3740		

Table 4. Statistical analysis of luciferase activity assay for the mouse HTR1A promoter constructs. Statistical analyses used a repeated measures one-way ANOVA analysing the effects of transcription factors on each expression vector individually of the other. Cell lines analyzed and tests are as indicated. Post hoc analyses were performed when the main effect of a RM one way ANOVA reached $p<0.05$ significance. Bold indicates statistical significance.

activity of MeCP2 on the promoter remained (Fig. 12B). Significant main effects of the mutations were also found ($F(3)=14.61$, $p= 0.0003$). Post hoc analysis indicated a de-repression in the full ($M= 265.7$, $SD=58.1$, $p=0.0014$) and m1&m2 ($M=225.1$, $SD=13.8$, $p=0.0007$), but not the m1 ($p=0.1563$) mutant, compared to WT ($M=141$, $SD=63.5$) mouse HTR1A promoter (Fig. 12A).

Deaf1 KO MEF

Transfected Deaf1 and MeCP2 had significant effects on mouse 5-HT1A promoter activity found in the Deaf1 KO MEFs (Table 4); post-hoc analysis using Dunnett's multiple comparisons test showed that MeCP2 enhanced promoter activity, while Deaf1 repressed WT mouse HTR1A promoter activity in MEFs. The repressor activity of Deaf1, and the Deaf1-MeCP2 combination was lost in the m1 mutant, as was the enhancer activity of MeCP2. In the full mutant MeCP2, Deaf1, and MeCP2 and Deaf1 together significantly reduced basal gene expression, through an unknown mechanism. The m1&m2 mutant was no longer regulated by Deaf1 or MeCP2 (Fig. 12B). A significant effect of promoter mutations was found ($F(3)=18.93$, $p=0.0002$). Post-hoc analysis revealed that a significant de-repression was observed in the m1 ($M=155$, $SD=46.4$, $p=0.0083$), full ($M=249.2$, $SD=46.4$, $p=0.0003$), and m1&m2 ($M=148.1$, $SD=14.8$, $p=0.0012$) mutants compared to the WT ($M=100.9$, $SD=37.4$).

LTK

A significant effect of Deaf1, MeCP2, and their combination was observed (Table 4). Post-hoc analysis using Dunnett's multiple comparisons test revealed that all three conditions repressed gene activity for the WT mouse HTR1A vector. Significant effects of vector mutation compared to WT were also found ($F(3)=220.6$, $p<0.0001$). Post hoc revealed a stark loss of repression in the m1 mutant ($M=400$, $SD=40.9$, $p<0.0001$) where MeCP2 now had enhancer activity and Deaf1 had no activity. However, in the Full mutant there was no loss of repression

(p=0.3170); but MeCP2 enhanced gene activity while Deaf1 had repressor activity. The m1&m2 mutant showed a significant de-repression (M=929, SD=117.3, p<0.0001), and was no longer significantly affected by Deaf1 but was repressed by MeCP2 independently of Deaf1 (Fig. 12C).

RN46A

In the RN46A cells transcription factors had a significant main effect on WT and m1 HTR1A promoter activity (Table 4). Post-hoc analysis using Dunnett's multiple comparisons test showed that MeCP2 enhanced WT HTR1A promoter activity, while Deaf1 repressed it. The repressor activity of Deaf1 was lost in the m1 mutant, while the enhancer activity of MeCP2 was greatly attenuated. No significant activity of the transcription factors was observed in the full or m1&m2 mutants and post-hoc analysis was not performed. Significant activity of mutant compared to WT HTR1A promoter vector (M=953.0, SD=89.6) were found (F(3)=19.47, p<0.0001). Post hoc analysis revealed a stark de-repression of promoter activity was observed in the m1 (M=1435.4, SD=226.5, p=0.0007), full (M=1299.6, SD=241.7, p=0.0111), and m1&m2 (M=1591.4, SD=165.7, p<0.0001) mutants (Fig. 12D).

Summary

The data from luciferase assays are summarized in Table 5. However, for technical reasons the full mutant and LTK data were not included (as explained in the discussion). MeCP2 acted as an enhancer of mouse WT HTR1A promoter activity in all cell types. Deaf1 functioned as an enhancer of mouse WT promoter activity only in the HEK-293 and as a repressor of promoter activity in the MEFs and RN46A cells. The combination of Deaf1 and MeCP2 led to repression of mouse WT promoter in all cell types. Mutating the m1 led to a loss of Deaf1 repressor activity in the MEFs and RN46A cells, but Deaf1 enhancer activity in the HEK-293 cells remained. Mutating both the m1 and m2 led to a loss of Deaf1 enhancer and repressor activity.

	5-HT1A promoter Construct								
Expression vector	W.T.			m1			m1&m2		
Cell / Transcription factor	M	D	MD	M	D	MD	M	D	MD
HEK 293	**2.3+**	**3.4+**	**1.5-**	1	**3.5+**	1	1.2+	1	1.2+
MEF Deaf1 -/-	**1.7+**	**1.8-**	**2.4-**	1	1	1	1	1	1
RN46A	**2.0+**	**2.8-**	**3.5-**	1.1+	1	1	1	1	1

Table 5. Summary of –fold effect of Deaf1, MeCP2 or both on mouse HTR1A promoter activity. The indicated cell lines were co-transfected with the indicated mouse HTR1A promoter constructs (wild-type WT; Deaf1 site 1 mutant, m1; Deaf1-site1&2 mutant, m1&m2; figuratively shown in Fig. A5) and either Deaf1 (D), MeCP2 (M), or both Deaf1 and MeCP2 constructs (MD) and their respective empty vectors and significant changes analyzed (Table 4). Significant increases (+) and decreases (-) are shown as -fold changes (bold) normalized to each respective expression vector without Deaf1 or MeCP2 (1x). Note that the full mutant was not included due to the aberrant effects observed, which could be due to the activation of a dormant Deaf1 site. Due to low transfection efficiency the LTK cell results were not included in this summary.

Mutating either the m1 or m1&m2 resulted in a loss or strong attenuation of any effects of MeCP2 on promoter activity. Mutating either the m1 or m1&m2 led to a significant de-repression in most cell types.

MeCP2 cKO mice have increased 5-HT1A autoreceptor levels

To address the role of MeCP2 on 5-HT1A receptors *in vivo* and to avoid deleterious developmental effects of a global MeCP2 knockout, we used mice in which MeCP2 is knocked out in 5-HT neurons of the raphé nuclei of adult mice (Fig. A7; referred to as $MeCP2^{TPH2\ creERT2\text{-}flx/flx}$ or $MeCP2^{TPH2\ creERT2\text{-}flx/Y}$). These mice were produced by crossing $TPH2^{cre\text{-}ERT2}$ mice with a tamoxifen-inducible Cre that is only expressed in TPH2 containing neurons (i.e. serotonergic neurons) with MECP2 flx mice that possess flx sites, which flank exons 3 and 4 of MeCP2 rendering it non-functional. In order to produce the knockout in adulthood the mice were treated with tamoxifen at 8 weeks of age to delete the MeCP2 gene specifically in 5-HT neurons. Control mice possessed the same flx sites flanking MeCP2, but no cre enzyme to excise it ($MeCP2^{flx/flx}$ or $MeCP2^{flx/Y}$).

<u>DPAT induced hypothermia</u>

In order to test for effects of MeCP2 deletion on functional 5-HT1A autoreceptors response *in vivo*, $MeCP2^{TPH2\ creERT2\text{-}flx/Y}$ and $MeCP2^{flx/Y}$ control mice were treated with tamoxifen at 8 weeks of age and examined using the DPAT-induced hypothermia assay, which selectively measures the function of 5-HT1A autoreceptors on 5-HT neurons (Richardson-Jones et al., 2010). When treated with the 5-HT1A agonist 8-OH DPAT, $MeCP2^{TPH2\ creERT2\text{-}flx/Y}$ showed a significantly greater drop in body temperature at all times measured compared to $MeCP2^{flx/Y}$ (Fig. 13; Table 6). This result suggests that arresting the production of MeCP2 led to a greater amount of

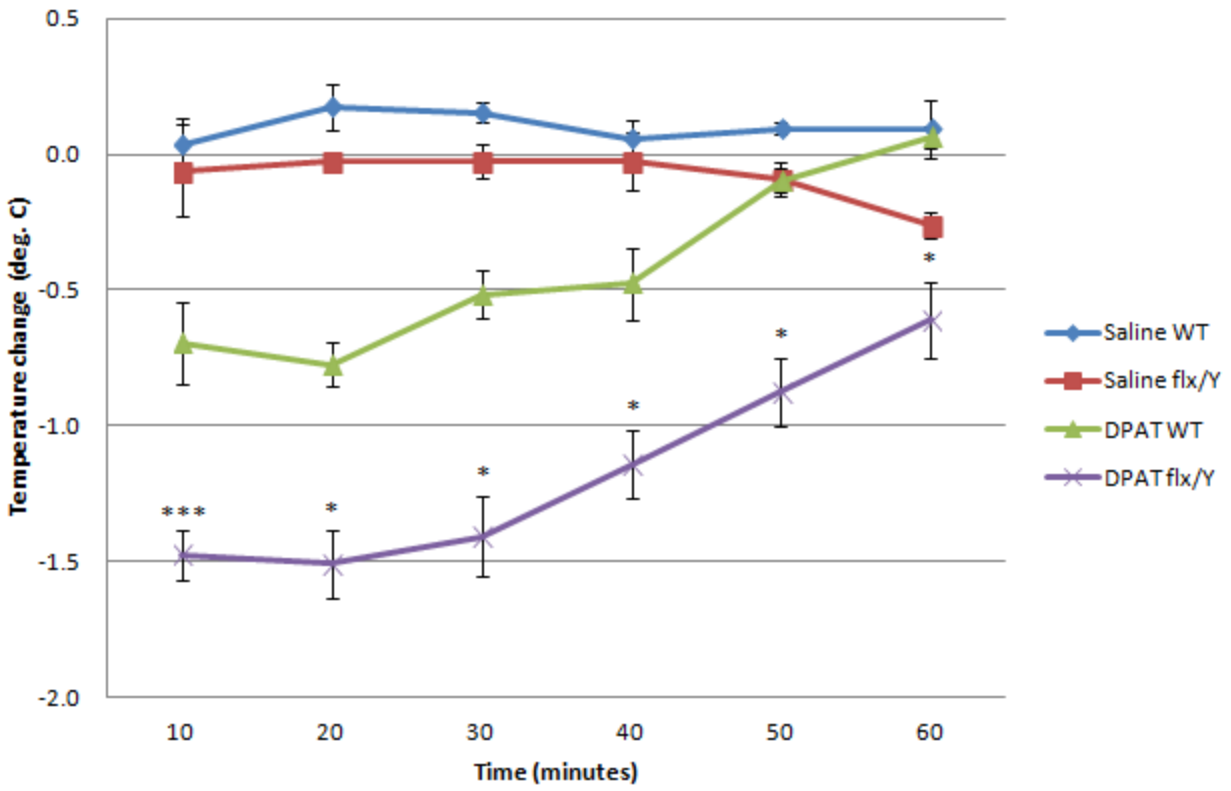


Figure 13. Enhanced DPAT-induced hypothermia in MeCP2 conditional KO mice. Average thermal change over time was measured by anal temperature probe comparing tamoxifen-treated male MeCP2$^{\text{TPH2 creERT2-flx/Y}}$ conditional knockout (Flx/Y) and control MeCP2$^{\text{flx/Y}}$ (WT) mice with C57BL/6 background following 0.9% saline (vehicle) injection or 0.5 mg/kg 8-OH-DPAT challenge. Four (4) baseline temperature measurements were averaged from the temperature post injection was subtracted to provide temperature change. N=5 WT, N=3 Flx/Y cKO. Data are shown as mean ± SE and statistics presented in Table 6; **p<0.01; ***p<0.001.

Post Hoc **Time**	10min	20min	30min	40min	50min	60min
WT DPAT x WT Saline	>0.05	**0.001**	**0.001**	**0.001**	>0.05	>0.05
KO DPAT x KO Saline	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.05**
WT DPAT x KO DPAT	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.01**

Table 6. Enhanced DPAT induced hypothermia in conditional MeCP2 knockout mice. Statistical analysis of average temperature change over time for saline or DPAT-treated MeCP2 Control (WT) or homozygous conditional knockout (cKO) mice. Bonferroni corrected post-hoc statistical results of one-way ANOVA comparing DPAT vs. saline and WT DPAT vs. cKO DPAT. Significant interactions are bolded.

functional 5-HT1A receptors in 5-HT neurons. This is consistent with our data suggesting that MeCP2 is recruited to the Deaf1 repressor site and enhances Deaf1 repression: thus, without MeCP2 present, Deaf1 repression would be attenuated, leading to increased levels of 5-HT1A receptors.

Deaf1 KO mice show greater 5HT1A autoreceptor response

In order to better understand the role of Deaf1 in regulating mouse HTR1A gene expression *in vivo*, a Deaf1 KO mouse line was created (Czesak et al., 2012). The loss of Deaf1 was shown to significantly increase the number of 5-HT1A/TPH2-positive neurons in the dorsal raphé (Czesak et al., 2012; Fig. 14). To test whether 5-HT1A autoreceptor function in the raphé is increased, we again used the DPAT-induced hypothermia assay, in which hypothermia is proportional to the levels of the 5-HT1A autoreceptor (Richardson-Jones et al., 2010). Deaf1 KO, HET, and WT mice were given 8-OH DPAT I.P., which led to a significant drop in body temperature upon injection. While there were no differences between the Deaf1 HET and WT mice, the Deaf1 KO mice had a significantly greater drop in body temperature compared to Deaf1 WT from 20 min to 40 min (Fig. 15A, Table 7A). When segregating the data by gender, only the Deaf1 KO males showed significantly greater drop in body temperature compared to the WT males (Fig. 15B, Table 7B); the females alone did not show a significant difference (Fig. 15C, Table 7B).

When performing DPAT injections, I observed that the KO mice appeared to be more anxious then the WT mice. We therefore began behavioural tests in these mice.

Deaf1 KO mice do not show depressive/anhedonic phenotype

To examine the behavioural effects resulting from the loss of Deaf1 and increase in functional 5-HT1A receptors in the raphé nuclei we performed several behavioral tests. To examine anxiety-like behaviours, Christine Luckhart performed the elevated plus maze (EPM), the open field (OF) test, and the light dark (LD) test (summarized in discussion). Christine Luckhart analysed depression-like behaviours using the tail suspension (TS) and the forced swim (FS) tests (summarized in discussion). I performed the sucrose preference (SP) tests, which is a of anhedonia, where having no preference for sugar water is seen as an indication of anhedonia (Borsini et al., 2002). While some mice showed a clear preference for sucrose and others a preference for water, no clear preference between the genotypes or genders was identified ($F(7)=0.9673$, $p=0.4656$) (Fig. 15). Thus the Deaf1 -/- mice did not show anhedonia-like behaviour despite the increase in 5-HT1A autoreceptor levels.

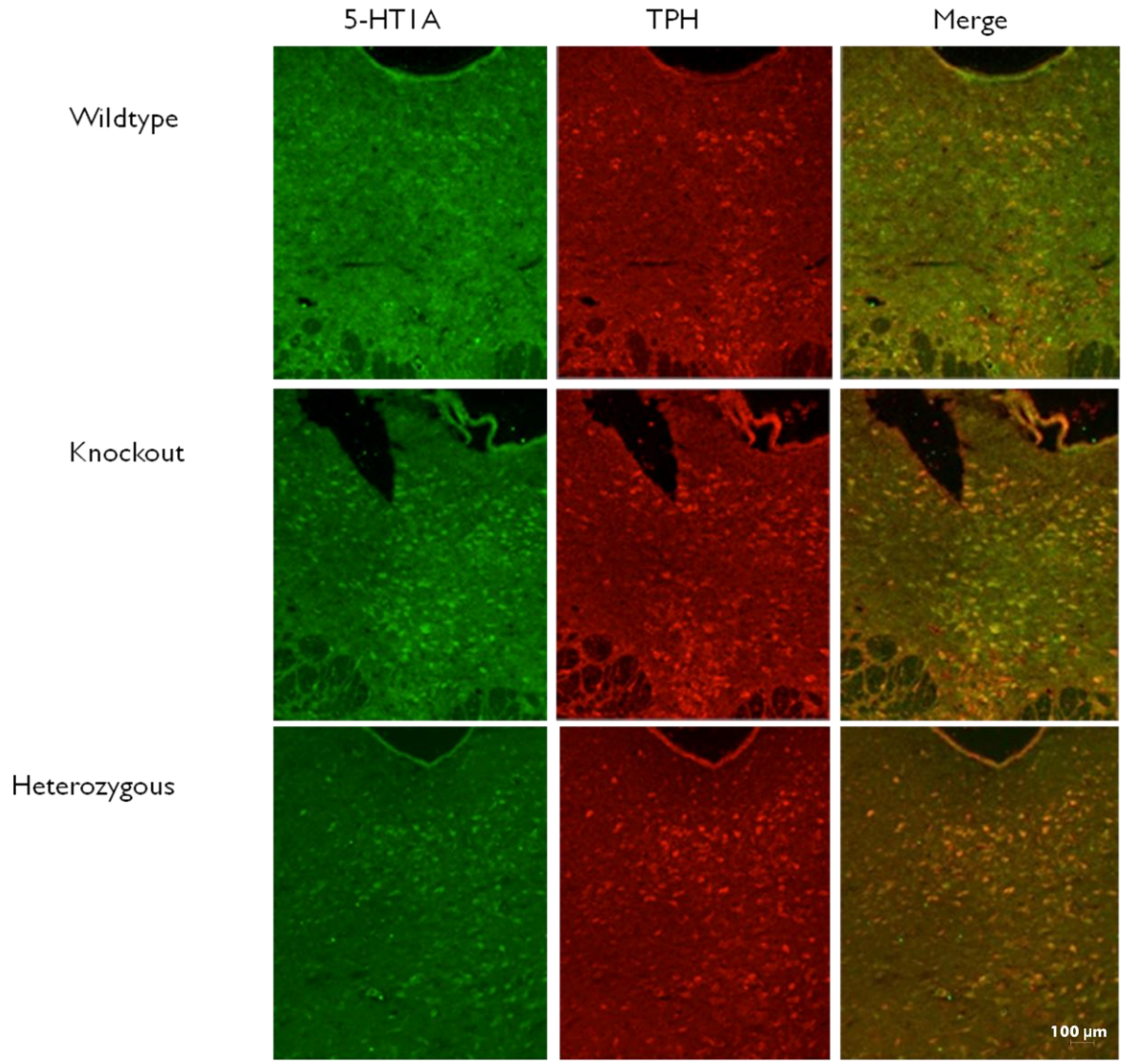


Figure 14. Loss of Deaf1 increases the number of 5-HT1A positive cells. Immunofluorescent staining of the dorsal raphe nuclei for 5-HT1A receptor (rabbit anti-5-HT1A, 1:50), TPH (sheep anti-TPH, 1:1000), and merged in the third row with slices from Deaf1 wild-type, knockout and heterozygous mice. Scale bar = 100 µm. From Christine Luckhart, 2014.

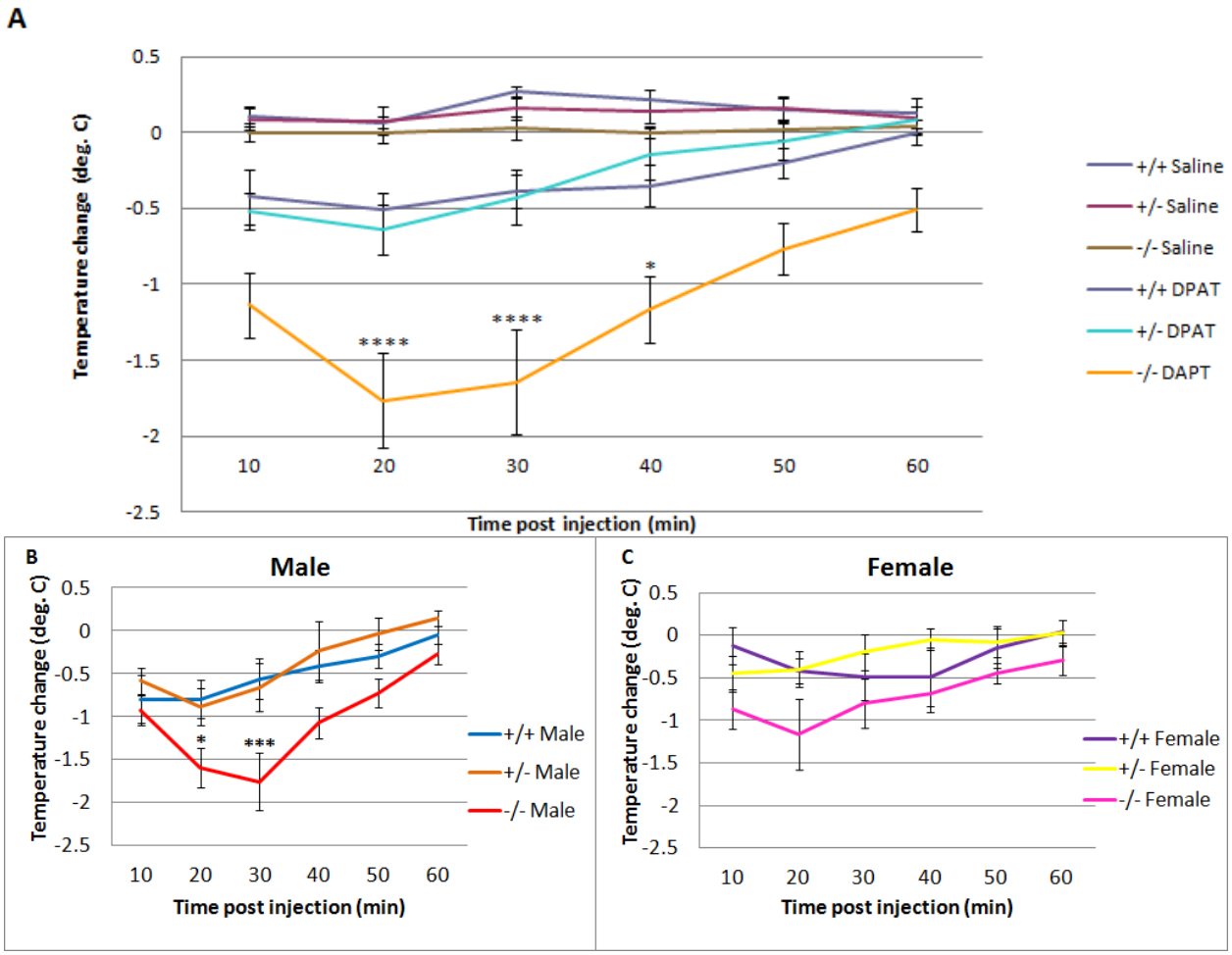


Figure 15. Enhanced DPAT induced hypothermia in Deaf1 knockout mice. (A) Average thermal change over time measured by anal temperature probe for Deaf1 wild type (+/+; n=9), heterozygous (+/-; n=8) and homozygous knockout (-/-, n=11) on C57BL/6-Balb/C background following 0.9% saline (vehicle) or 0.5 mg/kg 8-OH-DPAT (DPAT) injection following 4 baseline temperature measurements, which were averaged before the temperature post injection is subtracted to give temperature change. N=9 WT, N=8 Het, N=11 KO. Mice were segregated by sex in **(B) Male** with N=6 WT, N=4 Het, N=7 KO and **(C) Female** with N=3 WT, N=4 Het, N=4 KO. *<0.05; **<0.01; ***<0.001; ****<0.0001

A

Post Hoc

Time	10min	20min	30min	40min	50min	60min
WT DPAT x WT Saline	0.2244	0.1225	**0.0433**	0.1435	0.8472	> 0.9999
HET DPAT x HET Saline	*0.088*	**0.0165**	0.104	0.979	0.9988	> 0.9999
KO DPAT x KO Saline	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.001**	*0.0816*
WT DPAT x KO DPAT	0.1074	**< 0.0001**	**< 0.0001**	**0.0354**	0.3706	0.5418
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	0.9995	> 0.9999	> 0.9999

B

Post Hoc

Time		10min	20min	30min	40min	50min	60min
Male	WT DPAT x KO DPAT	0.9983	**0.0387**	**0.0005**	0.1409	0.5918	0.9656
Female	WT DPAT x KO DPAT	0.1644	0.1734	0.9382	0.9915	0.9524	0.9024

Table 7. Enhanced DPAT induced hypothermia in Deaf1 knockout mice. (A) Statistical analysis of complete DPAT-induced hypothermia data set. Average temperature change over time for saline or DPAT-treated Deaf1 wild type (WT), heterozygous (HET) and homozygous knockout (KO) were analyzed using Sidak corrected post hoc statistical results of one-way ANOVA comparing DPAT vs. saline and WT DPAT vs. KO DPAT and HET DPAT. **(B) Statistical analysis of segregated male and female data** Sidak corrected post hoc statistical analysis of one-way ANOVA for WT DPAT vs. KO DPAT. Significant interaction are bolded, trends are italicized.

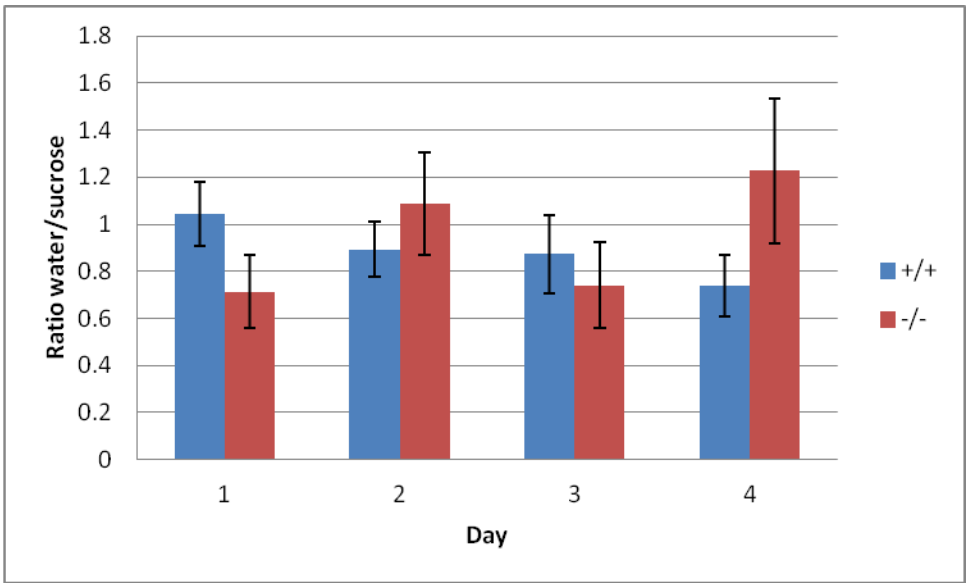


Figure 16. Deaf1 KO mice show no sucrose preference compared to WT. The amount of water from either the sucrose water or regular water bottles was weighed at noon (12:00pm) daily for four (4) consecutive days. The water weight was then divided by the sucrose weight to give a ratio where any value above one (1) would indicate a preference for sucrose and any value under one (1) an aversion to sucrose. WT male n=3 and female n=4 were combined; KO male n=4 and female n=3 were combined (n=7 per genotype).

Discussion

Depression is a complex disorder that results from the interplay of genetic and environmental factors (Meaney and Szyf, 2005; Sun et al, 2013). To better understand how the cell can integrate both genetic and environmental factors we looked at two major transcriptional regulators (Deaf1 and MeCP2). Deaf1 had been identified previously as an important transcriptional regulator of the 5-HT1A gene whose actions are blocked by the 1019-G allelic variant in humans (Lemonde et al., 2003; Czesak et al., 2006; Czesak et al., 2012). While MeCP2 is a methyl binding protein that reads epigenetic marks, which have been suggested to be important in the development of mood disorders such as stress reactivity and depression (Weaver et al., 2004; McGowan et al., 2009, Meaney and Szyf 2005; Ramocki et al., 2009). Therefore we addressed whether MeCP2 and Deaf1 interact, how they interact, and what functional effects this might have. To further support the theory that Deaf1 is an important transcriptional regulator of the HTR1A promoter we explored the functional and behavioural effects of knocking out Deaf1 in mice. To better convey that MeCP2 is important in regulating the HTR1A promoter and functional 5-HT1A receptor levels in an animal model, we explored the functional effects of conditionally knocking out MeCP2 only in serotonergic raphé neurons.

MeCP2 and Deaf1 interact

We showed that MeCP2 and Deaf1 interact by co-immunoprecipitation studies in transfected SKN-SH cells where MeCP2 was co-precipitated with Deaf1 and vice versa (Fig. 7). A weak signal identified as MeCP2 was present in the Co-IP when Deaf1 was absent. This could be due to insufficient washing or tag interactions. To demonstrate that this interaction does occur *in vivo* we performed Co-IPs in SKN-SH cells, Deaf1 WT and KO hippocampal extracts, and Deaf1 +/+ and -/- MEFs. MeCP2 was co-precipitated with Deaf1 when Deaf1 was present (SKN-

SH, Deaf1 WT hippocampal extracts and +/+ MEFs), but not when it was absent (Deaf1 KO hippocampal extracts and -/- MEFs; Fig. 8). Streaking was observed on the western blot, considering that MeCP2 and Deaf1 interact with DNA, these likely are due to DNA smears. These results suggest that MeCP2 interacts with Deaf1, however a major caveat is that we were unable to detect Deaf1 in the immunoprecipitates, probably reflecting a technical limitation of the antibody used. Because staining with our homemade rabbit polyclonal Deaf1 antibody led to high IgG background in the Western blot of immunoprecipitates, we used a mouse monoclonal antibody to human Deaf1 for the Western blot. While this antibody did recognize Deaf1 in the input from w.t. but not KO Deaf1 tissues, it failed to detect Deaf1 in the immunoprecipitates. It is possible that the Deaf1 epitope recognized by the monoclonal antibody is no longer recognized following the immunoprecipitation with the polyclonal antibody, even though it is recognized in the input. For example, the epitope may become modified (e.g., oxidized, phosphorylated, ubiquitinylated etc.). In particular, several amino acids that can be phosphorylated and ubiquitinylated were identified in the sequence of the antigenic Deaf1 peptide used to raise the monoclonal antibody. In this light, Deaf1 interacts with and is phosphorylated by GSK3beta at multiple sites that remain to be identified (Pilot-Storck et al., 2010). Further tests would be required to identify the problem. Another possibility is that MeCP2 recognizes a variant of Deaf1 that lacks the epitope of the monoclonal antibody. For example, a truncated splice variant of mouse Deaf1, DF1-VAR1 (50 kDa) that lacks the C-terminal portion of Deaf1 has been shown to bind to functionally antagonize full-length Deaf1 transcriptional activity (Yip et al., 2009; Yip et al., 2015). Preferential MeCP2 binding to this antagonistic Deaf1 isoform could relieve inhibition of Deaf1 and explain Deaf1 enhancer activity. Future co-immunoprecipitation studies could examine whether the MeCP2 binds to DF1-VAR1.

We also addressed whether Deaf1 can bind to its site in the mouse 5-HT1A promoter, and whether MeCP2 is also present. Previously Deaf1 had been shown to bind to the human and mouse HTR1A Deaf1 elements using *in vitro* electrophoretic mobility shift assays (Lemonde et al. 2003; Czesak et al. 2012), but this had never been tested *in vivo*. Therefore, ChIP assays were done in Deaf1 +/+ MEFs where we found a significant enrichment of the HTR1A promoter region where Deaf1 binds when pulling down with either Deaf1 or MeCP2 (Fig. 9). These ChIP results were substantiated in tissues by doing the same assay in Deaf1 WT raphé, hippocampus, and PFC extracts, and obtaining similar enrichments (Fig. 10). To demonstrate the specificity of Deaf1 binding we performed the same assay in Deaf1 -/- MEFs and Deaf1 KO raphé, hippocampus, and PFC extracts, which lack Deaf1 protein, where we observed no enrichment in this HTR1A promoter region (Fig. 10 and 9), despite being able to pull down for Histone H1 (Fig. 9). These results show for the first time that Deaf1 binds to the mouse 5-HT1A promoter in fibroblasts as well as in brain regions enriched in 5-HT1A receptors *in vivo*.

Despite the efficient pull-down of Deaf1 in the ChIP assay, we had difficulty in detecting Deaf1 in these tissues using Western blot (Fig. 5, 6). However, this may reflect that the polyclonal antibody we generated to full-length Deaf1 did not appear to recognize denatured Deaf1 (on Western blot) as well as it recognized non-denatured Deaf1 in immunoprecipitates. By contrast the mouse monoclonal antibody was able to recognize Deaf1 in Western blot (input lanes, Fig. 8), although it may not recognize all forms of Deaf1.

Unexpectedly, there was no HTR1A promoter enrichment in the Deaf1 -/- MEFs or Deaf1 KO brain extracts when pulling down with MeCP2 (Fig. 9, 10). This would suggest that MeCP2 binding to this region of the promoter is dependent on Deaf1 binding. As shown by yeast-one hybrid assays MeCP2 alone does not bind to the human Deaf1 site unless it is methy-

lated, suggesting that this site may not be methylated in the mouse tissues (Fig. 3). However, luciferase assays in the Deaf1 -/- MEFs suggest that MeCP2 can have functional effects on HTR1A promoter activity, without Deaf1 present (Fig. 11C and 12B). This could be due to MeCP2 binding to other methylated sites in the HTR1A promoter, particularly in the CpG-rich proximal promoter, which is partially methylated *in vivo* (Le Francois et al., 2015). Interestingly, other research has shown that Deaf1 is unable to bind its recognition site (TTCG DNA motif) when methylated (Jensik et al., 2014), but Deaf1 binding its site and driving β-gal activity was unaffected when methylated in the yeast-1 hybrid methylation assay (Fig. 3) and upon methylation of Deaf1 and HES sites in SKN-SH cells (Fig. 4). Furthermore, in EMSA we did not find any effect of methylation on Deaf1 binding to the human HTR1A Deaf1 site (Millar and Albert, unpublished data). Since MeCP2 was detected on western blot from SKN-SH cells extract (Fig. 5B), MeCP2 may enable Deaf1 to bind to the methylated site.

While we have shown that Deaf1 and MeCP2 interact, the domain(s) responsible remain to be discovered. These could be uncovered by mutations of important domains in both Deaf1 (i.e. SAND, MYND, or LIM) and MeCP2 (MBD, TRD etc.). Deaf1 has been shown to interact with other proteins and itself via its SAND domain (Vulto-van Sifhout et al., 2014; Jensik et al., 2012). Interestingly, mutations of Deaf1's SAND domain in humans have been implicated in intellectual disability (Vulto-van Sifhout et al., 2014) and mutations of MeCP2's MBD and TRD are implicated in Rett syndrome (Hite, Adams and Hansen, 2009). Interestingly, the SAND domain is present in the DF1-VAR variant, while the NLS, helix-loop-helix, nuclear export sequence and MYND domains are deleted (Yip et al. 2009). Recent studies have also shown that certain Rett syndrome mutations actually disrupt MeCP2 phosphorylation and interaction with the NCoR repressor complex (Ebert et al., 2013; Lyst et al., 2013). Additionally, antidepressant

treatment resulted in phosphorylation of MeCP2 (Hutchinson et al., 2012A; Hutchinson et al., 2012B). MeCP2 phosphorylation and dephosphorylation also plays an important role in BDNF transcription and learning (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006; Cheng and Qui, 2014). However, less is known about the functional relevance of Deaf1 post-translational modification. Consider also, that in our co-IPs with endogenous Deaf1 and MeCP2 we identified Deaf1 in the input but not in any of the pull-downs (Fig. 8), suggesting that the Deaf1 antibody may not have recognised this form of Deaf1. Thus it would also be important to consider whether post-translational modifications, such as phosphorylation, are necessary for Deaf1-MeCP2 interaction.

MeCP2 and Deaf1 affect HTR1A expression

After demonstrating that Deaf1 and MeCP2 interact, we explored the functional effects of either Deaf1 or MeCP2 or both on HTR1A promoter activity.

Deaf1 affects HTR1A promoter activity

In agreement with previously published data we found that Deaf1 can regulate the C-1019 (aka: 1128C) but not the G-1019 allele of the HTR1A promoter (aka: 1128G) (Fig. 11, Table 3; Lemonde et al., 2003; Czesak et al., 2006). In order to study some aspects of Deaf1's effects on the HTR1A promoter we turned to a mouse model. To demonstrate the functional specificity of Deaf1 on the mouse HTR1A promoter we showed that the Deaf1 -/- MEFs, which express no Deaf1 (Fig. 5B) had significantly higher promoter activity, which was returned to that of the Deaf1 +/+ MEFs upon co-transfection with Deaf1 (Fig. A6). In agreement with these results, Czesak and colleagues showed that Deaf1 no longer had any effects on promoter activity on a shorter 924 WT mouse HTR1A promoter that lacks both Deaf1 sites (Czesak et al., 2012). To substantiate previously published data using the human 5-HT1A promoter we also showed

that Deaf1 has repressor activity on mouse HTR1A promoter activity in the RN46A cells. However, we found that Deaf1 enhanced transcription from the mouse HTR1A promoter in HEK-293 cells, in contrast with previously published results showing repression (Czesak et al., 2012); this could be due to changes after passaging for several years or a different sub-clone. Consistent with this, Deaf1 enhancer activity instead of repressor activity at the 1128C HTR1A promoter construct has been observed previously in HEK293T cells (Pilot-Storck et al., 2010).

To determine which of the two putative Deaf1 sites in the mouse HTR1A promoter, if either, was important for Deaf1's activity we mutated the first site (m1) and both sites (m1 and m2). We also deleted the entire region between the m1 and m2 sites (61-bp), to create the full deletion mutant. However, the full deletion could have affected the HES1/5 binding site, which is located just after the Deaf1 site. The full deletion mutant also had unexpected effects that might be explained by the artificial generation of new transcription factor sites and therefore was not included in the final summary table (Table 5). The LTK cells had a relatively low transfection efficiency of 40% and very low levels of HTR1A promoter activity especially for the human constructs, preventing reliable analysis of the effects of transcription factors on HTR1A promoter activity and so were omitted from the summary table.

We observed that Deaf1 activated the mouse HTR1A promoter in the HEK-293 cells, and this effect was still present when the m1 site was mutated, but lost upon mutation of both sites (m1&m2), suggesting that the m2 site contributes to enhancer activity of Deaf1. In cells where Deaf1 has repressive activity (MEFs and RN46A cells, as well as LTK) mutating m1 eliminated Deaf1 repressor activity. This suggests that while Deaf1 binding to m2 is responsible for its enhancer activity on HTR1A gene transcription, Deaf1 binding to m1 is responsible for Deaf1's repressor activity consistent with our previous results implicating m1 in Deaf1 repression (Cze-

sak et al., 2012). Interestingly, the putative Deaf1 sites appear to act independently, since mutation of m1 did not affect Deaf1 enhancer activity.

While Jensik and colleagues have shown that Deaf1 can bind to variably spaced TTCG motifs, they stopped testing after the two TTCG motifs were separated by 11 nucleotides, which is far from the 40 nucleotides separating both mouse HTR1A Deaf1 sites. However, the secondary structure of the DNA helix may bring these sites physically proximal to each other. Furthermore, the mouse promoters presented in this project, consisted of a GTCG and CTCG motif. While Jensik and colleagues analysis of the Deaf1 binding site specifies a preference for a TTCG motif, they also showed that Deaf1 binds to a G or C -TCG and confirmed that Deaf1 binds to the HTR1A m1 site in EMSA, but with less affinity (Jensik et al., 2014).

There was also a significant increase in baseline promoter activity of the m1 mutant in the Deaf1 -/- MEFs and RN46A cells (Fig. 12B, D) and in LTK cells (Fig. 12C, and a non-significant (0.15) effect in the HEK-293 cells where Deaf1 had enhancer rather than repressor activity (Fig. 12A). Also, there was also a significant increase in baseline promoter activity for the m1&m2 mutant (and full mutant) in all cell types (Fig. 11). These increases could result from the loss of Deaf1's repressor activities on m1.

MeCP2 and DNA methylation affects HTR1A promoter activity

A part of the Deaf1 consensus binding site is a CpG site, which can be methylated. In the yeast one-hybrid system, MeCP2-GAL4AD was only able to enhance the activity of the methylated human 26bp C-1019 expression vector, an effect that was significantly reduced in the G-1019 variant, suggesting that without Deaf1 present, MeCP2 mainly binds to the methylated C(-1019) site. To examine Deaf1-MeCP2 function in a mammalian system, we demonstrated that MeCP2 increases both human (1128C) and mouse (984) HTR1A promoter activity when co-

transfected in HEK293 cells (Fig. 2). In performing several luciferase assays in HEK-293, Deaf1 -/- MEFs and RN46A cells we were able to replicate and substantiate these findings, although MeCP2 appeared to have greater enhancer activity at the mouse HTR1A promoter (Table 3, 5). Contrary to the yeast one-hybrid assay the 1128-G variant did not appear to significantly affect MeCP2 effects on gene expression (Table 3, under M). This suggests that, while MeCP2 may bind to the 1128C/G polymorphic site, it may regulate other sites in the human HTR1A promoter to yield its enhancer activity (see below). Note also that the vector used in the yeast one-hybrid system consisted of the human 26-bp Deaf1 site alone, while the vector used in luciferase assay contains the entire promoter sequence from -1128bp to the translational start site (TSS) and includes the Deaf1 site.

In analysing the mouse HTR1A promoter constructs, we observed that MeCP2 increased WT HTR1A promoter activity in all three cell types (HEK-293, Deaf1 -/- MEF, and RN46A; Table 5 under M). MeCP2 enhancer activity was lost in the m1 and m1/m2 mutants, suggesting that it requires the m1 element. Since the enhancer activity was observed in the Deaf1 -/- MEFs, it appears to be independent of Deaf1, in contrast to our ChIP results where MeCP2 binding appears dependent on Deaf1 (Fig. 9, 10). It is possible that the transfected HTR1A vector was methylated, enabling MeCP2 activity and that the promoter was not methylated in the context of chromatin in the ChIP assay, preventing MeCP2 binding in the absence of Deaf1. The methylation of the m1 and m2 sites would need to be determined experimentally to resolve this possibility. Alternatively over-expressing MeCP2 in the transfected cells may result in binding to low affinity unmethylated DNA binding sites. It is also important to consider that the transfected HTR1A luciferase constructs lack chromatin structure, which under normal conditions may prevent MeCP2 binding. It is also important to note that Deaf1 interacts with chromatin remodelling

protein interacting partner LMO4 (Joseph et al., 2014). Thus, in some circumstances, Deaf1 may be important in remodelling chromatin to permit MeCP2 binding to the promoter region. However, these potential explanations would need to be demonstrated experimentally.

In contrast, mutation of the human C(-1019)G did not affect MeCP2 enhancer activity, suggesting that MeCP2 acts at a different site. Alternatively, since inhibition from Deaf1 is lost in the mouse HTR1A mutant constructs, we may be observing a ceiling effect, where promoter activity can no longer be increased by MeCP2. For this reason and to better establish MeCP2's role, it would be important to test MeCP2 KO MEFs or knock down MeCP2. Since MeCP2 is an activator at the HTR1A receptor, we would expect to see greater baseline activity in the WT cells with MeCP2 compared to KO cells without. If MeCP2 can no longer have any effects due to the mutations of the m1 and m2 sites we would not expect any changes when we add MeCP2 in MeCP2 KO cells. However, if MeCP2 can bind to other sites in the HTR1A promoter then we would expect transfection of MeCP2 in MeCP2 KO cells to have enhancer activity similar to those that are maintained in the human 1128C/G vector.

To examine the effects of methylation on HTR1A promoter activity we transfected SKN-SH cells with a reporter construct of the human HTR1A 26-bp element where Deaf1 binds, which was either unmethylated, or methylated at particular sites. Methylation led to a significant increase in promoter activity, suggesting that a transcription factor that recognizes these marks (e. g., MeCP2) affected gene transcription. Endogenous MeCP2 was also detected in the SKN-SH cells (Fig. 5B). Furthermore, this activation occurred independently of Deaf1, which was able to repress gene activity when co-transfected. While Deaf1 repression of a methylated site is contrary to previous research (Jensik et al., 2014), Jensik and colleague's only tested this by EMSA assay where only Deaf1 (and no other transcription factors) was used to bind to the TTCG motif.

Furthermore in competition EMSA assay, methylation of the C(-1019) site did not affect its binding to Deaf1 (Czesak et al., unpublished data). In future experiments this could be repeated where MeCP2 is removed and subsequently added to demonstrate if this effect is dependent on MeCP2. It is possible that MeCP2 can primarily bind to the methylated promoter and/or depends on Deaf1 to bind to the unmethylated promoter.

Deaf1 and MeCP2 interact to differentially regulate HTR1A promoter activity

Lastly we wanted to explore the functional activity of the Deaf1-MeCP2 interaction. Initially, we performed a yeast one-hybrid assay and showed that MeCP2 acted synergistically with Deaf1 to enhance its effects of the C-1019 allelic variant. Since Deaf1 and MeCP2 have different effects on gene transcription we hypothesized that their interaction may be responsible for the differential regulation of HTR1A promoter activity observed in different cell types (Czesak et al., 2006; Fig. 11, 12) and in certain pre- and post- synaptic areas in mice (Czesak et al., 2012) and humans (Parsey et al., 2006b; Parsey et al., 2010).

In the human 1128-C construct, Deaf1 repressed gene activity while MeCP2 enhanced activity; their combination led to repression in all cell types, suggesting that Deaf1 has a dominant effect on MeCP2 activity, converting it to repression. However, in the 1128-G allelic variant we observed that Deaf1 lost all activity, while MeCP2 maintained its enhancer activity in the presence of Deaf1. This suggests that Deaf1 and MeCP2 work together to repress promoter activity when Deaf1 binds at the 1128-C allele. Consistent with this, in Deaf1 -/- MEFs, repression of the 1128-C human HTR1A promoter was greater for the Deaf1-MeCP2 combination than for Deaf1 alone, despite the enhancer effect of MeCP2 on its own (Table 3). Similarly, in HEK-293 cells, where Deaf1 is an enhancer at the mouse HTR1A, adding MeCP2 led to a significant repression of mouse HTR1A activity, apparently converting Deaf1 enhancer to repressor activity.

Additionally, in the Deaf1 -/- MEF and RN46A cells we observed a stronger inhibition of mouse HTR1A activity (-2.4 vs. -1.8 fold and -3.5 vs. -2.8 fold, respectively) when MeCP2 and Deaf1 were co-transfected vs. Deaf1 alone. These findings suggest that MeCP2 is able to specifically enhance the repressor activity of Deaf1. Further testing and statistical analysis would be necessary to test this hypothesis. Overall our results suggest that MeCP2 interacts with Deaf1 to enhance its repressor activity.

EMSA assays would need to be performed to see if this relationship is competitive (with Deaf1 binding instead of MeCP2) or synergetic (both would bind) in the human vector. Again knocking down or out MeCP2 may enable us to observe Deaf1's activity without MeCP2. From this initial data one could hypothesise that Deaf1 has enhancer activity without MeCP2, but repressor activity when it is able to interact with MeCP2. As MeCP2 is a ubiquitously expressed protein, Deaf1 and/or MeCP2 may require PTMs to be able to interact.

MeCP2 influences HTR1A expression in vivo

As discussed previously, this is the first evidence showing that MeCP2 interacts with Deaf1 and also binds to the HTR1A promoter region in a Deaf1 dependent fashion. From the luciferase assays we have observed that MeCP2 not only enhances the transcription of the HTR1A gene, but may also modulate Deaf1's effects on the HTR1A promoter region (Fig. 11, 12). Therefore we conditionally knocked out MeCP2 in serotoninergic raphé neurons at 8 weeks of age. We then challenged these mice with 8-OH DPAT, a selective 5-HT1A receptor agonist that induces hypothermia through activation of 5-HT1A autoreceptors (e. g. Richardson-Jones 2010). Considering that MeCP2 enhances Deaf1's repressor activity in luciferase assays, the loss of MeCP2 in the dorsal raphe would likely lead to an increase in HTR1A promoter activity and

functional 5-HT1A receptor levels. Richardson-Jones et al. (2010) observed a significantly greater drop in body temperature in their 1A-high mice compared to their 1A-low mice. We also previously observed that 8-OH DPAT reflected higher 5-HT1A levels in the Deaf1 mice. Thus an increase in functional 5-HT1A receptors would result in a greater decrease in body temperature. As hypothesized there was a significantly greater drop in body temperature in the MeCP2 $^{TPH2\text{-}Cre\text{-}Y/flx}$ males at all times (Fig. 13). Due to the availability of mice this experiment could only be performed in males. Similarly, we are in the process of breeding mice for immunostaining experiments necessary to substantiate these findings.

Overall these experiments demonstrate a potential for MeCP2 to regulate Deaf1's activity, which should be explored further by immunostaining for 5-HT1A receptors. Furthermore, it would be important to characterize these MeCP2 $^{TPH2\text{-}Cre\text{-}Y/flx}$ mice behaviourally, similar to what will be discussed with regards to the Deaf1 mouse line. Considering that Deaf1 has different roles pre-synaptically and post-synaptically, which we hypothesise are modulated by MeCP2, it would be important to create a mouse line where MeCP2 and/or Deaf1 can be conditionally knocked out in post-synaptic 5-HT1A expressing neurons. These experiments would lead to a better understanding of how Deaf1 regulates the HTR1A promoter, where MeCP2 and Deaf1 interact, and the effects of MeCP2 on Deaf1.

Deaf1 has functional and behavioural effects in vivo

In order to better demonstrate the role of Deaf1 in regulating the mouse HTR1A promoter, a Deaf1 KO mouse line was created (Czesak et al., 2012). In agreement with previously published data we observed a significant increase in the number of 5-HT1A receptors and TPH2-positive neurons in the dorsal raphe as well as a significant decrease in post-synaptic regions

(hippocampus and PFC) (Czesak et al., 2012). This increase in the number of 5-HT1A receptors in the dorsal raphe has also been identified in humans (Parsey et al., 2006b; Parsey et al., 2010; Lemonde et al., 2004) as has the decrease in 5-HT1A receptor density in the prefrontal cortex (Drevets et al., 2000; Sargent et al., 2000; Neumeister et al., 2004).

To demonstrate that the loss of Deaf1 does lead to functional changes in the serotonin system we performed DPAT-induced hypothermia. In agreement with Richardson-Jones et al. (2010), we observed a significant decrease in body temperature in all mice upon injection with the 5-HT1A agonist 8-OH-DPAT (Fig. 14). We also observed that the Deaf1 KO mice had a significantly greater drop in body temperature compared to the WT. This suggests an increase in functional 5-HT1A receptor levels in the raphé and is substantiated by immunostaining (Czesak et al., 2012; Fig.13). When stratifying males and females, only male Deaf1 KOs had a significantly greater drop in body temperature then WT. The variability in female temperatures in response to DPAT likely result from the effects the menstrual cycle has on 5-HT1A expression and levels (Klink et al., 2002A; Klink et al., 2002B). Recent evidence has shown sex differences in serotonin and response to stress, which are modified by gonadectomy and hormone replacement in rat (Goel et al., 2014). Testosterone treatment has increased serotonin transporter binding in transgender individuals (Kranz et al., 2014). Furthermore, 5-HT1A binding differences in the raphé are significantly greater in males (132%) than females (10.7%), demonstrating greater sensitivity (Kaufman et al., 2015A; Kaufman et al., 2015B). Nevertheless, women are also twice as likely to develop depression compared to men. Lastly, epigenetic modifications occur in a sexually dimorphic fashion (Gregg et al., 2010). These data suggest that sex steroid hormones can have a significant effect on serotonin neurotransmission, which may have affected our results here and account for the observed sex difference.

While the Deaf1 -/- mouse line was not the easiest or best model to work with due to partially penetrant embryonic lethality in the C57BL6 background of the global knockout (Hahm et al., 2004), we nevertheless performed behavioural testing. Overall, the loss of Deaf1 in male KO increased anxiety-like behaviour in most tests (EPM, LD), and reduced time spent in anxiety causing environments in most tests (OF, LD) compared to the WT (Luckhart, 2014). Vulto-van Silfhout and colleagues (2014) noted similar difficulties in using full Deaf1 KOs and instead used a conditional Deaf1 knock out ($Deaf1^{fl/fl;\ Nes\text{-}Cre}$), where Deaf1 was only knocked out in the brain using a Nestin-Cre mouse. In agreement with our results, they also demonstrated that nestin-cre conditional Deaf1 KO male spent significantly more time in the close arms of the EPM and traveled a smaller distance in the EPM (compared to controls). These mice also travelled less to the center zone in the open field. However, the heterozygous mice ($Deaf1^{+/fl;\ Nes\text{-}Cre}$) showed no differences in behavioural tests. Vulto-van Silfhout and colleagues, did not however test anxiety in female mice or directly relate these behavioural effects to changes in the serotonin system (Vulto-van Silfhout et al., 2014).

Similarly to the Deaf1 KO males, Deaf1 KO and HET females showed a significant anxiety-like phenotype in the EPM, but not in the OF or LD tests compared to the WTs (Luckhart, 2014). Both males and females nevertheless showed increased freezing behaviour in all tests compared to WT, perhaps unduly increasing time spent in anxiety-causing areas of these tests (Luckhart, 2014).

Considering that the Deaf1 KO mice have higher levels of 5-HT1A autoreceptor and lower levels of serotonin in the raphé (Czesak et al., 2012) we would expect a depression-like phenotype. To analyse this we performed the TS test and FS test, which are more accurately used to measure antidepressant activity (Garcia-Garcia et al., 2013; Albert et al., 2014). However, the

Deaf1 KO male and female showed no significant differences compared to the WT in the TS or FS tests (Luckhart, 2014). As a result, we performed the sucrose preference test, which is more specific to depression-like behaviours (i. e. anhedonia-like) and similarly found no significant differences (Fig. 16). While these tests may not effectively model depression in human, where major depression has been associated with the G(-1019) HTR1A allele (Albert et al., 2011), Richardson-Jones and colleagues demonstrated that high 5-HT1A autoreceptor expressing mice similarly demonstrated no overt depression phenotype. However, when the mice were subjected to repeated mild stress the high 5-HT1A expressing mice displayed decreased mobility in the FS and TS compared to their low 5-HT1A counterparts (Richardson-Jones et al., 2010). In addition environmental factors influence the development of depression via changes in gene methylation and expression (Sun et al., 2013), which can be influenced by gene polymorphisms (Albert and Lemonde, 2004). Likewise, it is also possible that the above mentioned compensatory mechanisms may counteract the loss of Deaf1 or higher levels of serotonin, which may be difficult to uncover unless the system is stressed. Thus it would be important to consider the effects of stress on depression in future experiments. Overall these behavioural results lend support to the model that Deaf1 influences HTR1A transcription which results in functional effects in the mice.

Overall conclusions

Taken together, these results in the mice, our luciferase expression assays, where the loss of Deaf1 (in the Deaf1 -/- MEFs; Fig. A5) and/or the loss of the mouse #1 Deaf1 binding site (in the Deaf1 -/- MEFs and RN46As; Fig. 12) led to an increase in HTR1A transcription support the model that Deaf1 is an important inhibitory transcription factor in regulating the HTR1A promoter activity. Our luciferase expression assays where Deaf1 led to an increase in gene transcription in the HEK-293 (Fig. 12), an effect that is lost in the m1&m2 mutant, as well as immu-

nostaining results in the mice showing that loss of Deaf1 in certain post synaptic regions (PFC) leads to a decrease in 5-HT1A receptor also demonstrate Deaf1's important enhancing effects on HTR1A promoter activity in post-synaptic brain regions. Our ChIP results where Deaf1 specifically enriched the HTR1A promoter region also provide support for the above models (Fig. 9, 10). However, better animal models, such as conditional Deaf1 KO, are needed to clearly demonstrate these effects without confounding factors (severe effects of full Deaf1 KO and adaptation to the mutation). Mice models to examine the role of the HTR1A promoter would also be useful. For example, a m1&m2 mutant HTR1A mouse knock-in model would be useful to mimic what occurs in the human 1128-C/G polymorphism and to determine the role of these sites and Deaf1 binding on HTR1A promoter activity.

Our results show that MeCP2 does interact with Deaf1, which recruits MeCP2 to its site on the HTR1A promoter, suggesting an important role for MeCP2 in HTR1A promoter regulation and depression. Furthermore the augmented DPAT-induced hypothermia observed in the MeCP2 cKO suggests that MeCP2 has a functional role in regulating HTR1A expression in 5-HT neurons in vivo. This could be better supported by immunostaining and electrophysiological experiments showing that the loss of MeCP2 leads to a greater 5-HT1A response to 5-HT1A agonists (8-OH-DPAT). We demonstrated (via luciferase and yeast one hybrid assays) that MeCP2 can regulate HTR1A transcription and enhance Deaf1's effects. The effects of methylation on Deaf1 and HES binding sites also provide a possible mechanism as to how environmental and genetic factors can interact by recruiting MeCP2 to different sites on the promoter. This may also help us to better understand mechanistically how environmental factors can affect depression. One possible mechanism, as suggested here, is through gene methylation and how methylation in turn affects MeCP2 and Deaf1 interaction.

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Appendices

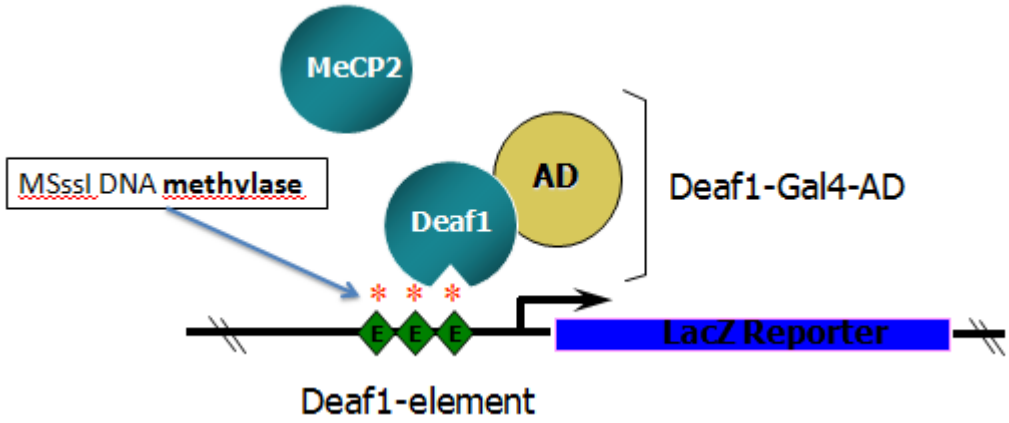

Figure A1. Graphical representation of the Yeast-one hybrid methylation experiment. The model shows the p8op-3RC or -3RG construct containing 3 copies of the C- or G-allele of the HTR1A 26-bp Deaf1 element (E) is fused to LacZ reporter gene. This construct can be activated by transducting Deaf1-Gal4AD (pACT2-Deaf1), which binds the Deaf1 C-allele to induce βga-lactosidase, but not at the G-allele. The effect of MeCP2 on Deaf1 is tested by co-transducing pRS423-MeCP2 and Deaf1-GAL4; while the direct effect of MeCP2 is tested using MeCP2-Gal4AD (pACT2-MeCP2). The p8op-3RC (2 CpG sites/element) or -3RG (1 CpG site/element) constructs can be methylated (red *) by co-transducing pA7WA-MSssI DNA methyltransferase in the yeast 1 hybrid system. From Paul Albert.

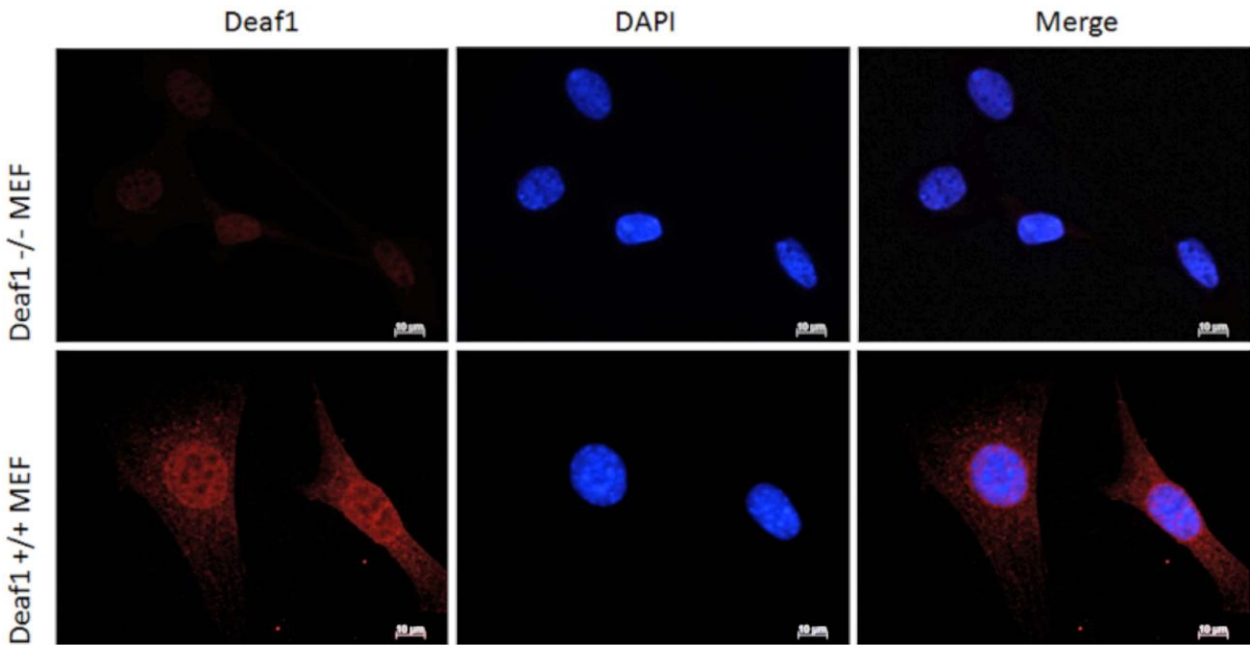

Figure A2. Specificity of the Deaf1 antibody. To verify the specificity of the Deaf1 antibody for Deaf1, immunostaining was performed on Deaf1 +/+ and -/- MEFs using 1:10, 1:25, 1:50 (1:25 shown) of the homemade rabbit Deaf1 IgG, probed with 1:1000 CY3 fluorochrome-conjugated secondary antibody against rabbit IgG (Jackson Immuno Research). DAPI was used to identify cell nuclei, merged in 3rd pannel. Scale bar 10μm**.**

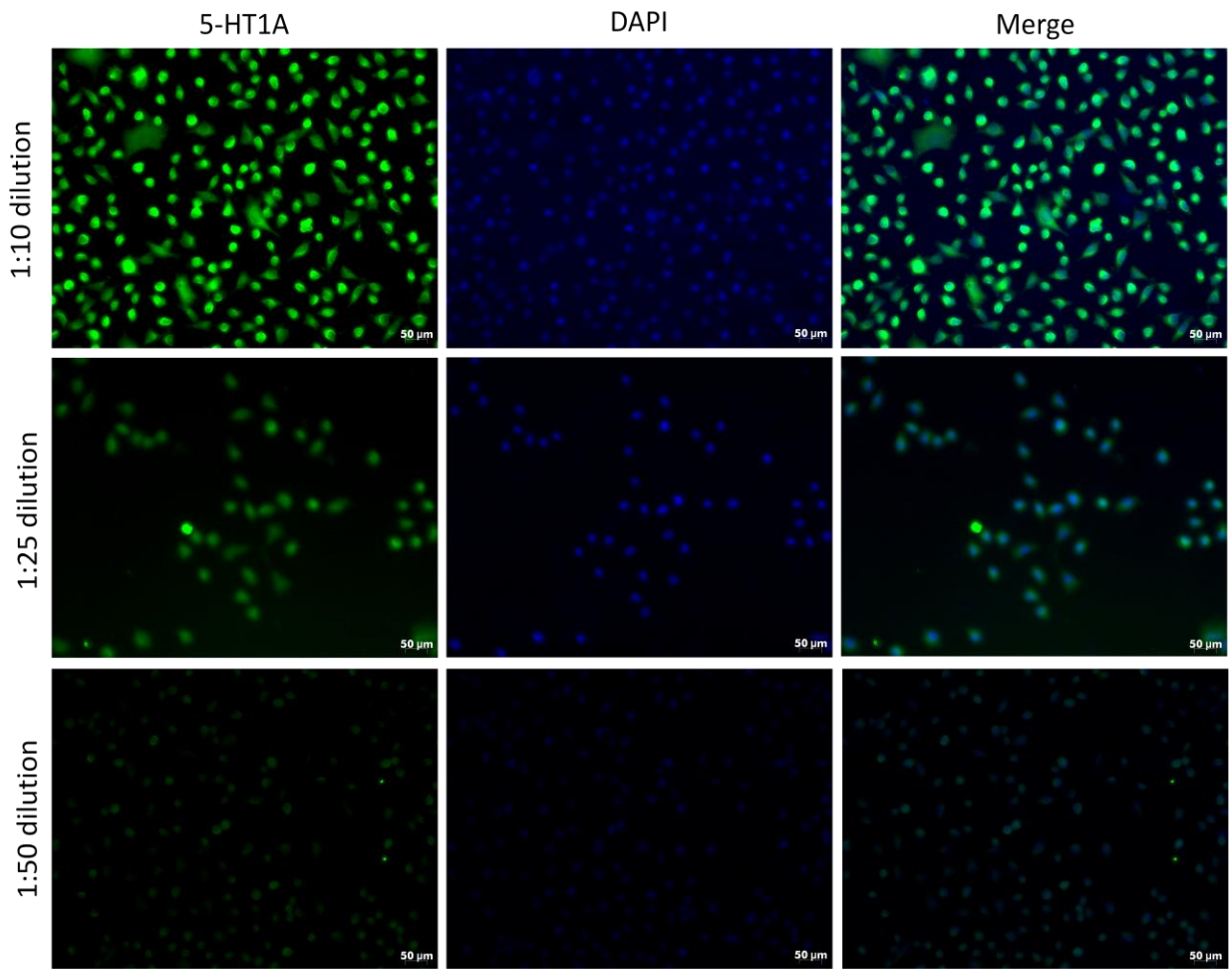

Figure A3. 5-HT1A antibody staining. To validate the 5-HT1A antibody, immunostaining was performed on 5-HT1A-expressing LTK cells with the homemade rabbit 5-HT1A IgG post-purification (1:10, 1:25, 1:50), followed by green fluorochrome-conjugated secondary anti-rabbit (Jackson Immuno Research; 1:1000), and DAPI staining, merged in third panel. Scale bar 50µm.

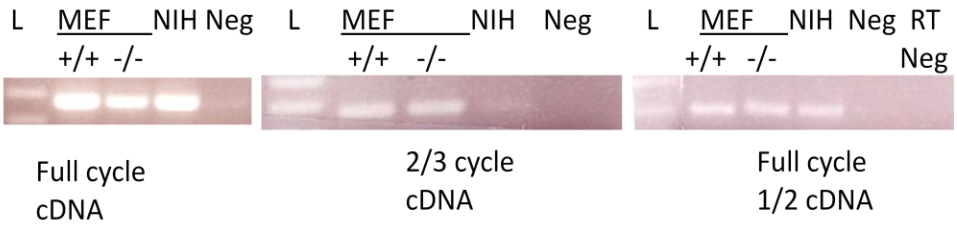

Figure A4. MeCP2 mRNA levels are similar in Deaf1 +/+ and -/- MEFs. RNA was harvested from MEFs and mouse NIH-3T3 cells, and used for RT-PCR analysis. The PCR reaction was performed at full cycle, 2/3 cycle and with half of the cDNA. In each case a similar level of PCR product was detected. The molecular size marker (L) 300 bp and buffer control (Neg) or MEF RNA reaction lacking RT (RT Neg) are shown as negative controls.

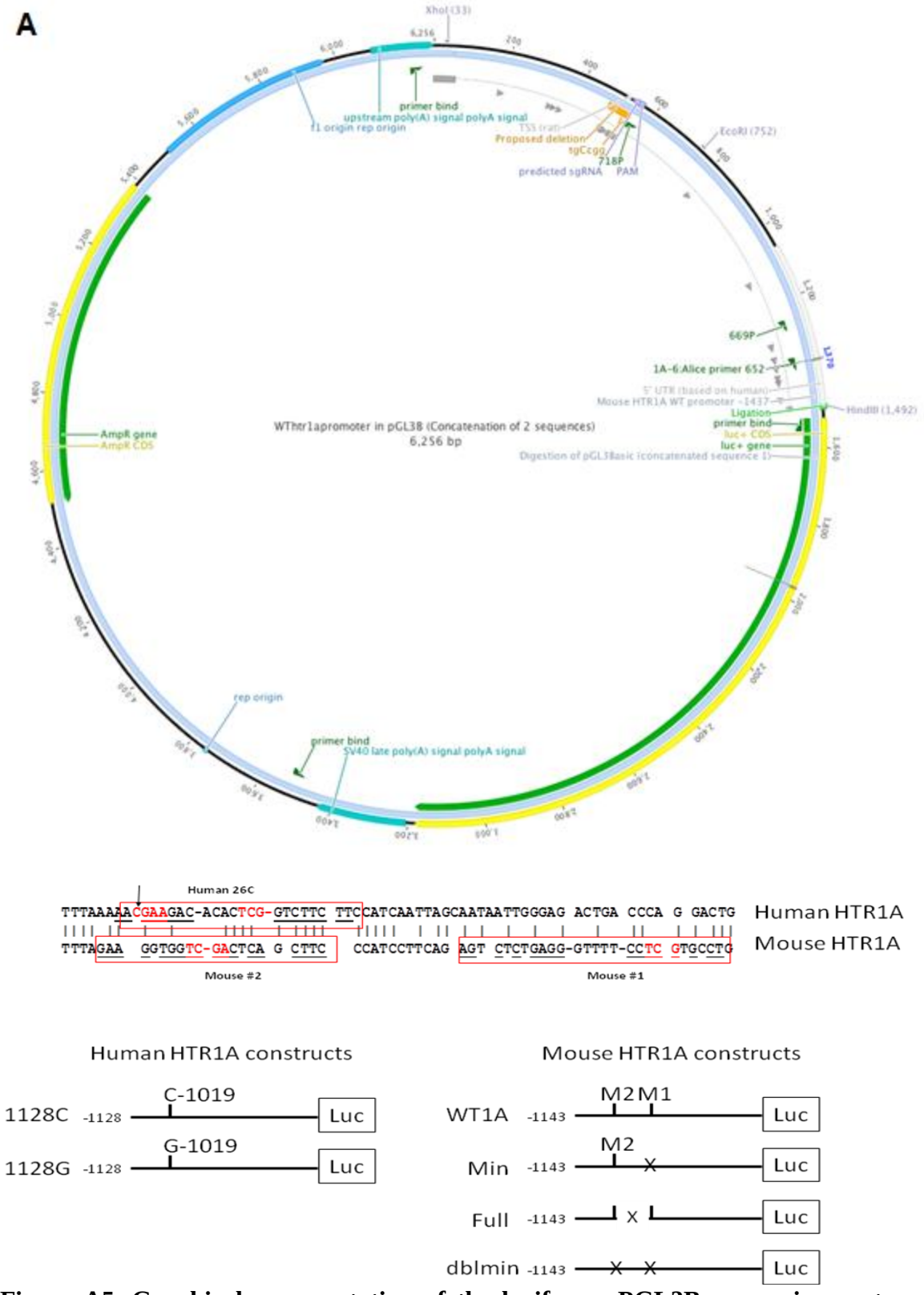

Figure A5. Graphical representation of the luciferase PGL3B expression vector and inserted mouse (A) and human (B) HTR1A constructs with the C/G-1019 variant; mouse #2 and #1 Deaf1 site (in pink) as well as proposed mutations. In the m1 mutant the CTCGTC is

changed to TGCCGG; In the full mutation both Deaf1 sites are deleted as denoted by proposed deletion, in the m1&m2 mutant the GTCGAC of the 2nd Deaf1 site and the CTCGTC of the first Deaf1 site were replaced with TGCCGG.

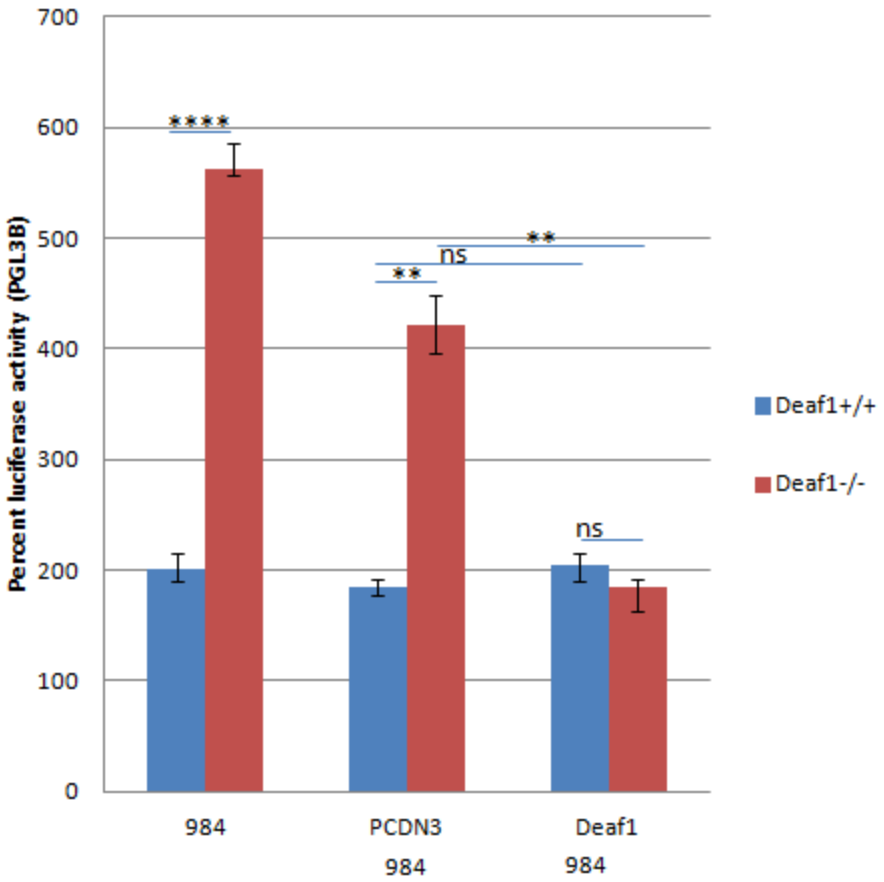

Figure A6. HTR1A promoter expression vector (984) is significantly more active in Deaf1 KO MEFs (red) compared to WT MEFs (blue). This over activity is lowered back to that of the WT upon co-transfection with Deaf1. Co-transfection with Deaf1 has no effects on 984 activity in WT MEFs. All luciferase values were normalized to B-gal and then normalized to the empty PGL3B vector. ns = non significant; **<0.01; ****<0.0001. See table A1 for statistical analysis.

RM one-way ANOVA		Post hoc			
		Expression vector	Transcription factor	MEF cell line	P =
F	15.58	984	None	WT vs KO	**<0.0001**
df	5	984	PCDNA3	WT vs KO	**0.0018**
p	**<0.0001**	984	Deaf1	WT vs KO	0.9991
		984	PCDNA3 vs Deaf1	KO	**0.0019**
		984	PCDNA3 vs Deaf1	WT	0.9989

Table A1. HTR1A promoter expression vector (984) is significantly more active in Deaf1 KO MEFs compared to WT MEFs. Statistical analyses using a repeated measures one-way ANOVA analysing the effects of transcription factors on each expression vector individually of the other. Bold indicates significance.

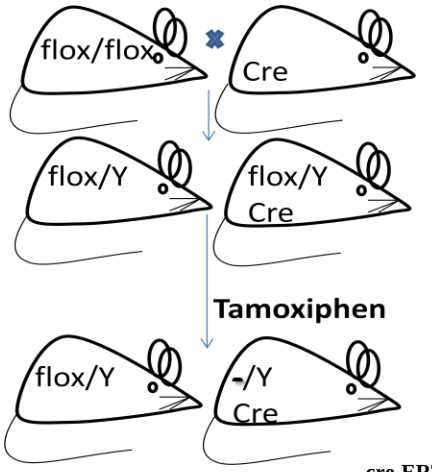

Figure A7. MeCP2-TPH2$^{cre\text{-}ERT2}$ mice breeding plan. Where flox represents MeCP2 surrounded by two flx sites, with no MeCP2 present on the Y chromosome, and cre represents TPH2$^{Cre\text{-}ERT2}$. All mice were given 180 mg/kg tamoxiphen daily injections for four days at 8 weeks of age.