Significance

Stress is an unavoidable facet of modern life, but dysregulation of the stress response can lead to severe negative consequences. Exposure to unavoidable stress has been implicated in many psychological disorders, such as depression[1], anxiety[2], and post-traumatic stress disorder[3]. Several classes of anti-depressants offer treatment options for these disorders, but the effects are often only slightly better than placebo[4], and highly variable[5], suggesting other factors have not been accounted for. Few genetic components have been found to account for and explain the psychological outcomes to a dysregulated stress response, and even the known loci cover only a small fraction of the variability[6].

The field of epigenetics examines the interaction of environmental stress and gene regulation to explain individual differences in stress responsivity. Epigenetic changes in the expression of the glucocorticoid receptor (GR) in response to stress has been particularly relevant for understanding the complexity of stress on cellular mechanisms through its role as a transcription factor[7]. However, the study of the GR has been mostly limited to the 5% of DNA that comprises protein-coding genes. Transposable elements (TEs) make up at least 50% of mammalian DNA, and are differentially transcribed in response to stress. TEs have been shown to be epigenetically silenced through trimethylation of histone 3 lysine 9 (H3K9me3)[8], putatively by the GR-regulated histone methyltransferase SUV39h2[9]. The necessity of this transcriptional silencing is unknown, but increases in TE transcription have been linked to . It is possible that GR, in addition to downstream silencing through histone methylation, also directly drives the expression of TEs. GR is also known to interact with TEs in cancerous cells[10], and it is likely that they contribute to the transcription of TEs in response to stress. Other steroid receptors are vital to the transcription of genes evolved from TEs, including androgen[11], and estrogen[12] receptors.

The proposed research uses an adrenalectomized rat model to investigate GR-mediated transcription of transposable elements in response to corticosterone injection. Found transcripts are expected to be uniquely regulated in the rat hippocampus. After transcripts are identified, over/under expression will reveal any functional significance to spatial memory impairment or anxiety—two robust stress responses. This research could provide a new perspective on the causes of psychological disorders, as well as new targets for treatments.

Stress

Stress is the biological reaction to environmental cues that are interpreted as a threat toward homeostasis or wellbeing, whether real or imagined. Stressors can be acute or chronic, physiological or psychological. Each stressor increases the allostatic load[13], or the cumulative deviation away from homeostasis in an organism. In normal situations, an individual will return to baseline after the stressor is overcome; however, if not allowed to return to baseline, the body will experience heightened subsequent responses, stacked on the previous stressors. Some stressors, notably when the individual feels in control, can provide physical and psychological benefits[14,15]. The inverse is that when an individual feels a lack of control, the physiological and psychological effects can be detrimental[16]. Likewise, overcoming a stressful situation of either type leads to resilience and preparedness for future uncontrollable events[17]. Positive and negative stressors are only separated by their circumstances and outcomes, but utilize the same common pathway.

The initiation of the mammalian stress response can involve many different brain areas; however, they all activate a common pathway, starting in the periventricular nucleus of the hypothalamus. Activation of parvocellular neurons releases corticotropin-releasing hormone and arginine-vasopressin into the pituitary portal. The two signaling molecules find their targets in the anterior pituitary and increase the production of proopiomelanocortin, which is then used to synthesize adrenocorticotropic hormone (ACTH). ACTH is released into the bloodstream and binds to receptors in the adrenal gland[18]. This is referred to as the HPA axis.

The adrenal gland produces corticosteroids (mainly corticosterone in rodents, and cortisol in

humans; CORT) throughout the day in circadian and ultradian rhythms, but most is bound by cortisol binding globulin (CBG). During a stress response, CBG is saturated and the level of free corticosteroids raises dramatically, making it biologically available. CBGs also release corticosteroids in response to physical stressors such as heat or changes in pH[19]. Free corticosteroids bind to and quickly saturate mineralocorticoid receptors, and activate glucocorticoid receptors (GR), considered the main stress hormone receptor. Upon activation, GRs in the cytosol translocate to the nucleus and serve as transcription factors. GRs have an inhibitory effect on stress-related gene expression in the PVN and anterior pituitary. This negative feedback shuts down the stress response at the source, and represents the main mechanism of restoring the HPA axis to baseline levels. GRs also have a longer influence through the upregulation of genes in the brain.

At the molecular level, the line between epigenetic mechanisms and the transcriptional machinery is not easily established, as the two processes are intimately intertwined, both structurally and functionally. Several epigenetic marks control the ability of transcription factors, such as GRs, to bind to DNA.

Histones are packaging proteins that form octamers called nucleosomes, and help transition DNA stands between tightly packed heterochromatin, or loose and easily accessible euchromatin. Each of the eight histones in the complex have a modifiable N-terminal tail which controls the chromatin remodeling. The tails can be acetylated, methylated, phosphorylated, or ubiquinated. The combinations of these marks has been referred to as the "histone code[20]," because they are highly specific to the cell type and state, and some, such as SUVRh2, only interact with one mark. Gene expression and epigenetic marks are cell and tissue specific, and changes observed in one area of the brain may not be the same in another brain structure[21].

Acute stress is a common occurrence, and brains are relatively well-equipped to respond to it. Short-term stress normally has few negative aftereffects, yet severe transient stressors can leave a lasting imprint. Fear conditioning is a reliable example, often being acquired in one trial. It can result in drastic and long-lasting epigenetic changes in several brain areas, including the amygdala[22], hippocampus[23], and mesolimbic dopaminergic system[24]. Several epigenetic regulators have previously been demonstrated to change in response to fear conditioning, such as methyltransferase expression[25].

In the hippocampus, stress, through GR activation, has been shown to affect spatial memory[26], excitatory and inhibitory neuron reactivity[27,28], neurogenesis[29], factors associated with neuronal plasticity[30], and epigenetic marks of several genes[31,32]. The glucocorticoid receptor itself is epigenetically regulated in the hippocampus in response to early life stress[33]. These widespread changes result in a web of regulatory interactions that are still not fully understood. Non-coding RNA (ncRNA), such as microRNAs[34,35], and long non-coding RNAs[36] in the hippocampus contribute to this diversity. A majority of known lncRNAs are derived from transposable elements[37].

Transposable Elements

Transposable elements (TEs) represent a new frontier for both epigenetics and genomics in the brain, and it has been established that they are regulated by stress[38], though the exact function of this regulation is still unknown. It is hypothesized that GR control of TE expression adds fine-tuned variability to neuronal responses to environmental stressors, and that their dysregulation can lead to the detrimental consequences of stress[39].

TEs fall into two main classes: retrotransposons (Class I) and DNA transposons (Class II). The three types of retrotransposons are long and short interspersed nuclear elements (LINEs and SINEs, respectively) and long terminal repeats (LTRs). Full-length retrotransposons are capable of multiplying in the genome via a "copy-paste" mechanism through translation of retrotransposon proteins, while functional SINEs and truncated retrotransposons rely on the translation of LINE-encoded endonuclease and reverse transcriptase. They are then reverse transcribed and inserted into the genome. SINES and LTRs LINEs are most commonly found in intergenic and scaffolding regions of DNA, whereas SINEs

are more frequently found in promoters[40], though not always. DNA transposons use a "cut-and-paste" mechanism to shuffle themselves throughout the genome. Both classes have few intact, inheritable genes left, but their remnants are scattered throughout the mammalian genome, comprising up to 50% of mammalian DNA.

TEs have several known mechanisms through which they influence cell activity. The first, and most easily identifiable through sequencing, is direct transposition, or their ability to move within the genome. If an insertion occurs within a functional gene, it can cause a loss of function, as has been documented in several dozen cases, with many more hypothesized[41]. A LINE transposition can also rearrange non-TE elements in its 3' UTR, including moving an entire protein-coding gene into another, or shuffling exons between genes, increasing genetic variability and contributing to the creation of new proteins[42,43]. LINEs can also function as anti-sense promoters of genes on the opposite strand[44]. In other cases, SINE transposition has been shown to rescue the function of a pseudogene in primates by lending a functional promoter to previously untranscribed DNA[45]. The synthesis of CORT itself is regulated by a promoter containing a SINE retrotransposon[46]. Transposition can lead to the creation of chimeric transcripts that are part TE, part gene, and/or part pseudogene, as is the case of the *SETMAR* methyltransferase[47]. The fusing of a DNA transposon to a methyltransferase (H3K36, a silencing mark) made the extant 1,500 *mariner* binding sites available to the new protein, a poignant example of the potential influence TEs can have to influence epigenetic regulation on a large scale. It is possible that other transcriptional machinery, such as GR, has made use of these readily available sites for regulation purposes.

Exaptation, or using sequences copied throughout the genome for regulatory purposes, has been predicted to compose 20% of all evolutionarily constrained regulatory elements[48]. Small pieces of deactivated TEs that are left over from ancient transposition events can be domesticated by the cell as a controlling element for gene expression[49]. These "leftovers" have different functional capabilities depending on where they are in relation to the gene. 25% of promoter regions include TEs, increasing in number further upstream of the transcription start site[50]. TEs in promoters have been shown to have cell type-specific effects on transcription[40,51], suggesting that their inclusion is not parasitic, but that they have been at least partially domesticated as a means to regulate gene expression[49]. The 3' UTR of protein-coding genes contain more TE fragments than in their promoters[50]. Interestingly, 3' polyadenylation of mRNAs may have originated with retrotransposon integration into protein-coding genes[40], and TEs can create cryptic polyadenylation signals, resulting in different length transcripts[52]. LTRs found in the 3' UTR of genes can reduce the transcription of that gene through epigenetic silencing[53], and LTRs found upstream of a gene can act as alternative promoters[54]. TE open reading frames included in translated peptides can change their solubility[55], resulting in different localization of proteins, and has been shown to have functional consequences[56]. A primate-specific SINE exaptation by the vitamin D pathway in an example of the powerful benefits

The third mechanism of TE influence on gene expression is through chromatin remodeling, or the interaction of proteins and DNA that changes its availability for transcription. TEs are present in higher concentration in scaffolding and matrix attachment regions than the overall genome[40]. They play an important role in the formation of heterochromatin, and may be evolutionarily responsible for telomeres and centromeres[57]. Heat shock proteins form stress granules and bind to satellite repeats in heterochromatin, thought to diminish aberrant transcripts during heat stress[58].

TE expression in the rat hippocampus was decreased in acute stress through H3K9me3 enrichment preferentially near retrotransposons[8,59,60]. Trimethylyation of H3K9me3 silences transcription through the recruitment of HP1 proteins[61] and the facilitation of heterochromatin[62], but not when H3K9 has already been acetylated[63], suggesting precise control over retrotransposon expression. H3K9 is trimethylated by the methyltransferase *Suv39h2*, which showed increased GR binding along with upregulation in response to stress[9].

GRs have been known to regulate retroviral elements for decades[64], and it is likely that in

addition to the silencing effects of GR-SUVR39h2 interactions, GRs utilize one or more of the previous methods of TE regulation in response to stress. Because TE transcription can also increase—even in epigenetically silenced regions—in response to stress[65], more research is needed to unravel the complex relationship between glucocorticoid receptors and their influence on and the function of TE expression.

Outline of Proposed Experiments

1. Do GRs drive the transcription of TEs?

GRs are the obvious target of investigation into a mechanism for stress-based influence of TE transcription due to their role as transcription factors. They are known to regulate gene transcription in response to stress, and chromatin remodeling[66]. Cell-specificity of GR binding depends on pre-determined availability of euchromatin[67], possibly explaining the hippocampal-specific nature of increased transposable element expression in response to acute stress. Only 40% of hippocampal GR binding is within genes or their promoters, with the remaining 60% lying in intergenic regions[68]. A simple motif search is not sufficient for finding GR binding sites, as 20% do not contain any form of a consensus GR response element[67]. Chromatin precipitation paired with next-generation sequencing (ChIP-Seq) is required to discover all GR binding sites in different biological conditions. It is expected that several TEs will be differentially regulated in the hippocampus response to corticosterone challenge in adrenalectomized rats.

1.1 What GR binding sites are different in acute stress vs. corticosterone challenge? Previous GR ChIP-Seq experiments in adrenalectomized rats used older sequencing technology and pooled hippocampi from three animals; a replication of this work would provide additional insight into the nature of GR binding variability between subjects. The technique will then be expanded to look at the GR binding differences in acute stress, for which other data already exists. Groups will consist of six rats each, in accordance with previous research. Part of this work will be testing newer GR antibodies and comparing them to established antibodies. Acute stress and corticosterone challenge should have different GR binding profiles.

1.2 Do GRs bind upstream from transcribed TEs? Using RNA-Seq datasets, functional transcription for both conditions will be compared to the ChIP-Seq data. The datasets from each condition discovered differentially expressed transcripts. Differentially expressed transcripts from each condition will be compared to the respective GR ChIP-Seq dataset, capturing any transcripts that are 1000 basepairs or less downstream from a GR binding peak. This will then be pruned down to TE transcripts, producing a list of differentially expressed TEs within 1000 basepairs downstream of GR binding. The tools for this analysis have already been piloted and suggests that this does occur[69]. This data will show the amount of transcription in acute stress that is purely GR mediated, and which TE transcripts are context dependent.

2. What is the functional significance of GR binding in relation to TEs?

Do transcribed TEs in the hippocampus have any functions? The most traditional, but least likely, is that they are translated into peptides or proteins. This is unlikely because the functional protein profile of stress is very well characterized. More likely the transcripts will belong to one of several classes of non-coding, regulatory RNAs. GR binding can initiate transcription, but it also plays a role in chromatin remodeling. What is unknown is whether GRs binding to TEs is part of a larger heterochromatin formation pathway.

2.1 Are there any identifiable motifs or consensus sequences in the transcribed TEs that can be found in *silico*? Do transcribed TEs have any known regulatory functions or translatable proteins? Previous studies have found that 83% of all known mammalian lncRNA contain at least one TE, most notably ERVs, but few SINEs or LINEs[70]. Identified transcripts will be analyzed with a database of known ncRNAs in rats. There are also identified micro-RNAs that are made from transposable elements[71]. TEs that have a change in regulation due to GR or stress could be known ncRNAs, though it

is also possible that the identified transcripts constitute new lncRNA genes that have yet to be discovered, which can then be annotated to contribute to the growing list of regulatory features. It is possiblel that unknown transcripts will contain recognizable elements, such as DNA binding sequences or pieces of other ncRNAs. TE transcripts will be examined for any clues as to their function, and possible regulatory targets. Transcripts will also be searched for open reading frames (ORF) using the NCBI ORF-Finder[72] to check for the possibility that the transcribed TEs are not encoding peptides or proteins. In concert with ORFs, the transcripts will be searched for the inclusion of a polyadenalyted tail, which would also hint at the possibility of translation.

2.2 Does GR work in concert with other transcription factors (TFs) to promote heterochromatin formation? TFs are hypothesized to interact with TEs in creating regions of heterochromatin[73], which is, in part, maintained by SUV39H-mediated methylation of histone H3[74], and preferentially around retrotransposons as reported previously in stress[9,75]. Because of GRs proposed influence on the upregulation of SUV39H2, it would be useful to look at GR binding to TEs away from any known transcripts to determine if GR is playing a role in the formation and maintenance of heterochromatin. GR ChIP sequences will be analyzed with Transcription factor Affinity Prediction (TRAP) software[76], which given a set of sequences, can determine which transcription factors will bind in that area. If GR peaks are also high for other transcription factors, the identified peaks can be checked for alternative transcription factor binding by using ChIP combined with qPCR, or by using data already available in the Gene Expression Omnibus[77]. GR binding away from transcripts may help recruit other transcription factors to promote heterochromatin formation.

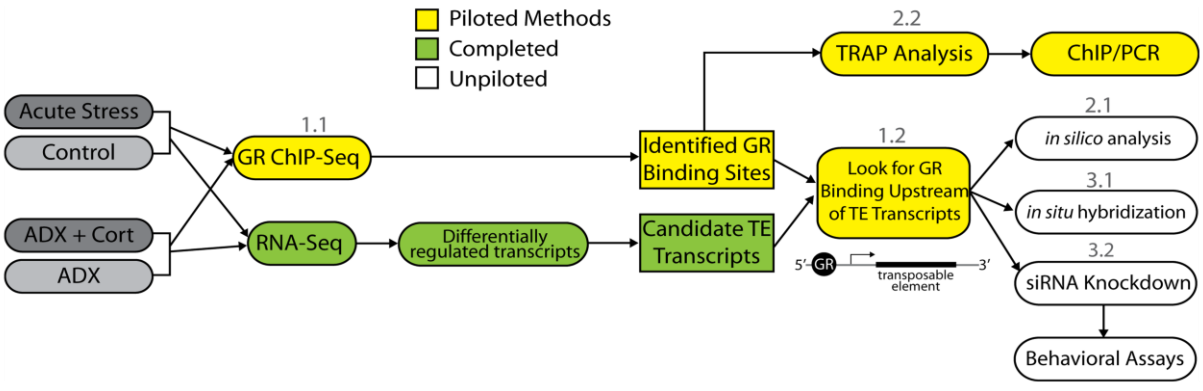
3. Anatomical distribution and behavioral studies

Once the function of GR-mediated transcribed TEs has been narrowed down, it is important to determine whether or not they have behavioral effects. An important subset of this is knowing where the transcripts are expressed in the brain.

3.1 Where are the identified TE transcripts expressed in the brain? *In Situ* Hybridization (ISH) will provide spatial visualization of the top candidate TE transcript. It is expected to be observed in the hippocampus, but could because GRs are found in almost every brain cell, it could be upregulated elsewhere. Determining if the transcript is hippocampus specific could provide answers to the hippocampus specific effects of h3K9me3 silencing of retrotransposon expression. If it is widespread, it could help explain the varied physiological symptoms of stress.

3.2 After figuring out where the transcript is expressed, it will be important to determine if the transcript contributes to common behavioral phenotypes in response to stress. Using silencing RNA (siRNA), the transcript can be effectively knocked down. Behavioral tests will be conducted on rats with siRNA, or scrambled for control, will be subjected tests of spatial learning (novel object test)[78] and anxiety (elevated plus maze, open field)[79]. Differences in stress behaviors should be apparent if the TE transcript has functional relevance to the pathology of stress-related disorders.

Figure 1. Flowchart of proposed experiments and methods.



Data Analysis

A significant portion of the proposed research involves building a powerful computer, installing the necessary tools, and maintaining the system. The Galaxy project will be the preferred UI for interacting with common NGS analysis tools such as the Tuxedo Suite for RNA-Seq analysis, and MACS for ChIP-Seq analysis. All of the software in this proposal has been tested with pilot datasets.

Methods

Animals. Male, adult Sprague-Dawley rats (aged 60 days) will be purchased through Charles River Laboratories. Upon arrival, rats will be observed for one week to check for illness or injury. They will be triple housed in the UMass Boston Integrated Sciences Animal facility in polycarbonate cages with *ad libitum* access to food and water. Humidity is kept at [35%] and temperature at [72° F]. Rats will be on a LD cycle of 12/12. Rats will be handled minimally during cage cleanings.

Adrenalectomy. Animals will be anesthetized with 3% isoflurane (Vet One, India) in a plastic induction chamber. Once unconscious they will be removed and shaved on both flanks, and then put back into the induction chamber. When they are unresponsive again they will be transferred to the operating bench, and isoflurane and oxygen flow is diverted to a nosecone placed over their nose. The shaved flank is rubbed with betadine swabs, and then a small incision (about an inch to an inch and a half) is made on one side, beneath the ribs. The adrenal gland is located and removed with forceps by pulling it away from the abdominal fat. The incision is sutured and the skin is autoclipped. The wound is swabbed with betadine again, and then the rat is given an injection at the incision site of .5% lidocaine and .25% bivuricane for pain management. The operation is repeated on the other side, and then the rat is transferred to an empty cage with a heating pad to recover. The rat is marked with a Sharpie on its tail for identification. The total time under anesthesia is around 15 minutes.

Injections. A complex of corticosterone and 2-hydroxypropyl-β-cyclodextrin (Sigma) is resuspended in 0.9% saline. Three days after adrenalectomy, half of the rats will be given an intraperitoneal injection of 0.250 mL corticosterone and returned to their home cage. The controls will be given an equal volume of 0.9% saline and returned to their home cage. All rats in a cage will be given the same treatment to minimize cross-contamination.

Tissue Harvest. Exactly one hour after injection, the rats will be removed from their cages and live decapitated using a guillotine. Trunk blood is collected and immediately cooled at 4° C until the end of tissue harvesting. The brain is removed and placed on a kimwipe wetted with 1x PBS. The cerebellum, frontal cortex, and both hemispheres of the hippocampus will be collected, transferred to separate Eppendorf tubes, and flash frozen on dry ice as they are removed. Samples will be stored at -80° C until further use. Blood is spun down at 3200 rcf at 4° C for 10 minutes. The plasma is then pipetted out into Eppendorf tubes and also stored at -80° C until further use.

ELISA. Corticosterone ELISA kits from Enzo Life Sciences will be used to assess relative corticosterone levels in blood plasma. Briefly, the plasma is diluted 40:1 and undergoes a steroid displacement procedure. After an hour incubation, standards and samples are added to a 96-well plate provided by the kit. After a series of incubations and washes, the reaction is stopped and read on a [brand, model] plate reader at 405 nm wavelength to obtain absorbance values. The samples are then fit to the standard curve to obtain absolute corticosterone concentrations.

RNA Extraction. Before extraction, the RNase free area of the lab bench is wiped down with 70% Ethanol, followed by ELIMINase (Decon Labortories). Other equipment that will be used for the extractions is wiped down with a kimwipe soaked in 70% ethanol (centrifuge, pipettes, tips boxes). RNA is extracted with an RNeasy extraction kit for lipid-rich tissues (Qiagen) according to the protocol provided by the manufacturer, with a few modifications. Briefly: samples are thawed in 1 ml of room temperature Qiazol lysis reagent (Qiagen). Tissue disruption is done with a desktop sonicator, using an alternating 2-second burst, 1-second rest at 40% amplitude power for 4 cycles, cleaning with Ethanol between samples. 200 mL of chloroform is added to each sample. The sample is shaken for 15 seconds,

then allowed to incubate at room temperature for three minutes. The sample is then spun down at 12,000 rcf for 15 minutes at 4° C. The aqueous top layer is pipetted into a new Eppendorf tube and combined with 1 volume of 70% ethanol and then vortexed. The solution is transferred into an RNeasy spin column, and spun for 30 seconds at 8,000 rcf at 4° C (same for all subsequent spins). The flow through is discarded, and the column is washed with kit-supplied washes. On the final wash the column is transferred to a new collection tube, allowed to air dry for 1 minute, and then RNA is eluted in 50 µL nuclease-free water. RNA is quality checked on a Nanodrop2000c. If RNA is to be sequenced, the samples are split in half for later PCR verification. Samples will be stored at -80° C until further use.

Reverse Transcription. RNA is thawed on wet ice. Components from the Quantitech Reverse Transcription kit (Qiagen) are thawed at room temperature and briefly spun down to collect all condensation off of the sides. The reverse transcription is done per the protocol provided by the manufacturer. Briefly: 1 µg of RNA is added to 1 ml of gDNA wipeout buffer and brought to a total volume of 14 µL in a .2 mL PCR tube. This is incubated at [30?] ° C for 15 minutes in a [brand, model] thermocycler, and then immediately placed on ice. Reverse Transcriptase, RT Buffer, and primers are added to the reaction to a total volume of 20 µL, then incubated in the thermocycler for [time]. The cDNA is quality checked on the Nanodrop2000c and stored at -80° C until further use.

Nanodrop. Both RNA and cDNA must be quality checked and quantified before moving to the next respective step for each. The Nanodrop2000c spectrometer (ThermoScientific) is cleaned with nuclease-free water and blanked. After blanking, 1 µL of water is measured to ensure proper blanking and the absence of contamination. 1 µL of sample is loaded onto the Nanodrop and measured. The machine is cleaned, and another 1 µL drop is added and measured. If the numbers are drastically different, a third sample will be measured. The ratio of 260/280 absorbance should be 2.0 ± .2 for RNA, and 1.8 ± .2 for cDNA. The ratio of the 260/230 absorbance should be .2 higher than the 260/230 ratio. Concentration for rat hippocampal RNA is usually around 1,500-2,000 ng/µL in 50 µL, producing an overall yield of 75-100 µg of RNA. cDNA concentrations are usually about 1,700 ng/µL in 20 µL, giving an overall yield of 34 µg.

RT-qPCR Primers. GAPDH primer sequences (Appendix A) were borrowed from previous research[80]. TE primers are not standard, and must be generated from scratch. Sequences of interest will be pasted into IDT DNA's PrimerQuest online application with default "RT-PCR" settings. Adjust the preferred amplicon size to 300 and generate primer sequences. For each primer of interest, use NCBI BLAST to check for specificity, where it does not bind with other regions in rats.

RT-qPCR. Each sample is diluted with nuclease-free water to a concentration of 1 µg / 11.5 µL H_2O, with a total volume of 80.5 µL for six wells per sample (three replicates each for control and target primers) in a new Eppendorf tube and placed on ice. Primers are diluted to 500 ng/µL, aliquoted, and placed on ice. SYBR green mastermix (Applied Biosystems) is aliquoted and placed on ice. A .1 mL PCR plate (Applied Biosystems) is placed on ice for the duration of loading. Each well receives 12.5 µL of SYBRgreen mastermix. Then 11.5 µL of each sample is loaded into 6 wells. 11.5 µL of H20 is added to the 6 no template control (NTC) wells. 1 µL of control primers are added to three of each sample well, as well as the NTC, and 1 µL target primers to the other three of each sample well. The plate is covered with a clear coversheet and spun down at 3500rcf for 5 minutes to eliminate bubbles. The plates are run on a StepOnePlus PCR machine (Applied Biosystems) using an amplification program that is 10 minutes 95°, and then 40 cycles of 1 minute 60°, 15 seconds 95°. After amplification a melt curve is generated.

RT-qPCR Analysis. Data is exported from the StepOnePlus machine to Microsoft Excel (Redmond, WA) and analyzed in Excel 2010. An average ΔCT is calculated for the control group, and then each target sample's ΔCT is compared to the control average. The mean difference of the target samples to the control average is the cycle difference. Two tailed t-tests of raw ΔCT values will be used to check for significant cycle differences.

RNA-Sequencing. After RNA extraction, samples will be placed on dry ice and delivered to the MGH Next Gen Sequencing (NGS) core. There samples will be quality checked independently on a Bioanalyzer. Library generation is done with a Ribozero kit by the sequencing core staff. The samples will be then run multiplexed on an Illumina HiSeq. Data will be transferred via a private Galaxy server.

RNA-Seq Analysis. All sequence data will be aligned to the rat genome (rn6) by the MGH NGS core. After RNA-sequencing data is aligned to the rat genome, the .bam files will be uploaded to the Galaxy Project. Using the Tuxedo suite of tools, cufflinks algorithms will be used to assemble transcripts and determine differential expression between groups. Cufflinks outputs a genomic ranges .bed file with the chromosome start and end locations of each differentially expressed transcript. To verify RNA expression, primers will be built according to the sequences to be tested, and RT-qPCR will be used to confirm the difference in RNA expression.

Chromatin Immunoprecipitation (ChIP):

Tissue Fixation. Tissue is harvested according to previous methods. Instead of flash freezing the brain tissue, it is chopped up into fine bits with a razor blade and added to a new Eppendorf tube with 1 ml of 1% formaldehyde (purchased as 16% formaldehyde in sealed ampoules, Thermo Scientific) in cold 1x PBS. The tissue is rotated on a rocker at room temperature for 4 minutes. Immediately after the fixation, .25 ml of glycine is added and rotated for an additional 5 minutes. The tissue is then briefly spun down (~5 seconds) and the formaldehyde aspirated off. The tissue is washed five times with cold 1x PBS, spinning down each time. After the last wash, all PBS is removed and the tissue is flash frozen with dry ice until it can be stored at -80°C.

Sonication. Sonication uses reagents and a modified protocol from the Covaris truChIP Chromatin Shearing Kit Tissue SDS. Samples are thawed on ice and resuspended in cell lysis buffer. They are desktop sonicated on low (40%) for 7 seconds, and then rotated on a rocker at 4°C for 20 minutes. Samples are then centrifuged for 5 minutes at 1700rcf. The supernatant is aspirated and washed with the provided wash buffer and spun down again. The wash process is repeated a second time, and then the sample is resuspended in the kid shearing buffer and left to sit on ice for 10 minutes. For each sample, 130 µL are transferred to a Covaris microtube, being careful not to introduce bubbles. The microtubes are loaded into a Covaris M-220 focused-ultrasonicator with a XTU tube holder (Covaris). The machine is loaded with nuclease-free water and allowed to reach temperature. The samples are sonicated at [sonication stats here] for [sonication time here]. After sonication, the sample is split into samples for further ChIP processing, PCR verification, and verification of fragmentation.

Sonication Verification. To check that sonication was successful, a portion of sonicated samples must be run on a gel. A mastermix containing 16 µL 1M TRIS HCl at 6.5 pH, 16 µL 2M NaCl, 8 µL EDTA at 8.0 pH, and 1 µL Proteinase K is added to each sample. Samples are left to incubate on a heated shaker at 65°C overnight. Samples are kept at 50°C until ready to be run on a gel. Gels are 1% agarose made with 1x TAE buffer and 1 µL ethydiam bromide. The gel is poured into the running box and allowed to set for half an hour. The comb is removed and the gel box is filled with addition 1x TAE buffer until the gel is completely submerged. It sets for another half hour. The DNA ladder is a mixture of 2.5 µL ladder and .5 µL loading dye. Load all 3 µL into the first well. 25 µL of sample is combined with 5 µL loading dye. 10 µL of each sample mixture is added to the other wells. Gel is run at 100 mV for 2 hours, or until loading dye is an inch away from the edge of the gel. After the run is finished, remove the tray and carry it to the ChemDock. Use the attached computer to make a UV image and check fragment length (Figure 2).

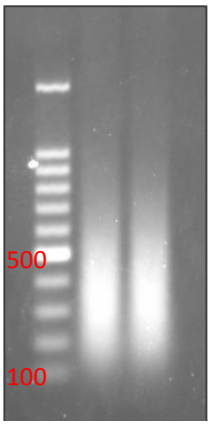


Figure 2. 300bp bands after sonication.

Chromatin Immunoprecipitation (ChIP) Sequencing. ChIP-Seq data will be aligned to the rat genome (rn5). The data will be uploaded to our local Galaxy server. Using Galaxy, the Model-based Analysis of Chip-Seq (MACS) algorithm will be used to detect binding peaks. Peaks are stored in a .bed

file with chromosome, start, end, and strand. The genomic sequences of peaks can be extracted into a .gff file, which can then be coverted to a .fasta format.

TRAP Analysis. The ChIP-Seq sequence files will be analysed with TRAP, which will scan each sequence and estimate the binding potential for a long list of transcription factors. This will both verify that ChIP pulled down DNA bound to GR, and will predict other TFs that may bind to those sequences. If other TFs are implicated, they can be further validated with ChIP-PCR.

Matching RNA-Seq transcripts to ChIP-Seq binding. An R script using the GRanges package of BioAnalyzer has been piloted with practice data. The window, set at 1000 basepairs, will match all GR peaks and RNA transcripts within that range. An file of transcripts is output in a .bed file, and sorted to include only unique transcripts. This list is further pruned to find only TE trascripts.

Acronyms: LINE, SINE, LTR, UTR, TE, GR, MR, ncRNA, ISH, ChIP, ChIP-Seq, NGS, TF, MACS

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