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Research Article

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Insights into the role of cis-regulatory elements of *5-HT2A* gene in gene expression and regulation: an *in silico* approach

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ABSTRACT

In multicellular organisms, coherent functioning of the central nervous system (CNS) and cellular diversity are driven by changes in high-fidelity gene expression. Crucial to these processes are cis-gene regulatory elements (CRE elements), which control transcription in response to chemical and physical stimuli. Variations in these components are a major contributor to several diseases in humans that result in particular phenotypic endpoints. The brain's neuropsychological processes are dependent on G-protein-coupled receptor (GPCR) activation, and various neuropsychiatric disorders are linked to GPCR dysfunction. The *5-HT2A* receptor plays a key role in many brain activities due to its neurobiological and signaling characteristics. The distinct topography of the *5HT2A* gene locus is outlined in this work, including the functions of CRE and regulatory elements. Further, the role of CRE elements in imprinting and methylation signatures' was investigated. Our findings indicate that the non-coding antisense RNA transcript (*HTR2A-AS1*) present in the locus may control the expression levels of the *HTR2A* transcript. This region's combinatorial DNA sequences include promoters, enhancers, silencers, and CTCF all of which may play a crucial role in the regulation of gene expression. Moreover, imprinting and epigenetic inheritance may be made possible by the distinct tapestry of chromatin architecture components, including H3K27ac, DNase I hypersensitivity, and CTCF binding regions found in the region. It is also possible that the non-canonical DNA structures and repetitive elements in the promoter region contribute to these functions and genomic stability. Together, the flanking regulatory elements and the gene-specific CRE contribute to the expression of the gene. They might function as plausible indicators for human illnesses.

KEYWORDS: Non-canonical DNA/RNA, *Cis*-gene regulatory elements (CRE elements), Long non-coding RNA (lncRNA), H3K27ac, DNase I hypersensitive sites

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INTRODUCTION

5HT2A Gene Organization-promoter Transcription and Translation

According to the most recent genomic annotation, the *5HT2A* gene (Gene ID: 3356; Chr-13q14.2) is composed of 5 exons and spans 65 kb. It is encoded from the antisense strand and undergoes alternative splicing at exon 2 (NCBI).The locus has a long non-coding RNA (lncRNA) gene *HTR2A-AS1*, which codes for two isoforms that are transcribed by alternative splicing from the sense strand. RNA expression analysis from the locus offers important insights into the genes of this region and their regulation (Ruble *et al.*, 2016).The genes are transcribed through the use of promoters, alternative transcription start sites known and novel exons and splicing (novel splice donor/acceptor sites). The gene's 5' flanking region contains a unique DNA domain that permits drug and cell-specific control. The basal promoter is essential for the transcriptional regulation of the receptor (Zhu *et al.*, 1995). Nonetheless, transcription can be triggered by different stimuli owing to many transcription initiation sites located at nucleotides -1157, -1137, -1127, and -496, respectively. Enhancers help to facilitate the regulation of the basal promoter and silencer sequences located across the genes aid this function which results in selective neuronal expression. Additionally, a stretch between -1.5 and -2.3 kb upstream of the translational start codon is shown to be drug-responsive (Toth, 1996). The area contains hallmark sequences for a number of

transcription factors, both cis, and trans including E-boxes, PEA3, Spl, and the cyclic AMP response element (CRE)-like sequence.

The adjustment of cell surface 5HT2A receptor levels is facilitated by the regulation of mRNA levels through multiple processes, including neuro-adaptive and gene regulatory mechanisms (Wohlpart & Molinoff, 1998). The timecourse and biphasic changes in the mRNA levels generated by agonists are supported by research evidence. Receptor mRNA levels rise in response to short-term exposure to 5-HT, but recover to control levels after extended exposure (Ferry & Molinoff, 1996). Numerous pharmaceutical medications, including antipsychotics, lower mRNA levels (Buckland *et al.*, 1996); additionally, persistent dextro-amphetamine treatment has been linked to region-specific expression alterations in the limbic and related areas (Horner *et al.*, 2011). Lastly, agonist-promoted 5-HT receptor mRNA regulation is made possible by cellular mediators such as protein kinase C through a post-transcriptional pathway. The locus also contains *HTR2A antisense RNA-1 (HTR2A-AS1)* gene with three exons. The genes in this class are identified by their length (>200 nt), inability to code for proteins, presence of non-canonical splice site signals, independence of transcriptional units, and capacity for alternative splicing. The post-transcriptional processing of RNAs from neighboring genes is influenced by long noncoding RNAs (lncRNAs). This is sometimes accomplished by chromatin modifications, RNA processing, and epigenetic changes in addition to direct interactions (RNA-RNA or RNA-DNA) (Werner *et al.*, 2009). Thus, various research results provide insight into the distinct arrangement and splicing processes, in addition the configuration of the promoter, flanking sequence, and regulatory components. Lastly, the influence of individual components or the total effect of all has an impact on the expression of genes.

Prominent Features of the Gene

Both genetic (imprinting) and epigenetic mechanisms tightly regulate the actions of different neurotransmitters, allowing for their high-fidelity CNS functions. Given that chemical signaling pathways amongst neurotransmitters exhibit functional commonalities, it is hypothesized that epigenetics plays a major role in these functions. Histone acetylation, which functions to activate the receptor and transporter, and DNA hyper-methylation in the promoter region, which inhibits the expresses and modifications in the levels of gene expression are caused by the histone deacetylases (HDAC) and DNA methyltransferases (DNMT) are examples of epigenetic methods that cells employ to enable these mechanisms. In this section, we provide a brief overview of these mechanisms in relation to normal and related pathologies of the brain.

Imprinting

Many metabolic and environmental factors influence the epigenetic process of genomic imprinting, which involves DNA methylation to silence genes (Singal & Ginder, 1999; Bird, 2002). Genomic imprinting is a sex-dependent marking of the DNA that fundamentally explains a parent-of-origin-dependent mechanism leading to the differential expression of a gene and ultimately the degree of gene expression (Monk *et al.*, 2019). Additionally, imprinting may be allele-specific and a source of variable gene expression within and between individuals (Reinius & Sandberg, 2015). In the brain, a significant number of imprinted genes are expressed. Several studies indicate that epigenetic factors are important in the regulation of the *5HT2A* gene and mediate genomic imprinting. First, differential imprinting is demonstrated in the adult brain (Bunzel *et al.*, 1998); second, methylated maternal chromosomes in human fibroblasts are shown to exhibit differential imprinting in the adult brain (Kato *et al.*, 1996); third, mono-allelic expression (Fukuda *et al.*, 2006) and finally the conservation of imprinting in eutherians (Das *et al.*, 2012). Genetic inheritances from both parents affect neurological, cognitive, and behavioral outcomes. Abnormal imprinting leads to a number of neuropsychiatric disorders, including neurodevelopmental disorder ASD (Badcock & Crespi, 2006), Kagami-Ogata syndrome (KOS) (Ogata & Kagami, 2016), and schizophrenia (SCZ) (Brucato *et al.*, 2014). Alterations in the expression of imprinted genes (fingerprints) have effects on the mother and her progeny. Epigenetic mechanisms mediated by the placenta in pregnancy can also impact these results. The receptor's roles in imprinting are supported by evidence linking it to child neurobehavioral outcomes (Lesseur *et al.*, 2014).

Developmental epigenetics

Gene expression is controlled by epigenetic regulation via RNA-based processes, histone modification, and DNA methylation (Kaidery *et al.*, 2013). One typical epigenetic marker that is frequently linked to CpG islands - regions with a high GC content - is DNA methylation (Antequera, 2003; Bergman & Cedar, 2013). Numerous neuropsychiatric illnesses have been linked to changes in HTR2A epigenetics: psychosis-related suicide (De Luca *et al.*, 2009); borderline personality disorder (Falkenberg *et al.*, 2011); bipolar disorder (BPAD) (Abdolmaleky *et al.*, 2011); and relapse-related behaviors in cocaine dependence (Land *et al.*, 2020). Three CpGs, positioned at -1439, -1420, and -1224 base pairs, respectively, are the center of methylation studies of the *HTR2A* promoter region (Wockner *et al.*, 2014). Additionally, hyper-methylation of CpG sites (at position 1438, near variation rs6311) or hypo-methylation (at position 102 of exon 1, near variant rs6313) is reported to cause region-specific decreased expression in the prefrontal cortex in SCZ (Abdolmaleky & Thiagalingam, 2011; Ghadirivasfi *et al.*, 2011). Placental methylation and fetal neurodevelopment are intimately related to serotonergic pathways. Several genes are functional components of this system because during the early stages of fetal brain development, the fetus depends on serotonin derived from the mother and placenta (commonly referred to as the "third brain"). Gene products influence placental implantation or function as mitogens (Rosenfeld, 2020).Infant neurobehavioral outcomes are dependent on *5HT2A* function (Paquette *et al.*, 2013). In conclusion, these studies demonstrate the transgenerational inheritance of these mechanisms and the roles that imprinting and epigenetics play in development and neurotransmitter gene regulation.

Basic Biology of 5HT2A GPCR-protein, Role in CNS, and in Human Physiology

Coherent brain functions are dependent on GPCRs. This class of proteins contains thousand-odd proteins constituting the largest group of signal transducers across the cell membrane (Azam *et al.*, 2020). All major neuromodulators and some fast neurotransmitters act through either a single GPCR or a family of GPCRs (Strokes & Piao, 2010). The receptor is localized to layers of the cortex, with high concentrations in layer 5 pyramidal neurons of the frontal, orbital, entorhinal, cingulate, piriform, insular, and deep layers of the cingulated cortex, also found in areas of the forebrain and basal ganglia (Mengod *et al.*, 2015). The receptors are found inside of cells, where they co-localize in intracellular vesicles with b-arrestins, also known as barrels (Zhang & Kim, 2017). Through the canonical G protein-dependent signaling coupled to Gαq/11, the 5-HT2A receptor detects extracellular effector molecules. This signaling then activates the intracellular second messenger inositol-1,4,5-trisphosphate (IP3), which is mediated by the enzyme phospholipase C (PLC) and the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2). Conformational changes brought about by ligand binding alter downstream effector activity and initiate signaling, which in turn releases intracellular calcium (Ca^{2+}) (Dorsam & Gutkind, 2007). Signaling results from the interaction between the receptor's i3 loop and serine-threonine kinase RSK2 (ribosomal 56 kinase-2) (Gelber *et al.*, 1999). Other organs that express the receptor include endothelial cells, platelets, and the gut. Cortical excitability, platelet aggregation, smooth muscle contraction, vasoconstriction and dilatation, inflammatory processes, and hormone signaling are few of the many physiological roles (Raote, 2007). The receptor's neurobiological roles include memory, cognition, and learning. Moreover, it mediates a number of cellular processes, including adenosine triphosphate (ATP) generation, neurogenesis, calcium level regulation, electron transport via the electron transport chain (ETC), and maintenance of the neuronal microtubule. Research indicates that increased receptor signaling results in a neurobiological (Ly *et al.*, 2018; Inserra *et al.*, 2021) as well as psychological (Nichols *et al.*, 2017; de Vos *et al.*, 2021) plasticity states, suggesting a psychedelic-like influence on CNS function. Numerous hallucinogenic substances function as receptor agonists to create psychoactive effects. Clinical research indicates that receptor blocking provides anxiolytic (Wojtas & Goembiowska, 2024), antipsychotic (Meltzer, 1999), and depressive (Kroeze & Roth, 1998) effects. Because 5-HT2A antagonists are effective at reducing both positive and negative symptoms of SCZ, they are considered atypical antipsychotics. Psilocybin and lysergic acid diethylamide (LSD), two psychedelic substances, have been linked to increased cognitive flexibility and creative thinking (Boulougouris & Robbins, 2008). Other psychoactive substances, such as ketamine and cannabis, boost brain plasticity to correct long-term synapse impairments brought on by stress. Genetic screenings, binding assays, and DNA or protein expression data support the notion that aberrant receptor function is implicated in a number of diseases, including autism spectrum disorders (Annamneedi *et al.*, 2023), obsessive-compulsive disorder (OCD) (Yin *et al.*, 2024), and SCZ (Nakao *et al.*, 2022). Cumulatively, several lines of research implicate the diverse role of the 5HT2A receptor in human physiology and several neurobiological functions in the brain.

Cis Elements and their Roles in Gene Regulation

The faithful beginning and regulation of transcription are significantly influenced by DNA promoter region components and architecture such as Non-coding DNA. Non-coding DNA segments with regulatory properties are known as cis-regulatory elements (CREs) or cis-regulatory modules (CRMs). Through DNA-protein interactions, the nucleus's regulatory "blueprint" or CREs regulate vital processes like transcription, replication, and recombination (Riethoven, 2010). Given their involvement in genome control at multiple levels, the significance of this information which is complex and degenerate in nature has lately been acknowledged. The promoter region, enhancers, insulators, and silencers are examples of cis-regulatory modules (CRMs), which make up the eukaryotic regulatory toolkit (Bylino *et al.*, 2020). Enhancers are the promoters of the promoter that allow the spatiotemporal tissue and cell type-specific gene transcription of the promoter; found close to a gene's TSS and serve as hubs for the assembly of the transcription machinery. The majority of the gene's regulatory elements will reside in the zone defined by insulators, also known as border elements. Gene activity is repressed by inhibitory regulatory elements or silencers. Thanks to multiple genome sequencing initiatives (1000 genome) cataloging and comprehensive analyses of sequences and structural motifs defining TSSs, promoters and CRE elements in metazoans, have been accumulating in recent years. Figure 1 illustrates schematic representation of the CRE elements (containing Enhancer, silencer, promoter-with TSS and TF binding sites). The CRE is suggested to enable this either by looping to their target promoter, creating repressive chromatin marks, and not allowing the transcription factor binding to non-cognate genes (Bansal *et al.*, 2014). CRE plays essential roles in the precision spatiotemporal patterning of gene expression necessary during development ensuring cell specificity. Response to the appropriate environment and their divergence is a common cause of evolutionary change (Chatterjee & Ahituv, 2017). To enable neural plasticity, learning and memory, and long-term memory, the brain's adult neurons need dynamic control of gene expression patterns for information storage and behavioral adjustments in response to various environmental signals. A remarkable range of cellular and functional variety arises from the precise control of these complex expression programs involving CRE in many neuronal classes (Carullo & Day, 2019). It has been documented that several diseases such as neuropsychiatric illness, cancer, and developmental abnormalities are caused by variations in the cis-regulatory regions (Douglas & Hill, 2014). Therefore, from the standpoint of clinical genetics, identifying and annotating the CREs in the human genome is a crucial objective.

Role of non-B DNA in the genome

Numerous secondary structures, including non-B-form DNA structures like the left-handed helix Z-form, cruciform

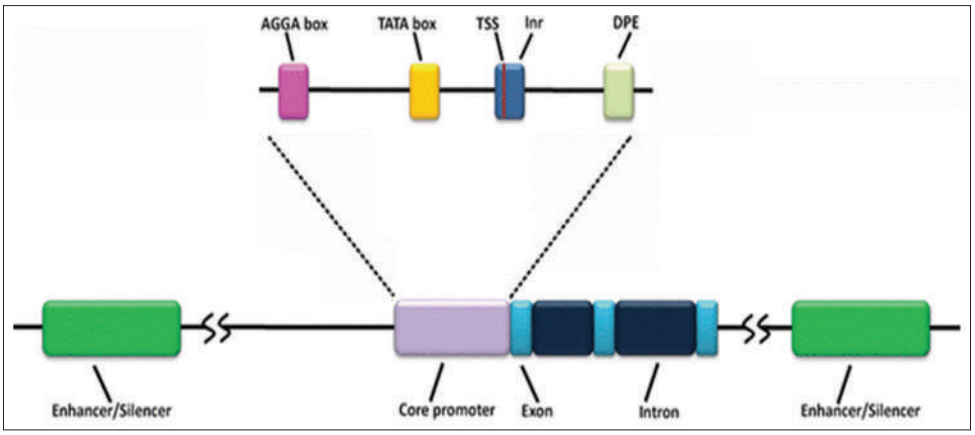

Figure 1: Schematic representation of the CRE elements (containing Enhancer, silencer, promoter-with TSS and TF binding sites

structure, triplex DNA, i-motif, and G-quadruplex, can be adopted by DNA and RNA sequences within the genome (Wadkins, 2000; Zhao *et al.*, 2019). RNA secondary structures include G-quadruplex (RNA-G4), non-canonical RNA, and stem-loops that produce A-form double-stranded RNA (Zhao *et al.*, 2010; Bevilacqua *et al.*, 2016). The upstream sequences of many mammalian genes share these distinctive secondary features. Non-B DNA plays contradictory roles in the cell; it controls transcription and replication related functions, but its presence is linked to increased mutagenicity and genomic instability (Poggi & Richard, 2021). These elements also drive a species' genetic and phenotypic evolution.

Implications from GWAS: role of non-coding variants in complex disorders

Deciphering the genetic etiology of complex disorders presents a significant challenge. A number of complex disorders have been linked to recognized disease risk loci, most of which are non-coding and include numerous variations in high linkage disequilibrium (LD); finding the causative variant within these variants is not an easy task (Zhong *et al.*, 2022). Despite not encoding proteins, transcripts play crucial regulatory effects on nearby genes adding another layer of complexity. Research in cell biology, driven by investigations in many model species, is providing additional evidence that the variations cause disease by interfering with CREs (Javierre *et al.*, 2016). The ability of CREs to operate over long distances makes gene hunting more difficult. It is well documented that genes are tightly bound to one another functionally and no gene is an island unto itself. The gene may be part of larger gene regulatory networks not necessarily by physical location in the genome (Lieberman-Aiden *et al.*, 2009). In conclusion, all of these studies point to the theme: cis-acting elements, with assistance from enhancers, silencers, and insulators, mediate gene transcription. Gene expression is changed by additional distinct DNA sequences (that have a tendency toward secondary structure) and also, DNA variations have a recognizable role. Several GWAS studies and other association genetic studies have implicated genetic risk variants lie within regions of the genome that are marked by specific enhancer marks (methylation marks such as H3K4me1, acetylation mark-H3K27ac, and chromatin mark - DNase I hypersensitive sites). Examples include Thrombosis (Stefanucci & Frontini, 2022), Cancer (Hennessey & Brown, 2021), Eye diseases (Soriano *et al.*, 2024) and neuropsychiatric diseases (Song *et al.*, 2019). Several variants are cell or tissue type specific as seen in pancreatic islets in type 2 diabetes (Khetan *et al.*, 2018). Furthermore, a number of complex neuropsychiatric disorders viz., Autism (Saeliw *et al.*, 2023), Addiction (Srinivasan *et al.*, 2021), and SCZ (Zhu *et al.*, 2024) have been linked to disruption of CRE-dependent transcription. Local chromatin loops, make it easier to link promoters with corresponding regulatory elements, which are frequently hundreds of kilobases away (Schoenfelder *et al.*, 2010). The CCCTC-binding factor (CTCF), an 11 tandem ZF (Zinc finger protein), is the best-characterized loop-forming factor in vertebrates (Lobanenkov & Zentner, 1990). The structural role of CTCF comprises promoter-enhancer connections, cohesin recruitment, chromatin barrier construction, insulator functions, and imprinting regulation (Cuddapah *et al.*, 2009). Important developmental processes like cell fate, differentiation, and maturation are controlled by CTCF-mediated chromatin structure, which has unique signatures for diverse cell types (Agrawal & Sridhar, 2021). Mutations of the CTCF coding sequence lead to diseases such as cancer (Debaugny & Skok, 2020), influenza (Allen *et al.*, 2017) and osteoporosis (Chen *et al.*, 2018). They are implicated in a spectrum of neuropsychiatric syndromes, intellectual impediments such as microcephaly, SCZ, and frontotemporal lobar degeneration. Finally, they also impact multiple-tissue disorders such as deletions in chromosome 16 (Dehingia *et al.*, 2022). Alternative DNA secondary structure (ssDNA) conformations, including left-handed helix Z-form, cruciform structure, triplex DNA, i-motif, and G-quadruples, can be adopted by the promoter's DNA. These sequences influence a number of gene expression processes and associated mechanisms, including TF binding, flexibility, and RNA polymerase docking. Thus, structural insight into this class of DNA from a genomics stand-point assumes significance.

Opportunities to investigate the role of CRE and regulatory components in nuclear regulation throughout normal and pathological processes are made possible by improvements in cell-based assays and genomics techniques aimed at interpreting

nuclear 3D architecture and bioinformatics. They make it possible to investigate the structure-function relationship of DNA and RNA secondary structure. Using the 5*HT*2A gene, the current *in silico* study is an attempt along this road.

MATERIALS AND METHODS

a. Analysis of Promoter Organization

The gene features were obtained from the genome browser (accessed on July 2024) and the ENSEMBL genome browser (accessed on July 2024). Using the NCBI gene (accessed in July 2024), the promoter sequences were retrieved (FASTA format) to enable several *in silico* analyses. The locus and surrounding regions up to 1 kb upstream flanking the promoter and exon 5 were physically scanned for CRE and regulatory elements and data, and images were recorded.

Using ENCODE tracks of the UCSC genome browser, repetitive DNA status around the promoter was analyzed.

b. Promoter, Enhancers, and Silencer elements were annotated on the Genecard server.
c. DNA secondary sequence analysis. DNA can adopt with different alternative conformations; this ensemble was analyzed using the Stitchprofile server.

RESULTS

The gene coordinates on the GRCh38.p14 (GCF_000001405.40) assembly covers 65 kb (5 exons), containing sequences that range from 46760156 to 47307540 (bp). Five enhancers and two silencers were found in the regions when the region surrounding the gene was physically examined using the NCBI genome browser (Figure 2a). Table 1 reports the genomic characteristics and functionalities of these components. The location of enhancers and silencers surrounding the 5HT2A gene locus is shown in Figure 2b. The enhancers (1, 2) bordering the promoter, the intronic enhancers (3, 4) in intron 4, and the enhancer 5 close to exon-5 are the relative positions of the enhancers aligned with the 5*HT*2A gene. The promoter contains silencer-A, while the intron 4 region has silencer-B. Numerous transcription factors with a variety of roles, including DNA binding, chromatin modelers, basal transcription, and architectural epigenetics, have sequence recognition sites in this region. Figure 2c shows the gene enhancer map, which indicates that the *HTR*2A and *HTR*2A-*AS1* genes are clustered together in a loop. Figure 3 shows the analysis of the promoter regions for enhancer and insulator markers using the Ensemble Browser (accessed on July 2024). High intensity peaks for H3K4me3, H3K27ac, and DNase I hypersensitivity sites were identified in the area. Additionally, the region has repetitive DNA elements marks with high peak enrichment for LINE and LTR and weak marks for LIM7 and MER5B. Finally, it was found that the promoter sequences were conserved in a number of mammals (Figure 4). Figure 5(a-d) summarizes DNA secondary sequence analysis. Figure 5a shows the stitch profile of the DNA secondary sequence. The data show that helices (0-600 bp and 800-1200 bp) suggesting (dsDNA) and potential melting loop openings (ssDNA) spanning the promoter between 300 and 1000 bp were found. A nonlinear curve indicative of unpaired base pairs was revealed by melting curve analysis (Figure 5b). Several peaks equal to 1 and above that suggested melted DNA base pairs were found by probability analysis (Figure 5c). Lastly, temperature analysis showed a plateau for sequences between 100 and 300 bp, as shown in Figure 5d, indicating melting cooperative sequences. A 14-bp inverted repeat DNA at bp (204-216) was found by non-B DNA analysis (Figure 6).

DISCUSSION

Basic and pharmacological research is largely motivated by the signaling and cell biology properties of the 5HT2A receptor. Numerous questions are raised by the widespread expression of the 5-HT2A receptor in various tissues, brain regions, and cell types (neurons and astroglial cells), as well as the specific regulatory mechanisms involved. These include a. how the receptor is regulated in different cell types; b. how it responds to a variety of exogenous and endogenous stimuli in both normal and abnormal disease states, and c. how pharmaceutical drugs and dosage affect the receptor. According to recent genomic research, non-coding genes are widely expressed and have a role in controlling the expression of the associated gene transcripts (Statello *et al.*, 2020). Antisense transcription can "rewire" regulatory networks, modify the architecture for protein complexes, and act as a fast-evolving regulatory switch. The locus contains the nested gene *HTR*2A *antisense RNA-1 (HTR*2A-*AS1)*, which is a non-coding RNA (ncRNA), 3 *HTR*2A-*AS1* exons overlap with the *HTR*2A intron. The gene's genomic characteristics include low guanine/cytosine content, a small open reading frame, two alternatively spliced isoforms found in the human brain and testes, and no homology to known proteins or RNAs. Previous research indicates that non-coding RNA (ncRNA) transcripts participate in epigenetic and chromatin changes, as well as post-transcriptional regulation of the sense or anti-sense transcript's RNA levels (Werner *et al.*, 2009). These observations are validated separately by the gene enhancer map data, which indicates that the two genes interact through enhancers. Hence, from mouse studies (suggesting conservation in the organization and regulation) and from above *in silico* experimental evidence, it could be hypothesized that the *HTR*2A-*AS1* gene has transcriptional and epigenetic roles.

Enhancers are found in areas of the genome that are free of nucleosomes, or open chromatin, and they are activated when one or more sequence-specific transcription factors (TFs) and coactivators attach to them. They control transcription and are found either in trans (on a different chromosome) or in cis (on the same chromosome) proximity to the gene (Ray-Jones & Spivakov, 2021). Enhancers activate transcription by several mechanisms, including modifying chromatin structure, relocating gene loci in the nucleus, or directly assisting in the recruitment of transcription complexes (Gibbons *et al.*, 2022). Figure 7a, b shows a schematic illustration of enhancer-mediated activated transcription. The following are the genomic characteristics of the six enhancers in the area: The GH13J046893 enhancer

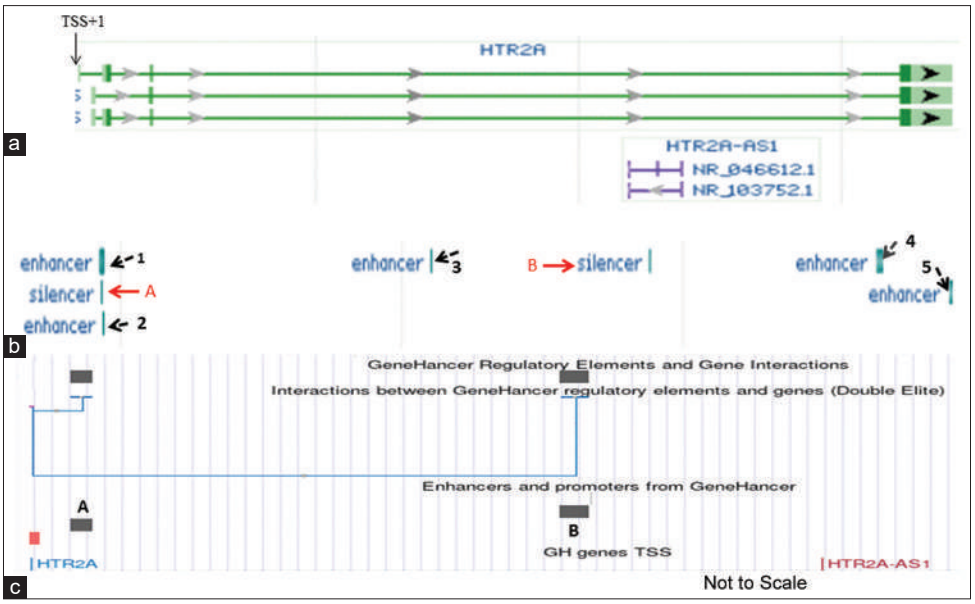

Figure 2: a) Depicting the 5HT2A (reverse strand) and HTR2A-AS1 gene with Enhancer and silencer locations around the region (arrow heads). (Source-NCBI gene). b) Arrangement of Enhancer/Silencer and c) GeneHancer regulatory elements/interations. (A-promoter, B-enhancer)

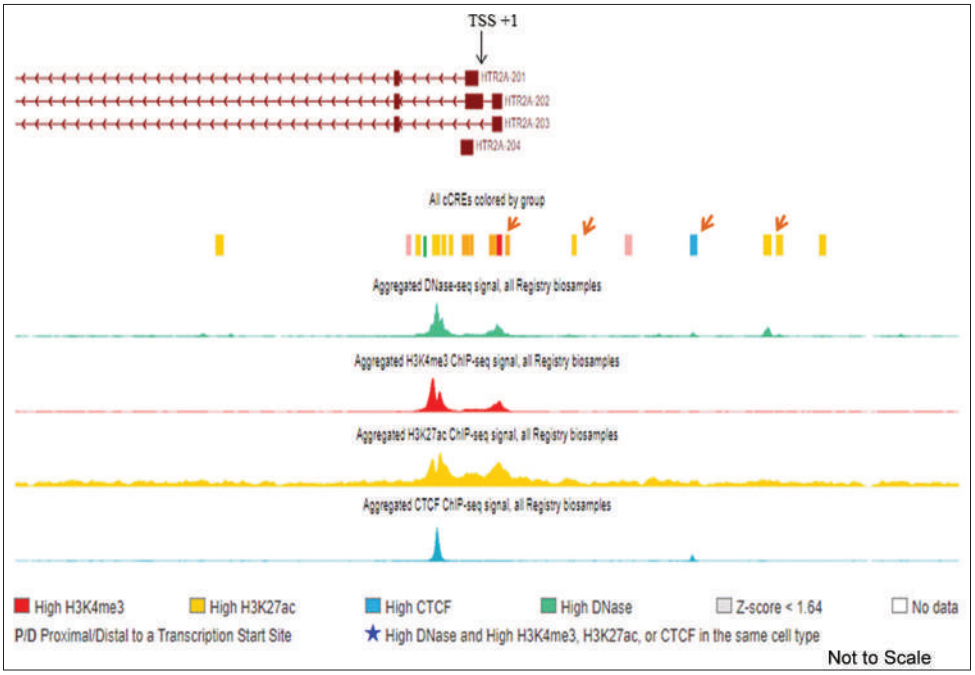

Figure 3: Promoter analysis for various CRE markers - H3K4me3, H3K27ac and DNase I hypersentivity

associates with the OCT4 and NANOG transcription factors and is marked by the H3K4me1 histone modification, which facilitates binding of the BAF complex and chromatin regulation. GH13J046982 a group 3 transcriptional regulatory area enhancer/silencer is largely or completely independent of the MED14 core Mediator complex component, but it does rely on the BRD2, BRD4, P300/CBP, and CDK7 co-factors. The enhancers GH13J046757, GH13J046866, and GH13J046795 enable chromatin accessibility. Last but not least, hESC H3K27ac enhancer GH13J046896 provides chromatin access and acts as a histone modulator. Anorexia Nervosa, a neurobehavioral eating disorder, has been linked to this enhancer (Malacards). Enhancers unique to the brain control the expression of genes throughout the neuronal life cycle. A subgroup of enhancers exhibits activity-dependent induction in response to neuronal activation and behavioral experience, indicating a regulatory role in transcription and brain function. Moreover, they ensure region-specific gene expression (Nguyen *et al.*, 2016). The analysis's findings support the additive or synergistic enhancer roles of cis-enhancers in the control of the locus in the brain. Differential regulation of cells and their response to stimuli, including medications, are made easier by this architecture.

Table 1: Genomic features and functions of enhancer elements

S. No.	Gene enhancer Identification a) Promoter/enhancer b) Enhancer c) Silencer	Distance from TSS (kb)	TF factors	Associated disease	Genes in the vicinity
1	a - GH13J046893 OCT4-NANOG-H3K4me1 hESC enhancer	+3.8	23 TFs	none	lnc-ESD-2 HTR2A HTR2A-AS1 ESDHSALNG0020226-108
2	b, c - GH13J046982 MED14-independent group 3 enhancer	-85.6	58 TFs	none	lnc-ESD-2 HTR2A HTR2A-AS1 ESD HSALNG0020226-108
3	b - GH13J046866 ATAC-STARR-seq lymphoblastoid active region 7714	+32.4	7 TFs	none	HTR2A CM034963-096 HTR2A-AS1 lnc-ESD-2 ESD HSALNG0020226-108
4	b - GH13J046757	+139.4	22 TFs	none	LOC107984563 FJ601684-101 HSALNG0096896 HTR2A ESD LRCH1 HSALNG0020226-108 lnc-ESD-1
5	b - GH13J046795	+101.0	188 TFs	none	ESD HTR2A lnc-ESD-1 LOC107984563 HSALNG0020226-108 LRCH1
6	a - GH13J046896	+1.6	3 TFs	Anorexia Nervosa	HTR2A lnc-ESD-2 HTR2A-AS1 ESD

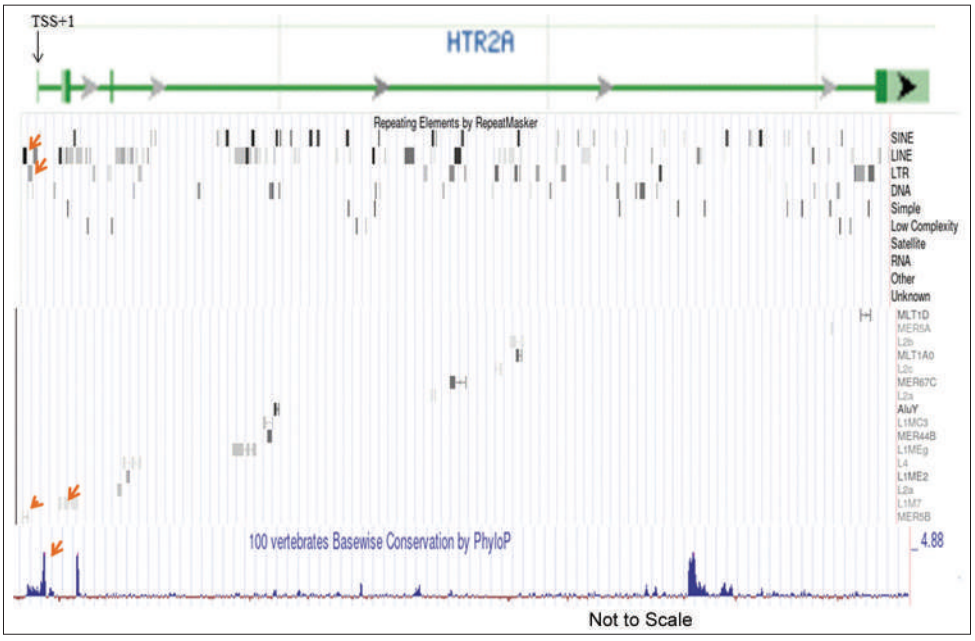

Figure 4: Repetitive DNA marks flanking the TSS and sequence conservation

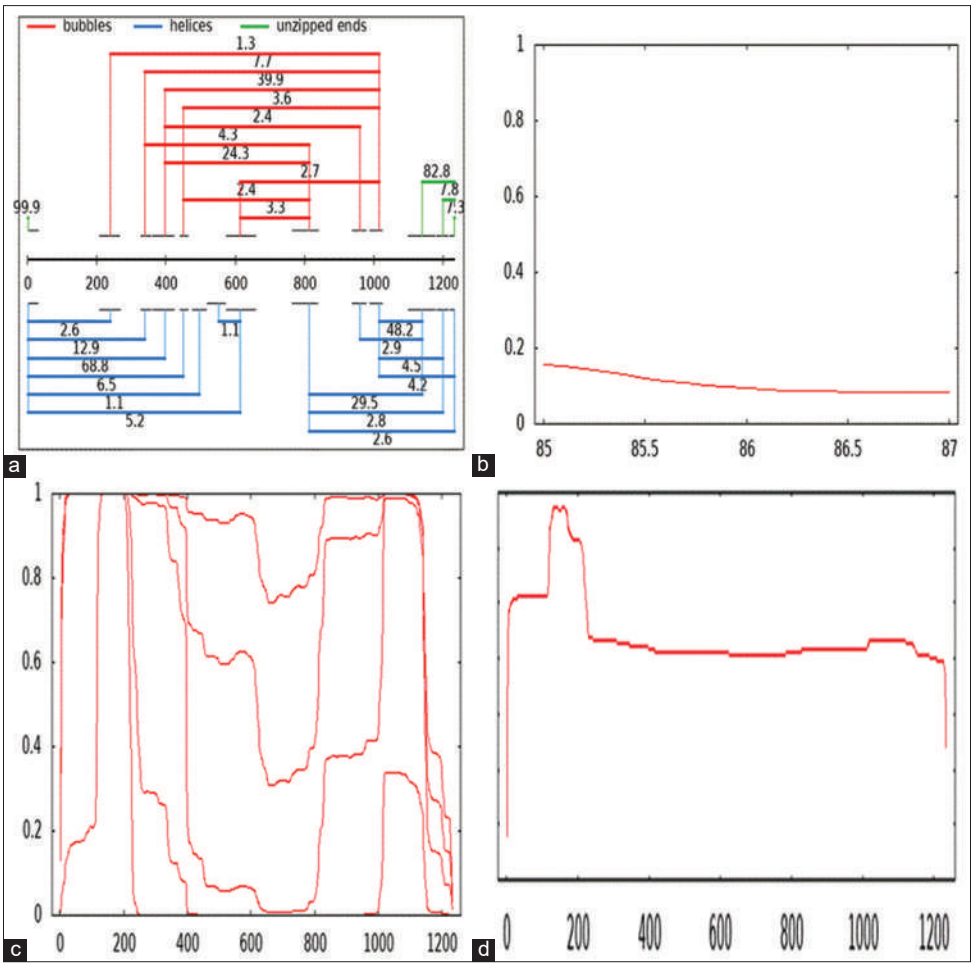

Figure 5: Analysis of the Promoter DNA sequence for DNA secondary structure (as a function of T-temperature at 80-90 °C). a) Stitch profile (Helicity-0.5, Dmax-3), b) Melting curve analysis, c) Probability profile (Helicity at 0.10, 0.25, 0.50, 0.75, 0.90) and d) Temperature profile (Helicity-0.5)

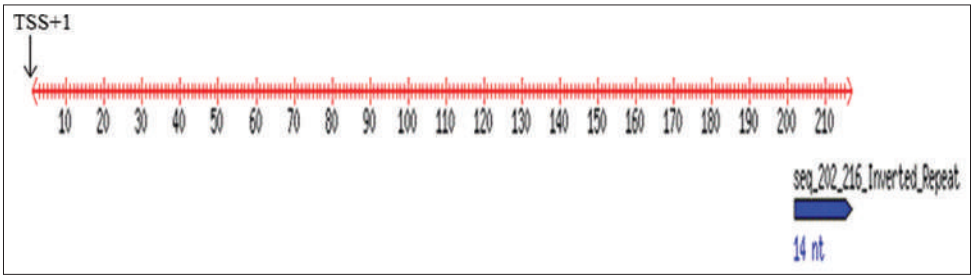

Figure 6: Non-B DNA analysis of the promoter region indicating a 14nt inverter repeat

There are two brain-expressed genes in the area (LRCH1, ESD). Gene esterase D (*ESD*) encodes the A serine hydrolase protein involved in sialic acid recycling. The gene is a genetic marker for Wilson's disease and retinoblastoma. Leucine-rich repeats and calponin homology domain proteins are encoded by the *LRCH1* gene (Leucine-Rich Repeats and Calponin Homology Domain). De Quervain disease and osteoarthritis are two conditions linked to the gene. A total of 188 TF factors bind to the locus and have a variety of activities, including basal transcription, DNA binding, and chromatin modelers/epigenetic regulators. In conclusion, these findings demonstrate the distinct genetic composition of the *5HT2A* gene, as well as the function of its flanking regulatory elements in gene regulation.

Histone modifications -H3K4me3 and H3K27ac, and DNase I hypersensitivity marks close to the promoter and exon1 indicate transcriptionally active chromatin (Beacon *et al.*, 2021). Figure 3 makes this clear, highlighting many H3K27ac marks and a high H3K4me3 mark in the region I marked. In addition to positively correlating with transcription, the concentration of H3K4me3 at active promoters close to TSS provides insight into their functions in DNA repair, cell fate determination, and epigenetic-mediated regulation. Both proximal and distal active enhancers

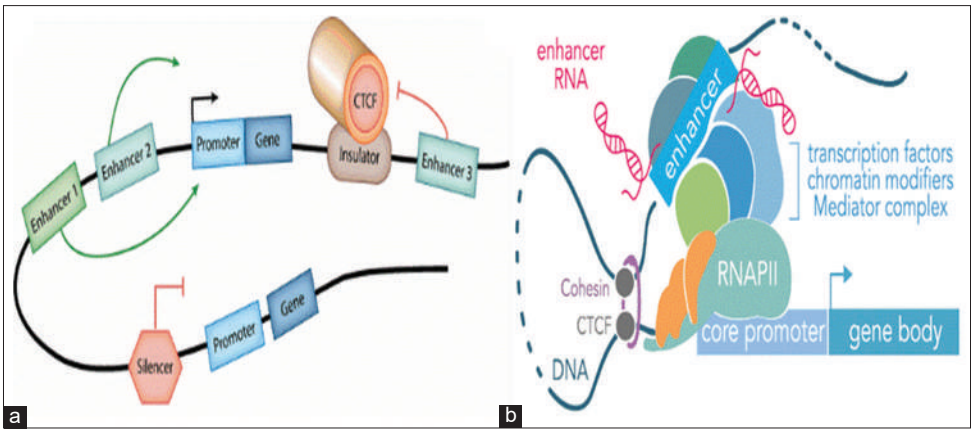

Figure 7: a) Schematic representation of the gene regulation involving enhancer, silencer and insulator CRE elements (Chatterjee & Ahituv, 2017). b) Gene expression mediated by Enhancers through enhancer-promoter looping (mediated by CTCF) enabling binding of TF, chromatin modifiers, mediator complex which enable docking of RNA poll II (Day *et al.*, 2019)

around TSS are shown by the H3K27ac markers. By preventing the distribution of the repressive histone mark H3K27Me3, H3K27ac promotes transcription. The CTCF proteins found in the area are sometimes referred to as "master weavers of the genome" because they facilitate promoter-enhancer loops and are found in intergenic and promoter-proximal areas. Based on cellular data, CTCF is considered a crucial, pleiotropic genome organizer that establishes a connection between intricate biological processes and higher-order chromatin structure (Phillips & Corces, 2009). CTCF facilitates multiple processes, including interacting with Pol II to initiate transcription and to permit the transcription factor-bound enhancers to the transcription start site (together with proximal regulatory elements). They also modulate messenger RNA (mRNA) splicing by controlling the rate of transcriptional elongation (Yadav *et al.*, 2023). Finally, through insulator activity, they block the interaction between adjacent gene enhancers and promoters.

The markers LINE and LTR for repetitive DNA are present in the receptor's promoter region. LINEs are retro-transposable, long- and short-interspersed, non-LTR (long terminal repeat) elements that use RNA intermediates to infiltrate new genomic locations. These elements are found in the genomes of many eukaryotes (Beck *et al.*, 2011). On the other hand, class I transposable elements (TEs) with long terminal repeats (LTRs) immediately flanking an internal coding region are known as LTR retrotransposons (Cordaux & Batzer, 2009). Their functions in the promoter and enhancer regions involve regulating gene expression by serving as binding sites for regulatory proteins (Liao *et al.*, 2023). Their functions include chromatin structural modification, gene splicing, and epigenetic modifications that impact gene transcription. However, genetic instability has been linked to aberrant LINE-1 and LTR element activity (Kazazian & Moran, 2017). Aberrant roles of repetitive elements can lead to disruption of CRE elements. In the brain, they are implicated in neurodevelopmental defects, neuroinflammation, and neurodegeneration, as well as neurological diseases (Blaudin de Thé *et al.*, 2018; Saleh *et al.*, 2019).

According to research, conserved structural motifs and non-B-DNA mark promoter sequences in mammalian genomes. These motifs have a variety of special biophysical and structural characteristics, including melting, stability, bendability, supercoiling, nucleosome positioning preference, and curvature (Duardo *et al.*, 2023). DNA molecules with varying lengths and/or base compositions would dissociate, or "melt apart," into their constituent single strands at varying temperatures, altering the thermodynamics. This is the underlying theory behind DNA secondary sequence analysis. Complementary repeats mispair as a result of denaturation/renaturation cycles, combining single-stranded loops and double-stranded DNA segments (Kovtun & McMurray, 2008). The promoter is demonstrated to have a distinct DNA profile as indicated in Figure 5, with potential melting loops (unpaired single-strand DNA) ranging in length from 300 to 1000 bp. The probability analysis, which showed multiple peaks equal to 1 and 1.1 suggestive of melted DNA base pairs, supports this observation, so does the nonlinear curve produced by melting curve analysis. Lastly, a plateau for sequences between 100 and 300 bp was found by temperature analysis, indicating the presence of melting cooperative sequences in the area. Different cellular reactions to physiological (pH, salt concentration, damage), cellular (TF), and chemical (drugs) stimuli can cause fluctuations in the shape of the DNA duplex (Metzler & Ambjörnsson, 2005). Non-B DNA analysis revealed a 14-bp inverted repeat in the promoter, these repeats are characterized by a single-stranded sequence of nucleotides followed downstream by its reverse counterpart. They have an impact on nucleosome location and DNA supercoiling degree. Further, they are also components of genomic instability, forming dumbbell-like DNA with hairpins capping both ends (Bowater & Brázda, 2022). Positive scores on the phyloP peaks imply conservation and slower-than-expected sequence evolution in the promoter. At the cellular level, enhancers evolve quickly and dynamically in response to their environment. In the genomes of mammalian species, the enhancers evolve quickly while the promoters evolve slowly (Villar *et al.*, 2015). Recently evolved lineage-specific enhancers such as those found in the present study dominate mammalian

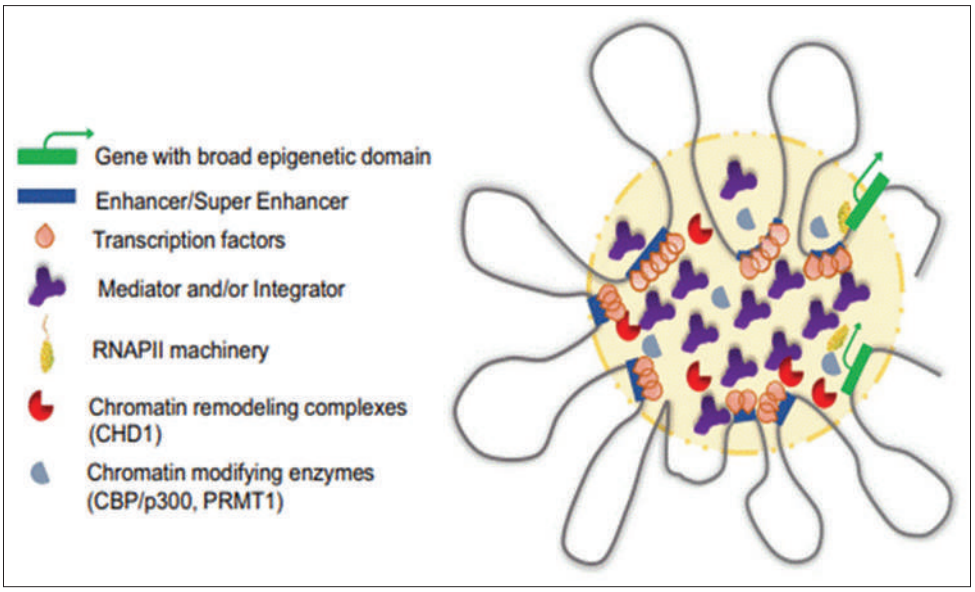

Figure 8: Schematic representation of a plausible Transcription hub containing enhancers, epigenetic marks, chromatin modifying enzymes, remodelers, mediator (and/or insulators), and cell-type specific TF. (Beacon *et al.*, 2021)

regulatory landscapes, which are under positive selection. Thus elucidating the functions of CRE and regulatory elements has relevance in neurobiology. It has relevance in identifying mechanisms of gene regulation in the CNS and also links non-coding regulatory and sequence variation to genetic risk for neuropsychiatric disorders.

CONCLUSION

Because of its importance in biochemistry, pharmacology, genetics, and cellular activities, the 5HT2A receptor is known as the "cynosure of brain". Because many mental illnesses have one or more dysfunctional GPCRs in their pathophysiology, drugs meant to treat mental illnesses primarily target GPCRs (Huang *et al.*, 2017). The careful regulation of several gene expression programs across different neuronal classes of cells in different brain sites leads to incredible cellular and functional diversity. The unique organization of HTR2A-AS1 antisense genes, which are transcribed from separate transcriptional units with cryptic promoters, demonstrates evolutionary conservation. The presence of these genes, which raise species fitness, is usually seen in intronic regions or near transcriptional start sites of nearby genes. Moreover, medications or external stimuli may cause alternative splicing, which may affect the transcription of the receptor. Research indicates that in several brain regions' alternative splicing patterns are significantly impacted by psychotropic and drug addiction substances (Nestler & Lüscher, 2019). In this endeavor, the regulators, CRE components, and promoter are important participants. This offers an extra benefit by allowing for the conditional, temporal, and cell-type control of the receptor. In conclusion, comprehending the functions of other cis-regulatory components and enhancers' structure-function will aid in clarifying the dynamics and mechanisms behind enhancer activity in certain brain regions in both health and disease in response to a variety of internal and external stimuli. Neuro-adaptive and gene regulatory processes control the gene's mRNA levels, which in turn affect the expression of cell surface receptors. Numerous pharmaceutical medications control the level of mRNA at the transcription level using time-course and biphasic mechanisms and post-transcriptionally via methods made possible by cellular protein kinase C. These controls may be particular to certain areas of the brain. It could be hypothesized that specific promoters and CRE elements are activated in response to such a stimulus, which could be response, dose, or cell-specific. The results of this study suggest that the CRE elements may play a part in these processes. The alternative and canonical promoters which contain drug-responsive binding sites are evidences to validate this proposition. The genomic arrangement could be part of self-regulatory circuits that allow genes to regulate their own expression since the region also has a CTCF binding site that directs intra-chromosomal loops, which also facilitates parent-specific imprinting. The results of the present study appear as of proof-of-principle to justify this notion.

Germline and somatic mechanisms of imprinting and epigenetic mechanisms enable imprint establishment and maintenance, respectively. In addition to being a crucial aspect of development, epigenetic regulation of gene expression permits heritable changes in the genome without changing the DNA sequence. The 3D architecture of transcription can be changed by DNA variations; additionally, the hub may change in response to epigenetic markers, DNA secondary structure, or specific types of repetitive DNA sequences. Previous studies suggest that the locus is tissue- and allele-specifically imprinted. Epigenetic mechanisms perhaps fill in the missing pieces of puzzle given that the brain shares a common theme of region-specific gene expression. The functions of CRE elements in promoting stable inheritance are supported by these findings. Observation of DNase I hypersensitivity marks, CTCF binding sites, and histone modifications (H3K27ac and H3K4me1) provides additional credence. Due to the multifaceted nature of gene expression, an

integrated biological model encompassing genetic, epigenetic, and chromatin variations in the context of developmental and environmental responses will be necessary to explain the *in nuclei* mechanisms and genetic associations with disease. A transcription hub of contacts is formed by the combinatorial toolkit of promoter, CRE, and regulatory elements interacting with the epigenetic domain, transcription factors, chromatin-modifying/remodeling enzymes, coactivators, mediator, and/or integrator complex. Spatiotemporal, cell-type, and conditional levels of gene regulation are facilitated by this configuration. Figure 8 shows a schematic illustration of a likely transcription hub linked to markers and mediators. A more thorough evaluation of the CRE elements, the impact of changes on gene expression, and the pathogenic role of these variations could result from such an analysis. It is currently thought that CRE impacts on gene expression account for a large portion of the genetic variation in human phenotypes, including vulnerability to diseases (Bray *et al.*, 2003). Numerous neuropsychiatric disorders, including ANK3 in BPAD (Rueckert *et al.*, 2013), SCZ (Fullard *et al.*, 2016), and paternal inheritance in autism (Brandler *et al.*, 2018), are linked to brain-specific cis-regulatory elements and transcriptional control. Since the CRE has tissue and allelic specificity, these studies provide empirical support for this hypothesis. Validation of the results in appropriate cell lines using CRE and chromatin mapping methods will facilitate unearthing finer 3D details.

In conclusion, information included in our genetic code, "blueprint," and the newly identified "CRE code," which has been shown to differ even among healthy people, are all related to human health. Many regulatory elements, which can be found anywhere in the genome, organize the expression of genes by physically interacting with distant genes. Numerous investigations have already established a connection between distinct chromatin architecture and gene expression patterns in both healthy and pathological conditions. These DNA connections can now be mapped because of the developments in chromatin mapping tools. Ultimately, such genomic data can be used to customize treatments and get a better understanding of disease states. They also serve as biomarkers of human disease in such genetically diverse populations.

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AUTHORS' CONTRIBUTION

Design, reviewing of literature, collation, conduction of *in silico* experiments, data analysis, drafting of manuscript Kiran Kumar H. B.; Sajeeda Niketh, reviewing of literature, collation, drafting of manuscript, data analysis. Rama thyloor, reviewing of literature, design, drafting of manuscript. Rajeev R Kolgi, reviewing of literature, collation of data from databases, manuscript drafting.

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