PROMOTION OR DESTABILIZATION OF G-QUADRUPLEX DNA STRUCTURES BY NEUROTRANSMITTER MOLECULES: A POSSIBLE APPROACH TO TREATING NEURODEGENERATIVE DISEASES?

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Abstract

The effect of various neurotransmitters on G-quadruplex DNA stabilization was analyzed using DNA melting experiments. The neurotransmitters tryptophol, serotonin, dopamine, and glutamate were studied with DNA sequences associated with the neurodegenerative diseases spinocerebellar ataxia type 36 and Unverricht-Lundborg disease. These molecules showed evidence of promoting G-quadruplex stabilization, destabilization, or structural transformation in both DNA sequences. Follow-up molecular docking studies showed that hydrogen-bonding and non π-stacking interactions with the π-system (π- π T-shaped and π-anion) were the primary modes of interaction between the neurotransmitters and DNA. These results show that neurotransmitters can potentially be used to develop treatments for different diseases.

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Introduction

G-quadruplexes are higher order DNA structures that are formed from guanine-rich (G-rich) nucleotide sequences. These structures are comprised of stacked tetrads, each of which arises from the planar association of four guanines in a cyclic Hoogsteen hydrogen-bonding arrangement. G-quadruplexes can be formed from one, two or four separate strands of DNA and are stabilized by small monovalent cations such as potassium (K^+) or sodium (Na^+).[1]

G-quadruplex DNA structures play key roles in gene transcription, translation, oncogene promoters, and protection of telomeres. The human genome consists of over 300,000 sequences that can form G-quadruplexes.[2,3] G-quadruplex structures, or G-quartets are located in the telomere region of the human chromosome and comprise of a repeating sequence of TTAGGG bases. Studies have shown that telomere shortening may be due to G-quadruplex-stabilizing ligands. Helicases such as the RecQ helicases WRN and BLM unwind G-quartets to assist in the proper replication of telomeric DNA.[2] Specifically, telomerase is believed to play an important role in the maintenance of telomeres in cancer cells. The binding of specific molecules to G-quadruplex DNA can be cytotoxic to tumor cells via telomerase inhibition. Therefore, there is biological interest in targeting G-quadruplex DNA as a therapeutic in the fight against cancer.

There have been several studies dedicated to the detection of G-quadruplex DNA using probes ranging from porphyrins, other aromatic molecules like ethidium and its derivates, carbazoles and even gold nanoparticles.[4-8] Some studies have also focused on promotion and destabilization of G-quadruplex DNA structures.[9] Herein, we report an initial study on the role a series of neurotransmitter molecules may play in the promotion or destabilization of G-quadruplex DNA structures associated with neurodegenerative diseases. Using DNA melting analysis and molecular docking simulations, we show that G-quadruplex DNA may alter their structures in the presence of neurotransmitters, and this can potentially lead to treatments.

Neurotransmitters are chemicals that are made within the body that allow neurons to communicate. Their role in the human body is to allow parts of the brain to perform different functions, such as neural growth and differentiation that occur in early human development.[10] Neurotransmitters reside in vesicles located at the axon terminals in the presynaptic gap. When an action potential is applied, the presynaptic terminal becomes depolarized, calcium enters through voltage gated channels binding to neurotransmitters and assists in releasing neurotransmitters into the synaptic gap where they can bind to corresponding receptors on the post synaptic cleft.

Serotonin (5-hydroxytryptamine) is a neurotransmitter that regulates many different physiological processes.[11] A lack of serotonin can negatively impact the immune system, resulting in diseases becoming increasingly harmful. Serotonin is also a precursor to the neural hormone melatonin, which is responsible for regulating circadian rhythms.

Tryptophol (indole-3-ethanol) is a compound that induces sleep in humans. Tryptophol can be formed in the liver after disulfiram treatment and also by a parasite that causes a form of sleeping sickness.[12] Structurally, both serotonin and tryptophol belong to a class of aromatic molecules known as indoles, which are organic heterocyclic molecules with the formula of C_8H_7N. Indoles and indole-derivatives have shown promise as DNA binding agents.[13,14] Catecholamines are a class of neurotransmitters that are synthesized in the brain and adrenal medulla. All catecholamines contain an amino group attached to a benzene ring with two hydroxyl substituents and are derived from the amino acid L-tyrosine. L- tyrosine is broken down into L-dopa via tyrosine hydroxylase. L-dopa is then broken down to dopamine by dopa decarboxylase. Dopamine is a catecholamine that contributes to motor function, mood, pain processing, sleep, stress response, and memory. It is commonly referred to as the "feel good" neurotransmitter because it is associated with pleasure and reward. It is synthesized in the central nervous system. There are threshold levels of dopamine in the body and dopamine is regulated through dopaminergic signal-

ing pathways.[15]

The fourth neurotransmitter in this study, glutamate, is a major excitatory neurotransmitter and like serotonin, it also acts as a chemical messenger by sending messages between neurons.[16] Glutamate plays a role in the sections of the brain that control learning and memory. In contrast with indole and catecholamine neurotransmitters, glutamate is a non-aromatic amino acid that would lack the ability to form π-stacking or other related interactions (π-anion, π- π T-shaped) that aromatic ligands like porphyrins and ethidium derivatives have shown.

There are several G-quadruplex DNA sequences associated with neurodegenerative diseases. Mutations in the $(GGC)_3$ sequence can cause Fragile X syndrome which is a dominant X-linked inherited disease. While there is no cure for fragile X syndrome researchers have found possible treatments.[17] Neurotransmitters in particular can reduce symptoms associated with fragile X syndrome. Zhang et al. studied the effects of γ-aminobutyric acid (GABA) on fragile X syndrome.[18] G_4C_2 is another G-quadruplex DNA whose overexpression in the intron of the C9orf72 gene has been linked to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[19] ALS is a neurodegenerative disease leading to loss of motor function and FTD is a common form of dementia in individuals 65 and younger. Recent studies have shown that various neurotransmitters and neural hormones can selectively interact with G-quadruplex DNA sequences associated with neurodegenerative diseases like Fragile X syndrome and ALS, as well as the c-MYC G-quadruplex DNA which offers promise for cancer treatments.[20,21]

The two sequences used in this study, 5'- GGCCTG -3' and 5'- CGGGGCGGGGCG -3', are associated with the NOP56 gene and the CSTB gene, respectively. The NOP56 gene, found on chromosome 20, provides instructions for making the nucleolar protein 56 that is found in the nucleus of neurons. This protein is mainly found in the neurons within the cerebellum, which coordinates movement. Expansion of the GGCCTG hexanucleotide repeat sequence of the NOP56 gene results in spinal cerebellar ataxia type 36 (SCA36), which causes progressive movement problems.[22] Expanded GGCCTG repeats may also induce a significant change in the expression of both precursor and mature Mir-1292 (microRNA 1292), which is located just 19 base pairs downstream of the repeat, and this may lead to upregulation of glutamate receptors in particular cell types, which would result in compromised signal transduction. Researchers have found that GGCCTG repeat expansion in intron 1 of NOP56 was the genetic cause of SCA36. Sequences containing GGC repeats are known to form G-quadruplex structures and Hirayanagi et al have report the presence of RNA G-quadruplexes in GGCCTG repeats and that porphyrins reduced SCA36 GGCCTG expansion-mediated cytotoxicity and improved cell viability.[23] Therefore, if neurotransmitters can inhibit the expansion and subsequent G-quadruplex formation, they may offer a potential therapeutic approach towards treatment of SCA36.

The CSTB gene, found on chromosome 21, provides instructions for making the cystatin B protein. This protein inhibits cathepsins that help break down certain proteins in lysosomes. When there is an increase in the number of copies of the dodecamer repeat in the CSTB gene, Unverricht-Lundborg disease occurs.[24] It is a rare form of epilepsy which usually presents symptoms between the ages of 6 and 15 years. It causes severe seizures and balance problems. The most common mutation is an unstable dodecamer repeat expansion in the CSTB promoter region. Dodecamer repeats of the CGGGGCGGGGCG sequence forms parallel-stranded G-quadruplex structures at physiological pH. These dodecamer repeats can lead to significantly lower CSTB mRNA levels in patients, which in turn leads to CSTB losing function as a cysteine protease inhibitor.[25] Also, stabilization of the dodecamer repeat expansions when stable G-quadruplex structures are promoted may provide treatment options. While the exact role of G-quadruplex formation in the pathology of Unverricht-Lundborg disease is uncertain, this study also aims to show the role neurotransmitters may play in inhibiting or promoting subsequent G-quadruplex formation.

Materials and Methods

Reagents

The DNA sequences referred to as NOP56: 5'-GGC CTG-3' and CSTB: 5'-CGG GGC GGG GCG-3'were obtained from Integrated DNA Technologies (Coralville, IA) and used without further purification. The concentrations of the DNA sequences were determined using UV-vis spectroscopy. The molar extinction coefficients (260 nm) used for the samples were 53,000 L mol^{-1} cm^{-1} (NOP56) and 112,000 L mol^{-1} cm^{-1} (CSTB). DNA stock solutions (3.25×10^{-4} M) were prepared in HEPES buffer (20 mM HEPES, 140 mM NaCl, pH = 7.0). The neurotransmitters were obtained from Millipore-Sigma and used without further purification. The concentration of each aqueous neurotransmitter stock solution was 1.0×10^{-4} M.

DNA melting

DNA melting experiments were carried out using a Cary 4000 UV-Vis Spectrometer with the DNA concentration at 27.8 μM, both with and without K^+, and with each neurotransmitter (11.1 μM, also with and without K^+). The concentration of K^+ in the samples was 5 mM (KCl). Following preparation, samples were annealed by heating to 90 °C and slow cooling to room temperature. The annealing process enables the DNA to explore all possible conformations before settling on the most stable form. For the melting experiments, samples were heated from 25 °C to 90 °C at a rate of 10 °C per minute, and the absorbance monitored at 295 nm. Each melting curve was then normalized for comparative purposes.

Molecular Docking

Molecular docking is a computational procedure used to determine what types of interactions were occurring between the neurotransmitter and the DNA molecule as well as which conformation of the molecule had the most thermodynamically favorable binding interaction.[26] Each ligand, or neurotransmitter, was optimized using Gaussion09 (B3LYP method and 6-31 G(d) basis set). Avogadro 1.2.0 was used to create the ligand pdb file from the optimized structure. DNA pdb files downloaded from the RCSB Protein Data Bank. For this study, DNA with the following IDs were used as they contained sequences that were the closest match to the sequences being studied: 2MJJ (CSTB) and 7VCK (NOP56). Python Molecular Viewer (PMV) was used to convert the pdb files into pdbqt files prior to docking. In this program, polar hydrogens

were added, and bonds were made rotatable. Autodock Vina 1.1.2 was used to dock the ligand to the DNA and calculate the binding affinities. The output file of the highest binding affinity was opened in Discovery Studio Visualizer Client 2019 (DSV) for visualization of the interactions within three-dimensional space.

Results and Discussion

DNA melting studies involve monitoring the change in absorbance as a function of temperature. For G-quadruplex DNA, this change is monitored at 295 nm, and the absorbance profile is used to determine whether or not the structure is being stabilized or destabilized. G-quadruplex DNA shows a decrease in absorbance, or hypochromism, at 295 nm, and that is used as confirmation of G-quadruplex formation.[27] Depending on the absorbance profile, G-quadruplexes can be further characterized as parallel, anti-parallel, or hybrid.[28] An increase in absorbance, or hyperchromism, on the other hand, would indicate destabilization of the G-quadruplex structure.

Figure 1 shows the melting curves for each DNA sequence, both in the presence and absence of potassium, and also in the presence of each neurotransmitter (with and without potassium). Low concentrations of potassium ions (5 mM) are required for G-quadruplex formation, usually in conjunction with 140 mM Na^+.[29] The potassium concentration in extracellular fluid is in the 3.5 – 5 mM range. In both NOP56 and CSTB sequences, hyperchromicity is observed from zero absorbance in the absence of K^+, indicating that G-quadruplex structures are not forming at first but above 50 °C, CTSB is showing evidence of G-quadruplex formation, while the high temperature hypochromicity is relatively small for NOP56 without K^+. When K^+ is added, both sequences show evidence of G-quadruplex DNA formation, with a higher starting absorbance in the case of CSTB followed by hypochromism above 50 °C. Hyperchromicity starting from a higher absorbance is indicative of the presence of parallel-stranded G-quadruples sequences.[28] In NOP56, hyperchromicity from a starting absorbance of zero indicates the absence of G-quadruplex structure, but this reverses above 60 °C. However, the G-quadruplex structure destabilizes above 70 °C. This raises the question of what happens when the neurotransmitters are added in place of K^+, and in the presence of K^+.

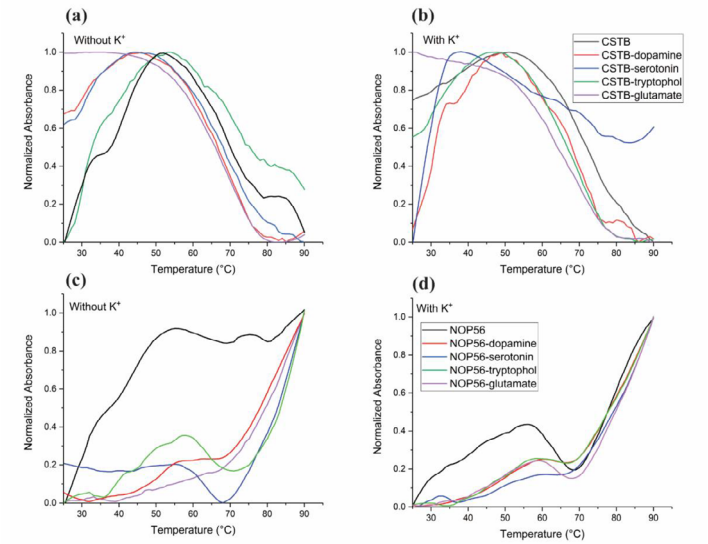

Figure 1. **Figure 1:** Melting profiles of CSTB (a and b) and NOP56 (c and d) showing primary interactions between the neurotransmitters and each DNA sequence.

In CSTB, all four neurotransmitters are promoting G-quadruplex formation in the absence of K^+. The key difference lies in the patterns at low temperature. With glutamate, the only non-aromatic neurotransmitter, CSTB appears to form a hybrid or antiparallel G-quadruplex structure as the absorbance is the highest at first and remains flat through 50°C. With dopamine, there is no evidence of any G-quadruplex present at the outset, similar to the control CSTB sequence. However, both serotonin and tryptophol appear to have promoted the formation of a parallel G-quadruplex structure in the absence of potassium, as the starting absorbance is high and there is hyperchromicity at low temperatures, followed by hypochromicity. The evidence of parallel G-quadruplex structures under certain conditions is consistent with previous studies that have shown repeats of the CGGGGCGGGGCG sequence forming parallel-stranded G-quadruplex structures at physiological pH.

When the neurotransmitters are added in the presence of K^+, the melting profiles change with the exception of glutamate. The evidence of parallel G-quadruplex structures with serotonin and tryptophol is no longer there but appears with dopamine and the control (no neurotransmitter) sequence. This would indicate that the addition of certain neurotransmitters is destabilizing the G-quadruplex structure in the presence of K^+. Despite these changes, there appears to be evidence of G-quadruplex formation at higher temperatures. Overall, G-quadruplex formation in CSTB with the different neurotransmitters has been observed under different conditions, with glutamate promoting hybrid G-quadruplex formation even in the absence of K^+.

In NOP56, hybrid or antiparallel G-quadruplex structures appear to be present when serotonin is added in the absence of K^+, but the structure destabilizes at high temperatures. None of the other neurotransmitters appear to have promoted G-quadruplex formation at the outset, but tryptophol and the control sequence show evidence of G-quadruplex formation above 55 °C, followed by destabilization at higher temperatures. Glutamate and dopamine do not promote G-quadruplex formation.

In the presence of K^+, NOP56 shows clearer G-quadruplex formation in the absence of neurotransmitters, and when glutamate is added, G-quadruplex structures are forming. In both cases, there is destabilization at higher temperatures. There is no evidence of G-quadruplex formation when any of the three aromatic neurotransmitters are added, which indicates that when serotonin and tryptophol are added to NOP56 in the presence of K^+, G-quadruplex destabilization is occurring. Therefore, it appears that with the exception of glutamate, none of the neurotransmitters are stabilizing G-quadruplex structures.

The question that follows these observations has to do with the nature of interactions between the DNA sequences and the neurotransmitters. There are a variety of experiments that can be carried out to determine binding between a ligand (neurotransmitter) and receptor (DNA), including fluorescence spectroscopy and Raman spectroscopy.[1,20,21] Fluorescence spectroscopy has been used in the past to analyze concentration-dependent binding of biomol-

ecules to a fluorophore, whereby the steady increase (or decrease) in fluorescence emission of the fluorophore can be analyzed using one of many binding models to determine binding constants.[21] A steady increase in fluorescence emission intensity is attributed to the intercalation of ligands between DNA bases (π-π stacking), a common occurrence with aromatic ligands like porphyrins and ethidium derivatives. Intercalation can disrupt the DNA structure, whereas other forms of binding may not. Raman spectroscopy, more specifically SERS (surface enhanced Raman scattering) has also been used to determine whether or not a ligand is preferably binding to the target biomolecule over the SERS substrate, which is usually created from gold or silver nanoparticles that have been aggregated by a cation.[5,20,21]

Follow-up experiments with NOP56 and CSTB DNAs and each of the neurotransmitters were inconclusive as there was no trend in the fluorescence emission of the neurotransmitters when the DNA concentration was steadily increased. Therefore, the aromatic neurotransmitters were not intercalating between the bases or G-tetrads. SERS experiments also did not show any change in the Raman intensity of the neurotransmitters when DNA was added, with the exceptions of dopamine-CSTB (decrease, indicating evidence of some binding) and serotonin-CSTB (increase, indicating the opposite). This was counter to our recent work with other neurologically-relevant G-quadruplex sequences.[20,21] Given that there are groups on these neurotransmitters that could interact with the DNA sequences, molecular docking studies were carried out to determine what these interactions could potentially be, and the results are summarized in Table 1.

In all cases, the interaction is thermodynamically favorable as the binding energies are negative. With CSTB, hydrogen-bonding and π- π T-shaped interactions are taking place with the three aromatic neurotransmitters. π- π T-shaped interactions take place between the π-electron clouds between two aromatic groups but in a T shaped, or perpendicular, manner. CSTB had unfavorable donor-donor interactions with both dopamine and tryptophol between an adenine and an OH group. However, the CSTB sequence studied experimentally did not contain an adenine group and the docking studies were carried out with the closest match available in the Protein Data Bank. Glutamate, being non-aromatic, only interacted through hydrogen-bonding but there was one unfavorable interaction between a guanine and an NH group. The interactions between the DNA sequences and neurotransmitters are shown in Figure 2.

NOP56 also interacted with serotonin and dopamine via hydrogen-bonding and π- π T-shaped interactions, and with glutamate via hydrogen-bonding. With tryptophol, NOP56 interacted via hydrogen-bonding and π-anion interactions, which is a form of noncovalent bonding interactions.[30] The binding energies for NOP56 were slightly higher than those for CSTB. There were unfavorable interactions between cytosine and the NH group on glutamate, and thymine and an OH group on dopamine. The docking studies indicate that these DNA sequences are entering into favorable interactions with the neurotransmitters, without any π-stacking interactions that would indicate intercalation. Intercalative interactions would have showed up in fluorescence experiments as mentioned earlier, and the docking results support the premise that the interactions are different than what has been observed with other types of molecules.

In conclusion, neurotransmitters can affect the structural integrity of G-quadruplex forming DNA sequences by either promoting the formation of G-quadruplex forms in the absence of monovalent cations like K^+ and also destabilize the structure in certain cases. The addition of K^+ destabilizes G-quadruplex in NOP56 in presence of tryptophol and serotonin, while promoting it with glutamate or no neurotransmitter. In the case of CSTB, the addition of K^+ alters the type of G-quadruplex, from parallel to hybrid, or vice-versa, depending on the neurotransmitter. Hydrogen-bonding and π- π T-shaped interactions are the most common forms of interactions, and there is no evidence of intercalation between the aromatic neurotransmitters and the two DNA sequences. Collectively, these results show that neurotransmitters have the potential to be considered as possible therapeutics to treat neurodegenerative diseases like spinal cerebellar ataxia type 36 and Unverricht-Lundborg disease.

Table 1: Summary of DNA-neurotransmitter interactions determined using molecular docking.

DNA (PDB ID)	Neurotransmitter	Binding Energy kcal.mol^{-1}	Type(s) of Interactions
CSTB (2MJJ)	Serotonin	-6.0	π- π T-shaped with cytosines and guanine, and H-bonding
	Dopamine	-5.6	π- π T-shaped with an adenine and cytosine and H-bonding plus unfavorable interaction between an adenine and OH
	Tryptophol	-6.2	π- π T-shaped with cytosines and guanine, π-donor H-bond with cytosine, one unfavorable interaction between an adenine and OH, and H-bonding
	Glutamate	-5.8	H-bonding with one unfavorable interaction between a guanine and NH
NOP56 (7VCK)	Serotonin	-4.9	π- π T-shaped with guanines and H-bonding
	Dopamine	-4.8	π- π T-shaped with guanines, H-bonding, and one unfavorable interaction between a thymine and OH
	Tryptophol	-4.7	π-anion with guanine, π-donor H-bond with thymine, and H-bonding
	Glutamate	-5.0	H-bonding with one unfavorable interaction between a cytosine and NH

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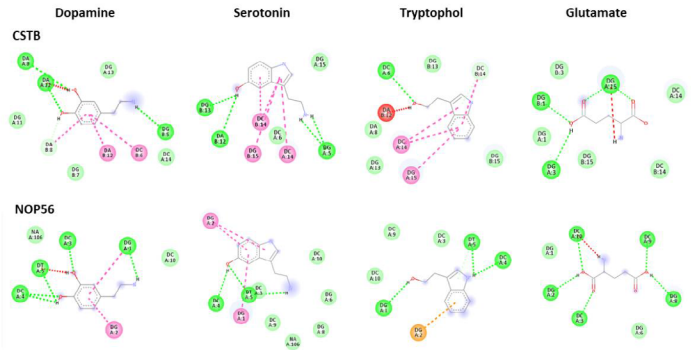

Figure 2. Interactions between the DNA sequences and neurotransmitters as determined via molecular docking calculations. Each interaction is color-coded as follows: dark green – hydrogen bonding, light green – π donor hydrogen bond, orange – π-anion, pink – π- π T-shaped, red – unfavorable donor-donor. The interactions are summarized in Table 1.

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