Hypothesis: Regulation of neuroplasticity may involve I-motif and G-quadruplex DNA formation modulated by epigenetic mechanisms

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ABSTRACT

Recent studies demonstrated the existence *in vivo* of various functional DNA structures that differ from the double helix. The G-quadruplex (G4) and intercalated motif (I-motif or IM) DNA structures are formed as knots where, correspondingly, guanines or cytosines on the same strand of DNA bind to each other. There are grounds to believe that G4 and IM sequences play a significant role in regulating gene expression considering their tendency to be found in or near regulatory sites (such as promoters, enhancers, and telomeres) as well as the correlation between the prevalence of G4 or IM conformations and specific phases of cell cycle. Notably, G4 and IM capable sequences tend to be found on the opposite strands of the same DNA site with at most one of the two structures formed at any given time. The recent evidence that K^+, Mg^{2+} concentrations directly affect IM formation (and likely G4 formation indirectly) lead us to believe that these structures may play a major role in synaptic plasticity of neurons, and, therefore, in a variety of central nervous system (CNS) functions including memory, learning, habitual behaviors, pain perception and others. Furthermore, epigenetic mechanisms, which have an important role in synaptic plasticity and memory formation, were also shown to influence formation and stability of G4s and IMs. Our hypothesis is that non-canonical DNA and RNA structures could be an integral part of neuroplasticity control via gene expression regulation at the level of transcription, translation and splicing. We propose that the regulatory activity of DNA IM and G4 structures is modulated by DNA methylation/demethylation of the IM and/or G4 sequences, which facilitates the switch between canonical and non-canonical conformation. Other neuronal mechanisms interacting with the formation and regulatory activity of non-canonical DNA and RNA structures, particularly G4, IM and triplexes, may involve microRNAs as well as ion and proton fluxes. We are proposing experiments in acute brain slices and *in vivo* to test our hypothesis. The proposed studies would provide new insights into fundamental neuronal mechanisms in health and disease and potentially open new avenues for treating mental health disorders.

I-motif and G-quadruplex

While double helix DNA structure (B-form) is the most common conformation, a variety of other forms are known to exist *in vitro* [1–5]. Some forms also appear to exist *in vivo* and were shown to have physiological significance [6]. Of those, G-quadruplex (G4) and intercalated motif (I-motif or IM) appear to be the most interesting. G4 is found in G-rich regulatory regions of the genome and was demonstrated to exist *in vivo* in several studies [6,7]. There is also evidence of its involvement in regulatory pathways [8].

IM, a form occurring primarily in C-rich regulatory regions and are characterized by C:C+ pairs. Hence IMs form more easily at lower pH. However, IMs can form at normal pH and are also affected by other factors, including ionic strengths (K^+, Mg^{2+} concentration in particular), sequence, DNA anti-helicity torsional strain, and molecular crowding. A recent study provides a strong indication that IM too exists *in vivo* and has a physiological role [1].

Many IMs occur in strands complimentary to G4 sequences and vice versa. There is some evidence of cases when G4 and IM can form simultaneously in the opposing strands, but there appears to be more evidence that one forms to the exclusion of the other (in the opposite strand), which may represent a bi-directional regulatory mechanism [2,5].

Cell cycle

The levels of both IM and G4 appear to change depending on the phase of cell cycle, which appears correlated with changes in gene activity/transcription patterns. A minimum of IM was reported to occur at G0/G1 boundary and the maximum at G1/S boundary [6]. On the other hand, G4 formation appears to occur predominantly in S phase [9].

IM has been tentatively linked with promoters of several proto-oncogenes (such as c-myc, Bcl-2), which play a role in cell cycle and apoptosis [10]. This appears to be consistent with its higher occurrence at G1/S boundary when many proto-oncogenes are active [11].

G4 and methylation

Methylation appears to affect the formation and stability of G4 and thereby modulate the effect of G4-capable sequences on transcription regulation [12,13]. Tsukakoshi and colleagues found that the binding of

the transcription factor SP1 to the G4-forming DNA sequence in the promoter of VEGF gene can be altered by CpG methylation. They showed the methylation state of the G4 sequence to correlate with changes in G4 structure/topology and VEGF expression, indicating the role of G4 conformation and its methylation state in transcription regulation.

In another study, CpG methylation was also shown to affect thermostability of G4 in the VEGF promoter [8]. This effect was dependent of the specific CpG position within G4 sequence as well as the presence of K^+, Na^+, Mg^{2+} ions. Also, CpG methylation in G4-forming sequence of MEST gene promotor was shown to interfere with polymerase chain reaction by stabilizing the G4 formation and thus causing the polymerase arrest [14].

Furthermore, using G4-ChIP-sequencing of human genome, Shi-Qing Mao and colleagues found that most G4 structures are formed in regions containing unmethylated CpG islands (CGIs). Additionally, they showed co-localization of G4s and DNMT1 binding at CGIs, in which G4s restrain DNMT1-mediated methylation. Mao and colleagues proposed that G4s' role involves conserving gene expression function of the corresponding sites by shielding them from methylation through isolating and constraining DNMT1 activity [15].

I-motif and methylation

Fairly little is known about the role of methylation in formation, stability and physiological role of IMs. *In-vitro* evidence indicates that cytosine methylation at position 5 increases the pH of mid-transition to IM form (by $+0.11$) whereas hydroxy-methylation reduces it (by -0.2). If this effect holds *in vivo*, then methylation may facilitate IM conformation *in vivo* while hydroxy-methylation could have the opposite effect [16].

Another *in vitro* study (using oligonucleotides) reported that CpG methylation of IMs altered the energetic barrier of dsDNA < - > IM transition. For some IM sequences, methylation had stabilizing effect, which was reduced or reversed by molecular overcrowding [17].

Hypothesis: IMs and G4 may play a regulatory role in neuroplasticity

Synaptic plasticity represents a mechanism in neurons whereby information input is turned into long-lasting changes in neuronal connectivity essential to memory, learning, cognition and adaptive behavior. The underlying mechanisms include a variety of activity-dependent molecular systems, such as histone modification, DNA methylation and nucleosome remodeling, which in turn alter and co-ordinate gene expression involved in long-term potentiation (LTP), long-term depression (LTD), and synaptic scaling, the key physiological "algorithms" involved in memory formation and other neural functions [18–21].

There is emerging evidence that IMs occur *in vivo* and their formation varies depending on the phase of cells cycle. Also, putative IM patterns are found in many of the genes involves the regulation of cell cycle and apoptosis (see *Cell Cycle* section above).

G4 have been well demonstrated to occur *in vivo*, appear to have some role in transcription regulation and their formation also varies depending on the phase of cell cycle (see *Cell Cycle* section above).

Also, IM and G4 patterns often occur together in the opposite strands but tend to be mutually exclusive conformations (in most cases), i.e. either one or the other form occurs at any given time. This appears to provide a molecular mechanism for a bi-directional regulatory switch controlling a potentially large set of genes whose regulatory sequences, such as promoters and enhancers, include IM/G4 patterns. Such mechanism could have a major role in a co-coordinated, potentially bi-directional regulation of the substantial number of genes involved in progression through phases of cell cycle [2,5].

While cell division and hence cell cycle regulation is of central importance for many tissues and cell types, terminally differentiated neurons generally do not divide. In adult brain, *de novo* neurogenesis occurs at fairly low rates (if at all - still some controversy) and may be limited only to certain areas (such as hippocampus) [22].

As opposed to other cell types, adult neurons adapt to changing conditions not via cell division or death (apoptosis) but by forming new neuronal connections (neurite growth/new synapse formation) and/or eliminating existing ones (synaptic pruning) or LTP and LTD, correspondingly [18–21].

Notably, many of the genes (including several proto oncogenes) involved in regulating cell cycle and apoptosis also play a role in LTP and LTD. In a sense, this is not surprising: while neurite growth, as opposed to cell growth, does not require nuclear DNA replication and cell division, both require an increase in energy production, protein synthesis, biogenesis of ribosomes and mitochondria, etc. [23]. Conversely, both apoptosis and synaptic pruning require activation of autophagy, inhibition of energy production, protein synthesis and so forth [24,23].

There appear to be many similarities in the regulatory pathways and mechanisms involved in cell division/apoptosis and LTP/LTD. In the light of the emerging evidence of the role of IM and G4 in regulating the genes involved in the control and progression of cell cycle and apoptosis, we propose that IM and G4 also have a regulatory role in neuroplasticity, including LTP, LTD, and synaptic scaling. In particular, one of the well-researched IM sequences is in Bcl-2 promoter [25,26]. Bcl-2 is known to be involved in apoptosis and autophagy, partly by inhibition of mTOR pathway [27]. Notably, mTOR pathway was shown to be involved in various aspects of neuroplasticity, including LTP, LTD and memory consolidation [28–30]. This putative new mechanism may not only improve our understanding of neuroplasticity regulation but also open a new direction in drug development for neurological and mental health conditions (see *Clinical implications*).

There is evidence that some neurodegenerative diseases may be caused by aberrant G4 formation interfering with transcription. The most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is the hexanucleotide repeat expansion (HRE), (GGGGCC)n, in C9orf72 gene. Haeusler and colleagues showed that the molecular mechanism of that pathology is based on HRE forming DNA and RNA G4s with distinct structures giving rise to aberrant variants of RNA•DNA hybrids (R-loops) [31]. The structural polymorphism in HRE causes a repeat-length-dependent accumulation of aberrant transcripts aborted in the HRE region. The transcribed HRE repeats were shown to bind to ribonucleoproteins (specifically nucleolin) in a conformation-dependent manner contributing to nucleolar stress and initiating cascades leading to ALS/FTD.

Tyrosine hydroxylase is the enzyme involved in the limiting step of catecholamine biosynthesis, the conversion of tyrosine to DOPA, which has been shown to play important roles in the nervous system functions, including learning, memory, synaptic plasticity, cognition, reward, motor function and others. Tyrosine hydroxylase mutations and variations have been associated with several neurological and psychiatric disorders including Parkinson's disease and Schizophrenia [32,33]. The regulatory regions of tyrosine hydroxylase gene appear to include and be regulated by G4 and I-motif structures [34,35].

Hypothesis: The regulatory effects of DNA methylation on neuroplasticity may, in part, occur via methylation-induced changes in the stability/formation of IM and G4

There is growing evidence that epigenetic mechanisms play a major role in neuroplasticity [36]. In particular, DNA methylation/demethylation appears to be especially important in inducing and maintaining long-lived circuitry changes, such as those associated with long-term memory, learning, habits, addiction, fear extinction and so forth [36–39].

If IM and G4 indeed play a role in neuroplasticity regulation, then

methylation/demethylation of DNA in IM/G4 sequences could modulate the regulatory signals by facilitating or hindering the formation on these non-canonical conformations.

This may occur in one of the following ways:

Methylation state may alter the stability of IM and/or G4 directly

Methylation, hydroxy-methylation or de-methylation of IM may alter its stability, increasing/decreasing its prevalence *in vivo*. For example, based on *in vitro* data mentioned above, methylation of IM pattern (via DNMT enzymes) may increase IM formation whereas hydroxy-methylation of methylated cytosine (via TET enzymes) would have the opposite effect. Similar methylation-related effects may be expected for G4. For example, CpG methylation appears to affect G4 stability and transcription factor binding to VEGF promoter [12].

Methylation state may affect formation of IM or G4 in one strand, thereby preventing the formation of the regulatory (transcription-factor binding) G4 or IM, correspondingly, in the opposite strand.

This is based on findings that IM and G4 patters often co-occur in opposing strands and the formation of one appears to usually suppress the other and vice versa. Therefore, if IM (or G4) sequence in a sense strand has a direct regulatory role (e.g. binds a transcription factor), then methylation of G4 sequence (or IM, correspondingly) in anti-sense strand, may have indirect regulatory affect by suppressing the IM (or G4) conformation in the sense strand.

IM and/or G4 may impact neuroplasticity by affecting MeCP2 function

MeCP2 is a regulatory factor that has a role in neuroplasticity. MeCP2 binds to the common DNA methylation site CpG, which changes the expression of multiple genes. MeCP2 has essential role in early CNS development as well as maintenance role in mature adult neurons where it modulates both synaptic development and function. The ability of MeCP2 to bind CpG site may be affected when the CpG is within or close to IM or G4 conformation.

A less direct but intriguing possibility is that MeCP2 transcription, splicing or translation may be affected by IM and/or G4 formation. Bagga and colleagues showed that Rett syndrome mutations resulting in the decreased MeCP2 protein expression was associated with a conserved G4. They also identified G4s that could regulate MeCP2 pre-mRNA alternative splicing in either negative or positive way [40].

IM may be involved in learning and memory regulation and affected by non-CpG (particularly CpC) methylation via DNMT3A

While the majority of methylated cytosines are found at CpG sites, non-CpG methylations (CpA, CpT and CpC) also occur and can induce gene silencing or activation depending on the region. However, non-CpG methylation appears to be restricted to a few specific cell types, notably neurons and glial cells. Non-CpG methylation in mammals is performed by DNMT3A and DNMT3B members of DNA methyl-transferase enzyme family [41].

In terminally differentiated neurons in adult brains, DNMT1, DNMT3A and DNMT3B appear to be active. DNMT3A appears to be the specific subtype required for normal de novo learning and memory. Forebrain specific DNMT3A knockout mice, but not DNMT1 knockout mice, had altered LTP and synaptic plasticity in the affected areas and showed learning and memory deficits [42].

IM is a C-rich pattern that usually includes repeating $C[C]_n$ clusters providing a potential target for CpC methylation specifically by DNMT3A (or DNMT3B). Considering the role of DNMT3A in learning, memory and likely other related instances of LTP, we propose that DNMT3A may act, in part, by methylating cytosines inside IM patterns, facilitating IM formation and thereby modulating expression.

It is notable that whereas non-CpG methylation is higher in the brain than in most other tissues (where it is minimal), different neuron types vary by level and distribution of both CpG and non-CpG methylation, possibly reflecting functional differences [43]. It is unclear whether and which TET enzymes have any specificity in regard to de-methylating CpG vs non-CpG sites, DNA sequence or conformation, such as IM or G4. Investigating this possibility appears worthwhile.

Hypothesis: The regulatory effects of DNA methylation on neuroplasticity may by modulated by IM and G4, whose formation may interfere with epigenetic signaling pathways, such as methylation/demethylation and histone acetylation/deacetylation activity.

Above we hypothesized that DNA methylation may affect the formation of IM and G4 and thus interfere with signal transduction and gene expression involved in neuroplasticity. We believe that the opposite may also take place, i.e. the formation of G4 or IM may modulate the activity of epigenetic mechanisms, making the connection between G4/IM and epigenetic modifications somewhat bi-directional. There is emerging evidence pointing to such possibility. In a recent study, Mao and colleagues found that G4 can bind to and thus inhibit the activity of DNMT1 enzyme leading to reduced level of DNA methylation, particularly at and near the binding site [15]. If so, the factors inducing G4 formation would presumably reduce DNA methylation levels, potentially altering gene expression. In most, albeit not all cases, DNA de-methylation upregulates the expression of directly affected genes. Furthermore, considering that IM and G4 tend occur in the opposing strands of the same sequence and their formation tends to be mutually exclusive, IM may have the opposite effect to G4 on DNA methylation of the corresponding sites. Hence, the factors that induce IM formation would tend the suppress G4 formation in the opposing strand and reduce the inhibitory binding for G4 to DNMT1, leading to greater DNMT1 activity and increased DNA methylation.

A recent study by Shioda and colleagues indicates that such effects not only occur *in vivo* but are relevant to the neuronal functions and potentially to neuroplasticity [44]. The researchers demonstrated that ATRX, a chromatin remodeling protein, acts by binding to G4 of the imprinted Xlr3b gene, regulating its expression. In turn, Xlr3b binds to dendritic mRNAs and its overexpression impairs dendritic transport leading to synaptic dysfunction. ATRX gene mutations cause alpha-thalassemia X-linked intellectual disability (ATR-X) syndrome. Notably, ATRX was shown to bind to G4s in Xlr3b together with DNMTs. Furthermore, the levels of DNMT1 and DNMT3A, the DNMT subtypes involved in adult neuroplasticity, were decreased (relative to wild type) in ATRX-deficient mutant mice in G4-rich regulatory site for Xlr3b.

While there is already emerging evidence that G4s and indirectly IMs can affect DNMT enzymes and thus alter local methylation levels, it is conceivable that other enzymes involved in epigenetic modifications may bind to and be inhibited/activated by G4s or IMs. It appears worthwhile to research the effects of G4s and IMs on the activity of ten-eleven translocation methyl cytosine dioxygenases (TET family), histone deacetylases, histone acetyltransferases, histone methyl-transferases and other enzymes and factors involved in epigenetic modifications occurring during neuroplasticity.

All in all, the relationship between G4, IM and epigenetic modifications in the context of neuroplasticity is likely to exists, be complex and vary depending on neuron subtype, external signals, phase of cell cycle and other variables. This is consistent with the recent findings that epigenome in neurons is not fixed but is in a state of equilibrium dynamically maintained by multiple mechanisms and shifted in different directions depending of a particular type of neuroplasticity, such as during LTP, LTD and synaptic scaling.

Hypothesis: The regulatory activity of microRNAs in neuroplasticity and elsewhere may be modulated by their effects on the formation and stability of DNA G4s and IMs. G4 formation in microRNA transcripts may also have a regulatory role.

Micro RNAs (miRNAs) are known to have multiple regulatory roles in all tissues, including the brain. Abnormal levels of various miRNAs were found in many neurological and mental health conditions [45]. There is evidence that DNA G4 conformation may hinder the DNA binding activity of relevant miRNAs thus affecting their regulatory function [46]. Similar effect of miRNA on DNA IM formation appears possible as well. Hence cellular events that affect the transition of DNA to/from IM and/or G4 conformation could affect signaling mediated by some miRNAs.

Also, G4 in miRNA transcripts were shown to affect their processing and maturation, which may modulate regulatory function of miRNAs [47,48].

Hypothesis: RNA G4 may play a key role in neuroplasticity through translation regulation and splicing control

Translation regulation plays a major role in synaptic plasticity, memory formation and related circuitry changes and occurs via multiple mechanisms [49–51]. Notably, dendrites contain a large amount of locally pre-positioned ribosomes and mRNA ready for rapid onset on translation occurring during LTP [52–54]. We propose that RNA G4s may play important role in regulating translation during neuroplasticity.

There is considerable evidence that G4s can form in RNA and may be more stable than their DNA counterparts *in vitro* [55,56]. There is also evidence that RNA G4s play a role in translation regulation by, for example, interfering with initiation or causing early termination of translation [57,58]. RNA G4s also may be involved in the regulation of alternative mRNA splicing [59]. The genomes of eukaryotes, including humans, appear to have a large number of transcribed sequences of putative RNA G4s [60]. However, Guo and Bartel have recently reported that the majority of G4 regions in eukaryotes *in vivo* exist in the unfolded state at any given time [56]. Notably, the model RNA G4 regions unfolded in eukaryotes became folded when ectopically expressed in *Escherichia Coli*. Guo and Bartel suggested that eukaryotes have the machinery that globally unfolds the majority of RNA G4s in order to suppress their ability to interfere with translation, whereas bacteria, lacking such machinery, have undergone evolutionary depletion of G4-forming sequences. The mechanism of active RNA G4 unfolding in eukaryotes remains to be determined and appear to involve RNA-binding proteins, such as hnRNP family [61,62] and certain helicases (the enzymes unwinding DNA and RNA), such as *Pif1* [63].

In the light of the above, it appears that, on one hand RNA G4-forming sequences are common in eukaryotes, are thermodynamically stable and can easily form spontaneously while, on the other hand, their formation is actively controlled and at least partly suppressed in most cases most of the time. We propose that such situation produces a metastable, actively maintained equilibrium suitable for a fine control of translation (and perhaps also splicing), which is crucial for the regulation of neuroplasticity. For example, an external signal or a miRNA could reduce the expression or impair the binding ability of an G-rich-RNA-binding protein, leading to increased formation of G4s, reduced translation in dendrites and eventually LTD. A similar mechanism that increases the unfolding of certain G4s may lead to LTP. The potentially significant role of RNA G4s in neuronal function and neuroplasticity appears to be supported by the evidence associating transcribed G4-forming sequences with a number of mental health disorders [64].

Fragile X syndrome (FXS) is the most-frequent heritable syndrome of mental insufficiency. It is caused by the expansion of (CGG)n repeats in the FMR1 gene leading to the loss of the corresponding protein FMRP. FMRP is involved in regulating the translation of specific neuronal messenger RNA (mRNA) targets and contains an arginine-glycine-glycine (RGG) RNA-binding domain shown to bind with G4 forming mRNA structures [65]. RGG motif of FMRP also modulates its association with polysomes indication the role in translation regulation [66].

Additionally, MAP1B, PP2A and Shank1 genes that are essential for neural development have been shown to harbor one or more G4s in their 50-UTRs and/or 30-UTRs and their expression was shown to be upregulated by the absence of FMRP, indicating that FMRP plays pivotal a role in neonatal brain development [67,68].

Hypothesis: Neuronal ion and proton fluxes may produce some of their regulatory/signaling by affecting the formation and stability of RNA G4, IM, and triplex structures

The formation of many non-canonical nucleic acid forms, such as IM, G4 and triplexes is affected by pH and ion concentrations, particularly potassium and magnesium [69–74]. Physiological variations in these parameters may exert some of their regulatory effects by facilitating or inhibiting the formation of these non-canonical structures.

Ion fluxes, including fluxes of potassium and magnesium ions, have a particularly large role in neurons. The greatest changes in neuronal ion concentration presumably occur in the cytosol, where they may affect RNA (and perhaps mit-DNA) conformation [75]. RNA G4 formation under physiological conditions is well established and there is considerable evidence supporting its regulatory significance (as we discussed above). Furthermore, potassium ions were shown to stabilize RNA G4 whereas magnesium and calcium ions have the opposite effect [71]. Similar effects except in the opposite regulatory direction appear to occur in RNA triplexes, with potassium inhibiting and magnesium facilitating their formation [73,74,76].

We propose that RNA G4 and RNA triplex conformation changes may be one of the mechanisms by which cytosolic ion fluxes associated with neuronal signaling exert their action. Similar considerations may also apply to pH changes. Proton fluxes/gradients appear to have a major regulatory role in neurons and glia [77].

Pre-synaptic activity is known to change extracellular pH in CNS. Extracellular pH changes affect neurons via acid-sensing ion channels (ASICs). However, extracellular pH changes may also directly alter intercellular pH in neurons and glia [78]. Neuronal signaling also appears to be associated with pH changes in intracellular compartments, such as endosomes [79] and mitochondria [80], and potentially with cytosolic proton fluxes [81]. The regulatory role of neuronal proton fluxes may involve, in part, the direct effect of pH on the stability of cytoplasmic non-canonical nucleic acid structures, such as mitochondrial DNA IMs and RNA triplexes [82,83]. It is unclear if pH or ion concentration changes in the nucleus are sufficient for regulatory effects via DNA IM/G4 but this may be worth investigating.

Clinical implications

New class/category of drugs for neurological and psychiatric conditions

The conformation and stability of both IM and G4 can be altered by specific chemical agents, which makes them a potential drug target. Some of the relevant chemical agents could be small molecules absorbed via GI tract after oral administration and capable of crossing blood-brain barrier.

For example, a pregnanol derivative and a cholestane derivative have been shown to target IM and affect Bcl-2 expression presumably regulated via IM pattern sequence [84,85]. G4 targeting agents is already a promising area of anti-cancer drug research. However, while designing drugs that target specific G4s is crucial for developing effective treatments, it is a challenge due to the topological similarity of the skeleton of diverse G4s. Nevertheless the next-generation G4 ligands are being developed [86,87]. In a recent study, Shioda and

colleagues reported promising results indicating that drugs targeting G4s may have potential as a treatment option. They administered 5-aminolevulinic acid (5-ALA), which reduced the abnormal over-expression of Xlr3b gene in the mouse model of alpha-thalassemia X-linked intellectual disability (ATR-X) syndrome. They demonstrated that ATAX suppresses expression of Xlr3b by binding to G4 and DNMTs, which does not occur in the ATAX deficient mutant mice. 5-ALA normalized Xlr3b expression presumably because its metabolites, protoporphyrin IX and hemin, bind to G4s interfering with the binding of ATAX and DNMTs. This appear to be an early indication of the potential that the drugs targeting IMs and G4s may have in neurological and psychiatric conditions [44].

Ideas/methodology for testing the hypotheses

Acute brain slices

Detecting IM, G4 and triplex formations and observing their dynamics in acute brain slices during/after induced LTP/LTD via specific anti-IM or anti-G4 antibodies in DNA and RNA [6,88] and fluorescence resonance energy transfer (FRET) [89].

Identify IM, G4 and their dynamics in brain slices during neuroplasticity, and LTP/LTD in particular, may also be feasible via nuclear magnetic resonance (NMR) spectroscopy [86,90]. NMR has been used to assign non-exchangeable protons in the nuclear magnetic resonance spectra of small DNA/RNA molecules, in which the groups of protons in specific sugars or bases are first detected by scalar couplings (COSY), and then correlated spatially based on the degree of cross-relaxation due to the Overhauser effect (NOESY). H-NMR may be used to monitor intracellular pH, which is a factor in IM and G4 formation [91]. However, H-NMR alone may be unsuitable or inadequate *in vivo* and in cell (including active brain slices) for DNA/RNA conformation resolution unless the other specific nuclei, such as ^{13}C, ^{15}N or ^{19}F, are also used. Manna and colleagues have recently developed a new method suitable for both *in vitro* and in-cell analysis of G4s using conformation-sensitive fluorescent nucleoside analog probe and ^{19}F NMR labeling [92]. We hypothesize that these and other innovative uses of NMR spectroscopy could provide the means for dynamically monitoring and distinguishing in-cell non-canonical DNA/RNA conformations while also measuring the intracellular characteristics affecting their stability, such as pH levels [93–95].

Homology/pattern search for putative IM and G4 in genes (including regulatory regions) involved in neuroplasticity.

Test the effects of anti-cancer drugs such as peptidomimetic ligands (PBP1 and PBP2) targeting G4s (daunomycin, PBP2) and IMs (PBP1) on LTP and LTD [27,96].

Test the effects of de-methylation/methylation drugs (especially targeting DNMT3A) on IM/triplex formation and G4 dynamics and stability in neurons by utilizing *in vitro* nuclear magnetic resonance (NMR) spectroscopy (methods mentioned above).

In vivo

Detect IM and G4 and monitor their dynamics in the process of new memory formation (e.g. fear conditioning/habitual avoidance and after its extinction or drug treatment) using specific anti-IM or anti-G4 antibodies [6,88].

In the human genome, it has recently been demonstrated that G4s are associated with CGI hypomethylation where they inhibit DNMT1 enzymatic activity and thereby protect certain CGIs from methylation [15].

Detect *in vivo* DNA methylation in putative IM/G4 in genes (including regulatory regions) involved in neuroplasticity (ideally at different stages of LTP/LTD, etc.). It would be ideal to determine specific methylation sites and whether it is CpG or non-CpG methylation. Further investigation into disorder specific IM/G4 formation and DNA methylation may help develop targeted treatments with lower side effects.

Investigate the proposed use of qPCR to detect methylation via effect on IM and G4 [13].

Test the effects of IM and/G4 disrupting drugs such as peptidomimetic ligands (PBP1 and PBP2) targeting G4s (daunomycin, PBP2) and IMs (PBP1) on neuroplasticity involved in mental health disorders such as ASD and OCD mice models [27,96].

Find physiologically active miRNAs likely to bind to IM, G4 and triplex forming sites (from literature and by homology search). Test their effects with or without drugs on the stability on these non-canonical structures *in vivo* [27].

Conclusions

In summary, we hypothesize that non-canonical DNA and RNA structures are an integral part of neuroplasticity control via gene expression regulation at the level of transcription, translation and splicing. We propose that the regulatory activity of DNA IM and G4 structures is modulated by DNA methylation/demethylation of the IM and/or G4 sequences, which facilitates the switch between canonical and non-canonical conformation. Other neuronal mechanisms interacting with the formation and regulatory activity of non-canonical DNA and RNA structures, particularly G4, IM and triplexes, may involve microRNAs as well as ion and proton fluxes. The studies we propose could yield new insights into the essential neuronal mechanisms involved in memory and behavior while possibly generating novel approaches for drug and treatment development for a broad spectrum of mental and neurological illnesses.

Competing interests statement

All author declare that they have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Additional literature

Special Journal Issue on IM/G4: https://www.mdpi.com/journal/molecules/special_issues/G-quadruplexes_i-motif_DNA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.04.003.

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