Evaluation of i-Motif Formation in the Serotonin Transporter-Linked Polymorphic Region

Bryony N. Thorne,[a] Bart A. Ellenbroek,[b] and Darren J. Day*[a]

Neuropsychiatric disorders such as major depressive disorder (MDD) arise from a complex set of genetic and environmental factors. The serotonin transporter (SERT) is a key regulator of synaptic serotonin (5-HT), and its inhibition is an important pharmacological target for treating MDD. The SERT-linked polymorphic region (5-HTTLPR) contains two major variants (short and long) that have been implicated in modulating susceptibility to MDD by altering the level of expression of SERT. Both variants contain C-rich repeats that conform to consensus i-motif folding sequences. i-Motifs are quadruplex DNA structures that have been proposed to have a role in transcription regulation. With spectroscopic techniques, we demonstrate that both alleles are able to form i-motifs at acidic pH, and at neutral pH under conditions of molecular crowding. This highlights the potential for i-motif formation to contribute to transcriptional regulation of the serotonin transporter, with a potential role in the pathophysiology of neuropsychiatric disorders.

Neuropsychiatric disorders such as major depressive disorder (MDD) are complex conditions which can be attributed to a combination of genetic and environmental factors. Although the pathophysiology of MDD remains to be fully elucidated, the role of the monoamine, serotonin (5-hydroxytryptamine; 5-HT) in the regulation of changes in cognition, emotions, and mood associated with MDD is well established.[1] Synaptic concentrations of 5-HT are regulated by the serotonin transporter (SERT), which facilitates reuptake of 5-HT into the presynaptic terminal.[2,3] SERT is encoded by the *SLC6 A4* gene, within the promotor region of which is the serotonin-transporter-linked polymorphic region (5-HTTLPR) that contains a variable number tandem repeat (VNTR).[4] There are two main allelic variants of this region that differ by a 44 bp insertion/deletion giving rise to the short (S) and long (L) allelic variants.[5] The S-allele shows reduced transcription of SERT and has been associated with an increased likelihood of developing depression, although other polymorphisms within this region might also contribute to expression.[6] A recent highly powered study by Border et al. has cast doubt upon the functional significance of these polymorphisms,[7] thus, whether or not the allelic variants are associated with MDD is now more controversial than ever. However, it is beyond contention that SERT has an important role in the aetiology of MDD, such that pharmacological inhibition of SERT by selective serotonin reuptake inhibitors remains an important therapy for treating MDD.[8]

i-Motifs (intercalated motifs) are noncanonical secondary DNA structures that can form in cytosine-rich sequences of single-stranded DNA.[9,10] i-Motifs are formed by two intercalated hairpins in a quadruplex structure, stabilised by hemiprotonated cytosine-cytosine base pairing.[11] As such, i-motif formation is favoured at acidic pH, however, molecular crowding, negative superhelicity of the DNA double helix, and localised melting by transcription factors have all been shown to facilitate i-motif formation at neutral pH.[12,13] That i-motifs naturally occur in the cell nucleus is supported by studies using an i-motif-specific monoclonal antibody, adding to a growing body of evidence that their formation may have a role in cellular regulation.[14]

Here we report the identification of sequences within the VNTR region of the 5-HTTLPR that might form i-motifs on the C-rich strand, and G-quadruplexes on the G-rich strand. We demonstrate that both alleles of the 5-HTTLPR fold into i-motifs at neutral pH, highlighting i-motif formation in this region as a potential regulatory mechanism for SERT expression.

To investigate i-motif formation in 5-HTTLPR, the region surrounding the deletion in 5-HTTLPR was synthesised as two oligonucleotides (Figure 1) to represent the allelic variants (VNTR-S and VNTR-L). Cytosine-rich regions that are able to form i-motif structures under acidic conditions are also able to

Figure 1. Nucleotide sequences of VNTR-L (left) and VNTR-S (right) of the 5-HTTLPR repeat. Poly-C tracks are indicated by grey shading, oligonucleotide sequences are shown in red boxes, and the repeat units deleted in the S-allele are shown in bold.

[a] *B. N. Thorne, Dr. D. J. Day*
School of Biological Sciences, Victoria University of Wellington
Kelburn Parade, Wellington 6012 (New Zealand)
E-mail: Darren.day@vuw.ac.nz
[b] *Prof. B. A. Ellenbroek*
School of Psychology, Victoria University of Wellington
Faculty of Science
Wellington 6012 (New Zealand)

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form i-motifs in the presence of silver ions.[15] i-Motif formation was determined by the reduction of silver-stabilised i-motifs to form silver nanoclusters (AgNCs). The luminescence of AgNCs is highly dependent upon the oligonucleotide sequence that serves for the scaffold of the AgNCs.[15,16] VNTR-S and VNTR-L both formed multiple AgNCs with highly similar excitation and emission spectra, suggesting that similar i-motif structures were produced (Figure S1 in the Supporting Information).

To confirm that silver-stabilised i-motifs can be formed by VNTR-S and VNTR-L, circular dichroism (CD) spectroscopy was undertaken. DNA sequences that fold into an i-motif conformation undergo a highly characteristic bathochromic shift in the CD spectra relative to unfolded DNA, with a region of negative ellipticity around 260–270 nm.[17] Figure 2 shows that the addition of $AgNO_3$ in 1.4 to 32 molar excess induced i-motif formation for the VNTR-S and VNTR-L oligonucleotides in a Ag^+ concentration-dependent manner.

A similar bathochromic shift indicative of i-motif formation was observed at acidic pH (Figure 3) for both VNTR-S and VNTR-L, however, this shift was not observed for an oligonucleotide with a similar base pair composition of random sequence not conforming to a consensus i-motif sequence. These data support the hypothesis that the VNTR sequences form i-motifs. Figures 2 and 3 show that similar CD spectra for VNTR-S and VNTR-L were obtained in the presence of Ag^+ and at acidic pH, thus suggesting that the two sequences produce similar i-motif structures.

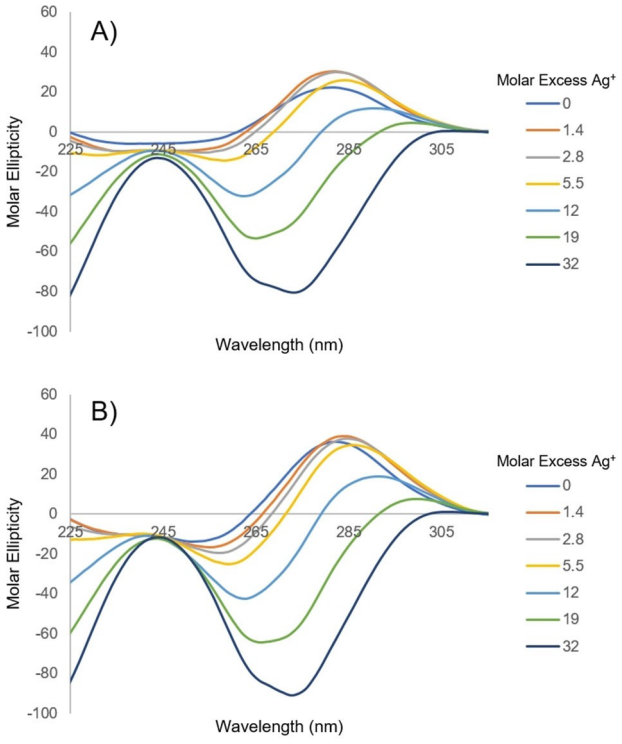

Figure 2. CD spectra of A) VNTR-S and B) VNTR-L oligonucleotides in the presence of $AgNO_3$. Oligonucleotide concentrations were 10 μM in 10 mM MES buffer (pH 6.5) with the following molar excesses of silver: 0, 1.4, 2.8, 5.5, 12, 19, and 32.

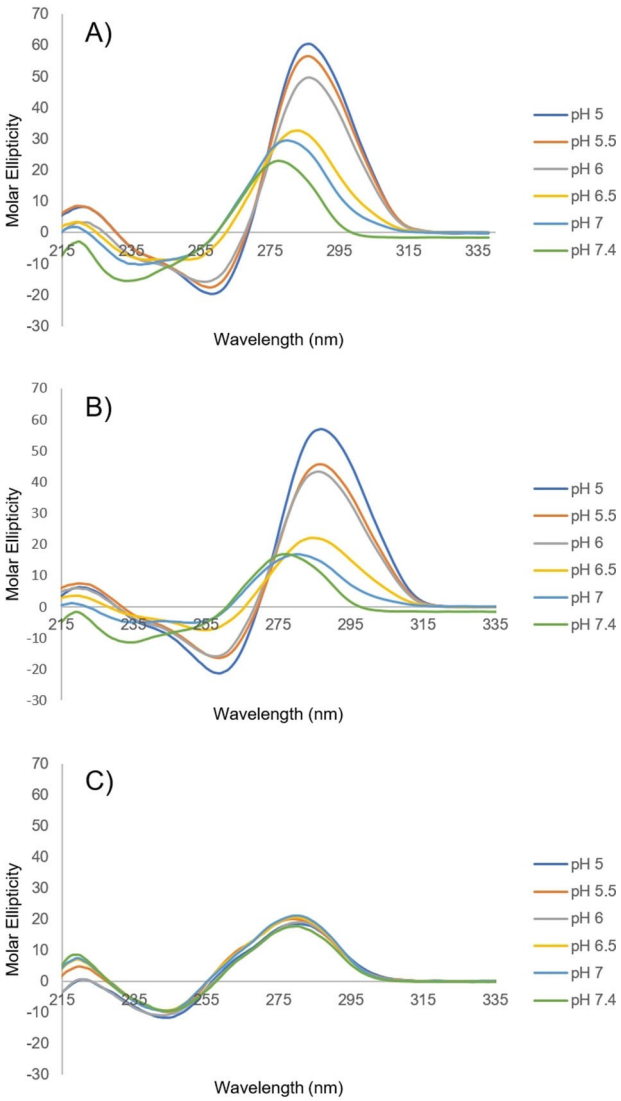

Figure 3. CD spectra of A) VNTR-S, B) VNTR-L, and C) random oligonucleotides at pH 5–7.4. Oligonucleotide concentrations were 10 μM in 100 mM cacodylate buffer at each pH.

CD melting experiments were undertaken to investigate the thermal stability of i-motifs formed by VNTR-S and VNTR-L at pH 5.[18,19] Figure 4 shows CD spectra of both oligonucleotides as a function of temperature. The bathochromic shift which is characteristic of i-motif formation is observed between 65–75 °C for both VNTR-S and VNTR-L, thus indicating that i-motif structures formed by both oligonucleotide variants have similar thermal stabilities.

Having shown that the 5-HTTLPR VNTR can form i-motif structures at acidic pH, we then sought to determine whether these structures may form under more physiologically relevant conditions such as might occur in the cell nucleus. Studies have shown that high concentrations of components found in the cell nucleus can lead to i-motif formation due to molecular crowding at neutral pH.[12,14,17] Experimentally, this can be mimicked by the use of high concentration poly(ethylene glycol) (PEG) to induce molecular crowding.[12,13] Figure 5 shows

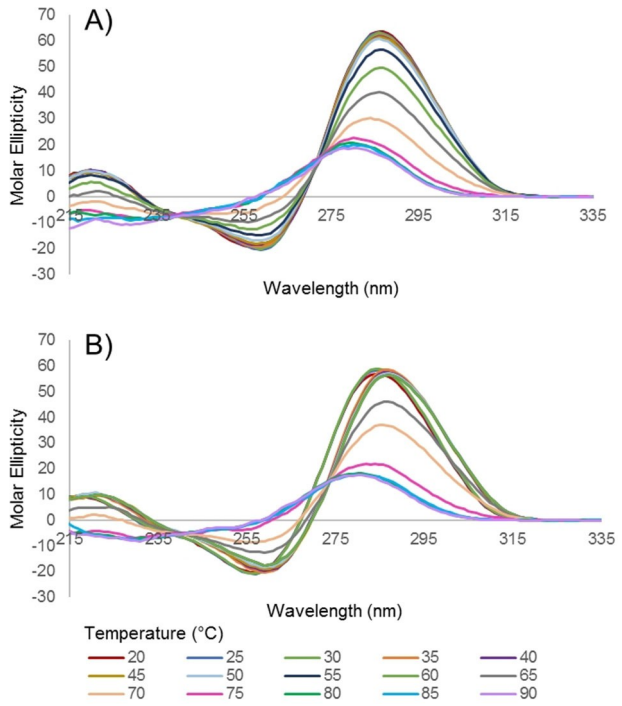

Figure 4. CD spectra of A) VNTR-S and B) VNTR-L at pH 5 captured between 20 and 90 °C. Oligonucleotide concentrations were 10 μM in 100 mM cacodylate buffer (pH 5).

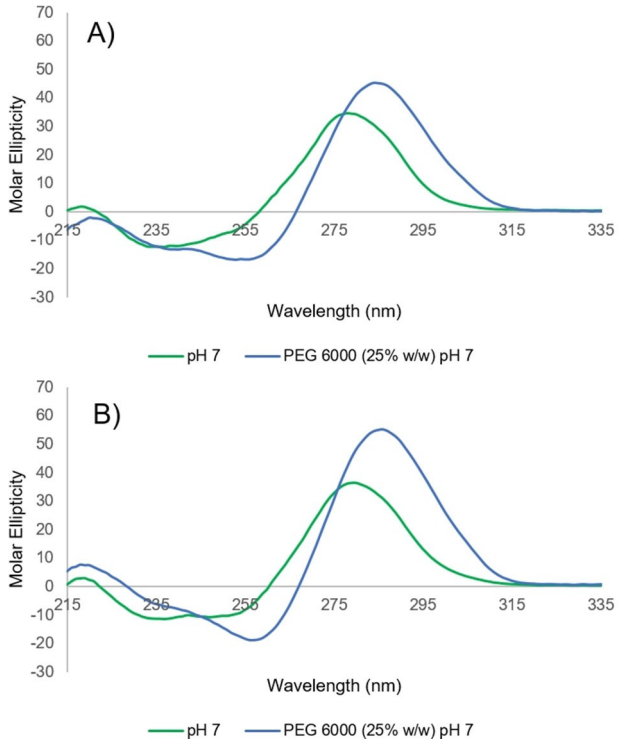

Figure 5. CD spectra of A) VNTR-S and B) VNTR-L oligonucleotides in the presence of PEG 6000. Oligonucleotide concentrations were 10 μM in 100 mM cacodylate buffer (pH 7), and 5 μM in 50 mM cacodylate buffer with PEG 6000 (25% *w/w*).

the CD spectra for VNTR-S and VNTR-L in the presence of 25% PEG 6000 at pH 7.0, both VNTR-S and VNTR-L formed i-motifs at neutral pH with molecular crowding. Figure 6 shows CD melting spectra of i-motifs formed by both oligonucleotide variants at the same conditions of neutral pH and molecular crowding. At pH 7 in the absence of PEG 6000, i-motif formation did not occur (Figure S2), however, molecular crowding induced the formation of i-motif structures for both oligonucleotide variants. VNTR-S and VNTR-L showed very similar thermal denaturation profiles, with the i-motif structure forming between 35–40 °C. Shown in Figure 7, transition profiles for fraction of i-motif as a function of temperature were generated using the molar ellipticity at 286 nm:

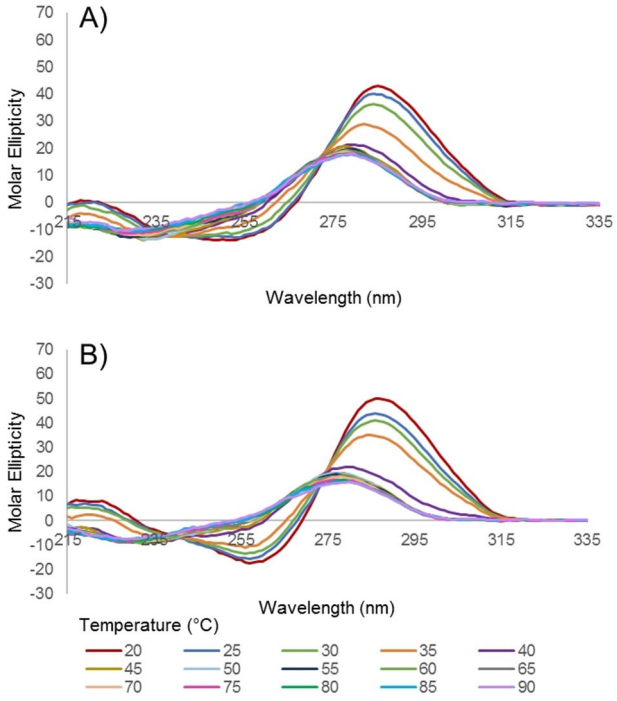

Figure 6. CD spectra of A) VNTR-S and B) VNTR-L in the presence of PEG 6000 (25% *w/w*) at 20–90 °C. Oligonucleotide concentrations were 5 μM in 50 mM cacodylate buffer (pH 7).

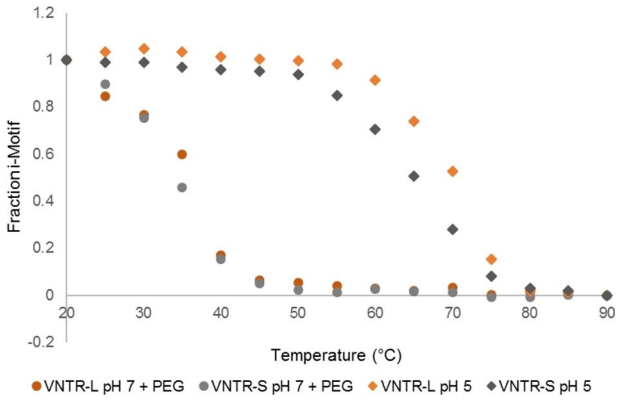

Figure 7. Transition profiles for fraction of i-motifs as a function of temperature for the spectra in Figures 4 and 6.

$$\text{Fraction i}-\text{motif} = \frac{\theta_{286,\,x\,^\circ\text{C}} - \theta_{286,\,90\,^\circ\text{C}}}{\theta_{286,\,20\,^\circ\text{C}} - \theta_{286,\,90\,^\circ\text{C}}}$$

Where $\theta_{286,\,x\,^\circ\text{C}}$ is the molar ellipticity at 286 nm at a given temperature, $\theta_{286,\,20\,^\circ\text{C}}$ is the molar ellipticity at 286 nm at 20 °C, and $\theta_{286,\,90\,^\circ\text{C}}$ is the molar ellipticity at 286 nm at 90 °C.[19,20]

Under conditions of molecular crowding, VNTR-L formed i-motif structures more readily and a greater ellipticity was observed. While this difference was reproducible between experiments, it is unclear whether this is attributable to the VNTR-L existing in a conformation that more readily forms i-motifs. Similarly, it is unclear if this observation has biological relevance for the propensity to form i-motifs *in vivo*.

The present data indicate that the 5-HTTLPR region at the SERT promotor is capable of forming i-motif structures, as has been demonstrated for a number of other genes.[17,21–23] The demonstration of i-motif formation at neutral pH under conditions of molecular crowding implies the putative formation of i-motifs *in vivo*, however, direct supporting evidence of their formation *in vivo* is lacking. Although small differences in i-motif formation were observed between the two allelic variants, the extent to which this may affect transcriptional regulation is unclear. This observation might have been influenced by the use of synthesised oligonucleotides with limited lengths and it remains possible that more significant differences would be seen if a greater proportion of the surrounding sequence was incorporated into the analysis. However, as both oligonucleotide sequences represent alleles from within a repeat sequence, this is unlikely.

Although the physiological function of i-motifs remains to be fully elucidated, it has been suggested that they may act as transcriptional regulators, as putative i-motif forming sequences are found clustered in regulatory regions of the genome,[10] particularly within oncogene promoting regions where they are speculated to repress transcription.[13,14,21,22,24,25] For example, transcription factors binding to i-motifs in the promotor regions of *BCL-2* and *H-RAS* have been shown to facilitate unfolding of the i-motif, with the unfolded state promoting gene expression.[21,22] While decreased expression of SERT has been proposed to be associated with neuropsychiatric disease, the genetic link remains unclear, particularly around potential mechanisms as to how the S- and L-alleles may lead to alterations in SERT expression[3]

The data in this study collectively supports the hypothesis that under physiological conditions, both alleles of 5-HTTLPR region may be able to form stable i-motifs. Whether or not the regulation of SERT expression is modulated by i-motifs formed by the S- and L-allelic variants is yet to be determined. Regardless, the formation of i-motifs by the promoter region of SERT highlights a new and promising area of research for MDD, especially as studies aimed at targeting i-motif forming sequences are already underway.[10]

Experimental Section

Oligonucleotides: Custom DNA oligonucleotides were purchased from Integrated DNA Technologies (Singapore) to represent VNTR-S (TTC ACC CCT CGC GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CTG CAG CCC TTC CAG CAT CCC CCT GCA CCT CTC CCA GGA TCT CCC C) and VNTR-L (TGC ACC CCC AGC ATC CCC CCT GCA GCC CCC CCA GCA TCT CCC CTG CAC CCC CAG CAT CCC CCC TGC AGC CCT TCC AGC ATC CCC C) of the 5-HTTLPR sequence, as well as a random oligonucleotide sequence with 60% GC content to represent a non-i-motif forming sequence (TCG GGC ATG TTT CCC TTG TAG GTG TGA GGC CAC CTG GCT TCG CGC CGT GGT CCC AAA GGA AAA CCT ATG GAC TTT GTT CCG GGT GG). Stock oligonucleotides were resuspended in Tris·EDTA (TE) buffer (10 mM, pH 8.0) to a final concentration of 100 μM for silver nanocluster synthesis, and 250 μM for CD spectroscopy. Two independent oligonucleotide syntheses were used for pH titrations and molecular crowding conditions.

Silver nanocluster synthesis: Nanoclusters were synthesised in 400 μL of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (10 mM, pH 6.5) containing 2 nmol of the oligonucleotide stock solution. $AgNO_3$ was added with rapid mixing to final concentration of 100 μM $AgNO_3$ and the mixture was incubated in the dark at 4 °C for 30 min. Unbound silver was removed by filtration through a 30 000 kDa molecular weight ultracentrifugal filter (Amicon) as per the manufacturer's instructions. 400 μL of MilliQ water was then added to the product before it was concentrated again, prior to dilution in 100 mM MES buffer to a final volume of 300 μL. Silver ions bound by the DNA were reduced to silver nanoclusters by the slow addition of 100 μL of 100 μM $NaBH_4$ while mixing vigorously. The reaction mixture was stored at 4 °C in the dark overnight, and AgNC formation was assessed by fluorescence spectroscopy the following day.

Fluorescence spectroscopy: Prior to fluorescence spectroscopy, the AgNC reaction mixture was diluted to approximately 1:100 in 100 mM MES buffer. Fluorescence excitation and emission measurements were obtained using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) using a 10 mm path length quartz cuvette, 10 nm slit width for excitation and emission, and were collected at a scan speed of 600 nm/min.

Circular dichroism spectroscopy: CD spectra were recorded on a Chirascan Plus spectrophotometer (Applied Photophysics) over a range of $\lambda = 200$–350 nm at 20 °C, using a 2 mm path length quartz cuvette and a scan speed of 120 nm/min. Scans were conducted in triplicate then averaged and background corrected against a blank containing all reagents except oligonucleotides.

I-motif formation was assessed in the presence of silver ions, at a range of pH values between 5 and 7.4, and under molecular crowding conditions. CD melting experiments were conducted at 5 °C intervals between 20–90 °C.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: gene expression · i-motif · major depressive disorder · nucleic acids · serotonin transporter

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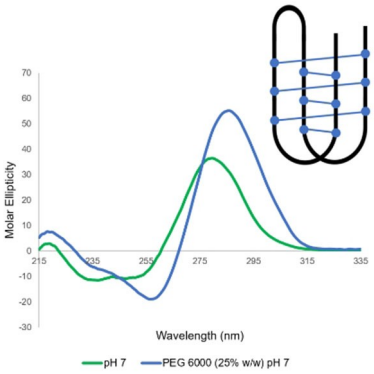
COMMUNICATIONS

Regulating transport: i-Motifs are secondary DNA structures that seem to have a role in transcription regulation. Here we show the formation of i-motifs in two allelic variants within the promotor region of the serotonin transporter. Polymorphisms within this region have been linked to neuropsychiatric disorders, and we highlights i-motif formation as a potential regulatory mechanism for the serotonin transporter.



*B. N. Thorne, Prof. B. A. Ellenbroek, Dr. D. J. Day**