A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo

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Polymorphic regions consisting of a variable number of tandem repeats within intron 2 of the gene coding for the serotonin transporter protein 5-HTT have been associated with susceptibility to affective disorders. We have cloned two of these intronic polymorphisms, Stin2.10 and Stin2.12, into an expression vector containing a heterologous minimal promoter and the bacterial LacZ reporter gene. These constructs were then used to produce transgenic mice. In embryonic day 10.5 embryos, both Stin2.10 and Stin2.12 produced consistent β-galactosidase expression in the embryonic midbrain, hindbrain, and spinal cord floor plate. However, we observed that the levels of β-galactosidase expression produced by both the Stin2.10 and Stin2.12 within the rostral hindbrain differed significantly at embryonic day 10.5. Our data suggest that these polymorphic variable number of tandem repeats regions act as transcriptional regulators and have allele-dependent differential enhancer-like properties within an area of the hindbrain where the 5-HTT gene is known to be transcribed at this stage of development.

Methods

Plasmid Construction. Stin2.10 and Stin2.12 polymorphisms were obtained from the DNA of nonaffectected and bipolar individuals, respectively, by PCR by using the following oligonucleotides (27, 28): 5′-GTCAGTATACAGGCTGCGAG-3′ and 5′-TGTTCCTAGTCTTACGCCAGTG-3′ (MWG Biotec, Ebersberg, Germany). The PCR products were cloned into pCR2.1 (Invitrogen) and several clones of each construct were sequenced on a LI-COR 4000L DNA sequencer (MWG Biotec) to ascertain PCR fidelity. Correct sequences were then subcloned into the NotI–SpeI site of the heterologous promoter (human β-globin promoter) LacZ expression vector pGZ40 (which has been...

Abbreviation: 5-HT, 5-hydroxytryptamine or serotonin; CNS, central nervous system; En, embryonic day; n, VNTR, variable number of tandem repeats; βgal, β-galactosidase; FP, floor plate.

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shown not to express LacZ in embryos on its own) to produce p29Stin2.10 and p29Stin2.12 (Fig. 14; ref. 29). Before oocyte injection, both constructs were removed from their plasmid backbones by restriction digestion with NotI/SalI.

Transgenic Mice. Transgenic mice were generated as described (30). DNA constructs were injected at a concentration of 2 ng/μl into the pronuclei of one-cell embryos derived from superovulated (CBA × C57BL/6)F1 females. Injected eggs were transferred into pseudopregnant CD1 females. Transgenic embryos were harvested at E8, E9, E10.5, E11.5, E12.5, and E13.5 and assayed for βgal activity (30).

PCR Screening of Transgenic Lines. Tail-tip DNA was extracted by incubating mouse tail-tip biopsy material overnight in 500-μl tail-tip buffer (200 μg/ml-1 proteinase K (Roche Molecular Biochemicals)/300 mM sodium acetate, pH 7/1% SDS/10 mM Tris, pH 8/1 mM EDTA, pH 8). After incubation, biopsy samples were frozen and then centrifuged at 13,000 × g at 4°C for 10 min. After the removal of 1 μl of the supernatant and its dilution into the pronuclei of one-cell embryos derived from superovulated (CBA × C57BL/6)F1 females. Injected eggs were transferred into pseudopregnant CD1 females. Transgenic embryos were harvested at E8, E9, E10.5, E11.5, E12.5, and E13.5 and assayed for βgal activity (30).

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Vibratome Sections. Vibratome sections were produced from embryos stained for LacZ to ascertain the exact locations of internal staining sites.

LacZ-stained embryos were washed twice in PBS and left overnight in 4% (vol/vol) sucrose/PBS. Embryos were then incubated for 6 h in 20% (vol/vol) sucrose/PBS then transferred into BSG solution [14% (vol/vol) BSA/20% (vol/vol) sucrose/0.5% gelatin in PBS] and incubated overnight with gentle agitation. On the next day, the embryos were embedded in fresh BSG solution mixed with 2.5% (vol/vol) glutaraldehyde, and 100-μm thick sections were cut with a Vibratome series 1000.

Discussion

Transgene Expression in the Embryonic Hindbrain. We have shown in this study that two polymorphisms of the 5-HTT intron 2 VNTR region, Stin2.10 and Stin2.12, which are correlated with susceptibility to depressive disorders, act as strong and consistent positive transcriptional regulatory elements. Moreover, they were found to differ in the strength of their transcriptional inducing abilities within the developing rostral hindbrain at E10.5, where Stin2.12 seemed to be significantly stronger than the Stin2.10 polymorphism. These findings are supported by in vitro data that show that Stin2.12 was found to act as a significantly more potent positive regulator of marker gene expression than the Stin2.10 polymorphism when transformed into embryonic stem cells deprived of leukemia inhibitory factor (31). Several lines of research may serve to put these findings into context.

One study used in situ hybridization to show that, at E11, transcripts of the 5-HTT gene are also present in the rostral hindbrain FP just caudal of the mesencephalic flexure (14). We suggest that the Stin2.10/12 transcriptional regulator elements within the 5-HTT gene contribute to its transcriptional regulation within the rostral hindbrain. One reason that has been suggested to explain why 5-HTT is expressed in the hindbrain comes from the observation that little or no 5-HT is produced in the young rodent embryo (10, 11, 14), a situation that may also exist in the human embryo. Therefore, one role for the 5-HTT protein in development could be the concentration of 5-HT in the rostral hindbrain from maternal sources where it can then act as a morphogen (1–4, 6, 7). Several other studies have shown that 5-HT neurons first develop in the rostral hindbrain in a cluster designated B5–9 (32–35).

It is intriguing that, in addition to our findings, two other groups have identified the rostral hindbrain as being significant in terms of the expression of 5-HTT mRNA and the eventual location of 5-HTT cell clusters. Therefore, we hypothesize that a link may exist between the expression patterns produced by the Stin2.10/12 transcriptional regulator elements, the expression of 5-HTT mRNA, and the eventual hindbrain localization of 5-HT neuron cluster B5–9. We suggest that the difference in levels of expression in the rostral hindbrain between the two polymorphisms used in this study might provide a clue as to how these
Fig. 1. (A) A limited restriction map of the p29Stin2.10 and p29Stin2.12 constructs used in this study. h\(\beta\)g, human \(\beta\)-globin minimal promoter sequence; LacZ, \(\beta\)gal gene; pA, simian virus 40 polyadenylation sequence. (B–J) Expression patterns of the LacZ gene in p29Stin2.10 transgenic embryos at E9 (B–D), E10.5 (E–G), E11.5 (H), and E12.5 (I and J). B, E, H, and I represent side views of the embryos; C, F, and J are viewed ventrally; D and G are viewed caudally. Fb, forebrain; Mb, midbrain; Hb, hindbrain. Sc, spinal cord; Fp, FP; Ov, otic vesicle; r1–5, rhombomeres 1–5; H, heart.
polymorphisms may contribute to depressive illness. Several groups have provided data that show that inappropriate expression of certain genes such as epidermal growth factor, protein tyrosine phosphatase, and interleukin-6 (36–38) within the brains of transgenic animals results in abnormal CNS development. Thus, inappropriate levels of 5-HTT expression in the rostral hindbrain during development may be produced if the gene is driven by a stronger or weaker than normal positive transcriptional regulator. This situation may have the effect of increasing/decreasing local morphogenic 5-HT levels and leading to possible aberrant 5-HT neuron development. Because of the association of 5-HT neurons in the generation of different behavioral states (13), the situation described in this study may have repercussions for the emotional well being of an individual in later life. It is intriguing in this context that the rostral raphe nuclei, derived from rostral hindbrain, have been shown to send axons into the forebrain and cerebral cortex where they have been suggested to have a function in emotional modulation (34).

Stin2.10 and 2.12 Transcriptional Regulation in the Midbrain and Neural Tube FP. With an absence of data showing expression of 5-HTT mRNA in the embryonic midbrain, rhombomeres 4 and 5, and spinal cord FP, it is not easy to reconcile these expression patterns produced by both constructs in the embryo with any known developmental process. However, several studies have shown that the proximity of sequences such as chromatin-anchoring sites and DNA hypersensitivity sites often influence the normal function of transcriptional control elements (39–42). The expression patterns may therefore reflect the removal of regulatory influences on the Stin VNTR elements once isolated from their normal promoter environment.

We have shown that a VNTR within intron 2 of the 5-HTT gene produces expression in the rostral hindbrain, where serotonergic neurons are known to differentiate and 5-HTT is known to be expressed (32–35, 43, 44). However, this VNTR also produces expression in the midbrain, where dopaminergic neurons derive (13, 32, 33). It is interesting in this context that a structurally similar VNTR region close to the human dopamine transporter (DAT1) gene has been identified (45). In addition, several studies have also discovered correlations with different polymorphisms of the DAT1 VNTR and a susceptibility to drug addiction, schizophrenia, attention deficiency disorder, and chronic depression (46–49). Therefore, expression of the marker gene driven by the 5-HTT VNTR regions in the midbrain might hint at the existence of an overlap in the regulation of expression of the 5-HTT gene and the determination of dopaminergic cell clusters where the DAT1 gene may play a role. In agreement with this hypothesis, the DNA sequence of the 5-HTT VNTR contains extensive homology to that found within the DAT1 VNTR (31). The relationship between these VNTR sequences and their ability to support gene expression warrants further study.

The FP expression described in this paper is interesting with respect to 5-HTT expression where it is known that exposure of chick embryos to 5-HTT inhibitors, such as cocaine and antidepressants, leads to abnormal development of the FP and the notochord (50). It may be significant therefore that Stin2.10/12, situated in intron 2 of the 5-HTT gene, should drive expression in the spinal cord FP. Although no FP expression of 5-HTT has been reported, one study reveals the expression of 5-HTT by immunohistochemistry in the spinal cord at E12 in the rat (14). If Stin2.10 was shown to drive 5-HTT expression in the FP, it would not be unreasonable to extrapolate the role 5-HTT might play within the neural tube in modulating morphogenic 5-HT levels and neural tube dorsoventral axis determination.

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