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Decreased mRNA Expression of Netrin-G1 and Netrin-G2 in the Temporal Lobe in Schizophrenia and Bipolar Disorder

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Abstract

The membrane-bound axon guidance molecules netrin-G1 (NTNG1) and netrin-G2 (NTNG2) play a role in synaptic formation and maintenance. Non-coding single nucleotide polymorphisms (SNPs) in both genes have been reported to be associated with schizophrenia. The main aim of this study was to determine if NTNG1 and NTNG2 mRNA expression is altered in schizophrenia or bipolar disorder, and/or influenced by disease-associated SNPs. NTNG1 and NTNG2 mRNAs were examined in the medial and inferior temporal lobe using in situ hybridization and RT-PCR in the Stanley Medical Research Institute array collection, and in rat hippocampus during development and after antipsychotic administration. NTNG1 mRNA isoforms were also examined during human brain development. For NTNG1, the G1c isoform was reduced in bipolar disorder and with a similar trend in schizophrenia; expression of four other NTNG1 isoforms was unchanged. In both schizophrenia and bipolar disorder, NTNG2 mRNA was reduced in CA3, with reductions also found in CA4 and perirhinal cortex in bipolar disorder. The SNPs did not affect NTNG1 or NTNG2 mRNA expression. Both NTNG1 and NTNG2 mRNAs were developmentally regulated, and were unaltered by haloperidol, but NTNG2 mRNA was modestly increased by clozapine. These data implicate NTNG1 and NTNG2 in the pathophysiology of schizophrenia and bipolar disorder, but do not support the hypothesis that altered mRNA expression is the mechanism by which genetic variation of NTNG1 or NTNG2 may confer disease susceptibility.

Keywords

gene expression, hippocampus, in situ hybridization; isoform; netrin-G1, netrin-G2, splice variant; temporal cortex

INTRODUCTION

Netrin-G1 (NTNG1) and netrin-G2 (NTNG2), also known as laminet-1 and laminet-2, are vertebrate-specific axon guidance molecules. NTNG1 and NTNG2 undergo alternative

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DISCLOSURE/ CONFLICTS OF INTEREST

Dr Eastwood: My research is funded by the UK Medical Research Council and NARSAD. I declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

Professor Harrison: My group's research is supported by the UK Medical Research Council, Stanley Medical Research Institute, NARSAD, Wellcome Trust, and an unrestricted educational grant from GlaxoSmithKline. In the past three years I have received honoraria for giving educational lectures or chairing scientific meetings from Bristol Myers Squibb, GlaxoSmithKline, Janssen, Lilly, Merck, Sanofi and Servier pharmaceutical companies, and I have been a scientific advisor to Curidium, Janssen and Wyeth. There are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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splicing, generating 10 known isoforms for NTNG1 which mostly differ in the number of laminin-type epidermal growth factor (EGF)-like domains they contain (Nakashiba et al, 2000; Meerabux et al, 2005; Figure 1), whilst for NTNG2, 3 different isoforms have been identified (Nakashiba et al, 2002). Unlike classical secreted netrins, NTNG1 and NTNG2 are bound to the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor, and do not bind to known netrin receptors such as UNC-5H and DCC (Nakashiba et al, 2000, 2002; Yin et al, 2002). Instead, NTNG1 and NTNG2 bind to specific ligands called netrin-G1 ligand (NGL-1) and netrin-G2 ligand (NGL-2) respectively (Kim et al, 2006; Lin et al, 2003). Both NGL-1 and NGL-2 are membrane-bound post-synaptic proteins containing an intracellular PDZ (postsynaptic density-95/discs large/zona occludens-1) binding domain thought to be important in effecting downstream signaling events, and a leucine-rich repeat extracellular domain which forms a horseshoe-shaped hook with which it binds to its pre-synaptic partner (Biederer, 2006). Together with their ability to promote neurite outgrowth and regulate synapse formation (Lin et al, 2003; Kim et al, 2006; Nakashiba et al, 2000, 2002), the structural characteristics of NTNG1 and NTNG2 and their ligands suggest that they may have functional similarities to the more extensively characterized neurexins and neuroligins, known to be important in synapse formation, function, and regulation of the balance between excitatory versus inhibitory inputs (Chih et al, 2005; Dean and Drebach, 2006; Lise and El-Husseini, 2006; Varoqueaux et al, 2006).

Schizophrenia is postulated to be a disorder of connectivity (Andreasen, 1999; Stephan et al, 2006) and of the synapse (Frankle et al, 2003; Mirnics et al, 2001; Moises et al, 2002) due, at least in part, to aberrant neurodevelopment (Lewis and Levitt, 2002; Marenco and Weinberger, 2000), with decrements in the number of synaptic contacts and/or synaptic activity being proposed as the possible substrate underlying altered functional connectivity in the disorder (Stephan et al, 2006; McGlashan and Hoffman, 2000). Of note, many of the recently identified potential susceptibility genes for schizophrenia play a role in synapse formation, functioning and plasticity (Harrison and Owen, 2003; Harrison and Weinberger, 2005). Recently, single nucleotide polymorphisms (SNPs) in the genes for NTNG1 and NTNG2 have been reported to be associated with schizophrenia (Aoki-Suzuki et al, 2005; Fukasawa et al, 2004). Although these associations have yet to be replicated, the role of NTNG1 and NTNG2 in synapse formation and function enhances interest in these observations. As observed for other schizophrenia susceptibility genes (Harrison and Weinberger, 2005), the NTNG1 and NTNG2 SNPs associated with the disorder are either intronic and/or within untranslated regions, suggesting that any disease susceptibility they may confer occurs by altering some facet of the expression of the genes. NTNG1 mRNA isoform expression has been reported to be decreased in the dorsolateral prefrontal cortex in schizophrenia (Aoki-Suzuki et al, 2005). Here we have determined if NTNG1 and NTNG2 mRNA expression is influenced by schizophrenia and/or schizophrenia associated SNPs. To address diagnostic specificity, subjects with bipolar disorder were included, and we examined a brain region implicated in both disorders, the temporal lobe, including the hippocampal formation and adjacent cortices (Arnold, 2000; Harrison, 1999, 2002, 2004; Vawter et al., 2000). Additionally, we determined if NTNG1 and NTNG2 mRNAs are developmentally regulated, and examined their expression in adult rat hippocampus after treatment with the antipsychotic drugs haloperidol and clozapine.

METHODS AND MATERIALS

Different methodologies were used to examine NTNG1 and NTNG2 mRNA expression, their choice based upon previous findings, the question to be examined, and their relative abundance. Firstly, as the distribution of these mRNAs in the human temporal lobe had not been described, we used *in situ* hybridization histochemistry (ISHH) with oligonucleotide probes (specific for each gene and designed to detect all known GPI-anchored splice

variants) to determine their regional and cellular distributions. ISHH was also used to examine NTNG1 and NTNG2 mRNA expression in the rat hippocampus during brain development, and in adult rats after antipsychotic treatment. To compare NTNG2 mRNA expression between diagnostic groups, ISHH using the same 'pan' probe was also employed, since (a) this allows subfields to be examined separately, and (b) the most significant disease-associated SNP, rs1105684, is in the 5' untranslated region; this location suggests that the SNP is more likely to regulate total NTNG2 mRNA than it is to affect alternative splicing. In contrast, for NTNG1, based on the previous study (Aoki-Suzuki et al, 2005), we predicted that any change in its expression occurring in schizophrenia or related to the disease-associated SNP would affect the relative abundance of NTNG1 isoforms, not total NTNG1 mRNA. Moreover, our pilot ISHH experiments showed a limited distribution of the mRNA. Hence, we used RT-PCR with primers designed to detect all known NTNG1 GPI-anchored splice variants; in this way, the relative abundance of all NTNG1 transcripts expressed in the temporal lobe could be explored in one PCR reaction, including less abundantly expressed transcripts which may not have been detected using less sensitive techniques. RT-PCR was also used to examine the developmental regulation of NTNG1 mRNA isoforms in human brain.

Netrin-G1 and netrin-G2 mRNA in situ hybridization histochemistry (ISHH)

Post mortem tissue from 35 subjects with schizophrenia, 34 with bipolar disorder, and 35 healthy comparison subjects was provided by the Stanley Medical Research Institute (SMRI) array collection (Table 1; see also http://www.stanleyresearch.org/programs/brain_collection.asp). Frozen sections (14μm) at the level of the mid-hippocampus, containing the hippocampal formation, perirhinal and inferior temporal gyri, were pretreated for ISHH (Eastwood et al, 2000), and stored at −20°C until use. ISHH for NTNG1 and NTNG2 mRNAs was carried out using ^{35}S 3'-labelled oligonucleotide probes complementary to the human sequences (NTNG1: bases 1771-1810, Genbank accession number NM_014917.2; NTNG2: bases 1442-1480, Genbank accession number NM_032536.1) and established protocols (Eastwood et al, 2001). Sections were hybridized overnight at 37°C in hybridization buffer (Eastwood et al, 2000) containing 1 million cpm purified labeled probe and 50mM dithiothreitol. Post-hybridization washes were carried out in 1× standard saline citrate for 3 × 20 minutes at 60°C, followed by 2 × 60 minute washes at room temperature. For NTNG2 mRNA, duplicate sections were run concurrently from all subjects. For NTNG1 mRNA, single sections from a subset of 4 male control subjects (age: 20-54 years; post mortem interval (PMI): 6-27 hours; brain pH: 6.25-6.78) were hybridized for regional and cellular distribution analysis. Hybridized sections were placed against Kodak Biomax MR film (Amersham, Little Chalfont, UK) alongside ^{14}C microscales (NTNG1: 28 days; NTNG2: 21 days) and subsequently dipped in autoradiographic emulsion (LM1, Amersham), exposed for 16 weeks, developed, and stained with cresyl violet. Experimental controls consisted of ISHH with sense orientation probes, and incubation in the presence of 50 fold excess unlabelled probe.

For the rat developmental studies, tissue was acquired from male animals sacrificed (in accordance with UK Home Office guidelines) from six litter groups obtained from timed pregnancy Sprague-Dawley rats (Harlan Olac, UK) at embryonic day 19 (E19), postnatal day 3 (P3), P7, P14, P22 and P42 (Eastwood et al, 2005). Each time point contained between 3-5 animals. For the drug treatment study, adult male Sprague-Dawley rats (8 weeks, 250-300g; 8 in each group) were administered 1mg/kg haloperidol, 25mg/kg clozapine, or saline by intraperitoneal injection once daily for 2 weeks (Law et al, 2004). No significant differences were found between the groups in their percentage weight gain over the two weeks, and the drug treated rats did not appear to be sedated. Animals were sacrificed 6 hours after the final dose by lethal injection of pentobarbitone. For both rat studies, frozen

coronal sections (15μm) were cut at the level of the dorsal hippocampus, collected onto Superfrost plus slides (VWR, Lutterworth, UK), pretreated (Eastwood et al., 2001), and ISHH performed for NTNG1 and NTNG2 mRNAs as described above. Triplicate sections were run concurrently for each transcript, and hybridized sections placed against Kodak Biomax MR film (Amersham) alongside ^{14}C microscales for the following times: NTNG1: 21 days; NTNG2: 14 days. Hybridized sections were subsequently dipped in photographic emulsion (LM1, Amersham), exposed for 4 weeks, developed and counterstained with cresyl violet.

Image analysis of ISHH autoradiographs

For the human study, NTNG2 mRNA autoradiographs were measured using an MCID Elite version 7.0 image analysis system (Interfocus, Haverhill, UK), calibrated to the ^{14}C microscales and corrected for non-specific signal. Measurements were taken over the granule cell layer of the dentate gyrus (DG), pyramidal cell layer of CA4 and CA3, and deep cortical layers of the perirhinal and inferior temporal cortex. Note that we use CA4 to refer to the invagination of the pyramidal cell layer into the 'jaws' of the dentate gyrus (Duvernoy, 1989) and not to the polymorphic layer [hilus] (see Insausti and Amaral, 2004).

For the rat studies, NTNG1 and NTNG2 mRNA autoradiographs were measured as described above. For E19 rats, measurements were taken over the entire hippocampal formation, whilst for all other animals, measurements were taken over the DG granule cell layer and the pyramidal cell layer of CA3 and CA1. NTNG1 and NTNG2 mRNA measures during rat hippocampal development were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Eastwood et al, 2005).

Netrin-G1 mRNA reverse- transcriptase PCR (RT-PCR)

Total RNA was isolated from a section adjacent to those employed for NTNG2 mRNA ISHH, using Tri-Reagent (Sigma, Poole, UK) and established methods. RNA integrity number (RIN) was determined using an Agilent Bioanalyzer 2100 and RNA 6000 Nano chips (Agilent Technologies Wokingham, UK). Following pilot studies to determine the linear range of detection, 700 ng of total RNA from each subject was DNAse treated and reverse transcribed using 200 units of MMLV (Promega, Southampton, UK), 30 ng oligo(dT) primer, 0.5mM each dNTP (Promega) and 10 units RNAsin (Promega). Duplicate 30 ng aliquots of the resulting cDNA samples were subjected to NTNG1 RT-PCR using PureTaq Ready-To-Go PCR beads (Amersham) and published primers (Aoki-Suzuki et al, 2005; Meerabux et al, 2005). Touchdown PCR amplification was performed as follows: an initial denaturing step at 94°C for 2 min, followed by 20 amplification cycles; 30 sec at 94°C; 45 sec at 70°C initially (minus 1°C at each cycle); 45 sec 72°C, followed by 15 amplification cycles; 30 sec 94°C; 45 sec 55°C; 45 sec 72°C, and a final extension step at 72°C for 7 min. PCR products were measured using an Agilent 2100 Bioanalyzer and DNA 1000 chips (Agilent Technologies).

To examine changes in the relative expression of NTNG1 mRNA isoforms between fetal and adult human brain, RT-PCR was performed as described above, using commercially available mRNA samples (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) taken from adult temporal lobe (pooled from 8 male/female Caucasians, aged 32-61 years), adult hippocampus (pooled from 20 male/female Caucasians aged 18-54) and from fetal brain (pooled from 21 male/female Caucasians aged 26-40 weeks).

Netrin-G1c isoform quantitative real-time PCR

To corroborate the above method, G1c transcript expression was also measured by quantitative real-time PCR (AB 7900HT, Applied Biosystems, Warrington, UK) using

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previously published primers and a G1c isoform-specific minor groove binding probe (Aoki-Suzuki et al, 2005). This was carried out in a subset of subjects for whom RNA was available (controls: n=28; age: 45 ± 8 (mean ± SD); PMI: 31 ± 13; RIN: 4.8 ± 1.6; schizophrenia: n=24: age: 44 ± 9; PMI: 29 ± 15; RIN: 5.2 ± 1.6; bipolar disorder: n=27; age: 47 ± 11; PMI: 36 ± 20; RIN: 4.9 ± 1.8). Triplicate reactions (each containing 10 ng of cDNA template) were performed for all these subjects concurrently on a single plate. The mean absolute measures were calculated using a standard curve of serially diluted pooled cDNA and sequence detector software (SDS version 2, Applied Biosystems).

Netrin-G1 and netrin-G2 genotype determination

DNA was extracted from cerebellar tissue using a Nucleon ST kit (Tepnel Life Sciences PLC, Manchester, UK). The most significantly associated SNP for each gene (as reported by Aoki-Suzuki et al, 2005 and Fukasawa et al, 2004), was chosen for genotyping (NTNG1: rs1373336; NTNG2: rs1105684). The NTNG1 SNP tags a 54 kb haplotype block that extends 43 kb upstream to intron 3 and spans the alternatively spliced region of the gene; the NTNG2 SNP tags a 16 kb haplotype block (Haploview, v.3.32). Genotype was determined using Applied Biosystems Taqman pre-designed SNP genotyping assays in accordance with manufacturer's instructions. Genotype reproducibility was greater than 99%.

Statistical analysis

All statistical analyses were performed using SPSS version 13 software. One sample Kolmogorov-Smirnov tests were used to verify that the data were normally distributed.

For the human post mortem studies, two-way analysis of covariance (ANCOVA) was performed to look for overall effects of diagnosis and isoform (NTNG1) or subfield (NTNG2), and their interaction. Subsequently, one-way ANCOVAs were performed for each isoform (NTNG1) or subfield (NTNG2), with planned comparisons between diagnostic groups explored using least significant difference (LSD). Effects of genetic variation on NTNG1 and NTNG2 mRNA expression were also examined using ANCOVA. For all the ANCOVAs, three covariates were chosen, *a priori*, based upon their frequent influence upon mRNA detection (e.g., Barton et al, 1993; Harrison et al, 1995): age, post mortem delay, and RIN (Imbeaud et al, 2005). RIN was chosen as the measure of RNA quality instead of brain pH, formerly widely used, since RIN is a direct rather than proxy measure, and explains a greater proportion of the variance (Lipska et al, 2006; Stan et al, 2006, and our unpublished observations). In addition to these primary analyses, we also carried out secondary analyses to assess the robustness of our findings: in particular, we re-ran the ANCOVAs, limiting the covariates to those that had a significant influence for each dataset (i.e. subfield or isoform) as determined by Spearman correlations ($p<0.05$). The latter were also used to examine relationships with other continuous variables (e.g. inpatient time, lifetime antipsychotic exposure). Finally, the potential influence of smoking, alcohol and substance misuse, as rated by the SMRI, was evaluated using ANOVA. Smoking was classified into three categories (smokers, ex-smokers, or non-smokers), while six categories were used for alcohol and substance use (little or none, social, moderate past, moderate present, heavy past, heavy present). All experiments were performed (by S.L.E.) blind to all clinical information; P.J.H. retains the SMRI code.

For the rat hippocampal development data, ANOVA was used to determine if there was a significant overall effect of age on the expression of each transcript for each region examined. Where significant effects of age were detected, Dunnett t-tests were used to determine if NTNG1 and NTNG2 mRNA expression at each postnatal age differed from that when each the expression of each transcript was first detected. In addition, post hoc Scheffe

comparisons were made between each age group and every other group to detect periods of transient changes in relative expression.

For the rat antipsychotic administration study, a two-way ANOVA was used, with drug and hippocampal subfield as factors. Subsequently, one-way ANOVAs were performed for each subfield, with differences between drug treated and control animals explored using LSD. In all instances significance was set as P=0.05.

RESULTS

Regional and cellular expression of netrin-G1 and netrin-G2 mRNAs in human temporal cortex

Using pan probes specific for each gene, NTNG1 and NTNG2 mRNAs were detected in the human temporal cortex. Minimal background signal was seen for sections incubated in the presence of excess cold unlabelled probe, or after hybridization with probes in the sense direction (data not shown).

Regionally, no NTNG1 mRNA signal was detected in the hippocampus proper, with low to moderate signal observed over the subiculum, parahippocampal gyrus, and perirhinal and inferior temporal cortex (Figure 2A). Examination of dipped sections revealed that NTNG1 mRNA was expressed in occasional hippocampal interneurons (Figure 3A), and some subicular pyramidal neurons (Figure 3B). In the perirhinal and inferior temporal cortex, NTNG1 mRNA was mostly localized over layer 3 neurons (Figure 3C).

NTNG2 mRNA was abundant in DG, CA4 and CA3, and the deep layers of the perirhinal and inferior temporal cortex. Signal was moderate in CA1, minimal in the subiculum, and variable in the parahippocampal cortex (Figure 2B). At the cellular level, NTNG2 mRNA was concentrated over DG granule cells and pyramidal neurons in each subfield, but was not convincingly observed over putative interneurons (data not shown). In the perirhinal and inferior temporal cortex, NTNG2 mRNA was strongly expressed by neurons in deep layer 5 and layer 6 (Figure 3D).

In summary, both NTNG1 and NTNG2 mRNAs are predominantly expressed by excitatory neurons in the human temporal cortex, but are concentrated in different cell populations and areas of the temporal lobe, and with NTNG1 mRNA also being expressed by some putative inhibitory neurons. Overall, NTNG2 mRNA is more abundantly expressed than NTNG1 mRNA.

Regional and cellular expression of netrin-G1 and netrin-G2 mRNAs in rat hippocampal formation

As in the human, in the rat hippocampus NTNG2 mRNA was more abundant than NTNG1 mRNA, though some species differences in the distribution of NTNG1 and NTNG2 mRNAs between rats and humans were found. For example, in contrast to humans, NTNG1 mRNA was expressed at relatively high levels in the adult rat DG (Figures 3E and 4D). In addition, at the cellular level in the adult rat, NTNG1 mRNA was localized over the pyramidal layer of CA1 and granule cells of the DG, and was also expressed by hippocampal interneurons (Figure 3E). Expression of NTNG1 mRNA in CA1 was especially prominent during early hippocampal development (Figures 4A-D).

As observed in humans, NTNG2 mRNA was more abundantly expressed than NTNG1 mRNA, though it was expressed at relatively high levels throughout most hippocampal subfields (Figures 4E-J), with NTNG2 mRNA concentrated over granule cells of the DG

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(Figure 3F) and pyramidal neurons in CA3 and CA1, but not convincingly over putative interneurons (Figure 3F).

NTNG1 isoform expression during human brain development

Developmental changes in NTNG1 isoform expression were examined in humans using total RNA from fetal brain and adult hippocampus and temporal cortex. Semi-quantitative RT-PCR detected several PCR products of the predicted sizes. In fetal brain and adult temporal cortex, these corresponded to G1c, G1d, G1e and G1m whilst in adult hippocampus, an additional band corresponding to G1a was detected (data not shown). The four NTNG1 isoforms that were detected in all samples examined (G1c, G1d, G1e, G1m), expressed of a percentage of their sum abundance, is shown in Figure 5. Whilst G1c, G1d, G1e and G1m are all detected from early brain development onwards, the relative expression of each isoform changes in adulthood (Figure 5). Of note, the relative abundance of G1c and G1d shifts with development, with G1c becoming the predominant isoform in adult hippocampus and temporal cortex.

Developmental changes in netrin-G1 and netrin-G2 mRNAs in rat hippocampus

Developmental shifts in expression of NTNG1 and NTNG2 mRNAs were seen in the rat hippocampus (Table 2), with a profile that differed between subfields and transcripts. NTNG1 mRNA was first detected reliably at P3. In the DG, NTNG1 mRNA was weakly expressed until P14, with significant increases between P14 and P22 ($P=0.046$), and between P22 and P42 ($P<0.001$). In CA1, NTNG1 mRNA decreased between P7 and P14 ($P=0.005$). For NTNG2, mRNA signal decreased steadily in CA1 from E19 through to P14, but increased in DG between E19 and P3 ($P=0.016$), and again between P22 and P42 ($P=0.037$). In CA3, NTNG2 mRNA increased between P3 and P7 ($P=0.001$), after which it decreased, reaching significance between P14 and P22 ($P=0.002$).

Netrin-G1 mRNA isoform expression in schizophrenia and bipolar disorder

Using the same semi-quantitative RT-PCR method as utilized in the study of NTNG1 isoform expression during human brain development described above, five NTNG1 PCR products (G1a, G1c, G1d, G1e and G1m) were readily detectable in adult human temporal lobe from the Stanley Medical Research Institute array collection (Figure 6)

Two-way ANCOVA (with age, post mortem delay and RIN as covariates) demonstrated a significant effect of diagnosis ($F_{2, 497} = 3.88$, $P=0.021$) and isoform ($F_{4, 497} = 276.59$, $P<0.001$), and a significant diagnosis-by-isoform interaction ($F_{8, 497} = 2.25$, $P=0.023$), on NTNG1 mRNA. Subsequent one-way ANCOVAs revealed a significant effect of diagnosis upon G1c mRNA, but not for any other isoform (Table 3). As shown in Figure 7, G1c mRNA was significantly decreased in bipolar disorder subjects as compared to controls, with a similar trend in schizophrenia that became significant ($P=0.037$) if the analysis was repeated with the omission of the one outlier (defined as > 2 SDs from the group mean). We also quantified G1c mRNA in a subset of subjects using qPCR to assess the quantitative nature of our RT-PCR method. A significant correlation was detected between G1c mRNA measures using cDNA obtained from the same reverse transcription reaction for each subject ($r=0.844$, $P<0.001$). These findings, together with the absence of any change in the expression of the other NTNG1 isoforms examined, indicate that decreased G1c mRNA is unlikely to be due to any chance difference between the groups in the amount of starting mRNA used.

The abundance of all NTNG1 isoforms correlated with RIN ($R\geq0.245$, $P<0.013$), and all except G1e also correlated with brain pH ($R\geq0.294$, $P<0.003$), but there were no correlations with age nor post mortem delay ($R\leq-0.138$, $P\geq0.165$). As described in the Methods, for our

secondary analyses we therefore repeated the ANCOVAs using only RIN, and pH for all isoforms except G1e, as the covariates. This retained the significant diagnosis-by-isoform interaction, but the reduced G1c mRNA expression in bipolar disorder became non-significant (data not shown). NTNG1 isoform expression did not correlate with lifetime antipsychotic drug exposure nor total inpatient time in either subjects with schizophrenia or bipolar disorder, except for G1e mRNA expression in schizophrenia, which correlated significantly with lifetime drug exposure (r=−0.453, P=0.007). Alcohol, substance misuse and smoking had no significant effects upon NTNG1 isoform expression (data not shown).

Netrin-G2 mRNA expression in schizophrenia and bipolar disorder

Two-way ANCOVA (with age, post mortem delay and RIN as covariates) detected significant effects of diagnosis ($F_{2, 494}$ = 3.707, P=0.025) and subfield ($F_{4, 494}$ = 191.27, P<0.001) upon NTNG2 mRNA, but with no diagnosis-by-subfield interaction ($F_{8, 494}$ = 0.57, P=0.801). Nevertheless, one-way ANCOVAs were performed for each subfield, and showed that diagnosis had a significant effect on NTNG2 mRNA in CA4 and CA3, with a trend in the perirhinal cortex, and no changes in DG or inferior temporal cortex (Figures 7B-F). In CA4 (Figure 7C) and perirhinal cortex (Figure 7E), NTNG2 mRNA was reduced in bipolar disorder as compared to control subjects, whilst in CA3 (Figure 7D), NTNG2 mRNA was decreased in subjects with bipolar disorder and schizophrenia.

Significant inverse correlations between age and NTNG2 mRNA were detected in CA4 (R= −0.234, P=0.017), CA3 (R=−0.209, P=0.033) and the inferior temporal gyrus (R=−0.306, P=0.002). Brain pH correlated with NTNG2 mRNA in most subfields (R≥0.216, P≤0.033) except DG (R=0.129, P=0.192), whilst RIN correlated with NTNG2 mRNA signal in all subfields (R≥0.202, P≤0.047). When the ANCOVAs were repeated including only the significantly correlated variables as covariates, the significant effects of diagnosis seen in CA4, CA3 and perirhinal cortex remained, and no additional significant effects emerged.

Lifetime antipsychotic exposure correlated inversely with NTNG2 mRNA in DG (r=−0.336, P=0.049), CA4 (r=−0.448, P=0.007), CA3 (r=−0.382, P=0.024) and inferior temporal cortex (r=−0.38, P=0.032) in schizophrenia. Total inpatient time did not correlate with NTNG2 mRNA. Neither smoking nor alcohol had any significant effects upon NTNG2 mRNA in any subfield. Substance misuse affected NTNG2 mRNA in CA4 ($F_{5, 61}$=4.209, P=0.002) and CA3 ($F_{5, 61}$=4.476, P=0.002), with expression being lower in subjects with no history of misuse.

Schizophrenia associated SNPs and netrin-G1 and netrin-G2 mRNA expression

Genotypes for the two SNPs examined, rs1373336 and rs1105684, were in Hardy-Weinberg equilibrium in each diagnostic group. NTNG1 and NTNG2 mRNAs were not influenced by the respective disease associated SNPs examined, and no genotype-by-diagnosis interactions were detected (Table 4).

Effects of antipsychotic drug treatment on netrin-G1 and netrin-G2 mRNAs in rat hippocampus

Two-way ANOVA showed that 2 weeks' antipsychotic drug treatment did not affect NTNG1 mRNA expression ($F_{5, 42}$ =0.521, P=0.598), but did influence NTNG2 mRNA ($F_{8, 63}$ = 4.49, P=0.015). Subsequent one-way ANOVAs demonstrated that NTNG2 mRNA was unaffected by haloperidol, whilst small but significant increases in NTNG2 mRNA were detected in the DG and CA1 with clozapine (Table 5).

DISCUSSION

The main findings of this study are: (1) NTNG1 and NTNG2 mRNAs have distinct regional and cellular distributions in the human medial and inferior temporal lobe. (2) Both genes are differentially expressed during rat hippocampal development, and NTNG1 isoforms are developmentally regulated in human brain. (3) Of the five NTNG1 mRNA isoforms detected, G1c mRNA was selectively decreased in bipolar disorder and schizophrenia. (4) NTNG2 mRNA expression was decreased in CA3 in bipolar disorder and schizophrenia, and additionally in CA4 and perirhinal cortex in bipolar disorder. (5) No association was found between NTNG1 and NTNG2 mRNA expression and the two schizophrenia-associated SNPs examined. (6) NTNG1 and NTNG2 mRNAs in the rat hippocampus were unaffected by haloperidol administration and modestly increased by clozapine. These findings add to the candidacy of NTNG1 and NTNG2 involvement in the pathophysiology of schizophrenia, and also suggest a role in bipolar disorder. Decreased NTNG1 and NTNG2 mRNA expression may contribute to the synaptic involvement noted in both disorders, but do not shed any light on the molecular basis of their putative genetic association.

Distribution of netrin-G1 and netrin-G2 mRNAs in the human temporal lobe

To our knowledge, the distribution of NTNG1 and NTNG2 mRNAs in the human temporal lobe has not been previously described. As reported in rodents (Kim et al, 2006; Nakashiba et al, 2002; Yin et al, 2002) and monkey (Miyashita et al, 2005), NTNG2 mRNA was the more widely and abundantly expressed (Figure 2). Minimal NTNG1 mRNA signal was detected in the hippocampus proper (Figure 2A), except for expression by occasional scattered non-pyramidal neurons (Figure 3A), while NTNG2 mRNA was reliably detected in granule cells of the DG and in the pyramidal cell layers of CA4 and CA3. In the perirhinal and inferior temporal cortex, wherein both genes are expressed, NTNG1 mRNA was localized over layer 3 (Figure 3C), whilst NTNG2 mRNA expression was limited to layer 5/6 neurons (Figure 3D). Although it is difficult to distinguish deep layer 5 neurons from those in layer 6, the reported expression of NTNG2 mRNA by layer 6 neurons in monkey cortex (Miyashita *et al*, 2003) makes it likely that neurons we observed expressing NTNG2 mRNA are also layer 6 neurons.

In summary, both regionally and cellularly, NTNG1 and NTNG2 mRNA expression was essentially non-overlapping. As previously suggested for rodents (Kim et al, 2006; Nakashiba et al, 2002), the differential expression of NTNG1 and NTNG2 mRNAs in the human temporal lobe implies non-redundant, distinct functions for these axon guidance molecules.

Netrin-G1 and netrin-G2 mRNA expression is developmentally regulated

NTNG1 and NTNG2 mRNA expression was examined during rat hippocampal development from E19 through to P42, a period of time roughly corresponding in humans from the later part of the first trimester of pregnancy through to early adulthood (Clancy et al, 2001). Our data show that NTNG1 and NTNG2 mRNAs are developmentally regulated. Both NTNG1 and NTNG2 mRNAs were expressed during early hippocampal development, though that for NTNG1 was not detected in the hippocampus until P3. The difference in the timing between the first detection of NTNG1 and NTNG2 mRNAs could be taken as supportive of different functional roles for NTNG1 and NTNG2, but may also reflect a lack of sensitivity for ISHH to detect the less abundantly expressed gene. Regardless, the expression of both genes during early hippocampal development is consistent with other findings indicating the importance of NTNG1 and NTNG2 in guiding synapse formation and neurodevelopment (Kim et al, 2006; Nakashiba et al, 2002), and suggests that NTNG1 and NTNG2 may exert

some of their pathogenic influence on schizophrenia and bipolar disorder during this time period.

We also examined the relative expression of NTNG1 mRNA isoforms in human fetal and adult brain and confirmed previous findings that these isoforms are developmentally regulated (Meerabux et al, 2005). In both studies, G1d is the predominant isoform in fetal brain, whilst G1c is the most abundantly expressed isoform in adult brain; the expression of G1e mRNA is also reduced in adult as compared to fetal brain, whilst G1a mRNA expression increases during brain development. Functional differences between the various NTNG1 isoforms have not been explored, though the suggestion that differences in the number of EGF-like domains regulates the quantity and variety of binding partners (see Mitsui et al, 2002) implies that EGF domain number differences between NTNG1 isoforms may confer different binding properties or potencies. For G1c and G1d, an additional distinction between these isoforms is the inclusion of an unknown domain encoded by exons 6 and 7 in the latter. As such, the higher G1d mRNA expression in fetal brain may indicate that the function of this domain is particularly related to developmental events.

Netrin-G1 and netrin-G2 mRNA expression in schizophrenia and bipolar disorder

To date, only one other study has examined NTNG1 and NTNG2 mRNA expression in schizophrenia (Aoki-Suzuki et al, 2005), and found total NTNG1 and NTNG2 mRNA expression to be unchanged in orbitofrontal cortex and the superior temporal gyrus in a small Australian brain series (six controls versus six schizophrenics). Both transcripts were also unaltered in the dorsolateral prefrontal cortex in schizophrenics from the SMRI array collection (Aoki-Suzuki et al, 2005); the authors did not report the bipolar disorder subjects in this cohort. Further examination of specific NTNG1 isoforms in the dorsolateral prefrontal cortex found that G1c and G1d were decreased in schizophrenia. Our finding of a trend decrease in G1c mRNA in the temporal lobe in schizophrenia (significant if the outlier is omitted) is consistent with Aoki-Suzuki and colleagues, but we did not replicate their finding of decreased G1d mRNA in schizophrenia. Furthermore, we report here that NTNG2 mRNA is decreased in the temporal cortex in schizophrenia and bipolar disorder, the expression of NTNG2 mRNA having been unchanged in the cortical regions examined by Aoki-Suzuki and colleagues.

These differences in results may be due to the fact that the two studies examined different brain regions; methodological differences may also contribute to the discrepancies. For example, our use of ISHH allowed us to detect changes in NTNG2 mRNA that were present in some but not all subfields, effects that would have been diluted using qPCR on homogenized tissue. Furthermore, Aoki-Suzuki and colleagues did not take into account the methodological variables discussed below, and did not include all subjects in each diagnostic group (in some instances, it appears that as many as 10 subjects were omitted due to amplification failure).

As in any study of this kind, many factors other than diagnosis *per se* may underlie our findings of disease-related changes in NTNG1 and NTNG2 expression. We attempted to control for several known confounders (e.g. RIN, age, pH, post mortem delay) using ANCOVA, and our results were robust to these factors. Factors to which patients and controls were differentially exposed in life, notably prescribed, recreational and illicit drugs, are more difficult to control for. Neither smoking nor alcohol use had a significant effect upon NTNG1 or NTNG2 mRNA expression, whilst substance misuse was found to increase NTNG2 mRNA expression, making it unlikely that these factors account for reduced NTNG1 and NTNG2 mRNA expression in schizophrenia and bipolar disorder. Regarding antipsychotic medication, for NTNG1 mRNA isoforms, no correlations were detected with lifetime exposure, and antipsychotic drug treatment in rats did not alter hippocampal

NTNG1 mRNA expression. Whether medication affects the NTNG2 mRNA results is less clear: NTNG2 mRNA was negatively correlated with lifetime antispychotic exposure in schizophrenia, suggesting an effect; on the other hand, no correlation was seen in bipolar disorder subjects, and in rats, antipsychotics either did not affect (haloperidol) or increased (clozapine) NTNG2 mRNA. Moreover, NTNG2 mRNA reductions remain significant in bipolar disorder when the analyses are repeated with lifetime fluphenazine equivalents included as a covariate (data not shown). The negative correlation in the schizophrenic patients may in fact index illness severity. Comparable information for the major drug classes received by the bipolar disorder patients, notably mood stabilizers and antidepressants, was not available, and so we cannot assess whether such medications might have confounded our findings.

Netrin-G1 and netrin-G2 and the pathophysiology of schizophrenia and bipolar disorder

Most pathophysiological models of schizophrenia propose that the disorder is one of the synapse and of connectivity (Andreasen, 1999; Frankle et al, 2003; McGlashan and Hoffman, 2000; Mirnics et al, 2001; Moises et al, 2002; Stephan et al, 2006). Data indicative of altered synaptic density and/or function in the temporal lobe in schizophrenia has come from post mortem studies of synaptic protein expression as well as ultrastructural findings (for reviews see Harrison and Eastwood, 2001; Honer et al, 2000; Honer and Young, 2004) and although there are fewer data in bipolar disorder, synaptic alterations also appear to be present (Eastwood and Harrison, 2000; Fatemi et al, 2001; Harrison, 2002; Knable et al, 2004; Torrey et al, 2005; Vawter et al, 2002). Our finding of decreased G1c and NTNG2 mRNAs provide further indications of altered synaptic protein gene expression in the temporal lobe in both disorders. Additionally, the roles of NTNG1 and NTNG2 in synapse formation and maintenance (Nakashiba et al, 2000, 2002; Yin et al, 2002) imply that the reductions may not only reflect, but be causally related to, the synaptic changes.

The cortical synaptic pathology of schizophrenia, and probably that of bipolar disorder, affects glutamatergic (Eastwood and Harrison, 2000, 2005; Harrison and Eastwood, 1998; Sawada et al., 2005) as well as GABAergic (Heckers et al, 2002; Knable *et al*, 2002; Lewis et al, 2005) synapses. Our finding that NTNG1 and NTNG2 mRNAs are predominantly expressed by excitatory neurons suggests that their decreased expression in schizophrenia and bipolar disorder may particularly affect glutamatergic synapses, whilst the fact that NTNG1 mRNA is also expressed by some hippocampal interneurons suggests that it may also be relevant for the GABAergic changes noted in the disorder. However, the identity of the post-synaptic neuronal populations affected by the NTNG1 and NTNG2 alterations is unclear, since there is no information regarding the cellular distribution of NGL-1 and NGL-2 in human brain. Investigation of the expression of NGl-1 and NGL-2, using both ISHH and immunocytochemical approaches, will be required to clarify the post-synaptic partners in these neural circuits.

In the case of NTNG2, the suggestion that its decreased expression may primarily impact on glutamatergic synapses is supported by *in vitro* studies that have examined the effect of over- and under-expression of its ligand NGL-2, and the discovery that NGL-2 binds to the first two PDZ domains of PSD-95 (Kim et al, 2006). NGL-2 coated beads induce the aggregation of PSD-95 and associated proteins, including the NMDA receptor subunit NR2A, whilst siRNA knockdown of NGL-2 reduces the number of excitatory but not inhibitory synapses in cultured neurons (Kim et al, 2006). Furthermore, over-expression of NGL-2 increases the number of PSD-95 positive, but not negative, dendritic protrusions (Kim *et al*, 20066). These data, together with the importance of PSD-95 in glutamatergic synapse formation and receptor recruitment (Garner et al, 2000; Kim and Sheng, 2004), indicate that adhesion between NTNG2 and NGL-2 is required for maintenance of excitatory synapses and recruitment of NMDA receptors to the synapse. Thus, decreased

NTNG2 mRNA in schizophrenia and bipolar disorder may contribute particularly to the NMDA receptor-mediated glutamatergic synaptic alterations observed in both disorders (Harrison et al, 2003; Law and Deakin, 2001; McCullumsmith et al, 2007; Scarr et al, 2003).

As NTNG1 and NTNG2 are expressed by different excitatory neuron populations (see Figures 2 and 3), their altered expression will affect different glutamatergic synapses. The widespread distribution of NTNG2 suggests that it may modulate excitatory synaptic circuits throughout the hippocampus and inferior temporal lobe, whereas the predominant localization of NTNG1 mRNA to layer 3 perirhinal and inferior temporal cortex pyramidal neurons (Figures 2A and 3C) implicates corticocortical connections. As functional differences between the alternatively spliced NTNG1 isoforms have yet to be examined, no comment can be made as to the significance of the fact that G1c but not the other NTNG1 isoforms was decreased in schizophrenia and bipolar disorder.

Schizophrenia associated netrin-G1 and netrin-G2 SNPs are not associated with mRNA expression

SNPs in the genes for NTNG1 and NTNG2 reported to be associated with schizophrenia are either intronic and/or within untranslated regions, suggesting that any disease susceptibility they may confer occurs by altering some facet of the expression of the genes. Our data showing that NTNG1 or NTNG2 mRNA expression is not associated with schizophrenia-associated SNPs suggest that altered mRNA expression is not the mechanism by which genetic variation of NTNG1 and NTNG2 confers disease susceptibility, although the low minor allele frequency of the NTNG2 SNP examined (rs1105684: ~16% in our study) means we may have been underpowered to detect small effects of this kind.

Finally, although our interest in NTNG1 and NTNG2 was primarily with regard to schizophrenia, we found more significant changes in their expression in bipolar disorder. Possible linkage has been noted for the NTNG2 locus (9q34) with early onset bipolar disorder (Faraone et al, 2006) and with affective disorder (Venken et al, 2005), and for the NTNG1 locus (1p13.3) with psychosis and bipolar disorder (Cheng et al, 2006). It will therefore be of interest to determine whether the genes for NTNG1 and NTNG2 are associated with bipolar disorder.

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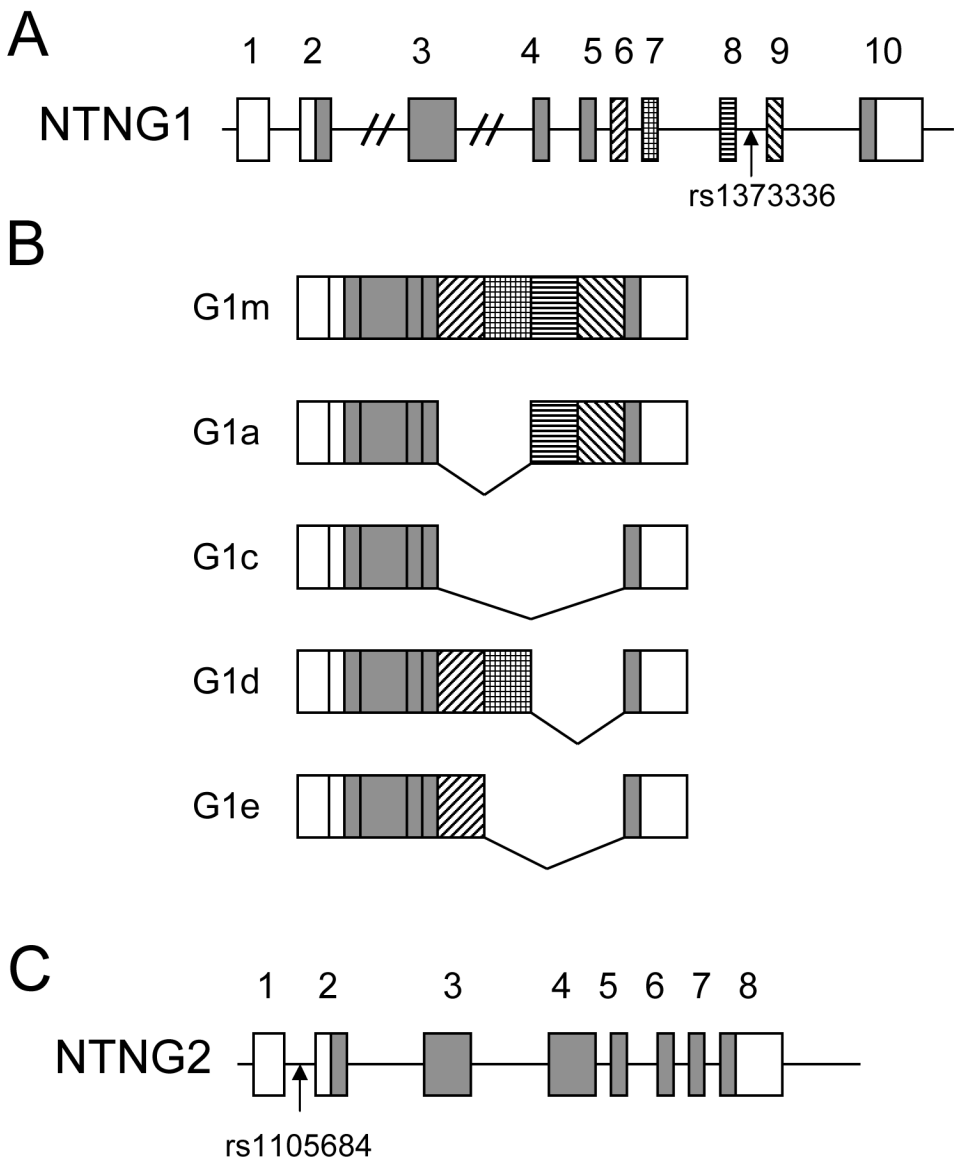

Figure 1.

A: Structure of the human NTNG1 gene, showing the alternatively spliced exons 6-9, and the position of SNP rs1373336. The SNP tags a haplotype block that extends as far upstream as the third intron. The forward primer used to amplify G1 isoforms was located in exon 4, with the reverse primer, and the ISHH probe, in exon 10. B: NTNG1 splice isoforms expressed in human brain. C: Structure of the human NTNG2 gene and location of SNP rs1105684. The ISHH probe spanned the exon 7/8 boundary, and is predicted to detect all NTNG2 splice variants based upon the known mouse transcripts.

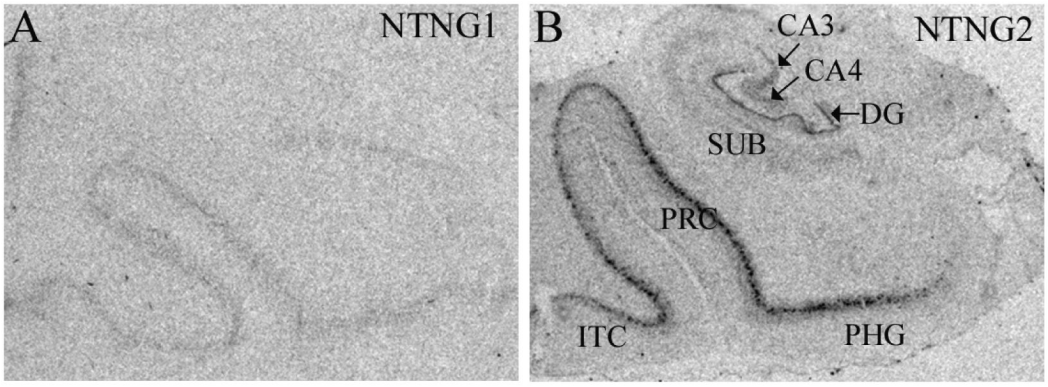

Figure 2.
Regional distribution of (A) NTNG1 and (B) NTNG2 mRNAs in the temporal lobe of representative control subjects. CA: cornu ammonis; DG: dentate gyrus; ITC: inferior temporal cortex; SUB: subiculum: PHG: parahippocampal gyrus; PRC: perirhinal cortex. Although NTNG2 mRNA was observed in the PHG of some subjects (as seen here), its variable expression between individuals precluded quantitative analyses.

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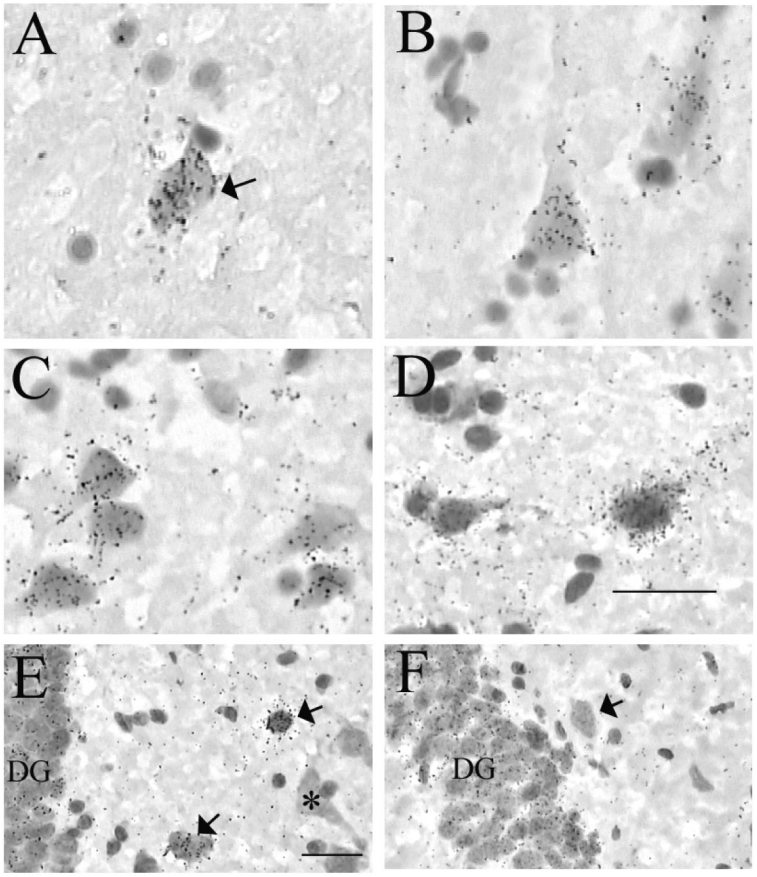

Figure 3.

Cellular expression of (A-C, E) NTNG1 and (D, F) NTNG2 mRNAs in (A-D) human temporal lobe and (E, F) P42 rat hippocampal formation. A: NTNG1 mRNA is expressed by occasional putative hippocampal interneurons (arrow), as observed here in the stratum oriens of CA3. B: NTNG1 mRNA over pyramidal neurons in the subiculum. C: NTNG1 mRNA expression by layer 3 perirhinal cortical neurons. D: NTNG2 mRNA in the perirhinal cortex is localized over deep layer 5/6 neurons. E: NTNG1 mRNA is expressed by granule cells of the rat DG, and by putative interneurons (arrows), but is not reliably detected over pyramidal neurons (asterisk). F: NTNG2 mRNA is expressed by granule cells of the rat DG, but is not reliably detected over putative hippocampal interneurons (arrow). Bars in (D) and (E) = 30 μm. Abbreviations as in Figure 2.

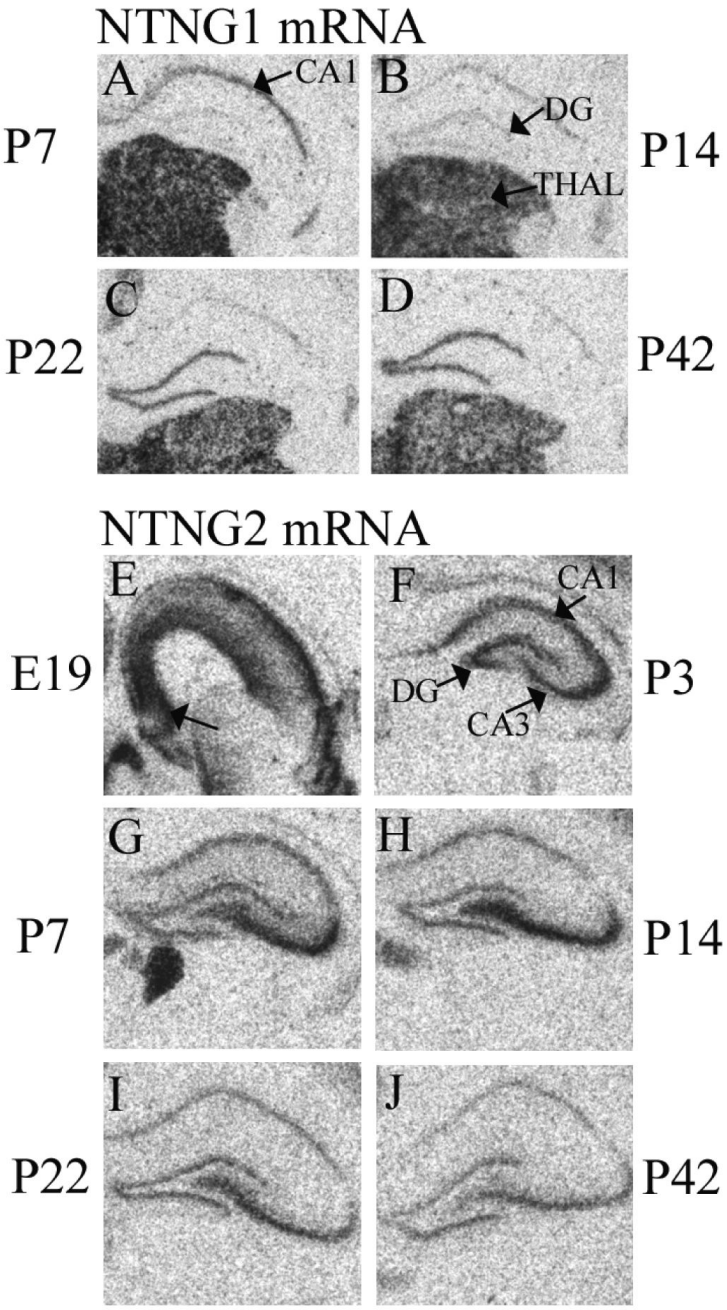

Figure 4.

Representative autoradiographic images demonstrating the regional distribution of (A-D) NTNG1 and (E-J) NTNG2 mRNAs in the developing rat hippocampus. Arrow in E indicates the location of the developing hippocampus. NTNG1 mRNA was not detected in the hippocampus at E19 (not shown), and was first detected in CA1 at P3 (not shown). At P7 (A) NTNG1 mRNA was also detected in the DG. Note that NTNG1 mRNA expression in the DG increases with age, whilst that in CA1 decreases. As previously reported (Nakashiba et al, 2000; Yin et al., 2002) NTNG1 mRNA is strongly expressed in the thalamus. NTNG2 mRNA was detected from E19 onwards and is robustly expressed in most hippocampal subfields. THAL: thalamus. Other abbreviations as in Figure 2.

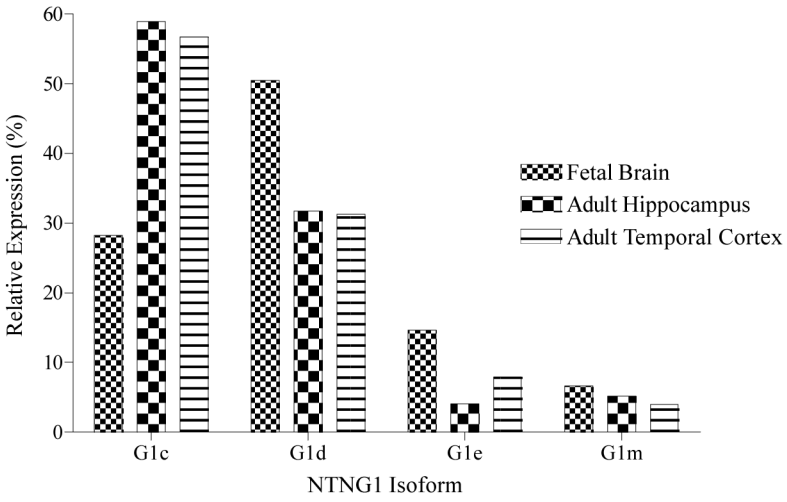

Figure 5.

Relative expression of NTNG1 isoform mRNAs during human brain development. Four isoforms (G1c, G1d, G1e, and G1m) were reliably detected in fetal brain and adult hippocampus and temporal cortex. The relative abundance (expressed as a percentage of the sum of all four mRNAs quantified) of G1c increased, and that of G1d decreased, between fetal and adult brain.

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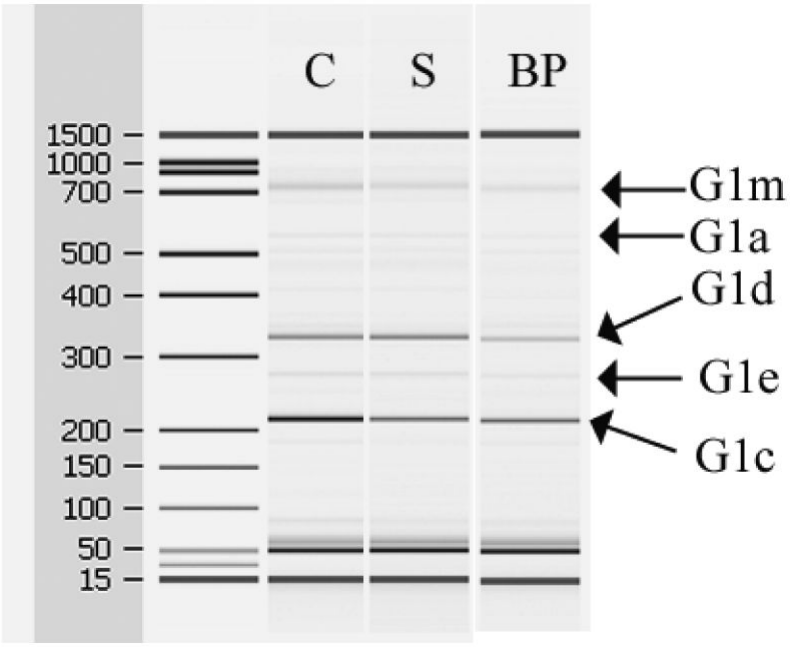

Figure 6.
Examples of gel-like images produced by the Agilent 2100 bioanalyzer for semi-quantitative analysis of NTNG1 mRNA using RT-PCR, showing a representative control subject (C), a subject with schizophrenia (S), and a bipolar disorder subject (BP). Bands of the predicted size (ladder on left) corresponding to the G1a, G1c, G1d, G1e and G1m isoforms were detected.

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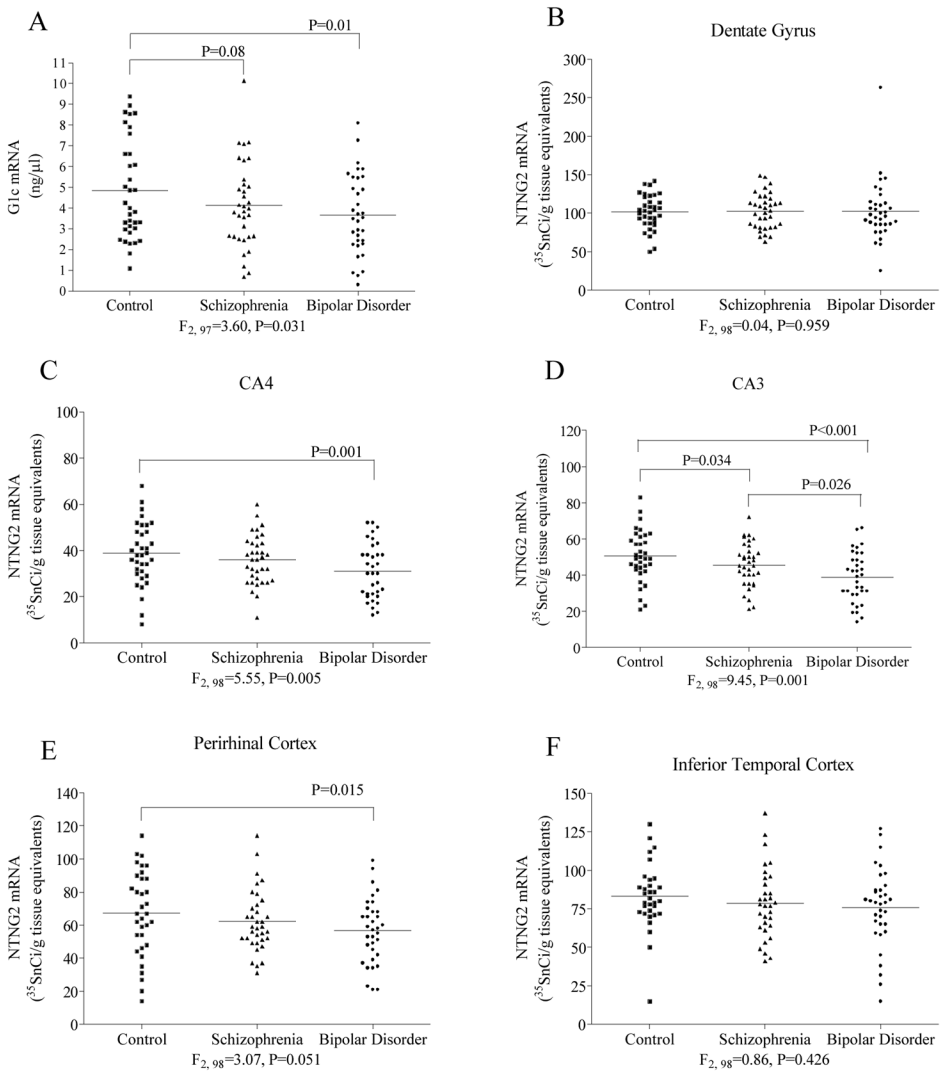

Figure 7.

Quantitative analyses of NTNG1 and NTNG2 mRNA expression in the temporal lobe in controls (squares), subjects with schizophrenia (triangles), and subjects with bipolar disorder (circles). A: G1c isoform of NTNG1 mRNA in homogenized temporal cortex tissue. One subject with schizophrenia was omitted from the G1c study due to amplification failure. B-F: NTNG2 mRNA in subfields of the temporal lobe.

Table 1

Demographics of subjects included in the study.

	Controls	*Schizophrenics*	*Bipolar disorder*
Gender (M:F)	26:9	26:9	16:18
Age (years)	44.2 ± 7.6	42.6 ± 8.5	45.4 ± 10.7
Brain pH	6.61 ± 0.27	6.47 ± 0.24	6.43 ± 0.30
RIN	4.72 ± 1.56	5.07 ± 1.52	4.89 ± 1.82
Post mortem delay (hours)	29.4 ± 12.9	31.4 ± 15.5	37.9 ± 18.6
Onset of psychosis (years)	-	21.3 ± 6.1	25.3 ± 9.2
Duration of illness (years)	-	21.3 ± 10.1	20.1 ± 9.6
Total inpatient time (years)	-	1.23 ± 2.25	0.53 ± 1.39
Lifetime fluphenazine equivalents (mg)	-	85000 ± 100300	10200 ± 23200
Alcohol use			
Little or none	18	10	4
Social	12	7	8
Moderate past	1	3	6
Moderate present	2	3	3
Heavy past	2	3	4
Heavy present	0	9	8
Not known	0	0	1
Drug Use			
Little or none	30	14	11
Social	4	4	4
Moderate past	0	3	6
Moderate present	1	3	3
Heavy past	0	3	4
Heavy present	0	6	6
Not known	0	2	1

Values are mean ± SD. A significant effect of diagnosis was detected for brain pH ($F_{2, 101}$ =4.17, P=0.018), with controls having higher pH than subjects with schizophrenia (P=0.043) or bipolar disorder (P=0.006). The groups were matched for all other demographic variables, except for post mortem delay, in which controls had a significantly shorter mean post mortem delay than subjects with bipolar disorder (P=0.028).

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Table 2

Summary of developmental changes in NTNG1 and NTNG2 mRNAs during rat hippocampal development.

Age		***E19***	***P3***	***P7***	***P14***	***P22***	***P42***
Number of animals		***3-4***	***3-53***	***3-4***	***3-5***	***4-5***	***4***
NTNG1	***Effect of age***						
DG	$F_{5, 19}$=28.30, P<0.001	-	0.030 (0.013)	0.029 (0.009)	0.037 (0.014)	0.074 (0.023)	0.189 (0.045)
CA1	$F_{5, 19}$=9.39, P=0.001	-	0.099 (0.025)	0.096 (0.019)	0.047 (0.028)	0.024 (0.004)	0.044 (0.013)
NTNG2	***Effect of age***						
DG	$F_{5, 18}$=7.42, P=0.001	0.231 (0.052)	0.333 (0.067)	0.219 (0.032)	0.137 (0.017)	0.133 (0.027)	0.220 (0.096)
CA3	$F_{5, 19}$=12.64, P<0.001	0.231 (0.052)	0.211 (0.038)	0.288 (0.007)	0.241 (0.020)	0.161 (0.013)	0.142 (0.028)
CA1	$F_{5, 19}$=17.39, P<0.001	0.231 (0.052)	0.173 (0.028)	0.126 (0.016)	0.082 (0.016)	0.080 (0.009)	0.096 (0.030)
NTNG1		***Change in expression relative to when first detected***					
DG		-	-	(↓)	(↑)	(↑)	↑ ‡
CA1		-	-	(↓)	↓ †	↓ ≠	↓ †
NTNG2							
DG		-	(↑)	(↓)	(↓)	(↓)	(↓)
CA3		-	(↓)	(↑)	(↑)	↓ †	↓ ≠
CA1		-	↓ *	↓ ≠	↓ ≠	↓ ≠	↓ ≠

Values and mean (SD), normalised to GAPDH mRNA. Post hoc comparisons between developmental age when mRNA is first detected (NTNG1: P3; NTNG2: E19) and subsequent age groups, Symbols in brackets denote non-significant changes.

* P<0.05

† P<0.02

≠ P<0.01.

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Table 3

NTNG1 isoform expression in the temporal cortex in controls and subjects with schizophrenia or bipolar disorder

	ANCOVA[a]	*Control*	*Schizophrenia*[b]	*Bipolar Disorder*
G1a	$F_{2, 97}$=0.56, P=0.571	0.16 ± 0.17	0.15 ± 0.17	0.16 ± 0.19
G1c	$F_{2, 97}$=3.60, P=0.031	4.83 ± 2.41	4.12 ± 2.07	3.66 ± 1.92 *
G1d	$F_{2, 97}$=0.03, P=0.971	1.35 ± 0.78	1.51 ± 0.89	1.44 ± 1.14
G1e	$F_{2, 97}$=1.74, P=0.181	0.26 ± 0.16	0.21 ± 0.13	0.22 ± 0.13
G1m	$F_{2, 97}$=1.24, P=0.294	0.68 ± 0.48	0.63 ± 0.45	0.56 ± 0.50

Values are mean (ng/μl) ± SD.

[a] With age, post mortem delay and RIN as covariates.

* P=0.01 as compared to controls.

[b] One subject with schizophrenia was omitted from the study due to amplification failure.

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Table 4

NTNG1 and NTNG2 genotype is not associated with mRNA expression.

		NTNG1 isoform mRNA expression (ng/μl) [a]				
Genotype (rs1373336)	N	G1a	G1c	G1d	G1e	G1m
C/C	25	0.14 ± 0.15	4.04 ± 2.00	1.24 ± 0.70	0.23 ± 0.15	0.54 ± 0.40
C/T	53	0.14 ± 0.15	4.09 ± 2.18	1.38 ± 0.95	0.23 ± 0.15	0.60 ± 0.51
T/T	25	0.20 ± 0.20	4.64 ± 2.35	1.74 ± 1.10	0.23 ± 0.15	0.76 ± 0.50
		NTNG2 mRNA expression (^{35}SnCi/g tissue equivalents)				
Genotype (rs1105684)	N	DG	CA4	CA3	Perirhinal Cortex	Inferior Temporal Cortex
A/A	74	100.9 ± 24.1	34.9 ± 12.9	44.3 ± 13.8	61.5 ± 20.6	78.1 ± 22.4
A/T	26	109.4 ± 37.2	36.3 ± 12.2	46.7 ± 14.8	63.6 ± 21.4	84.1 ± 29.6
T/T	4	79.6 ± 37.8	33.1 ± 14.2	44.5 ± 18.0	65.4 ± 37.4	69.9 ± 24.4

Values are mean ± SD.

[a] One subject was omitted due to NTNG1 RT-PCR failure.

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Table 5

Effect of antipsychotic drug treatment on NTNG1 and NTNG2 mRNAs in rat brain.

mRNA	*Area*	*Effect of drug*	*Saline* (n=8)	*Haloperidol* (n=8)	*Clozapine* (n=8)
NTNG1	DG	$F_{2,21}$=0.708, P=0.504	477 ± 31	451 ± 23	458 ± 54
	CA1	$F_{2,21}$=0.418, P=0.663	102 ± 23	108 ± 25	98 ± 17
NTNG2	DG	$F_{2,21}$=3.069, P=0.068	384 ± 42	394 ± 34	428 ± 37[a]
	CA3	$F_{2,21}$=0.727, P=0.494	328 ± 28	316 ± 28	334 ± 37
	CA1	$F_{2,21}$=2.236, P=0.132	152 ± 25	163 ± 23	175 ± 17[b]

Values are ^{35}SnCi/g tissue equivalents (mean ± SD).

as compared to saline controls.

[a] P=0.027

[b] P=0.047