COGNITIVE DOMAINS FUNCTION COMPLEMENTATION BY *NTNG* GENE PARALOGS

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ABSTRACT

Gene duplication was proposed by S.Ohno (1) as a key mechanism of a novel gene function evolution. A pair of gene paralogs, *NTNG1* and *NTNG2*, sharing identical gene and protein structures and encoding similar proteins, forms a functional complement subfunctionalising (SF) within cognitive domains and forming cognitive endophenotypes, as detected by Intellectual Quotient (IQ) tests (2). Both *NTNG* paralogs are associated with autism spectrum disorder (ASD), bipolar disorder (BD) and schizophrenia (SCZ), with unique non-overlapping segregation among the other 15 cognitive disorders (CD), emphasizing an evolutionary gain-dependent link between advanced cognitive functions and concomitant neurocognitive pathologies. Complementary expression and human brain transcriptome composition of the paralogs explains the observed phenomena of their functional complementarity. The lowest identity among *NTNGs* is found in a middle of encoded by them proteins designated as uknown (Ukd) domain. *NTNG1* contains anthropoid-specific constrained regions, and both genes contain non-coding conserved sequences underwent accelerated evolution in human. *NTNG* paralogs SF perturbates "structure drives function" concept at protein and gene levels. The paralogs function diversification forms a so-called "Cognitive Complement (CC)", a product of gene duplication and subsequent cognitive subfunction bifurcation among the *NTNG* gene duplicates.

INTRODUCTION

Complex behaviors arise from a combination of simpler genetic modules that either have evolved separately or co-evolved. Many genes and proteins they encode have been found to be involved in cognitive information processing with a single variant or a single gene generally accounting for only a partial phenotypic variation of a complex trait, such as cognition. Cognition as a quintessence of brain functioning can be viewed as a product of intricately interlinked networks generated by deeply embedded into it gene-nodes with specific or partially overlapping functions. The robustness of the cognitive processing towards its single elements genetic eliminations (to study their function) and its simultaneous fragility expressed in the multiple forms of neurological disorders manifest the existence of cognitive domains interlocked but SF within a unit of cognition formed upon these domains interaction. Previously, we have described a function of a pair of gene paralogs, *NTNG1* and *NTNG2*, involved in human IQ tests performance, and underwent hominin-specific evolutionary changes (2). Hereby, we report on these gene paralogs features focusing on underlying mechanisms of their function segregation and complementation within the cognitive domains.

RESULTS

The previously observed phenomena of functional complementation among the *NTNG* paralogs within cognitive domains (2) is also manifested in *NTNG*-associated human pathologies diagnosed in most cases (if only not in all) by a cognitive decline (Figure 1A-1 and A-2). Both genes are associated with BD and SCZ – devastating disorders sharing similar etiology (3) with genetic correlation by multivariate analysis of 0.590 (4), linked to human creativity (5), and characterized by impulsiveness as a common diagnostic feature (6). Recently found associations of both paralogs with ASD (7) supports the reported genetic correlation of 0.194 ASD/SCZ pair (4) and shared module eigengenes detected by PC1 among these two disorders (8). 12 *NTNG1*-linked CDs, ranging from AD to TS, span a broad spectrum of clinical features frequently involving reduced processing speed (PS) and verbal comprehension (VC, Figure 1A-1). As for *NTNG2*, working memory (WM) deficit and inability "to bind" events (perceptual organization, PO) are the most prominent diagnostic traits for the SLE and TLE patients (Figure 1A-2), with PN also being characterised by indolent behavior in 90% of the cases (9). Interestingly to note that association of the synapse-expressed *NTNG2* with both SCZ and autoimmune pathology (SLE) correlates with a recent finding that human complement component C4 is involved in the synapse elimination and SCZ development (10). Thus, both *NTNG* paralogs are associated with a variety of CDs and mostly in a non-overlapping manner, except for ASD, BD and SCZ characterized by shared and wide spectrum of cognitive abnormalities. The clinical etiology of the aforementioned diseases supports the deduced by IQ functional complementation existing among the *NTNG* paralogs (2) with (VC/PS) and (WM/PO) endophenotypic deficits being uniquely segregated within the associated cognitive pathologies.

Since both gene paralogs are expected to have identical gene exon/intron compositions but different in their intron lengths (11) we have reconstructed both paralogs

transcriptomes by re-processing the publicly available RNA-seq dataset (12) from healthy and SCZ human subjects superior temporal gyrus (STG) post-mortem brain tissue (Supplementary Table 1a=ST1a). A difference is noted instantly at the total expression levels (genes, exons, individual RNA transcripts) when two gene paralogs are compared (Figure 1B-1 and B-2). *NTNG2* amount (as a whole gene) is 5 times larger comparing to *NTNG1*; exons (2-5) are 3 times, exons (8-9) are 18 times and exon 10 is 4 times higher expressed for *NTNG2* than for *NTNG1*. The only two exons outlaying the prevailing amount rule for the *NTNG2* mRNAs are exons 6 and 7, expressed nearly at the same absolute level as for the *NTNG1* exon paralogs, making them highly underrepresented within the *NTNG2* transcriptome. Next, distinct non-alternating splicing modules are formed by exons (2-5) for *NTNG1* (Figure 1B-1), while exons (4-5) and exons (8-9) for *NTNG2* (Figure 1B-2). Two structurally identical RNA transcript paralogs (*NTNG1a* = G1a and *NTNG2a* = G2a) have been found to exist in both *NTNG* transcriptomes with G2a being expressed at 8-9 times higher level than G1a. *NTNG1* is uniformly presented across the all analysed 16 human samples by 2 more protein coding RNAs (G1c and G1d, detected previously in mice brain, Nakashiba et al., 2000) and by 2 non-coding intron (9-10) derived transcripts (Figure 1B-1). At the same time, *NTNG2* transcriptome is comprised of one extra potentially coding RNA (G2a-like with exon 2 spliced out but in-frame coding preserved) and 2 assumed to be non-coding RNAs with exons 6 and 7 retained along with preceding and following them introns. Quite interesting that these two latter transcripts are the only RNA species with *NTNG2* exon 6 and 7 retained (Figure 1B-2). Two more coding (G1f and G1n) and 4 more non-coding for *NTNG1* and 9 extra non-coding for *NTNG2* RNA species have been also assembled from the available reads but due to inconsistency in their appearance across all 16 STG samples they are not presented on the figure but summarized in the table (Figure 1C, for details refer to ST1d). Summarising above, quantitative and qualitative complementary differences is a

103 prominent feature characterising the brain RNA transcriptome of human *NTNG* paralogs.
104 However, no significant changes at the transcription level of neither whole genes, nor
105 individual exons, nor reconstructed RNA transcripts have been found when SCZ and healthy
106 subjects samples are compared.

107 Upon calling the presence of IQ-affecting SNPs (2) across all STG samples (ST1c) it
108 has been revealed that 15 out of 16 subjects were positive for the T-allele of rs2149171 (exon
109 4-nested), shown above to attenuate the WM score in SCZ patients, and making a comparison
110 among the allele carrier *vs* non-carrier impossible. Four healthy and three SCZ samples carry
111 a T-allele of rs3824574 (exon 3-nested, non-affecting IQ), and 1 healthy and 1 SCZ sample
112 each contains a C-allele of rs4915045 (exon10, non-coding part-nested, and non-affecting IQ).
113 Thus, among the eleven cognitive endophenotype-associated SNPs only 3 were possible to
114 call out of the available *NTNG* transcriptome.

115 Distinctly complementary nature of the *NTNG* paralogs segregation within
116 neurological disorders and RNA transcriptome usage in STG (Figure 1) has prompted us to
117 analyse both genes expression across the entire human brain. We have reconstructed both
118 genes expression profiles in the human brain areas over the life span from conception (pcw =
119 post-conception week) to mature age (30-40 yrs old) using the RNA-seq data from BrainSpan
120 (www.brainspan.org). Similarities and differences are easily noted when the age-dependent
121 phases of *NTNG1* and *NTNG2* expression profiles are matched (Figure 2). Based on the
122 visual inputs three distinct classifiers have been elaborated: 1. predominantly synchronous
123 (Figure 2A(1-4)), characteristic mostly for the cortical areas; 2. predominantly mixed and
124 asynchronous (Figure 2B), characteristic for the cerebellar cortex and subcortical formations;
125 and 3. anti-phasic (complementary, Figure 2C), characteristic for the MD of thalamus and
126 hippocampus. All analysed brain areas demonstrate an elevated level of *NTNG2* expression in
127 comparison to *NTNG1* except for thalamus (Figure 2C) with the largest difference observed

128 at the time of birth (35-37 pcw) or soon after (4 mo) for the synchronous classifiers (Figure
129 2A), oscillating increment values across the life span for the mixed (Figure 2B) and anti-
130 phasic (Figure 2C) classifiers. It is quite intriguing to note that essentially all brain areas
131 show a trend towards the expression difference being negated between the paralogs by
132 reaching the mature age of 30-40 yrs old (nearly or above the mean age used for the IQ
133 testing), except MD where the expression discrepancy is increased. Thus, the observed
134 functional complementation among the *NTNG* paralogs is supported by the anatomical
135 distribution of the genes in human brain and their expression pattern modality over the
136 human subjects lifetime.

137 A direct comparison of the *NTNG* paralogs shows not only identical intron-exon gene
138 structure (Figure 1B-1, 2B-2) but also closely matched exon sizes (Figure 3A). There are
139 three exons of identical sizes (exons 4, 8 and 9), another three exons differed by one encoded
140 amino acid (exons 3, 5 and 6) and there are exons of different sizes (exons 2, 7 and 10). In
141 terms of size the largest difference among the genes is visually presented by the introns:
142 intron (9-10) of *NTNG1* is 52.7 times larger its *NTNG2* paralogous intron with intron (6-7) of
143 *NTNG1* being only 1.43-times larger pointing towards non-equilibria process of non-coding
144 elements elaborations as the process of gene paralogs SF proceeded. Nevertheless, it can be
145 generalised that in average all *NTNG1* introns are several times larger their *NTNG2* analogs
146 (Figure 3A). We have shown previously that exons 6 and 7 are differentially used within the
147 brain *NTNG* transcriptome (Figure 1B-1 and B-2) and to explore their potential contribution
148 into the paralogs SF we have built identity matrices with these exons being excluded and
149 included (but still producing in-frame existing transcripts, Figure 3B-1 left and right panels,
150 respectively). Exclusion of both exons from the full-lengths transcripts (thus converting
151 *NTNG1m* to *NTNG1a* and *NTNG2b* to *NTNG2a*, respectively) increases the identity of DNA
152 on 2% (a relatively large effect since both exons together represent only 7.22 and 9.69% of

153 the total coding part of the full-length RNA transcripts, *NTNG1m* and *NTNG2b*, respectively).
154 This effect becomes even stronger when the encoded by these transcripts proteins are also
155 compared (Figure 3B-2). The spliced out Ukd protein domains (encoded by the exons 6 and
156 7) increases the proteins identity on 3.8% thus making the middle of both genes (and encoded
157 proteins) substantially more different among the both gene paralogs. To corroborate this
158 observation and to explore the importance of other protein parts we have directly compared
159 the sequences encoded by the full-length transcripts and producing Netrin-G1m and Netrin-
160 G2b (Figure 3C). Similarly to what has been shown on Figure 3B-1 and 3B-2, the lowest
161 identity (17.5%) is represented by the Ukd domain (encoded by the exons 6 and 7) and by the
162 preceding it exon 5 (a 3'-part of the LE1 domain). Two other areas also show a substantially
163 low identity, namely the N-terminus (it includes the protein secretory signal indicated by an
164 arrow) and the outmost C-terminus responsible for the unique feature of Netrin-Gs – the GPI
165 attachment. Thus, based on the percent identity comparisons among the Netrin-G paralogs it
166 can be predicted that there are several potential protein parts contributing to the paralogs SF.
167 As it has been reported by Seiradake et al. (13), identical gene and protein domain
168 compositions result in the identical structural motif with differences only in the spatial
169 arrangement of the loops facing the post-synaptic Netrin-G's interacting partners, NGL-1 and
170 NGL-2, respectively (Figure 3D). Loop I binding surfaces alignment (Figure 3C, blue color)
171 shows a high level of conservation (with at least 5 amino acids 100% conserved) among the
172 Netrin-G paralogs, indicating that it is unlikely to be responsible for the cognate ligand
173 binding specificity. Neither Loop II (Figure 5C, yellow color) nor Loop III (Figure 5C,
174 orange color) display a single conserved amino acid shared among the paralogous binding
175 interfaces (as it has originally been described in (13)). Thus the complementary pattern of the
176 pre-postsynaptic interactions mediated via specific Netrin-G/NGL pairs is reflected in the

177 reciprocally different sizes of the loops binding interfaces representing another element of the

178 *NTNG*-encoded protein paralogs SF.

179

DISCUSSION

Complementary contribution of *NTNG* paralogs into human cognitive pathologies. Involvement of the pre-synaptically expressed axon-localised *NTNGs* in SCZ diagnosis supports the established view of SCZ as a product of distorted trans-synaptic signaling (14), with a recent study also proving that axonal connectivity-associated genes form a functional network visualisable by fMRI (15), and that brain connectivity predicts the level of fluid intelligence (16, 17). Both *NTNGs* have been found to participate in the brain functional connectivity found by the parcellated connectome reconstruction (18). Most of the reported disease associations link *NTNG1* to SCZ with a variety of other neurologic pathologies (15 in total, Figure 1A-1), while *NTNG2* pathologic associations (6 in total, Figure 1A-2) are quite limited to those affecting WM or PO. Among them is SLE frequently characterized by WM deficit (19) and also known to represent schizoid-type abnormalities characteristic for autoimmune pathologies (20, 21). Immune activation is known to lead to altered pre-pulse inhibition (a key diagnostic trait for SCZ) reversed by antipsychotics (22). The three diseases associated with both paralogs (ASD, BD and SCZ) are also a primary focus of the recently initiated PsychENCODE project (23). It is also worth to mention the resemblance of the reported disease associations with the behavioral phenotypes of *Ntng1* and *Ntng2* gene knockout mice (24).

A gene content associated with attenuated IQ score often relates to numerous diseases, such as SCZ, ASD, depression, and others (see (25) for ref.; 26) with several of them also undergone positive selection following the human brain evolution (27). Despite the fact that global network properties of the brain transcriptome are highly conserved, among the species there are robust human-specific disease-associated modules (28) and human accelerated regions (HARs) - highly conserved parts of genome that underwent accelerated evolution in humans (29). HARs can serve as genomic markers for human-specific traits underlying a

204 recent acquisition of modern human cognitive abilities by brain (30) but that also "might
205 have led to an increase in structural instability… resulted in a higher risk for
206 neurodegeneration in the aging brain" (31), rendering our intellectual abilities genetically
207 fragile (32) and resulting in a variety of CDs. The role genomic context, epistasis (33), plays
208 in the evolution and pathology is manifested by frequently found disease-causing alleles
209 present in animals without obvious pathological symptoms for the host (34). Any CD is
210 characterized by general intellectual disability (GID) plus psychiatric symptoms. A genetic
211 perturbation-exerted behavioral cognitive deficit (BCD) in an animal model organism is a
212 poor match to a human CD *per se* due to very poor contextual resemblance between the
213 human GID and animal BCD together with the absence of interpretable psychiatric symptoms.
214 Usefulness of animals as psychiatric models is also compromised by the fact that
215 transcriptome differences within species tissues is smaller than among the homologous
216 tissues of different species (35, 36). No wonder that the compounds that "cure" mice models
217 consistently fail in human trials (discussed in (37)).
218 *NTNG* paralogs brain transcriptome intrinsic complementarity and possible mechanism for
219 the IQ-affecting mutation alleles effect. There is no global change at the mRNA level
220 between healthy subjects and SCZ patients (Figure 1B). This conclusion is supported by
221 previously published works stating that globally altered mRNA expression of *NTNG1* or
222 *NTNG2* is unlikely to confer disease susceptibility, at least in the temporal lobe (38), and
223 Brodmann's area (39). However, the original paper-source of the STG samples RNA-seq
224 along with many other genes (>1,000) found that *NTNG1* (but not *NTNG2*) falls within the
225 group of genes with significant alternative promoter usage ((12): ST6, p<9.05E-10 at FDR
226 <0.5) and *NTNG2* (but not *NTNG1*) clusters with genes (>700) with significant alternative
227 splicing change ((12): ST7, p<6.15E-12 at FDR<0.5) when SCZ and controls are compared.
228 Such GWAS observation adds an extra layer of complementary regulation to both *NTNG*

229 paralogs on a top of the described in the results section complementary usage rule for the
230 exons, formed by unspliced splicing modules, resulting transcripts and comprising them
231 exons (Figure 1B). Based on the available RNA-seq dataset it was almost impossible to
232 detect RNA with the matched position of *NTNG* SNPs used for the IQ testing (ST2c) except
233 for two coding exons located (rs2149171 and rs3824574) and exon 10 non-coding area
234 located but transcribed rs4915045 (in 2 out of 16 samples). This fact points towards indirect
235 effect of the IQ-affecting mutation alleles potentially associated with shorter (secretable)
236 isoforms generation (Prosselkov et al., unpublished) lacking two of the most prominent
237 *NTNG* features: GPI-link and the Ukd domain through an aberrant splicing factor binding.
238 The GPI-link is a hallmark of Netrin-G family members (40, 41) and lacking it the aberrant
239 Netrin-G isoforms are likely to mimic the action of their releasable ancestry molecules -
240 netrins, still being able to bind to their cognate postsynaptic ligand – NGL but without
241 forming an axonal-postsynaptic contact and potentially dominant negative consequences. The
242 Ukd domain of Netrin-G1, despite its so-far unknown function, is involved in lateral binding
243 to the pre-synaptically localised LAR modulating the binding strength between NGL-1 and
244 Netrin-G1 (42). Work is currently underway in search for a similar lateral interaction partner
245 for the Netrin-G2 Ukd domain. Inclusion of the Ukd-encoding domain exons 6 and 7 is
246 regulated by the Nova splicing factor (43) affecting the cortex Netrin-G1 exon 7 but not exon
247 6, and, simultaneously, Netrin-G2 paralog exons exhibiting an opposite pattern. In general, it
248 is tempting to speculate that deregulation of *NTNG* transcripts processing may have a role in
249 the brain-controlled cognitive abilities and associated CDs. Supporting such notion, a
250 decreased level of Netrin-G1c mRNA (exons 6-9 excluded, Figure 1B-1) has been reported
251 for BD and SCZ (38) with Netrin-G1d (exons 6 and 7 included but 8-9 excluded, Figure 1B-
252 1) and Netrin-G1f (a secretable short isoform consisted of domain VI only and lacking the
253 Ukd and GPI-link) being increased in BD, but not in SCZ, in anterior cingulated cortex (44).

254 Higher Netrin-G1d mRNA expression in fetal brain but low for the Netrin-G1c isoform in the
255 human adult (38) indicates different functionality of these two splice variants joggling with
256 the Ukd domain inclusion/exclusion pattern. And, according to our unpublished data, if
257 Netrin-G1 Ukd-containing isoforms are the dominant isoforms in adult mouse brain, Netrin-
258 G2 Ukd-containing isoforms are present only at the trace level (Prosselkov et al.,
259 forthcoming), resembling the human STG samples transcriptome pattern (Figure 1B-1 and B-
260 2). A similar "dynamic microexon regulation" associated with the protein interactome
261 misregulation has been reported to be linked to ASD (45).
262 Synchronous and complementary expression of *NTNG* paralogs in the human brain supports
263 the IQ-associated cognitive endophenotypes. Influential parieto-frontal integration theory (P-
264 FIT, (46)) states that general intelligence ("g") is dependent on multiple brain cortical areas
265 such as dlPFC, Broca's and Wernicke's areas, somatosensory and visual cortices (47). Despite
266 "g" is widely accepted as the only correlate of the intelligence, its unitary nature was
267 challenged by (48) claiming had indentified two independent brain networks (for memory
268 and for reasoning) responsible for the task performance, the idea later criticised for the
269 employed data processing approach (49). Higher IQ scores (a composite surrogate of "g")
270 have been reportedly associated with the fronto-parietal network (FPN) connectivity (50, 51).
271 Higher levels of *NTNG* paralogs expression within the cognition intensively loaded areas of
272 the brain and the distinct patterns of expression profiles (synchronous, asynchronous/mixed,
273 and complementary, Figure 2A) support associations of *NTNG1* and *NTNG2* with the
274 recorded cognitive endophenotypes (2). Based on the expression patterning over the human
275 life-span, among the total 16 analysed brain areas we found two falling under the same "anti-
276 phasic (complementary)" classifier (Figure 2C): HIP and MD. Adding more to that, MD is
277 the only brain area (out of the 16 presented) where *NTNG1* expression level exceeds that of
278 *NTNG2* making it a promising candidate for the phenomena of *NTNGs* SF explanation. Two

279 other brain areas classified by a synchronous paralogs expression deserve a special attention,
280 dlPFC and mPFC (Figure 2A-4). PFC circuitry has been known as a "hub of the brain's WM
281 system" (52, 53), which acts through direct HIP afferents (54) and has many connections
282 with other cortical and subcortical areas (55). mPFC may function as an intelligence-control
283 switchboard and lPFC, part of the FPN global connectivity, predicts the WM performance
284 and fluid intelligence (56). Interactions of the auditory recognition information fed by the
285 vPFC stream with the sequence processing by the dorsal stream are crucial for the human
286 language articulation (57; 58). The fact that both *NTNG* paralogs are extensively expressed
287 across PFC (Figure 2A-2 and A-4) pinpoints this area as a key for future molecular studies of
288 *NTNGs* and the human-unique symbolic communications. And PFC is not only implicated in
289 many psychiatric disorders, including SCZ ((59); see also (55) for ref.), but is also the only
290 brain structure unique to primates without known homologs in the animal kingdom (60).
291 Evolution of the protein paralogs encoded by the *NTNGs*. Forkhead box P2 (FOXP2) – a
292 ubiquitously expressed transcription factor that has been reportedly linked to the evolution of
293 human language through T303N, N325S substitutions when compared to a primate ortholog
294 (61), is 100% identical to Nea protein (62). FOXP2 regulates expression of multiple genes in
295 human and chimpanzee (63), and among them is an M3 gene brain module representative
296 responsible for general fluid cognitive abilities (26), *LRRC4C*, a gene encoding NGL-1, a
297 post-synaptic target of Netrin-G1. Similarly to FOXP2, Netrin-G1 is a 100% conserved
298 protein among the hominins with only 1 mutation found in chimpanzee which is absent in
299 marmoset (and other primates) and mice proteins (2). On the other hand, extinct hominins'
300 Netrin-G2 relative to modern human contains T346A point mutation (as per current version
301 of hg19), also found in primates and mouse and known as rs4962173 (dbSNP missense
302 mutation) representing an ancient substitution from Neandethal genomes found in modern
303 humans and reflecting a recent acquisition of the novel allele around 5,300 yrs BC. Nothing

304 is known regarding the functional significance of this mutation allele but biochemically a
305 substitution of alanine (A) on a polar threonine (T) could bring an extra point of post-
306 translational modification, e.g. a phosphorylation or glycosylation (NetPhos2.0 (64) assigns a
307 low probability score for the T346 to be phosphorylated but NetOGlyc4.0 (65) robustly
308 predicts it to be glycosylated, see SM). Another mutation, S371A/V, reflects a selective
309 sweep in Netrin-G2 protein from primates to hominins within a similar to T346A functional
310 context when a hydrophobic alanine (in chimpanzee, A)/valine (in marmoset, V) is replaced
311 by a polar serine (S) with a strong positive predictions for glycosylation but not
312 phosphorylation (see SM). This poses a question whether these two human-specific protein
313 substitutions associate with advanced cognitive traits as they may represent a hidden layer of
314 poorly studied so far protein glycosylation-associated regulatome known to affect the brain
315 function and diseases (66, 67). Adding more to this, T346 is nested on exon 5 just 20 nu away
316 from the affecting WM score rs2274855 mutation allele (2), and, together with S371A/V,
317 they are both located within the lowest percent identity area (exons (5-7)) of Netrin-Gs
318 (Figure 3C) and, proposedly, contributing to the *NTNG* duplicates SF. There are at least three
319 more protein parts potentially contributing to the gene paralogs specialised function
320 subdivision (based on the low identity scores, Figure 3C): the secretory peptide, the GPI-link,
321 and the outmost structurally elaborated unstructured loops (I-III) responsible for the
322 reciprocal binding of Netrin-Gs to their post-synaptic cognate partners, NGL-1 or NGL-2,
323 both containing a C-terminal PDZ-binding domain (68). An interesting finding was reported
324 in (69) reported a presence of SH3(PSD95) domain binding site (required for the
325 phosphatidylinositol-3-kinase recruitment) in mice Netrin-G2 (100% identical to human) but
326 not in Netrin-G1. The detected SH3 binding site overlaps with the Netrin-G2-loop III
327 responsible for the binding specificity to NGL-2 (12, 70, 71). A plausible working hypothesis
328 would be that while internalised (and being GPI-link naïve/immature) the pre-synaptic

329 Netrin-G2 is bound to SH3-PSD95 via loop III but as soon as being secreted extracellularly
330 (and being attached to the membrane) it is bound to post-synaptic NGL-2. Corroborating this,
331 in the absence of Netrin-G2 in the KO mice NGL-2 is unstable on the post-synaptic surface
332 and gets quickly internalised (24). We can only speculate regarding the potential importance
333 of PSD-95(SH3)-Netrin-G2-NGL-2 scaffolding loop interaction/competition but the ability
334 for Netrin-G1 to bind to SH3 has not been reported. Following this logic, Netrin-G1 should
335 have a similar binding partner via loop II.

336 The overall identical structural scaffold among the Netrin-G paralogs (Figure 3D) is
337 likely to represent an anciently preserved one of the primordial protein (encoded by a single
338 gene in the primitive urochordate *C.intestinalis*) and its contribution to the process of SF
339 among the *NTNG* paralogs goes against the "structure drives function" concept. It looks like
340 that it is not the "structure" but rather the "evolution" itself that drives a selection for the best
341 structural (or unstructural in our case) fit out of the available frameworks provided by the
342 gene duplicates to fulfill the emerged functional demand in a new ecological niche. The
343 intricate variability of phenotype is grounded by the conserved nature of genotype and
344 constrained by the "structure-function" limitations of the coding DNA and is only possible
345 due to permissive evolutionary continuing elaborations of non-coding areas able to absorb the
346 most recently acquired elements (having a potential to become regulatory at some point, e.g.
347 like HAR5 (30)) and carried over by the neutral drift. At the same time, the multiple protein
348 substitutions coinciding with the SF labor segregation phenomena among the Netrin-G
349 paralogs question their neutral nature. Both of them undergo a purifying selection from mice
350 to human through the reduction in size of non-coding DNA (introns) and encoded proteins
351 (the mice Netrin-G2 is 2 aa longer its human ortholog) further contributing to the host-
352 specific SF. Thus while the non-coding sequences are used to explore the evolutionary space
353 in time, the restrictive boundaries of the paralogs SF are determined by the protein

354 (unstructured) elements.

355 Molecular evolution of the Cognitive Complement (CC). Appearance of the neural crest (72),
356 an event that "affected the chordate evolution in the unprecedented manner" (73), multipotent
357 progenitor cells (74), and neurogenic placodes (suggesting a chemosensory and
358 neurosecretory activities (75)) in the first primitive urochordates/tunicates coincides with the
359 presence of *Ntng* precursor gene (ENSCING00000024925) later undergoing two rounds of
360 duplication events in fish and found to affect human cognitive abilities (2). *NTNG* paralogs
361 are expressed in the human neural crest-forming cells with *NTNG2* 10 times stronger than
362 *NTNG1* (76), both are differentially expressed in human comparing to chimpanzee and rhesus
363 monkey with *NTNG2* expression model showing stronger probability than *NTNG1* (77), and
364 both are stronger expressed in human telencephalon comparing to chimpanzee and macaque
365 (78). *NTNG1* has been classified as a brain module hub gene "whose pattern fundamentally
366 shifted between species" (18). Belonging to distinct modules of brain expression regulation
367 (78, 79), *NTNGs* are classified as "genes with human-specific expression profiles" (79). The
368 nearby gene ~260 kbp upstream of *NTNG2* is *MED27* (mediator of RNA polymerase II) has
369 been proposed to be associated with the evolution of human-specific traits (80). *NTNG1* has
370 also been reported among the "adaptive plasticity genes" (81) potentiating rapid adaptive
371 evolution in guppies (*NTNG2* was not found in the input RNA).

372 Complementarity among the *NTNG* paralogs and encoded by them proteins has been
373 reported previously: brain expression complementary pattern (in almost self-exclusive
374 manner) defined by the 5'-UTR-localised *cis*-regulatory elements (82); complementary
375 distribution within the hippocampal laminar structures (83); axon-dendrite synaptic ending
376 resulting in differential control over the neuronal circuit plasticity (84); mutually-exclusive
377 binding pattern to post-synaptic partners, NGL-1 and NGL-2, dictated by the protein
378 unstructural elements (13); alternative promoter usage *vs* alternative mRNA splicing (12) and

379 increased coefficient of variation (CV, ST1d) for *NTNG1* expression but not *NTNG2* in SCZ
380 patients (also reported in (85)); KO mice behavioral phenotypes and subcellular signaling
381 partners complementarity (24); "differential stability" brain modules expression (*NTNG1* is
382 expressed in the dorsal thalamus (M11) as a hub gene (Pearson's 0.92) while *NTNG2* is in
383 neocortex and claustrum module (M6, Pearson's 0.65)) (18); hypocretin neurons-specific
384 expression of *NTNG1* (but not *NTNG2*) as a sleep modulator (86); top-down *vs* bottom-up
385 information flows gating in mice and differential modality (Prosselkov et al., forthcoming),
386 and human IQ-compiling cognitive domains complementation (2). The current study reports
387 on *NTNGs* complementarity association with the CDs (Figure 1A); mRNA splicing pattern
388 complementary at the quantitative and qualitative levels via differential use of the middle-
389 located exons (Figure 1B); brain complementary oscillatory expression over the human life
390 span observed in the intensive cognitively loaded brain areas (Figure 2); AE of the paralogs-
391 segregated unique non-coding elements (Figure 3A); complementary pattern of the protein
392 orthologs (mice-to-human) protein sequence evolution. Such multi-level complementation is
393 likely to reflect a shared evolutionary origin from a single gene in a primitive vertebrate
394 organism 700 mln yrs ago and its subsequent functional segregation among the evolution-
395 generated gene duplicates in jawless fish, such as lamprey.
396 Occupying independent but intercalating functional niches, *NTNG1* and *NTNG2* do
397 not compensate but complement each other's function forming a "functional complement" of
398 genes. Half a billion yrs ago the doubled gene dosage led to the gradual SF and manifested in
399 a function complementation within the cognitive domains, at least in human. We would like
400 to coin such gene pair as a Cognitive Complement (CC).
401
402 CONCLUSION
403 The emerged functional redundancy, as an outcome of gene duplication, leads to function

subdivision and its bifurcation among the gene paralogs resulting in the paralogs SF. A functional compensation is known to exist among the evolutionary unrelated genes but has not been reported among the gene paralogs, more frequently characterized by the function complementation. Gene paralogs structural identity (at both, gene and protein levels) does not provide a substrate for function compensation but rather for complementation, perturbating "structure drives function" rule. A gene duplication event of a tunicate *NTNG* primordial gene and the subsequent process of its function specialisation (driven by the new ecological niches appearance and evolution) among the gene duplicates made them to SF into distinct cognitive domains in a complementary manner forming a CC. In our forthcoming work we are to describe how *Ntng* mice genes function resembles that of human orthologs (Prosselkov et al., forthcoming).

415 MATERIALS AND METHODS

416 Human brain *NTNG* transcriptome reconstruction. Relates to Figure 1B and 1-C. The original
417 source of the dataset was produced by ((12): E-MATB-1030) and the downloaded .bam files
418 used for the re-processing are listed in ST1a. All reconstructed transcripts are presented in
419 ST1d standalone Excel file. Two samples were excluded from the analysis due to failed "per
420 base sequence quality" measure, and zero expression level for *NTNG1a* and *NTNG1int(9-10)*
421 otherwise consistently expressed throughout other samples (ST1b). SAMtools software was
422 used for the SNPs calling from the available RNA-seq datasets (ST1c). For details refer to
423 SM.

424 Human brain expression profiling for *NTNGs* across the life span. The original source of data
425 was www.brainspan.org. All available samples were initially included into the analysis but
426 two of them excluded at a later stage (MD for 12-13 pcw and mPFC for 16-19 pcw) due to
427 high deviation (6-7 times) from the mean for other replicas. The mean expression values per
428 each brain area as RPKM were plotted against the sampling age. Profiles classification was
429 done visually considering the trend over the all plotted points as an average.

430 *NTNG1* (*NTNG1m*) and *NTNG2* (*NTNG2b*) full-length mRNA transcripts assembly. Relates
431 to Figure 3B. Human *NTNG1m* brain transcript has been reported previously (125) and we
432 have also confirmed its ortholog presence in the mice brain via full-length cloning (42). Since
433 NCBI contains only its partial CDS (AY764265), we used the RNA-seq-generated exons
434 (Figure 1B) to reconstruct its full-length and to generate an ORF of the encoded Netrin-G1m.
435 Similarly, human *NTNG2b* was reconstructed from the RNA-seq dataset and from Ensemble
436 as follows. Exon 5 sequence was deduced from ENST00000372179, other exons were from
437 ENST00000467453 (no longer available on the current version of Ensemble) except for exon
438 6 deduced by running three independent alignments against the human genomic DNA with
439 the mice 3'-intron (5-6), exon 6, and 5'-intron (6-7) concomitantly confirmed by the

440 generated full-length ORF for Netrin-G2b. The reconstructed protein was predicted to encode
441 587 amino acids, which is in a close proximity to the mice netrin-G2b ortholog of 589
442 residues (Prosselkov et al., forthcoming).
443 Full-lengths gene structures of *NTNG* paralogs reconstruction. Relates to Figure 3A. Both,
444 the obtained above from the STG brain samples RNA-seq and the reconstructed full-lengths
445 transcripts carrying all stably expressed exons were used to confirm the intron-exon junctions
446 positioning for *NTNG1* and *NTNG2*. Due to observed variability in the intron (1-2) and exon
447 10 sizes their boundaries were left unmarked.
448
449 SUPPLEMENTARY MATERIALS (SM)
450 Contain Supplementary Methods (RNA-seq of STG re-processing and SNPs detection) and
451 Supplementary Tables (ST1a-d, ST2) as a single compiled pdf file. Reconstructed RNA-seq
452 (.gtf) of the STG is presented as a standalone Excel file (ST1d). Also included: Netrin-G2b
453 predicted phosphorylation and *O*-glycosylation, Netrin-G1 *vs* Netrin-G2 Ukd alignment (87),
454 predicted secretory peptide cleavage and GPI attachments.
455
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461
462 COMPETING INTERESTS
463 Authors would like to express a lack of any competing interests associated with the work.
464

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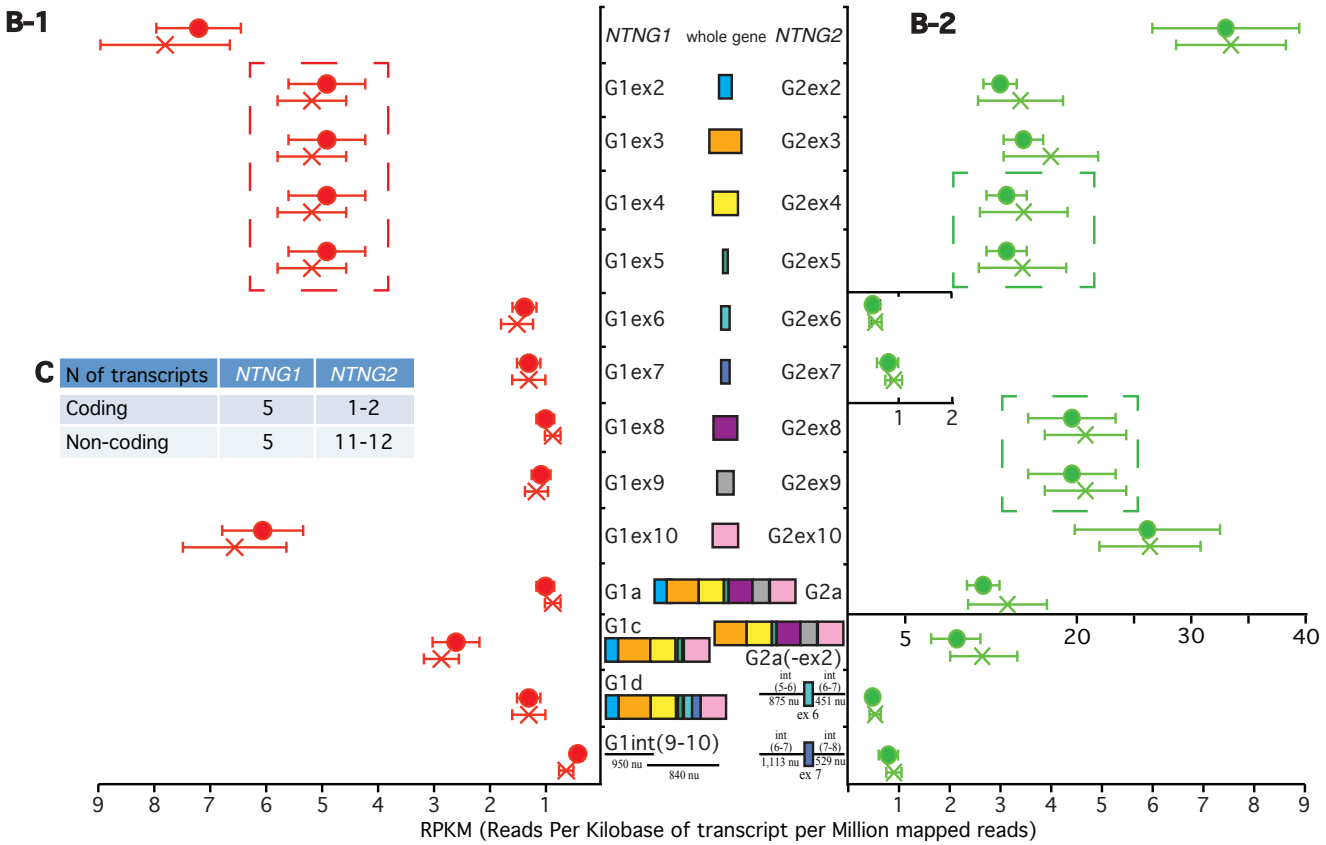
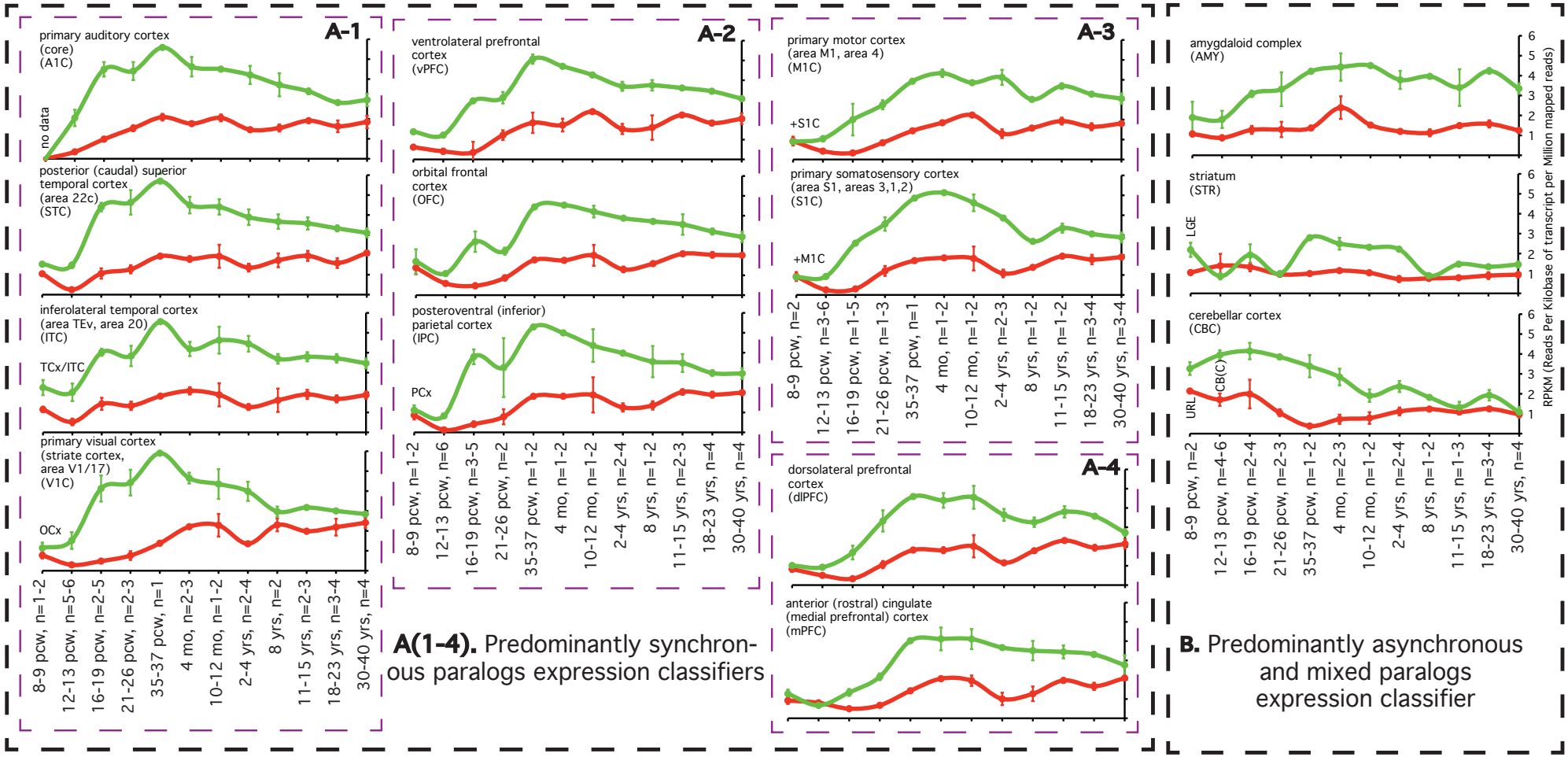
A-1 *NTNG1* reported associations	Cognitive disorder (CD)	A-2 *NTNG2* reported associations
7, 88-90, 91*, 92, 93	Autism sporadic disorder (ASD)	7; 93
38, 44, 94	Bipolar disorder (BD)	38, 44, 94
38, 39, 95-101	Schizophrenia (SCZ)	38, 39
102	Alzheimer disease (AD)	
(103-106)*	Amyotrophic lateral sclerosis (ALS)	
	Fronto-temporal lobe dementia (FTLD)	
107-109	Anorexia nervosa (AN)	
110	Dihydropyrimidine dehydrogenase deficiency (DPD)	
111	Intellectual disability (ID)	
112	Ischemic stroke (IS)	
113	Mental retardation (MeR)	
114*	Obsessive-compulsive disorder (OCD)	
115	Parkinson disease (PD)	
	Pseudopapillary neoplasm (PN)	9
116-118	Rett syndrome (RS)	
	Systemic lupus erythematosus (SLE)	107
	Temporal lobe epilepsy (TLE)	119
120*	Tourette syndrome (TS)	



C

N of transcripts	*NTNG1*	*NTNG2*
Coding	5	1-2
Non-coding	5	11-12

Figure 1. *NTNG* paralogs complementation within neurological disorders and brain transcriptome. (**A-1, A-2**) Reported cognitive disorders associations for *NTNG1* and *NTNG2*. *denotes rather an indirect association via a direct interaction with the research target. (**B-1, B-2**) RNA-seq of the STG of healthy (circle) and SCZ (cross) human subjects. The original dataset was produced by **Wu et al. (12)**, accession number E-MTAB-1030 on ArrayExpress (**ST1a**) and reprocessed as described in **SM**. Five *NTNG1* and four *NTNG2* transcripts, consistently expressed across all 16 human samples are shown. Two samples (one healthy and one SCZ) have been omitted due to unsatisfactory quality of reads and expression profiling (**ST1b**). For the SNPs calling by SAMtools see **ST1c**. Data are presented as a mean RPKM±SEM. (**C**) Total number of the assembled transcripts across all samples for both paralogs (see **ST1d** for the completely reconstructed *NTNG* transcriptome). Dash-outlined are co-spliced exon clusters.



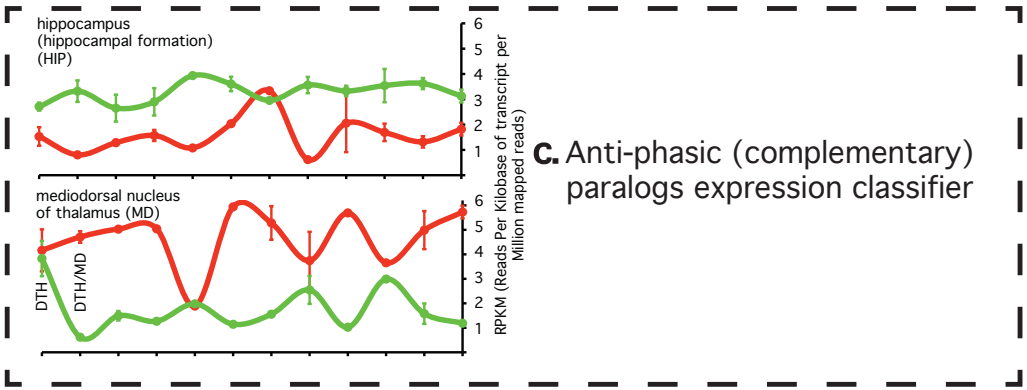


Figure 2. *NTNG* paralogs expression dynamics classification (A-C) in the human brain across the life span. A (1-4): further subdivision of the classifier. RNA-seq data are from the BrainSpan (www.brainspan.org) presented as a mean±SEM. TCx = temporary neocortex; OCx = occipital neocortex; PCx = parietal neocortex; LGE = lateral ganglionic eminence; CB(C) = cerebellum(cortex); DTH = dorsal thalamus; URL = upper (rostral) rombic limb; pcw = post-conception week. Two data points (MD, 12-13 pcw, and mPFC, 16-19 pcw) for *NTNG1* expression were omitted as they were 6-7 times different from the mean of other replicas. All processed brain samples are listed in **ST2**.

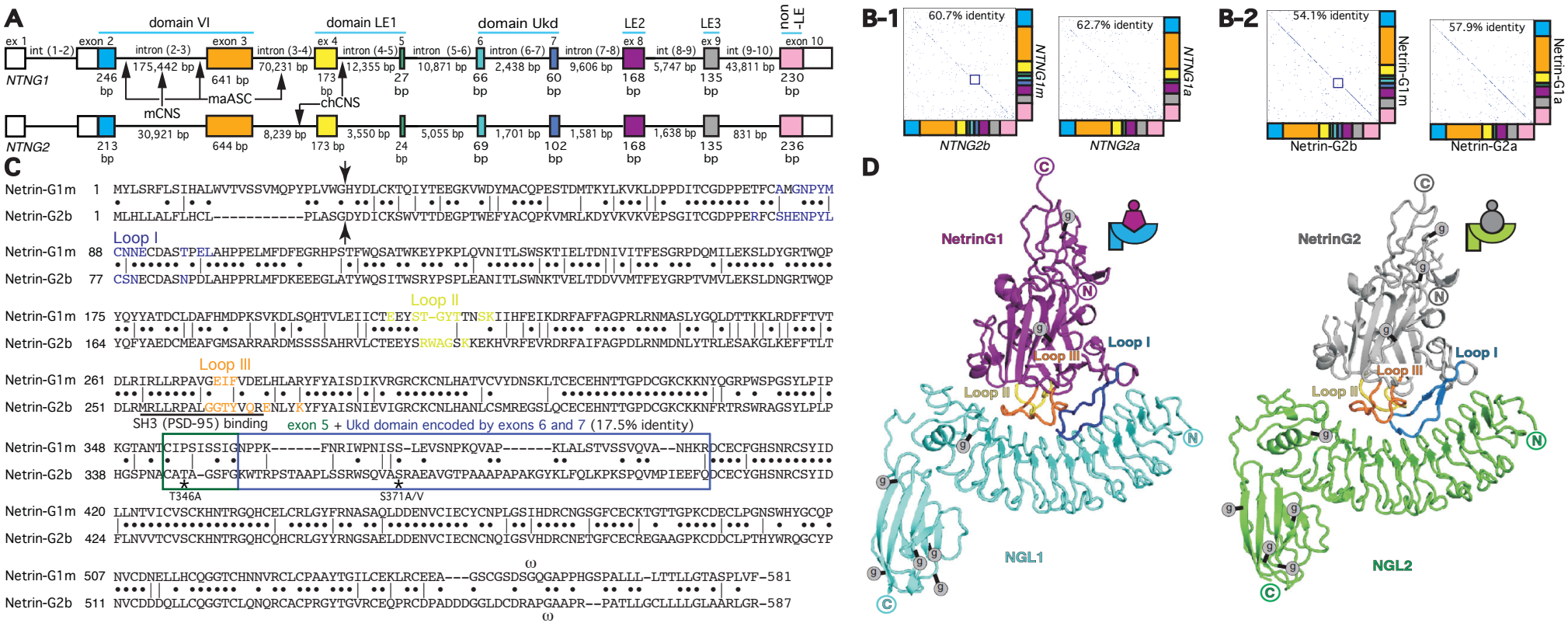


Figure 3. Human *NTNG* paralogs DNA and protein sequence comparisons and "structure-function" rule incongruency. (**A**) Identical gene structures with different sizes of introns. RNA-seq data from **Figure 1B** were used to precisely deduce the exon/intron junction boundaries. The sizes of exons 1, 10 and introns (1-2) are not indicated due to observed among the splice transcripts lengths variability (see **ST1a** for details). Arrows indicate location of CNS = conserved non-coding sequences underwent accelerated evolution in human compare to mice (mCNS) and chimpanzee (chCNS), as per **(124)**; and ASC = anthropoid-specific constrained regions in human compare to marmoset (maASC), as per **(121)**. (**B**) Identical exonal composition of the longest *NTNG* encoded RNA paralog transcripts and corresponding proteins with relatively high percent of identity among them dependent on the included/excluded Ukd domain (**B-2**) encoded by the exons 6 and 7 (**B-1**). Notably, the protein sequence represents higher percent of the paralogs difference than encoded it DNA. The matrices were obtained by GeneJockey II (Biosoft). (**C**) Protein alignments for the longest human *NTNG* encoded proteins, Netrin-G1m and Netrin-G2b, with Loops I-III highlighting binding sites for their cognate post-synaptic binding partners NGL-1 (*Lrrc4c*) and NGL-2 (*Lrrc4*), respectively, as determined by **Seiradake et al. (13)**. Arrow indicates a putative secretory cleavage site location, as calculated by SignalIP (**122**), the blue rectangle delineates the area of the lowest identity (3'-domain LE1+Ukd domain); ω – denotes a point of putative GPI-attachment, as predicted by Big-PI (**123**). PSD-95 interaction site via the SH3-binding domain ((**69**), as determined for mice Netrin-G2) overlaps with the Loop III NGL-2 binding surface. Two stars indicate a modern human (T346A) and a hominin-specific (S371A/V) amino acid substitutions (**2**). **(D)** Identical structural motif of the Netrin-G1/NGL1 and Netrin-G2/NGL2 complexes as per (13). The figure's reproduction is covered by the Creative Commons license.