Molecular Evolution of Social Behavior-Related Candidate Genes (*DRD4* and *GRIN1*) in Apes

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Chapter 1: Introduction & Background

Introduction

As naturalist and philosopher Peter Kropotkin noted in 1902, "it must be said that sociability, action in common, mutual protection, and a high development of those feelings which are the necessary outcome of social life, are characteristic of most monkeys and apes" (p. 32). Apes certainly demonstrate a range of prosocial behaviors, from food-sharing (Silk et al., 2013; Kopp & Liebal, 2016; Sánchez-Amaro et al., 2021) to adoption (Boesch et al., 2010; Morrison et al., 2021) to coalition formation (Heras et al., 2016). These behaviors have been investigated mostly to better understand the evolution of the human species' place in the ape clade (Shultz et al., 2011). Humans exhibit intensified prosocial behaviors, represented in archaeological finds. For instance, Neanderthals practiced healthcare for their injured (Spikins et al., 2019), and, more generally throughout our evolution, there are signs of complex social structures, as illustrated by long-distance resource gathering, as well as deliberate mortuary practices (McBrearty & Brooks, 2000; Scerri & Will, 2023; Spikins et al., 2021). In times of crisis, even as close to home as the COVID-19 pandemic, humans have tended towards "disaster communism:" spontaneous collectivism arising from catastrophe (Desta, 2020; Protevi, 2013).

In this age of genomics, there has been an increasing interest to understand how genetics might influence variation in these behaviors, within the context of elucidating the mechanisms of various neurodevelopmental and psychiatric disorders, such as autism spectrum disorder (ASD) and schizophrenia (McGraw & Young, 2010); of studying the evolution of social behaviors in apes (Inoue-Murayama, 2009; Inoue-Murayama et al.,

2011; Kovalaskas et al., 2020; Staes et al., 2018, 2021); and combining the two in evolutionary neuroscience to understand the evolution of neurodevelopmental and psychiatric disorders (Berto et al., 2020). The genes associated with social behavior and cognition are likely under evolutionary pressures as displaying the proper behaviors is key in "securing a mate, parenting" (Ebstein et al., 2010). Other important non-reproductive behaviors and general other-regarding behaviors, which are also mediated by neuropeptides, such as allogrooming (Barelli et al., 2011), alloparenting (Glasper et al., 2019), and cooperative hunting (Samuni et al., 2018), can impact an individual's access to resources. Thus, inappropriate social behaviors may severely hinder an individual's fitness (Clutton-Brock, 2002).

1.1 Ape social systems

While it can be argued that humans exhibit intensified prosocial behaviors, as exemplified by our robust and variable cultures (Meder, 2013), the social structures of other apes cannot be discounted. The composition of social structures is largely dependent on ecological factors, such as food availability and competition (Meder, 2013). There are clear differences and similarities between species, though intraspecies variation can exist beyond these generalities.

Gibbons (genera *Hylobates*, *Hoolock*, *Nomascus*, *Symphalangus*), or "lesser apes," have long been thought to practice monogamy, though recent studies have shown intraspecific variation – the norm, however, is small units, usually made up of two pair-bonded individuals and offspring (Meder, 2013). These social units also engage in territory defense against other groups (Harding, 2012). In some gibbon species, males engage in offspring care (Fuentes, 2002) and there is evidence of affiliative behaviors in

these taxa, such as food-sharing (Sánchez-Amaro et al., 2021) and allogrooming (Barelli et al., 2011; Fuentes, 2002).

Orangutans (*Pongo*), a genus of three species (Bornean [*P. pygmaeus*], Sumatran [*P. abelii*], and the recently named Tapanuli [*P. tapanuliensis*]), seem to be more solitary than other apes (Delgado Jr. & Van Schaik, 2000; Roth et al., 2020), with male-female contact largely restricted to mating (Meder, 2013), within the framework of their polygynous mating system. Orangutan adults seldom spend time together and mothers spend most of their time with their offspring (Delgado Jr. & Van Schaik, 2000). High fruit availability is positively correlated with small party formation (not for the purpose of territory defense), which provide individuals with social benefits, such as protection for females against coercive males (Delgado Jr. & Van Schaik, 2000; Roth et al., 2020). Sumatran orangutans, who live in fruit abundant environments, are more gregarious and engage in social transmission of skills, including limited tool use (Delgado Jr. & Van Schaik, 2000). Generally, orangutans live in loose social structures and are less gregarious than other great apes (Delgado Jr. & Van Schaik, 2000; Meder, 2013).

While the two *Gorilla* species (*G. gorilla* and *G. beringei)* inhabit a variety of environments, mostly montane forests and lowland rainforests, they both live in stable polygynous communities, with a clear hierarchy among males (Meder, 2013). Upon reaching maturity, western lowland gorillas (*G. gorilla*), both males and females, usually disperse from their natal groups to form new communities, though mountain gorillas (*G. beringei*) also exhibit philopatry (Meder, 2013; Robbins & Robbins, 2018). Female dispersal is interesting as females will often join groups that already include a female relative, suggesting possible kin-recognition and affiliative relationships between female

gorillas (Bradley et al., 2007). Male aggression among gorillas is not related to territory defense, but rather to protect females from other males (Robbins & Robbins, 2018; Rosenbaum et al., 2016). Coalitionary violence has infrequently been documented in mountain gorillas against extra-group males (Rosenbaum et al., 2016). Gorillas show general tolerance (though violence, e.g. infanticide, has been observed [Robbins et al., 2013]) of changing social structure, such as groups transitioning from single male to multi-male and flexibility in philopatry and dispersal (Robbins & Robbins, 2018).

The social structures of the genus *Pan* have been of great interest as chimpanzees (*P. troglodytes*) and bonobos (*P. paniscus*) are our closest living relatives, and their behaviors may shed light on our own evolution (Staes et al., 2021). Despite both being species characterized by fission-fusion groups and female-biased dispersal, the social dynamics of bonobos and chimpanzees are markedly different (Garai et al., 2014). Bonobos show inter- and intragroup tolerance and females are known to mitigate feeding competition related tension through genito-genital rubbing (Meder, 2013). Female bonobos are far more affiliative than female chimpanzees, and can form coalitions against male bonobos (Kovalaskas et al., 2020; Meder, 2013). Male chimpanzees, on the other hand, form coalitions to defend their territories, which can lead to fatalities (Meder, 2013), and hunt cooperatively (Samuni et al., 2018). Chimpanzees, both male and female, are known to commit infanticide (Walker et al., 2021) – a behavior not observed in bonobos (Kovalaskas et al., 2020). However, both bonobos and chimpanzees are known to adopt and alloparent (Tokuyama et al., 2021). Additionally, both species show cognitive differences: bonobos outperform chimpanzees in tasks requiring an understanding of social causality, while chimpanzees excel at tool use tasks (Herrmann et

al., 2010). Garai et al. (2014) have posited that this increased social tolerance and decreased aggression in bonobos may be mediated in part by the *MAOA* and *MAOB* genes: bonobos exhibited more polymorphic diversity than chimpanzees at these genes. Nonetheless, bonobos inhabit more food-rich environments than chimpanzees, which may influence their aggressive and affiliative behaviors (Meder, 2013).

Differences between social structures and behaviors in apes largely reflect the various environments they occupy. At the proximate level, aspects of social behavior and behavioral tendencies can be influenced by genetic variations (Ebstein et al., 2010; Staes et al., 2021), suggesting that differential selection acting on genetic variation may play a role in the evolution of social behavior and cognition in apes. The genes of interest are those which act in the brain, specifically in neurotransmitter systems. The release and integration of these chemicals, such as oxytocin, in the neural circuitry have long been linked with sociality (Quintana et al., 2019).

1.2 Brain & behavior

The brain is the hub of social behavior, as it directs our thoughts and actions. There are key regions of the brain which regulate social cognition, such as the hippocampus, the amygdala, and the prefrontal cortex (Franklin et al., 2017; McGraw & Young, 2010; Staes et al., 2021). In particular, Von Economo neurons in the anterior insula and anterior cingulate cortex found in large-brained mammals with complex social systems are thought to have evolved to better integrate socially-relevant information (Stimpson et al., 2011). Moreover, several neurotransmitters are known to be involved in social behavior and they are mostly expressed in the brain, where information is

integrated and influences our actions within social contexts (Meyer-Lindenberg et al., 2011).

1.2.1 Neurotransmitters

Through the increasing power of genomics, many genes have been associated with social behavior, using genome-wide association studies, for instance (Ebstein et al., 2010). The majority of these are genes in neurotransmitter systems – a process by which certain chemicals are transmitted between neurons, regulating their activity (Gobrogge & Wang, 2011; Inoue-Murayama, 2009), and consequently influencing social behavior. There are six neurotransmitters often associated with social behavior: oxytocin, vasopressin, serotonin, dopamine, glutamate, and gamma-aminobutyric acid (GABA) (Table 1). There are many genes implicated in all of the neurotransmitter systems, including genes coding for receptors, transporters, and proteins with secretory and modulatory functions (Table 1). It is important to note that these systems often interact with each other (McGraw & Young, 2010; Staes et al., 2021).

Oxytocin and vasopressin have been implicated in the regulation of social memory, affiliative behaviors, and mate preference (Anestis et al., 2014; Fam et al., 2019; McGraw & Young, 2010; Staes et al., 2021). Notably, these neuropeptides are paralogous, originating from a 600 million year old duplication. Oxytocin only has one receptor, OXTR, while vasopressin has three, AVPR1A, AVPR1B, and AVPR2, and there is evidence of cross-reaction between one peptide and the other's receptors (Fam et al., 2019).

The serotonergic system is largely responsible for regulating aggression and impulsivity (Inoue-Murayama, 2009; Staes et al., 2021) and increased serotonin has been

associated with the ASD behavioral phenotype (Veenstra-VanderWeele et al., 2012). This system has a complex distribution of receptors, with seven families and several subtypes, which not only regulate serotonin, but influence the activation of other neurotransmitters, such as dopamine, glutamate, and GABA (Švob Štrac et al., 2016).

Dopamine plays a role in the reward system of the brain and acts on novelty-seeking, social cognition, and attachment (Ding et al., 2002; Ebstein et al., 2010; Staes et al., 2021). The dopaminergic system has five receptors, which are all G-protein coupled receptors (GPCR) (Beaulieu & Gainetdinov, 2011; Wang et al., 2017). Of the five receptors, D(4) dopamine receptor (DRD4) is the one highlighted in this study. This receptor is expressed throughout the brain, but most significantly in the amygdala, hypothalamus, hippocampus, and cerebral cortex (Seeman, 2009). DRD4 is known to inhibit adenylyl cyclase, an enzyme that plays important roles in the regulation of metabolism, olfaction, cell proliferation, and memory (Beaulieu & Gainetdinov, 2011; Taussig, 2013). DRD4 is, however, largely restricted in its role and exhibits considerable genetic variation (Ding et al., 2002; Inoue-Murayama et al., 1998; Livak et al., 1995; Seaman et al., 2000; Seeman, 2009; Wang et al., 2017).

Glutamate has been associated with the regulation of social behavior, including communication, and parental behaviors, as well as cognition and learning (Audet et al., 2018; Milenkovic et al., 2014; Sengar et al., 2019). It is the primary excitatory neurotransmitter of the nervous system (Bredewold et al., 2015; McEntee & Crook, 1993) and is highly conserved across species (Sousa et al., 2017). This system has three ionotropic receptor types: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors, and

kainate receptors (Purves et al., 2001). NMDA receptors have been studied for their association with the maintenance of social behavior, such as social play, communication, and memory (McEntee & Crook, 1993; Milenkovic et al., 2014; Zoicas & Kornhuber, 2019), though also for their roles in nervous system disorders, including epilepsy (Meldrum, 1994). Glutamate ionotropic receptor NMDA type subunit 1 (*GRIN1*) is one of the genes analyzed here and its protein function as an ion channel is dependent upon the formation of a dimer with GRIN2 in the hippocampus and neocortex (Sengar et al., 2019).

Finally, GABA has been linked with both socially-beneficial behaviors, such as exploration and grooming (DeLorey et al., 2008), but also with agonistic behavior (Gobrogge & Wang, 2011). GABA is the main inhibitory neurotransmitter of the nervous system and its receptors are either ionotropic or GPCR (Singer et al., 2016). This neurotransmitter helps regulate the development of the fetal brain, including neurogenesis and synaptogenesis (DeLorey et al., 2008), though it has a depressant effect on the adult neuromuscular system (Singer et al., 2016).

Many of the aforementioned neurotransmitters are closely associated with mate choice and mating systems (Babb et al., 2010; Fam et al., 2019). The social behaviors of apes are also influenced by ecology, which includes food and mate availability. The socioecological model places female sexual and reproductive behavior as incipient to social relations. This model posits that the availability of food and predation risk drives the gregariousness of females, which in turn impacts the distribution of males, as they seek to access females for reproductive purposes (Sterck et al., 1997; Wrangham, 1980). It would, therefore, be salient to consider the neurohormone gonadotropin and its role in

female sexual behavior as an avenue of investigation into the evolution of social behavior (Kovalaskas et al., 2020).

1.3 Aims of study

This study aims to expand on previous research on the genetic underpinnings of social behavior variation in apes within an evolutionary framework (Staes et al., 2021). However, the field of behavioral genetics is contentious for its association with biological determinism: that a particular genetic makeup could control a person's behavior and personality, reminiscent of eugenics (Blakey, 2020; Strier, 2018). I strongly stand against biological determinism, as it ignores the major impacts of environment and culture on behavior and further entrenches societal inequalities (see Lewontin, 2000; Lewontin et al., 1984). I am also critical of the "deficit model" applied to neurodevelopmental and psychiatric disorders, such as ASD (Dinishak, 2016): many of these "deficiencies" are simply part of normal human variation (see Grinker, 2021).

This study looks at the broad patterns of protein evolution associated with behaviors we know are important to ape survival and are variable across species. Ultimately this study aims to answer whether genes that are involved in these neurotransmitter processes show interesting patterns of protein evolution in the ape clade.

Chapter 2: Methods

2.1 Identifying candidate genes of interest

To identify genes potentially associated with variation in social behavior, I used two databases: Simons Foundation Autism Research Initiative (SFARI) Gene (https://gene.sfari.org/) (Banerjee-Basu & Packer, 2010) and Mammalian Phenotype Ontology Browser (MPOB) (http://www.informatics.jax.org/vocab/mp_ontology/MP:0005386) (Smith & Eppig, 2009). For SFARI Gene, I looked at genes implicated in neuromodulator systems, then browsed the cited literature for relevance. For MPOB, I focused on genes listed under the behavior/neurological phenotype, and browsed for key words associated with social behavior, such as grooming, prepulse inhibition, parenting, aggression, and communication (Ebstein et al., 2010). I then went through the associated literature, again focusing on genes linked to neuromodulator systems.

Of the ~30 candidate genes I identified (Table 1), I analyzed two: D(4) dopamine receptor (*DRD4*) (Ebstein et al., 2010) and glutamate ionotropic receptor NMDA type subunit 1 (*GRIN1*) (Milenkovic et al., 2014; Zoicas & Kornhuber, 2019).

2.2 Construction of dataset

I compiled the reference genome coding sequences, flagged as “Ensembl Canonical," of *DRD4* and *GRIN1* via the Ensembl genome browser (https://useast.ensembl.org/index.html) (Martin et al., 2023) for the following species: humans (*Homo sapiens*, genome GRCh38.p13), chimpanzees (*Pan troglodytes*, genome Pan_tro_3.0), bonobos (*Pan paniscus*, genome panpan1.1), western lowland gorillas (*Gorilla gorilla gorilla*, genome gorGor4), Sumatran orangutans (*Pongo abelii*, genome

Susie_PABv2), and white-cheeked gibbons (*Nomascus leucogenys*, genome Nleu_3.0). Rhesus macaques (*Macaca mulatta,* genome Mmul_10) were added as an outgroup species.

I visualized each gene (coding sequence only) in Jalview (Waterhouse et al., 2009) and used the "Alignment" option in the "Web Service" tool. I chose MAFFT (Katoh et al., 2002; Katoh & Toh, 2010) with the L-INS-i preset, with a gap opening penalty of 1.53 and gap extension penalty of 0.123. Sequences were then trimmed and refined by hand to ensure an open-reading frame. The final *DRD4* dataset consisted of six sequences with a length of 1,257 base pairs (419 codons). Bonobos were not included in this dataset as there was no *DRD4* sequence for this species in the reference genome in Ensembl. The final *GRIN1* dataset consisted of six sequences with a length of 2,763 base pairs (921 codons). Gibbons were not included in this dataset as the available sequence could not be satisfactorily aligned with the others (likely reflecting lower reference genome quality).

2.3 Selection analysis

I analyzed the molecular evolution of each gene using the methods available through HyPhy (Kosakovsky Pond et al., 2005) on the Datamonkey web server (https://datamonkey.org/) (Weaver et al., 2018). Negative selection refers to selection which is observed as a higher rate of synonymous than nonsynonymous substitutions ($dS > dN$), whereas positive selection is observed as more nonsynonymous than synonymous substitutions ($dN > dS$), leading to a diversification of the encoded protein. I looked for site-specific selection using FEL (Kosakovsky Pond & Frost, 2005), which identifies site specific pervasive positive/diversifying and negative/purifying selection, and MEME

(Murrell et al., 2012), which identifies site specific episodic positive/diversifying selection. I looked for branch-specific selection using aBSREL (Smith et al., 2015), which identifies branch specific positive/diversifying selection. Finally, I looked for gene-wide selection using BUSTED (Murrell et al., 2015), which identifies gene-wide episodic positive/diversifying selection. For each of these methods, I used a p-value threshold of 0.05. Analyses were run on a user-defined tree generated from 10kTrees (version 3) (https://10ktrees.nunn-lab.org/Primates/index.html) based on the well-resolved primate phylogeny (Arnold et al., 2010).

2.4 Determining effect of nonsynonymous changes

To determine whether nucleotide changes would affect protein function, the nucleotide sequences were first translated into amino acids and viewed in Aliview (Larsson, 2014) to identify conserved and variable regions of the protein. Species trees illustrating the amino acid changes were created using the trees from 10kTrees (Arnold et al., 2010) (Figs. 2 & 6).

Amino acid sequences (human for DRD4 and orangutan for GRIN1) were input into SNAP2 (https://rostlab.org/services/snap2web/) (Hecht et al., 2015) to predict whether the observed amino acid changes would likely have a functional effect. The strength of effect is on a range from -100 (no effect) to 100 (strong effect) and presented as a heatmap. I then used the AlphaFold predicted models (Jumper et al., 2021; Varadi et al., 2022) available on Ensembl for each gene in humans to determine where variable regions were in the protein, supplemented by UniProt entries (https://www.uniprot.org/) (The UniProt Consortium, 2023).

Chapter 3: Results

3.1 Genes of interest

By searching the SFARI Gene (Banerjee-Basu & Packer, 2010) and MPOB (Smith & Eppig, 2009) databases, I found 30 genes associated with variation in social behavior (Table 1). Many of these genes are associated with the behavioral phenotype of ASD and other neurodevelopmental and mental health disorders in humans (McGraw & Young, 2010). More generally, the evolution and variation of these genes could shed light on the genetic scaffolding of ape sociability.

Neurotransmitter system	**Gene**	**Links to behavior**	**References**
Dopamine	*COMT*	Associated with aggression and social skills	Li et al. (2016)[HS]
	DRD1	Associated with social cognition	Homberg et al. (2016)[RMS]
	DRD2	Regulation of impulsivity	Della Torre et al. (2018)[HS]
	DRD4	Regulation of altruism and sexual behavior	Ebstein et al. (2010)[HS]
Dopamine & GABA	*CNTNAP4*	Associated with prepulse inhibition and repetitive behavior	Karayannis et al. (2014)[RMS]
GABA	*GABRB3*	Associated with sociability and exploratory behavior	DeLorey et al. (2008)[RMS]
	PX-RICS	Associated with social interaction, including reciprocation, and social dominance	Nakamura et al. (2016)[RMS]
GABA & Glutamate	*BAIAP2*	Regulation of social interaction, including memory, and hyperactivity	Kim et al. (2020)[RMS]
Glutamate	*CYP39A1*	Possibly associated with prosocial neurophysiology	Blumer et al. (2022)[OMS]

	GRIN1	Associated with social interactions deficits, cognitive flexibility, and repetitive behavior	Milenkovic et al. (2014)[RMS]; Zoicas & Kornhuber (2019)[RMS]
	GRIN2A	Associated with social memory, social communication, and aggression	Zhao et al. (2009)[RMS]; Zoicas & Kornhuber (2019)[RMS]
Gonadotropin	*GnRH1*	Regulation of sociosexual behavior	Kovalaskas et al. (2020)[NHPS]; Parhar et al. (2016)[OMS]
	GnRH2	Regulation of sociosexual behavior	Kovalaskas et al. (2020)[NHPS]; Parhar et al. (2016)[OMS]
Oxytocin	*CAPS2*	Regulation of social interaction, including social memory	Fujima et al. (2021)[RMS]
	CD38	Regulation of social memory, vocalizations, and affiliative behavior	Lopatina et al. (2013)[RMS]; Quintana et al. (2019)[HS]
	GTF2I	Regulation of social anxiety, social communication skills, and extraversion	Crespi & Hurd (2014)[HS]
	OXT	Regulation of social memory	Ferguson et al. (2000)[RMS]
	OXTR	Associated with aggression, social memory, and prosociality	Fujima et al. (2021)[RMS] Staes et al. (2021)[NHPS]
Serotonin	*APBA2*	Associated with conflict resolution and depression	Sano et al. (2009)[RMS]; Yun et al. (2015)[HS]
	EN-2	Associated with sociability impairments, prepulse inhibition, fear learning and memory	Brielmaier et al. (2012)[RMS]
	HTR1A	Regulation of aggression	Staes et al. (2021)[NHPS] van den Berg et al. (2003)[OMS]
	HTR1B	Regulation of aggression	Saudou et al. (1994)[RMS]

	MAOA	Associated with aggression and antisocial behavior	Cohen et al. (2011)[HS]; Garai et al. (2014)[NHPS]
	SLC6A4	Associated with aggression, social impairment, and repetitive behavior	Veenstra-VanderWeele et al. (2012)[RMS]
Vasopressin	*AVPR1A*	Regulation of social interaction and pair-bonding	Babb et al. (2010)[NHPS]; Staes et al. (2021)[NHPS]
	AVPR1B	Regulation of aggression and social memory	Fam et al., (2019)[NHPS]; Pagani et al. (2015)[RMS]
N/A	*CYP19*	Regulation of female sociosexual behavior	Bakker et al. (2002)[RMS]
N/A	*ELP2*	Associated with developmental delays and vocalization impairments	Kojic et al. (2021)[RMS]
N/A	*HOXB8*	Regulation of grooming behavior	Greer & Capecchi (2002)[RMS]
N/A	*SPOCK3*	Regulation of anxiety and social novelty seeking	Yamamoto et al. (2014)[RMS]

Table 1: 30 candidate genes associated with social behavior. Bolded genes are those analyzed in this study (*DRD4* and *GRIN1*). Abbreviations: [HS] = human study; [RMS] = rodent model study; [NHPS] = non-human primate study, [OMS] = other mammal study.

The genes analyzed in this study are receptor genes of different neuromodulatory systems and are associated with different behaviors. *DRD4*, as part of the dopamine system, has been linked to variation in sexual and altruistic behavior in humans (Ebstein et al., 2010). *GRIN1* is one of the NMDA receptors of the glutamate system and is related to cognitive flexibility and the regulation of social behavior (Milenkovic et al., 2014; Zoicas & Kornhuber, 2019).

3.2 DRD4

3.2.1 Selection analysis

I analyzed via HyPhy a total of 419 sites for this dataset and found evidence of both negative/purifying and positive/diversifying selective pressures. FEL detected 26 sites under negative selection and two (codons 237 and 326) under positive selection (Tables 2, 3). Only eight of the 26 sites under negative selection are found between codons 220 and 330, while both sites under positive selection are in this region. FEL, which uses a fixed-effects model, assumes a constant rate of selection across the defined phylogeny (Kosakovsky Pond & Frost, 2005). To account for branch-specific selection rates, I also used MEME to identify sites under positive selection (Murrell et al., 2012), of which there were seven (Table 2): codons 26, 27, 237, 272, 288, 308, and 326 (Table 4). The two sites identified by FEL as being under positive selection were corroborated by MEME. From a site-specific perspective, *DRD4* has been more so under negative selection than positive selection, though there has been positive selection in the region from sites 220 to 330.

aBSREL	BUSTED	FEL	MEME
6 of 9 branches	no evidence	26 sites under N/P selection 2 sites under P/D selection	7 sites under P/D selection

Table 2: Summary of significant results of the HyPhy selection analyses of *DRD4* ($p \leq 0.05$).
Abbreviations: N/P = negative/purifying; P/D = positive/diversifying.

Site	α	β	LRT	p-value
237	0	5.342	6.275	0.0122
326	0	2.582	7.324	0.0068

Table 3: FEL analysis of site-specific positive selection in *DRD4* ($p \leq 0.05$). α = synonymous substitution rate (*dS*); β = nonsynonymous substitution rate (*dN*); LRT = likelihood ratio test statistic.

Site	α	β-	β+	LRT	p-value
26	0	0	14.28	8.46	0.01
27	0	0	5382.04	12.73	0
237	0	0	24.33	9.85	0
272	0	0	17.86	6.36	0.02
288	1.46	0	10,000	4.55	0.05
308	0.7	0.7	121.52	4.59	0.05
326	0	0	2.71	7.33	0.01

Table 4: MEME analysis of site-specific positive selection in *DRD4* ($p \leq 0.05$). α = synonymous substitution rate (*dS*); β- = non-synonymous substitution rate for the negative/neutral evolution component; β+ = non-synonymous substitution rate for the positive/neutral evolution component; LRT = likelihood ratio test statistic.

aBSREL analysis determined that there were two *dN/dS* (ω) classes across the phylogeny and identified branch-specific diversifying selection on six of the nine branches (Table 2). Four of them are species-specific: gorillas, gibbons, orangutans, and chimpanzees; the other two are ancestral nodes: *Gorilla-Pan-Homo* and *Pan-Homo* (Table 5). However, BUSTED did not find any evidence of gene-wide diversifying selection (Table 2).

Node	ω1	ω2	LRT	Test p-value
Gorilla gorilla gorilla	0	3.99	19.2216	0.0002
Gorilla-Pan-Homo LCA	0	100000	18.6827	0.0002
Nomascus leucogenys	0	3.3	13.0726	0.0035
Pan-Homo LCA	0	545	11.4113	0.0069
Pongo abelii	0	8.04	9.9178	0.0097
Pan troglodytes	0	16.3	10.0569	0.0113

Table 5: aBSREL analysis of branch-specific diversifying selection in *DRD4* ($p \leq 0.05$). ω1 = *dN/dS* rate 1*;* ω2 = *dN/dS* rate 2; LRT = likelihood ratio test statistic; test p-value = p-value corrected for multiple testing.

3.2.2 Effect of nonsynonymous changes

The D(4) dopamine receptor is largely conserved, except at the start of the protein and between positions 220 and 330 (Fig. 1). The latter is further investigated and labeled by me here as the "highly variable region" (HVR) of DRD4. Due to missing data from the start of the protein in gorillas and gibbons, parsimony with this dataset cannot resolve the order of certain amino acid changes (Fig. 2). The macaque sequence was taken to represent the ancestral sequence. The changes are mostly species specific, with the gorilla sequence having the most substitutions (Fig. 2). In this region, gorillas have two deletions, one of which is shared with chimpanzees. This is a four amino acid deletion from positions 324 to 327, consisting of one unit (QTPP) of a two-unit repeat found in all other sequences. The orangutan sequence has three single amino acid deletions. Humans and gibbons have fewer changes (relative to rhesus macaque) than the other apes, with humans being the most conserved. Interestingly, there is a three amino acid insertion common to the great apes, though this may be due to missing gibbon data rather than being hominid specific. In accordance with the aBSREL results (Table 5), there is

evidence of branch-specific diversification at the *Gorilla-Pan-Homo* LCA and

Pan-Homo LCA nodes (Fig. 2).

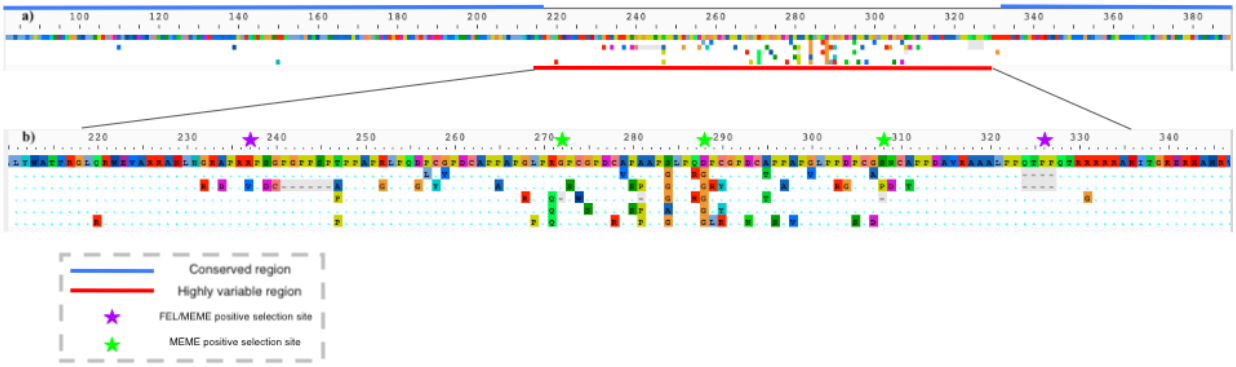


Figure 1: DRD4 amino acid sequences. From top to bottom: human, chimpanzee, gorilla, orangutan, gibbon, and macaque. Human sequence is set as reference and only non conserved amino acids are shown in the other sequences. **a)** overview of D(4) dopamine receptor (90-419); **b)** Highly Variable Region (HVR) of D(4) dopamine receptor with asterisks at positive selection sites identified by FEL and MEME (purple) or only by MEME (green).

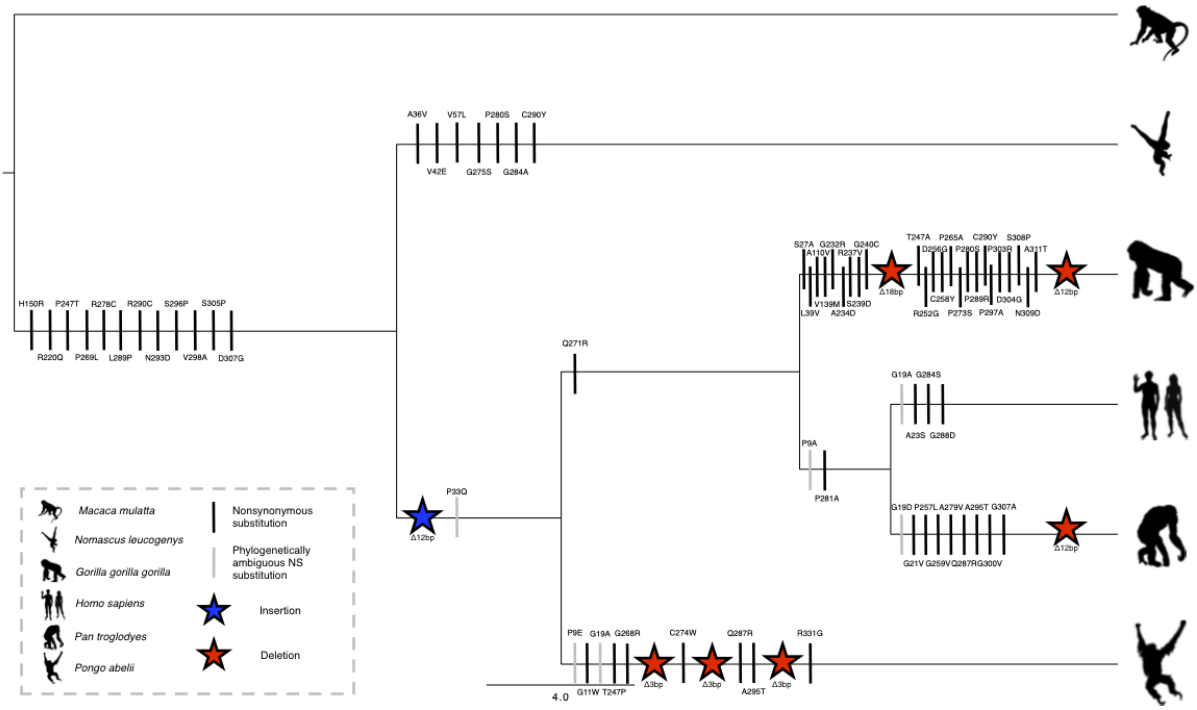


Figure 2: DRD4 protein variation in apes. Macaque is the outgroup species. Nonsynonymous substitutions are represented by a black line; those that are phylogenetically ambiguous are represented by a gray line. Their positions are indicated.

Insertions are represented by blue stars and deletions are represented by red stars. The number of basepairs inserted/deleted is indicated.

Once nonsynonymous substitutions have been identified, it must be determined whether they would have a functional effect on the protein. To do so, SNAP2 predicted whether amino acid changes in the human DRD4 sequence would impact the protein's function. While most of the protein is in red, which means that a change to any other amino acid at any given site would likely have a functional effect, the HVR is mostly blue and white (Fig. 3). SNAP2 predicted that changes in this region would likely only have a neutral effect or cannot reliably predict the effect. This suggests that the HVR may not be an important functional region of the D(4) dopamine receptor protein.

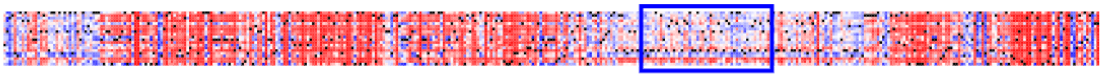
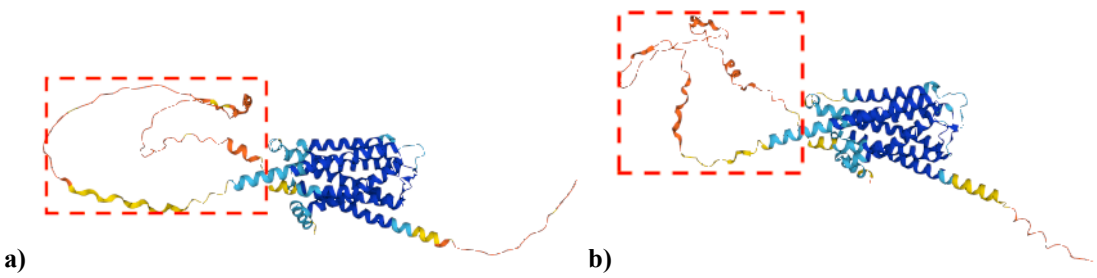


Figure 3: SNAP2 functional effects prediction score heatmap. The human amino acid sequence of DRD4 is on the x-axis and changes to other amino acids on the y-axis. Red represents a strong predicted effect; white represents an inconclusive prediction; blue represents a neutral predicted effect. The blue rectangle highlights the HVR.

To further investigate the role of this protein region, I used the resources on UniProt (The UniProt Consortium, 2023) for the human (UniProt Accession: P21917) and chimpanzee D(4) dopamine receptors (UniProt Accession: H2Q2V4). The proteins look overall similar, with the starkest difference in configuration being the HVR (Fig. 4). This region is not helical and has a low AlphaFold prediction confidence. According to Wang et al. (2017), positions 214-347, which include the HVR, are cytoplasmic. DRD4 has three other such regions, though these are only around 15 amino acids long.



a) b)

Figure 4: AlphaFold predicted 3D structure of DRD4. a) Human protein (P21917); **b)** Chimpanzee protein (H2Q2V4). The highly variable region is highlighted by the red rectangle. The color of the protein corresponds to the AlphaFold model confidence, with dark blue as very high and orange as very low. (Jumper et al., 2021; Varadi et al., 2022)

3.3 GRIN1

3.3.1 Selection analysis

I used HyPhy to analyze 921 sites for the *GRIN1* dataset and found scant evidence of positive selection (Table 6). As for the analysis of *DRD4,* two methods of site-specific selection were implemented in order to identify sites when it is assumed that selection is constant across the phylogeny (FEL) and when branch-specific selections rates are considered (MEME). Only two positive selection sites were identified: 203 (only by MEME; Table 8) and 209 (by both FEL and MEME; Table 7 & 8). These sites are located in an apparent 21 amino acid deletion in humans, from positions 190 to 210. FEL detected 67 sites under negative selection.

aBSREL	BUSTED	FEL	MEME
3 of 9 branches	no evidence	67 sites under N/P selection 1 site under P/D selection	2 sites under P/D selection

Table 6: Summary of significant results of the HyPhy selection analyses of *GRIN1* ($p \leq 0.05$).
Abbreviations: N/P = negative/purifying; P/D = positive/diversifying.

Site	α	β	LRT	p-value
209	0	2.841	5.521	0.0188

Table 7: FEL analysis of site-specific positive selection in *GRIN1* ($p \leq 0.05$). α = synonymous substitution rate (*dS*); β = nonsynonymous substitution rate (*dN*); LRT = likelihood ratio test statistic.

Site	α	β-	β+	LRT	p-value
203	0	0	32.69	6.27	0.02
209	0	0	5.75	6.56	0.02

Table 8: MEME analysis of site-specific positive selection in *GRIN1* ($p \leq 0.05$). α = synonymous substitution rate (*dS*); β- = non-synonymous substitution rate for the negative/neutral evolution component; β+ = non-synonymous substitution rate for the positive/neutral evolution component; LRT = likelihood ratio test statistic.

From a branch perspective, aBSREL analysis detected two rates of branch-specific diversifying selection on three of the nine branches of my phylogeny (Table 9). These are all species-specific branches: gorillas, chimpanzees, and humans. BUSTED found no evidence of gene-wide diversifying selection (Table 6).

Node	ω1	ω2	LRT	Test p-value
Gorilla gorilla gorilla	0	450	209.2065	0
Pan troglodytes	0	100000	274.3407	0
Homo sapiens	0.0622	429	109.9741	0

Table 9: aBSREL analysis of branch-specific diversifying selection in *GRIN1* ($p \leq 0.05$). ω1 = *dN/dS* rate 1*;* ω2 = *dN/dS* rate 2; LRT = likelihood ratio test statistic; test p-value = p-value corrected for multiple testing.

3.3.2 Effect of nonsynonymous changes

The GRIN1 protein is largely conserved across the ape clade, except for a 21 amino acid deletion in humans (Fig. 5), which corresponds to the complete exon 4 in bonobos and orangutans. A similar region is missing in chimpanzees, from positions 132 to 245. Gorillas also appear to have a large deletion from positions 420 to 564 (Fig. 5).

Given that the chimpanzee and gorilla reference genomes have been less studied than the human genome, these deletions may not reflect the biological reality of the protein, but rather missing data from the reference genome. There is only one nonsynonymous substitution in this dataset: it is a gorilla specific change at site I163V (Fig. 6).

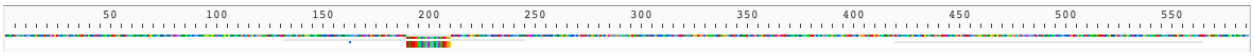


Figure 5: GRIN1 amino acid sequences. From top to bottom: human, bonobo, chimpanzee, gorilla, orangutan, and macaque. Human sequence is set as reference and only non conserved amino acids are shown in the other sequences.

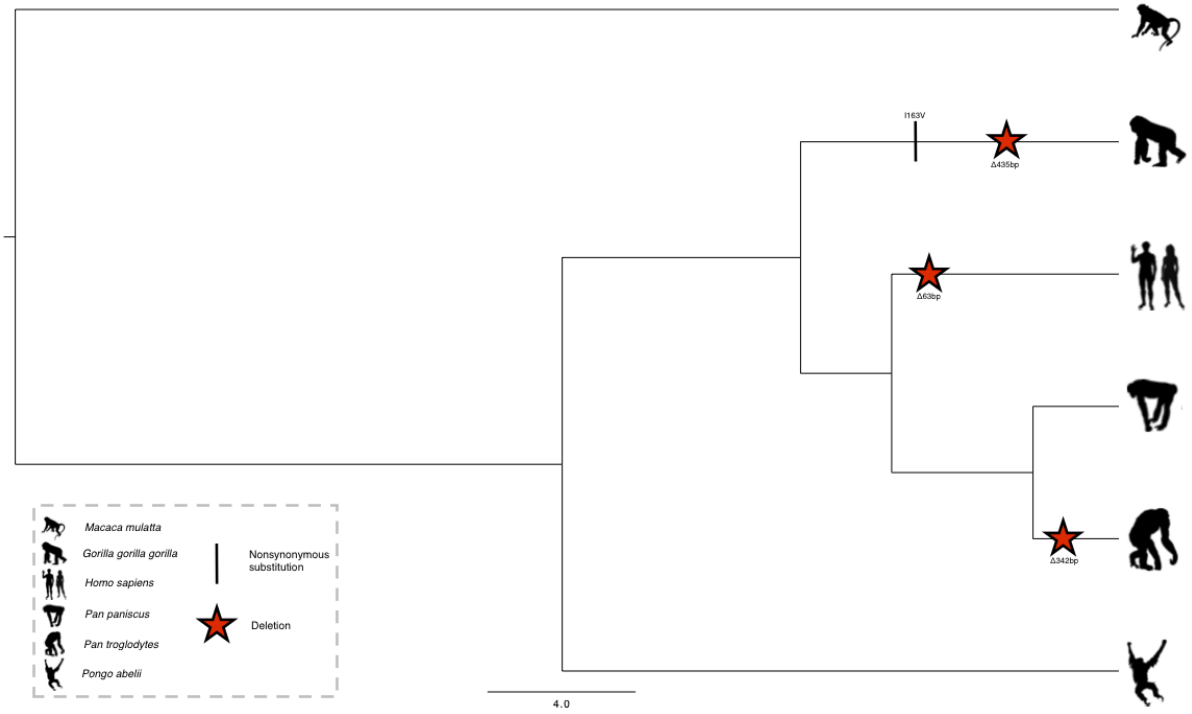


Figure 6: GRIN1 protein variation in apes. Macaque is the outgroup species. Nonsynonymous substitutions are represented by a black line. Their positions are indicated. Deletions are represented by red stars. The number of basepairs deleted is indicated.

SNAP2, with the orangutan sequence as reference, was used to determine whether these deletions would have a functional effect. While SNAP2 is not made to predict the effect of indels, I am making the assumption that regions in are red, i.e., where an amino acid change is confidently predicted to have a functional effect, or blue, i.e., where an

amino acid change is confidently predicted to not have a functional effect, can serve as a proxy for the impact of a deletion. GRIN1 is mostly red (Fig. 7a), meaning that much of the protein would be affected by amino acid changes. Of the 21 amino acids deleted in humans, a string of six (RNYENL) are predicted to impact the protein's function if changed (Fig. 7b).

a)

b)

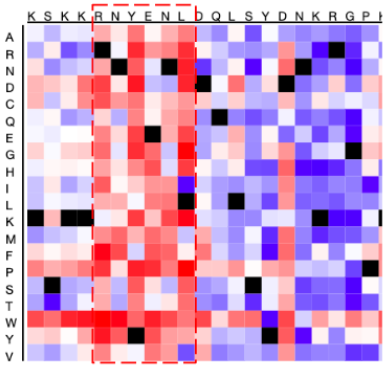
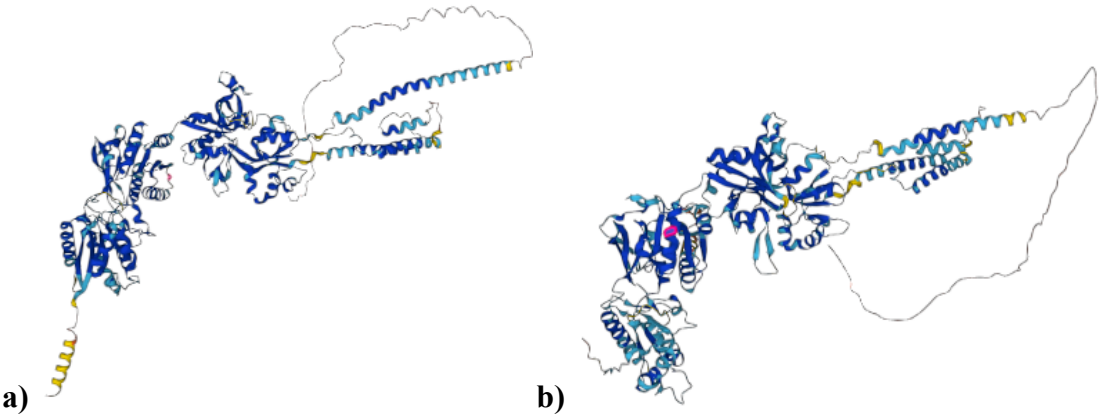


Figure 7: SNAP2 functional effects prediction score heatmap. a) Full SNAP2 heatmap; **b)** SNAP2 heatmap of positions 190 to 210, with the six likely to be functionally important highlighted by a red rectangle. The orangutan amino acid sequence of GRIN1 is on the x-axis and changes to other amino acids on the y-axis. Red represents a strong predicted effect; white represents an inconclusive prediction; blue represents a neutral predicted effect.

I used the UniProt resources (The UniProt Consortium, 2023) for both the human (UniProt Accession: Q05586) and orangutan (UniProt Accession: H2PU27) to identify the placement of the deletion. The proteins look overall similar, though amino acids 190 to 210 in orangutans make up a helix, which does not appear in the human protein. Positions 19 to 559 of the human protein are described as extracellular, by similarity to the mouse protein (UniProt Accession: P35439). Additionally, the subcellular location features of the human and orangutan proteins do not seem to align, with humans having

four transmembrane regions and orangutans only having three. The orangutan entry is more sparsely annotated.



a) **b)**

Figure 8: AlphaFold predicted 3D structure of GRIN1. a) Human protein (Q05586), with position 190 highlighted; **b)** Orangutan protein (H2PU27), with position 200 highlighted. The color of the protein corresponds to the AlphaFold model confidence, with dark blue as very high and orange as very low. (Jumper et al., 2021; Varadi et al., 2022)

Chapter 4: Discussion & Limitations

Discussion

This study analyzed the molecular evolution of two social behavior-related candidate genes in apes: *DRD4* and *GRIN1*. These are both receptor genes, but of different neurotransmitter systems and are associated with different aspects of social behavior. Previous studies have characterized the natural variations in DNA, amino acids, and expression of these genes in various species (Audet et al., 2018; Inoue-Murayama et al., 1998; Livak et al., 1995; Sousa et al., 2017); however, this study contextualizes these genes within social behavior and evaluates the evolution of these genes across the ape clade using modern bioinformatics methods.

1. DRD4

DRD4 is known to be polymorphic in exons 1 (Seaman et al., 2000) and 3 (Inoue-Murayama et al., 1998; Livak et al., 1995) in many primate species, including humans. The best known example of this is the seven-repeat (7R) allele of DRD4, which has been widely characterized as an allele associated with novelty-seeking behavior, and is found in approximately one-fifth of the human population, while the 4R allele is the more widespread (F = 0.651) (Ding et al., 2002). Ding et al. (2002) posit that this diversity indicates that *DRD4* is under positive selection in humans. While there were many nonsynonymous substitutions between ape species (Fig. 2), only seven of the 419 sites were significantly under positive selection and there was no significant signature of gene-wide diversification (Table 1). Within the context of the ape clade, there is no significant evidence of positive selection in humans (Table 5) (cf. Ding et al., 2002). In fact, all of the ape species except humans show evidence of branch-specific diversifying

selection. However, there is evidence of diversification in the African great apes, which then continued in the *Pan-Homo* last common ancestor (LCA), and the chimpanzee lineage (Table 5; Fig. 2). This could indicate that, since our divergence with the *Pan* genus, approximately 5 million years ago (Sarich & Wilson, 1967), the evolution of *DRD4* has been more constrained during human evolution than chimpanzee evolution. The future inclusion of the bonobo reference sequence could shed light on this trend.

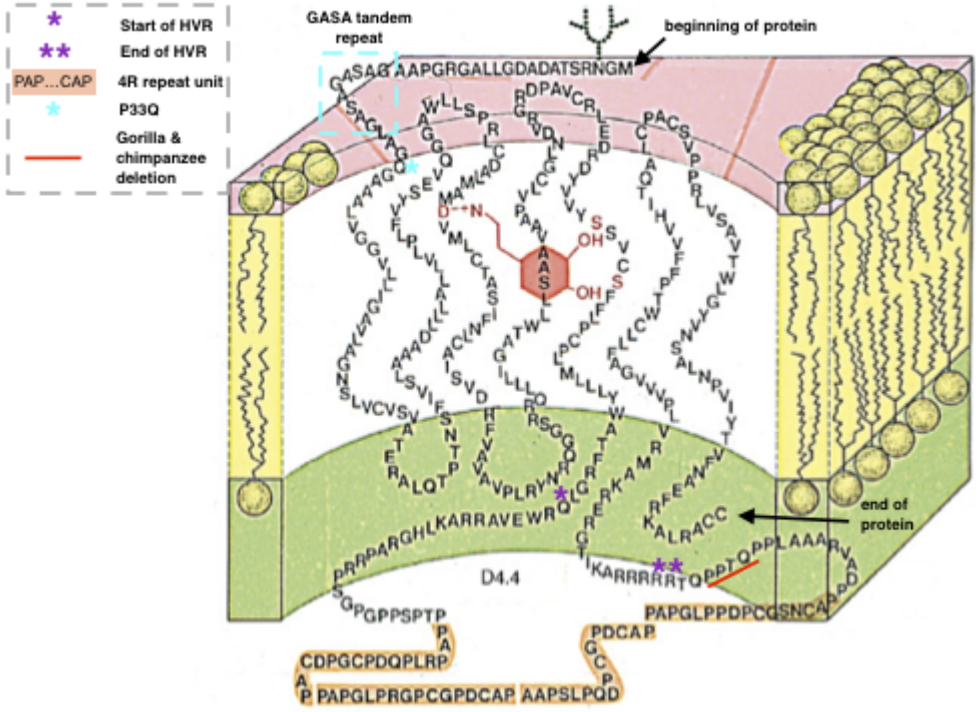


Figure 9: Schematic representation of the DRD4 protein. (modified from Seeman, 2009)

Two of the positive selection sites are found in exon 1, which includes a 12bp insertion in hominids (Fig. 2). This insertion exists as a tandem repeat unit (GASA) in humans, chimpanzees, and orangutans. This region is missing in the reference gorilla and gibbon sequences. Seaman et al. (2000) also note this variable repeat in the apes, except in chimpanzees and bonobos, which have respectively different monomorphic alleles. Bonobos are reported to have an identical exon 1 to humans with one repeat unit (Seaman

et al., 2000), and chimpanzees have two repeat units, though with two substitutions (G21V and S23A, compared to humans). At site 23, most primates have an alanine, whereas humans have a species-specific A→S substitution (Fig. 2), which SNAP2 predicts to have a neutral effect. Humans and orangutans both have polymorphisms of one, two, or three units, while gorillas only have one unit or two units with a substitution (S27A) (Seaman et al., 2000), the latter of which is present in the gorilla reference genome sequence (Fig. 2). The two gibbon species included in the Seaman et al. (2000) study only have one unit. Additionally, the P33Q substitution is specific to hominids (Fig. 2) (Seaman et al., 2000), occurring where the protein enters the membrane (Fig. 9) (Seaman et al., 2000). This may suggest a clade-specific functional change, which SNAP2 predicts to have a mild effect (score of 33, confidence of 66%) on the protein.

Five of the sites under positive selection are found in exon 3, specifically between sites 220 and 330, known in this study as the HVR of DRD4 (Fig. 1). The gorilla sequence has the most species-specific substitutions, followed by chimpanzee and orangutans with nine each, then gibbons, and lastly humans (Fig. 2). There likely are lineage-specific evolutionary pressures for this gene, which may be related to the social behaviors associated with *DRD4*, including sexual and prosocial behaviors (Ebstein et al., 2010). While apes show a range of these social behaviors (Boesch et al., 2010; Heras et al., 2016; Kopp & Liebal, 2016; Morrison et al., 2021; Sánchez-Amaro et al., 2021; Silk et al., 2013), gibbons and humans are the only apes to engage in semi-regular pair-bonding (Li et al., 2022; Schacht & Kramer, 2019) and they have the fewest species-specific substitutions. They are identical at sites 247 and 295, where at least two other ape species have nonsynonymous changes. This observation should not be taken as

a causal explanation of *DRD4* and monogamy, as other genes, such as the vasopressin receptors, have been shown to regulate pair-bonding in primates (Babb et al., 2010; Fam et al., 2019).

The *DRD4*-HVR is found in exon 3, coding for the third cytoplasmic loop of the protein (Fig. 9) (Livak et al., 1995; Wang et al., 2017) and it is unclear whether this variation results in an impact on the protein's function. SNAP2 predicts that changes from the human sequence to any of the other ape amino acids would either be neutral or have a weak effect (Fig. 3). *DRD4* is a G protein coupled receptor (Wang et al., 2017) and this group, while diverse, has a conserved structure of seven α-helices and four cytoplasmic loops (Yamashita et al., 2000). These loops serve important roles in the activation of the G protein (Yamashita et al., 2000) and the third loop particularly ensures coupling with the appropriate protein, despite nucleotide diversity of the receptor gene (Sadler et al., 2023). The third cytoplasmic loop seems to tolerate genetic variation of the receptor gene, corroborating the SNAP2 functional predictions. Nevertheless, in humans the 7R allele, found in exon 3, is associated with a different behavioral phenotype than the 4R allele (Ding et al., 2002), indicating that, while variation in this region does not impair the protein's function, it may alter how dopamine is processed in the brain.

This region of the gene also shows evidence of incomplete lineage sorting and/or convergent evolution. The most closely related species should have the most similar sequences; however, for example, at sites 287 and 295 chimpanzees share substitutions with orangutans (Q287R and A295T) and chimpanzees and gorillas both have a 12bp deletion, corresponding to a repeat unit (QTPP) (Fig. 2). The evolutionary relationships implied by these shared differences are incongruent with the species tree (Fig. 2), and

therefore, it must be considered that these may represent ancestral polymorphisms that became differentially fixed in the hominid species. Alternatively, the highly polymorphic nature of the HVR has been caused by species-specific mutations (Livak et al., 1995; Seaman et al., 2000), with homoplasy. The 7R and 4R allelic diversity in humans is due to recombination, linkage disequilibrium, and unequal crossovers (Ding et al., 2002), which emphasizes the complexity of mutation events in *DRD4.* This, however, can muddy the molecular evolution analysis of this gene.

DRD4 is the fourth receptor gene of the dopamine system and is highly polymorphic. This study has found that this gene is under positive selection in all ape lineages, except for humans. Despite numerous nonsynonymous substitutions and potential unequal crossovers (Ding et al., 2002), DRD4 remains functional in apes, as exemplified by the third cytoplasmic loop, which retains its G protein activation function even as its amino acid scaffolding differs radically. In humans, polymorphisms at *DRD4* are associated with differing social behaviors, such as novelty-seeking (Ding et al., 2002), suggesting that this gene may have impacted the behaviors of apes as they evolved and diverged from each other.

2. GRIN1

GRIN1 is an NMDA receptor gene for glutamate and has many splice variants. Both this study and previous studies (Goto et al., 2009; Sousa et al., 2017) show that this gene is highly conserved in primates. This gene is more than double the length of *DRD4* and has double the number of sites under negative selection, but only one third of the sites under positive selection (Table 6). *GRIN1* and *DRD4* are both under vastly different functional pressures. The isoformic nature of *GRIN1* complicates the analysis of its

molecular evolution, as apparent deletions may simply reflect splice variants. This is the case in this study. The Ensembl canonical human *GRIN1* transcript (ENST00000371561.8) has a 21 amino acid deletion compared to the other ape species. This region of the gene corresponds to the N1 cassette (Fig. 10) (Sengar et al., 2019), which is the fourth exon of this gene and is absent in certain isoforms. Other human transcripts (e.g., ENST00000371546.8) do contain this exon.

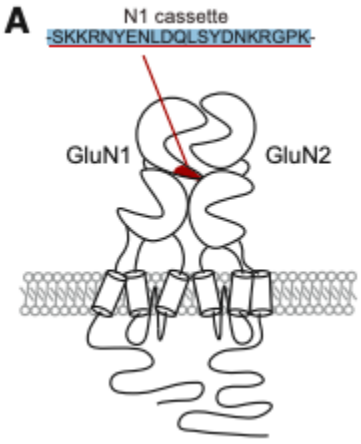


Figure 10: Schematic representation of the GRIN1 (GluN1)/GRIN2 (GluN2) dimer with the N1 cassette indicated. (Modified from Sengar et al. 2019)

While the N1 cassette is not necessary for the function of GRIN1, its absence confers "improved spatial memory acquisition and recall" (Sengar et al., 2019) in mice, without changing synaptic transmission. Approximately half of *GRIN1* transcripts lack the N1 cassette in the human brain, whereas this number is higher, at 62%, in rats (Sengar et al., 2019). It would be interesting to know the ratio of N1 cassette-lacking transcripts to N1 cassette-containing transcripts in the other ape brains, as it may shed light on the evolution of learning. In addition, the expression of *GRIN1* impacts the social behavior of rodents. The reduced expression of this gene was associated with altered social memory, social interaction, social communication, and sexual behavior (Zoicas & Kornhuber, 2019), though the N1 cassette was not implicated in these changes.

Ensembl lists several transcripts for the following great ape species: seven protein coding isoforms for humans, four for chimpanzees, five for bonobos, five for Western lowland gorillas, and five for Sumatran orangutans. The Ensembl canonical transcripts for chimpanzees and gorillas both contain large deletions (Fig. 6), which appear to be supported by the other transcripts of each species. However, Goto et al. (2009) sequenced a chimpanzee *GRIN1* (GenBank Accession: AB514214), which was identical to the human Ensembl canonical transcript. This disparity does bring into question the quality of the chimpanzee reference genome, raising a potential red flag on its use in comparative genomic analyses. The gorilla transcripts are similarly short – it is unclear whether this reflects a biological reality of the transcriptome or a poor quality reference. This does suggest that these branches may not actually be under positive selection, though the gorilla sequence was the only one to have a nonsynonymous substitution (I163V).

However, assuming that the large gaps in chimpanzees and gorillas are real, this could suggest that this gene is under some selective pressures against long GRIN1 proteins in chimpanzees and gorillas. Further studies would need to better characterize the *GRIN1*-associated transcriptome in these apes to assess this possibility. The canonical bonobo transcript also presented issues: sites 468 to 474 and sites 741 to 746 did not match the amino acid sequence of the other apes and were changed to Ns to control for poor sequencing. These do appear in all five bonobo transcripts, which could support their biological existence. That bonobos could have regions so different from the other apes' *GRIN1*, considering how conserved this gene is, does warrant further investigation. SNAP2 predicts these changes to have neutral or very mild effects on protein function. Due to these issues, the results of positive selection in apes should be interpreted

cautiously, as they may only reflect poor sequencing or splice variants, rather than molecular evolution. Overall, *GRIN1* needs to be better understood in nonhuman apes.

GRIN1 is a highly conserved gene across apes, underlining the likely functional importance of this gene in cognition, learning, and social behavior. Its splice variants, including transcripts lacking the N1 cassette, play various roles in the brain, and various mammals have different proportions of these transcripts. This has implications for the selective pressures on cognitive genes across mammals, as there may be an evolutionary trade-off between cognition and social behavior at play. The chimpanzee, bonobo, and gorilla reference transcripts proved to be problematic in this study, and the analysis of positive selection is likely skewed.

Limitations

Studying only two genes of different neurotransmitter systems, while allowing a look into the evolution and function of these genes and their proteins, offers a narrow view of the complexity of neurotransmitter systems and their associations with social behavior. Additionally, there were issues regarding the available reference genome sequences. For instance, *DRD4* was not available for bonobos, and the *GRIN1* transcripts of bonobos, chimpanzees, and gorillas included problematic regions, likely due to poor sequencing coverage, quality, or annotation.

Some lineages were also not well represented. For instance, gibbons, a diverse clade of 20 species, could only be represented by Northern white-cheeked gibbons. By using only the available reference sequences, intraspecific diversity was lost. Future studies would need to incorporate intraspecific data to better determine the patterns of protein evolution of these genes, as well as consider more genes and how they may

interact with each other. Additionally, this study only focused on apes, whereas adding more primate species to the analysis could provide additional evolutionary context, especially in regards to derived genetic changes.

This study has shown that neurotransmitter systems are under different selective pressures, likely owing to both their functions within the nervous system and the social behaviors they influence. Future research could further investigate these two genes and the 30 proposed to shed more light on the evolution of prosocial behavior among primates.

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