N-METHYL-D-ASPARTIC ACID RECEPTOR SUBUNIT NR2A REPEAT POLYMORPHISM AND SPORT CONCUSSION

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By

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Abstract

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During a concussion, mechanical forces cause neuron cell strain that initiates dysfunction through the indiscriminate movement of ions through protein channels. Extracellular glutamate binds with cell membrane proteins (e.g. NR2A), which exacerbates the Ca^{2+} ion influx and prolongs neuron dysfunction. Genetic variation may be a factor in regulating glutamate binding and therefore cell recovery time. The NR2A subunit of NMDA contains a variable (GT)n nucleotide tandem repeat (VNTR) within *GRIN2A* promoter region. This VNTR has been shown to regulate transcription levels in a length dependent manner, where longer repeat decreases transcription of the NR2A subunit. The purpose of this study was to determine the association of the *GRIN2A* VNTR and recovery (days) as well as concussion severity scores within concussed athletes.

The independent variable was VNTR (long allele vs. short allele). The primary dependent variable, recovery time, was defined as injury date to return to play (RTP) clearance date as determined by the physician. Participant RTP time was categorized as normal ($\leq$ 20 days) and prolonged (> 20 days). Secondary dependent variables were assessed at the initial evaluation and included vestibular ocular score, Balance Error Scoring System (BESS) score, and Immediate Post Concussion and Cognitive Testing (ImPACT) module scores. All 51 participants were athletes, comprised of 38 males and 13 females with a mean age of 18.69 ± 6.65. Participants were evaluated at a university concussion center. The standardized concussion evaluation consisted of cranial nerve, vestibular ocular tests, balance (Balance Error Scoring System), signs and symptoms (s/s), and neurocognitive (ImPACT) testing. Each participant was genotyped via saliva sample for the *GRIN2A* (GT)n repeat polymorphism (rs3219790).

Data analysis consisted of descriptive and inferential statistics. Chi-squares were used to assess the association between VNTR (long allele versus short allele) and concussion recovery (prolonged versus normal). Regression analyses were used to estimate extent to which non-genetic factors and genotype contributed to concussion recovery group assignment. One-way ANOVAs were used to identify any significant differences in vestibular ocular, BESS, and ImPACT module scores between long and short alleles. Primary potential non-genetic contributing factors were age, race, sex, concussion history, dizziness at time of injury, history of migraines, and history of ADHD. The alpha level was set at $p \leq .05$.

There were no differences between demographic or health history within the VNTR or recovery groups. There was a significant association ($x^2 = 4.01$, $p = 0.045$) between the VNTR group (dominant model: LL versus SS + LS) and recovery, where the chance of prolonged recovery was 4.3 times greater for carriers of the homozygous long allele. There were no differences in concussion severity scores between VNTR group and concussion severity scores. This was the first study to investigate the association of the (GT)n VNTR within *GRIN2A*. We established a DNA collection, estimation, and genotyping protocol of the (GT)n VNTR for 96 samples and demonstrated accuracy of this genotyping method. Clinically, athletes carrying the long allele genotype may be predisposed to prolonged recovery following a concussive injury.

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CHAPTER 1

N-METHYL-D ASPARTIC ACID RECEPTOR SUBUNIT NR2A REPEAT POLYMORPHISM AND SPORT CONCUSSION

Introduction

Concussion is defined as a traumatically induced transient disturbance of the brain brought on by complex pathophysiological processes (Harmon et al., 2013). Each year an estimated 1.6 to 3.8 million sports-related concussions occur (Langlios, Rutland-Brown & Wald, 2006), which results in billions of dollars in healthcare costs (Sosin, Sniezek, & Thurman, 1996). This is likely an underestimate since many more concussions are missed (Mansell et al., 2010). In a recent epidemiologic study, researchers found that concussions accounted for nearly 15% (1,056) of all sport-related injuries (Meehan, d'Hemecourt, Collins, & Comstock, 2011).

Researchers suggest that signs and symptoms (s/s) evaluation alone is not sensitive enough for return to play decisions and propose several different tests (e.g., neurocognitive assessment, vestibular ocular exam, balance exam) need to be used to ensure appropriate return to play decisions (Lau, Collins, Mucha, & Lovell, 2011; McCrory et al., 2013). Previous research has found that athletes report concussion s/s without having a diagnosed concussion (Mansell et al., 2010). Using a multi-tool approach to evaluate concussions has been reported to increase assessment sensitivity up to 90% (Broglio, Macciocchi, & Ferrar, 2007). Sensitive tests are necessary for not only the diagnosis of the concussion injury, but to follow the injury and determine the return to play time for an athlete. Eighty to 90% of athletes are returned to play within seven to 10 days, but up to 20% of athletes do not return to play for 21 or more days after injury (Iverson, Brooks, Collins, & Lovell, 2003; Lau et al., 2011; McCory et al.).

Prolonged concussion recovery is largely not specifically defined as it has been described as an injury persisting over ten, 14, or 21 days (Harmon et al., 2013; Iverson et al., 2003; Lau et al., 2011). Several risk factors that could contribute to a greater risk of prolonged recovery are age and sex (McCrory et al., 2013), reporting dizziness at the time of injury (Bruce & Echemdia, 2004; Lau et al.), history of previous concussions (Colvin et al., 2009; Schultz et al., 2004) and playing specific sports (e.g., football; Pellman et al., 2004). Recovery is also likely to be influenced by genetics because genes determine the structure and function of proteins involved in the cell's resistance and response to mechanical stress.

During a head impact, an external force causes head acceleration (Ommaya & Gennarelli, 1974). Head acceleration causes stress to the neuron resulting in cell strain or deformation (Gaetz, 2004; Grady et al., 1993). If these strains are large enough (greater than 10% to 20% beyond resting length), then the cell's structure and chemical environment can be altered (Costa, Hucker, & Yin, 2002; Gaetz). Athletes sustaining a concussion could have neuron dysfunction and exhibit both cognitive (e.g., difficulty thinking, difficulty concentration, feeling slowed down) and physical s/s (e.g., loss of consciousness, headache, dizziness, poor balance; McCrory et al., 2005), which is a result of neuron dysfunction (Giza & Hovda, 2001).

Neuron cell strain initiates dysfunction through the indiscriminate movement of ions (e.g., Ca^{2+}, K^{+}, Na^{+}) through protein channels and the release of the excitatory amino acid glutamate, which can lead to excitotoxicity (excess Ca^{2+} in the cell due to glutamate; Choi, 1987).

Extracellular glutamate binds to several receptors (e.g., NR2A) within the n-methyl-d aspartic acid (NMDA) channel, which exacerbates the Ca^{2+} ion influx and prolonged neuron dysfunction (Choi; Nicholls, Martin, Wallace, & Fuchs, 2001). Cellular uptake mechanisms following a head impact are unable to purge the excess glutamate, which enhances the Ca^{2+} ionic flux. This excitotoxicity promotes cytocellular edema (Katamaya et al., 1990; Strong et al., 2002), depletes energy stores (Anderson & Marmarou, 1992), and degrades structural proteins and the cell membrane (Choi, 1988) resulting in further neuron dysfunction.

There are two main glutamate receptors within the NMDA channel (Figure 1). Specifically, these glutamate receptors are within subunits NR2A and NR2B (Figure 2). When two glutamate molecules and two glycine molecules attach to their respective subunits it causes the NMDA channel to open and permit Ca^{2+} ions into the cell. For normal cell function intracellular Ca^{2+} levels must be maintained between 50 to 300 nM (Choi, 1987), in order to maintain resting membrane potential. To control resting membrane potion sustaining low intracellular levels of Ca^{2+} are critical. Following a concussive event, Ca^{2+} levels may increase 12% to 64% beyond normal levels (Osteen, Moore, Prins, & Hovda, 2004), and can remain elevated for up to 14 days (Giza & Hovda, 2001). This causes the membrane potential to be affected as well as make the neuron more vulnerable for more severe injury after a second impact (Giza & Hovda, 2001).

There are four main ways Ca^{2+} is regulated in cells: entry through the NMDA channel which is the predominant route; the Na^{+}/Ca^{2+} exchange; voltage gated Ca^{2+} channels; and release of Ca^{2+} from intracellular stores (Choi, 1987; Nicholls, Martin, Wallace, & Fuchs, 2001). When intracellular Ca^{2+} levels increase it leads to secondary effects that cause neuron cell dysfunction. Variation in the NMDA structure or production could influence the magnitude of neuron cell dysfunction following a traumatic mechanical event. In an athletic population, this NMDA variation could be a contributing factor to variability of concussion severity. If the brain is injured from a concussion, and a glutamate subunit is insufficiently produced by one of the NMDA genes (e.g., glutamate receptor, inotropic, N-methyl D-aspartate 2A encoded by; *GRIN2A*; Figure 3) the level of intracellular Ca^{2+} could be decreased. This would thus, limit the amount of secondary actions Ca^{2+} would cause. One proposed mechanism for reduced levels of NR2A subunit is a polymorphism within the promoter region of the gene that codes for the production of NR2A.

Variable nucleotide tandem repeats (VNTR) are genetic variations that occur when short nucleotide sequences (e.g., GT or CAG) are repeated within DNA a variable number of times (Roth, 2007). Within the NR2A gene (*GRIN2A*) there is a short (GT)n segment can repeat from 19 to 36 times in this upstream region. The (GT)n tract is located in the promoter region of *GRIN2A*, with 3'-end of the GT tract at position (-679) upstream of exon 1 (accession # AF443855; rs3219790; Figure 4). The genetic polymorphism of the (GT)n tract is associated with the expression level of *GRIN2A* (Inoue et al. 2010; Itokawa et al., 2003; Iwayama-Shigeno et al., 2005). The length of (GT)n repeat modulates *GRIN2A* expression level, with longer alleles (≥ 25 repeats) typically associated with poorer outcomes (e.g., Parkinson's disease, schizophrenia; Inoue et al.; Itokawa et al.; Iwayama-Shigeno et al.). Hypofunction of the NMDA receptors have produced behavior that is similar to cognitive problems observed in schizophrenia (Gilmour et al., 2012). Consequently, (GT)n repeat is hypothesized to be associated with hypofunction of the NMDA receptor due to the decrease in NR2A transcription (Itokawa et al.). This could lead to altered function of the NMDA channels and limit the amount of Ca^{2+} entering the cell. Less Ca^{2+} entering may lead to diminished amount of excitotoxicity and shorter recovery time following a concussion. In sports, this could be manifested in an athlete experiencing concussions and concussion s/s for longer or shorter periods of time. There has been no research performed on the association between NMDA subunit NR2A repeat polymorphism and concussion recovery.

Statement of the Purpose

The primary purpose of this study was to determine if there is an association between long and short (GT)n alleles and concussion recovery (days). The second purpose was to evaluate the association between long and short (GT)n alleles and evaluation concussion severity scores.

Aims and Hypotheses

Aim 1

To assess if the *GRIN2A* (GT)n long or short allele groups are associated with concussion recovery time.

Research Hypothesis for Aim 1

There will be no association between carrying the long or short (GT)n genotype and concussion recovery.

Specifically, the frequency of (GT)n VNTR will be similar in long versus normal recovery groups.

Aim 2

To assess if *GRIN2A* (GT)n long or short allele groups are associated with the initial concussion severity scores.

Research Hypothesis for Aim 2

There will be no differences between those carrying the long or short (GT)n genotype and concussion severity scores (i.e., positive vestibular ocular tests, BESS scores, and ImPACT scores). Specifically, concussion severity scores will be similar in the carriers of the short and long (GT)n VNTR.

Methodology

Research Design

A between subject design was utilized. The independent variable was the (GT)n VNTR. Participants were genotyped and grouped into having a long allele, short allele, or both a long and short allele. The long (GT)n allelic repeats were defined as ≥ 25 dinucleotide repeats. The short (GT)n allelic repeats were defined as < 25. This cut point was used in similar *GRIN2A* promoter (GT)n VNTR studies (Inoue et al. 2010; Itokawa et al., 2003; Iwayama-Shigeno et al., 2005).

The long and short alleles groups were analyzed using dominant, recessive, and codominant models. The dominant model consisted of two levels: 1. LL (homozygous long alleles) 2. SS + LS (homozygous short alleles along with heterozygous long and short alleles). The recessive model was also comprised of two levels: 1. LL + LS (homozygous long alleles along with heterozygous long and short alleles) 2. SS (homozygous short alleles). Lastly, the codominant model consisted of three levels: 1. LL (homozygous long alleles) 2. SS (short alleles) 3. LS (heterozygous long and short alleles).

The primary dependent variable was recovery time defined as injury date to return to play (RTP) clearance date as determined by the physician. Athletes suspected of concussion were referred to the center by a coach, parent, other physician, or certified athletic trainer. All patients enrolled in the study had concussion verified by the center physician during initial evaluation. Concussion was defined as the patient having a pathomechanical event followed by s/s (e.g., headache, dizziness) within the next 48 hours.

Participant RTP time was categorized as normal (≤ 20 days) and long (> 20 days). This criterion was based on the study performed by Lau et al. (2011) that defined prolonged recovery as greater than 21 days. The participants within the normal recovery group were tested at a mean 5 ± 2.69 days after the concussion injury. The participants in the prolonged recovery group were tested at a mean 62 ± 113.51 days after concussion injury. Secondary dependent variables were assessed at the initial evaluation and included vestibular ocular score, Balance Error Scoring System (BESS) score, and Immediate Post Concussion and Cognitive Testing (ImPACT) module scores. Initial evaluation scores were on average seven days when excluding the eight patients that were seen over a month after injury.

Participants

Fifty-one concussed patients were recruited from the Temple University Sport Concussion and Athletic Neurotrauma Center (Center). Thirty-eight of the participants were male with a mean age of 18 ± 4 years and 13 were female with a mean age 20 ± 12 years. These patients were referred to the Center because of a suspected concussion. All participants completed an Institutional Review Board approved informed consent or assent (Appendix A), Health Insurance Portability and Accountability Act (HIPAA; Appendix A), genotyping consent (Appendix B) and Genetic Information

Nondiscrimination Act (GINA) compliance forms (Appendix B), prior to data collection. Exclusionary criteria included any participant who presented with a non-sports-related concussion (e.g., fall, car accident).

Total allele counts within the 2 (group) x 2 (allele) chi-square analysis for concussion groups and control groups are presented in Table 1. The control group was unlabeled DNA from a general population. This was done to ensure similar frequencies of long and short alleles within the general population and the concussed population. There was no statistical significance between the frequency of the long or short allele between the control and concussed groups.

Instrumentation

Concussion Assessment Battery

Patients completed a standardized initial evaluation where the following was assessed: concussion injury characteristics including the date and time of injury, mechanism of injury, acute s/s (e.g., loss of consciousness, dizziness); patient history including concussion, migraine, learning disability (e.g., ADHD), and psychiatric history (e.g., depression; Appendix C).

Table 1. Association Between Concussed and General Population Allele Frequencies

Variable	Total Concussed Alleles	Total Control Alleles	X^2	*p*
LA	55	54	0.95	0.330
SA	47	34		

Note. LA (long allele), SA (short allele), X^2 (Chi-square), *p* (alpha level), *df* = 1.

An objective screening followed, which included the vestibular ocular assessments. Balance was assessed with the BESS test. Finally, the concussion assessment concluded with a computerized neurocognitive exam (ImPACT).

Vestibular Ocular Exam. The vestibular ocular exam began with the H-test, where the tester looked for smooth eye pursuits that were held at extreme points (i.e., extreme point high/low and left/right). An abnormal test resulted in saccades, nystagmus of the eye (e.g., jumping around), or abnormal ocular function (e.g., asymmetry of the pupil). The next exam was the vestibular ocular reflex, which consisted of two tests that lasted 15 seconds each. First, the participants performed a vertical pursuit test, where the participants' eyes focused on high and low objects, while moving the head up and down. Next, the horizontal pursuit test, where the participants' eyes focused on left and right objects, while the head rotated left and right. A positive or abnormal test resulted when the patient felt dizzy, nauseated, dazed, or vomited. The final assessment was the convergence test, where an object with writing on it was moved toward the participants' face at eye level from 40 cm. An abnormal test occurred when blurred or double vision ensued greater than 10 cm away, where normal is within three to 10 cm from the face. An abnormal response was recorded with a score value of one and a normal response was scored with a value of zero, allowing for a total score to be calculated. The total possible abnormal tests would result in a score of three.

BESS. The BESS test provided an objective measure of postural stability on a firm surface and a foam surface. The test involved a series of three stances: double-leg standing, single-leg standing, and tandem standing. Each test was performed for 20 seconds with the patients' eyes closed and hands on hips. Points were given when postural errors were made. Errors included lifting the hands off of the iliac crest; opening the eyes during the test; stepping, stumbling, or falling; moving the hip into more than 30° of flexion or abduction; lifting the forefoot or heel; or remaining out of the testing position for more than 20 seconds. Each position was tested on a firm surface and then on an Airex foam pad (Alcan Airex, Sins, Switzerland). The total numbers of errors made were summed to determine the subject's score, with a higher score demonstrating a lower level of postural control. The total number of errors for any single condition is 10, or 60 total possible errors. An escalation from baseline in the number of errors greater than three on any surface indicated a possible concussion. With no baseline scores, falling outside 3.37 ± 3.10 on a firm surface and 8.65 ± 5.13 on a foam surface indicated a potential concussion (Riemann, Guskiewicz & Shields, 1999). BESS test reliability has been recorded as r = 0.97, specificity as 91%, and sensitivity as 35% (Guskiewicz et al., 1997; Harmon et al., 2013).

ImPACT. ImPACT is a computerized concussion evaluation system (Iverson, Lovell & Collins, 2005). It includes three sections (i.e., demographic and health history, current s/s, and six neurocognitive test modules). The modules evaluated attention span, working memory, sustained and selected attention time, response variability, non-verbal problem solving, and reaction time. It took approximately 30 minutes to complete this exam.

In the first category, patients used the keyboard and an external mouse to input demographic and descriptive information through a series of easy to follow instructional screens. The questionnaire included demographics; years experience playing sport, history of alcohol and drug use, learning disabilities, attention deficit hyperactive disorder, major neurological disorder, and previous concussion history. The second category consisted of 22-concussion s/s that allowed patients to rate their current s/s state using a 7-point Likert scale. Patients would self-rate their concussion symptoms by clicking on a number between zero and six using an external mouse.

The third category consisted of six neurocognitive modules. The first module evaluated attention processes and verbal recognition memory utilizing a word discrimination paradigm. The second module measured attention processes and visual recognition memory that utilized a design discrimination paradigm. The third module evaluated visual working memory and visual processing speed through a distractor (choice reaction time) and memory (visual memory) task. The fourth module measured visual processing speed, learning, and memory. The fifth module measured choice reaction time. Patients were presented twice with a list of 12 words for 750 ms each time. Then, the athlete had to differentiate if the next 24 words were in that 12 word list by clicking yes or no. The sixth module measured visual motor response speed and working memory. Patient module scores during the initial evaluation were used as criterion data and compared to percentile scores from normative tables to determine presence of concussion. The same tester conducted all concussion assessments. ImPACT's reliability has been recorded as 0.88-0.94, sensitivity = 65%, and specificity = 80% (Harmon et al., 2013). Following the concussion assessment instructions for RTP were given or follow-up appointments for re-evaluation were given to the athlete from the physician (Appendix D).

DNA Collection

During the visit to the Center participants had their DNA collected using Oragene®•DNA Self Collection Kits (DNA Genotek, Ottawa, Canada; Appendix E) for genotyping. For a valid sample collection participants must refrain from eating, drinking, and smoking 30 minutes prior to saliva sample collection. Therefore, samples were collected at the end of their concussion evaluation to ensure collection validity. The saliva was transported to Jayne Haines Center for Pharmacogenomics Drug and Safety Center of Temple University School of Pharmacy (Philadelphia, PA) where it was stored at room temperature.

DNA Extraction

DNA was extracted from saliva collection containers according to manufacturer's standard methodology (Appendix E). In brief, saliva sample tubes were incubated in an air incubator at 50 °C for one hour. Next, 400 µL was transferred into a 1.5 microcentrifuge tube (Eppendorf, Barkhausenweg, Germany) labeled with the corresponding participant identification number. Sixteen µL of Oragene Purifier was added to the sample and mixed by inversion for three minutes by hand. Following the inversion, the tubes were incubated on ice for 10 minutes. Then, the samples were centrifuged for three minutes at 13,000 g. Two hundred µL of the clear supernatant was transferred into a new 1.5 microcentrifuge tube labeled with the corresponding participant identification number and the remaining sample was discarded according to OSHA regulations. The samples were withdrawn for DNA purification using the DNA Tissue Kit protocol implemented in EZ1 Biorobot software (QIAGEN, Valencia, CA). Samples, elution tubes, and cartridges were loaded into the BioRobot EZ1 workstation and the extraction process lasted approximately 20 minutes. The BioRobot EZ1 uses magnet bead technology for extraction of DNA from different sources including saliva (our tissue source). The last step of the protocol DNA was eluted to a final volume of 200 µL. The DNA solution was stored at 4 °C. All samples were coded according to the IRB approved protocol.

DNA Estimation

Purified DNA was quantified fluorometrically using a Quant-it PicoGreen dsDNA assay kit (Invitrogen, Grand Island, NY), according to manufacturer's instructions with modifications. Briefly, the sample DNA diluted to a final volume of 100 µL with 1 x TE buffer mixed with 100 µl of 1 x PicoGreen dsDNA reagent buffer in a 96- black well plate (Appendix E). Fluorescence data were collected by a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) fluorescence plate. DNA concentration in samples were calibrated from a calibration curve prepared from DNA standards provided with the Quant-it PicoGreen dsDNA assay kit (Invitrogen, Grand Island, Ny). Samples and standards were measured with the excitation and emission wavelengths set at 480 nm and 520 nm, respectively.

Optimization of Conditions for Polymerase Chain Reaction (PCR)

The region surrounding position (-975 to -776) in the promoter region of *GRIN2A* gene was amplified by PCR reaction. The forward (FWD), 5'-FAM-labeled forward (FAM-FWD), and reverse (REV) primers were synthesized by Eurofin Operon (Operon, Huntsville, AL) and used without further purification.

The sequences of primers shown in Table 2 were used as described in a previous publication (Itokawa, 2003). For optimization of PCR, the primers FWD (without FAM label) and REV were used. Optimization of PCR conditions was performed using the PCR Optimizer kit (Invitrogen, Grand Island, NY), per manufacturer's instructions. Quantity and purity of the reaction product was estimated by gel electrophoresis in 1.4% agarose. After electrophoresis at 100 V for one hour, the gels were inspected under UV light, and the images were recorded (Appendix E).

PCR Analysis of Genomic DNA

Analysis of genomic DNA was performed under the following conditions: The optimized reaction mix for amplification of the *GRIN2A* promoter fragment contained

Table 2. Sequence and Properties of the Primers used in PCR Reaction for Amplification of *GRIN2A* Promoter Region

Primer	Sequence	Length bp	Tm °C
FWD	5’-GAAGGAAGCATGTGGGAAATGCAG-3’	24	64.6
5’-FAM-FWD	5’-FAM-GAAGGAAGCATGTGGGAAATGCAG-3’	24	64.6
REV	5’-gtttcttGCTGGGTACAGTTATCCCCCT-3’	28	67.5

Note. Bp (base pairs), Tm (temperature), FWD (Forward), FAM (carboxyfluorescein), REV (reverse).

Buffer D (3.5 mM MgCl2 pH 8.5), 20 to 40 ng template (genomic DNA), 0.1 µM each primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The cycling conditions were 94 °C for two minutes, 35 cycles (94 °C for one minute, 55 °C for two minutes, 72 °C for one minute), followed by incubation at 72 °C for seven minutes. For all genotyping reaction, FAM-FWD/REV pair of primers was used in the PCR reaction (Appendix E).

Purification of the Reaction Mix and Genotyping.

The PCR products (5 µl) mixed with 2 µl of ExoSAP-IT reagent (Affymetrix, Santa Clara, CA) and incubated at 37 °C for 15 minutes followed by thermoinactivation at 80 °C for

15 minutes (Appendix E). The reaction mix was directly analyzed by capillary electrophoresis analysis (Genewiz, South Plainfield, NJ).

Cloning and Sequence Analysis of the Synthesized Promoter Fragment

PCR products generated from genomic DNA were cloned into the bacterial plasmid using TOPO technology (Invitrogen, Grand Island, NY). Briefly, the freshly prepared PCR products were incubated with pCR4-TOPO vector, and the product of recombination was used for transforming TOP10 *E. coli* strain. The colonies with recombinant plasmids were grown on LB agar at 37 ^{o}C overnight in the presence of 100 *u*g/ml ampicillin. Single colonies were collected from agar, and further grew in 2 ml liquid LB medium with 100 *u*g/ml ampicillin at 37 ^{o}C overnight. Recombinant plasmid DNA was isolated from 2 ml liquid medium using the QIAGEN miniprep kits (QIAGEN, Valencia, CA) per manufacturer's instructions, and sequenced with universal primers M13 forward (-20) and M13 reverse (Figure 5).

Pedigree Analysis of (GT)n Polymorphism

To validate the analytical method for (GT)n repeat genotyping, a pedigree analysis of (GT)n alleles in a three-generation family was performed. To this end, saliva was collected from six members of the family, and genotyping analysis was performed using the established protocols described above.

Analysis of Repeat Length Polymorphism

To identify (GT)n length polymorphism alleles, the fragment of *GRIN2A* promoter encompassing (GT)n tract was amplified with FAM-containing primers using genomic DNA from participants of the project and analyzed by capillary electrophoresis at single nucleotide resolution by a commercial provider. The length of (GT)n tract was determined by comparing with calibrators prepared from standard DNA, and validated by sequencing. The size of the fragments was estimated by Peak Scanner software v1.0 (Applied Biosystems, Foster City, CA) using internal standards. The number of GT dinucleotide repeats was calculated using the following equation: $n(GT)=(L - 167)/2$, where L is the length of the PCR fragment estimated in base pairs.

Validation of PCR protocol

Genetic analysis of short fragments within the genomic DNA is greatly facilitated by amplification of the interrogated DNA region using PCR reaction. Dinucleotide polymorphisms, including the (GT)n, are genotyped by PCR using the unique primers that flank the repeat. Then, genotypes can be determined from the length of the fragments by capillary electrophoresis. Unfortunately, analysis of dinucleotide repeats poses an experimental problem because of complex amplification pattern in the course of DNA polymerase reaction (Innocenti & Imle, 2005). The addition of terminal dA nucleotides at the ends of double-stranded PCR products catalyzed by Taq polymerase is also a well-studied phenomenon, which makes interpretation of results more cumbersome (Innocenti & Imle).

The origin of additional peaks is due to several reasons (e.g., incomplete denaturation of PCR product, slipped strand mispairing, or due to annealing of truncated products; Hauge & Litt, 1993). Experiments with direct sequencing of the PCR products demonstrated that different genotype length depends solely on the variations of the dinucleotide length (Hauge & Litt). This experiment rules out PCR recombination (Hauge & Litt). Therefore, the mechanism for additional peak formation is slipped strand mispairing (Hauge & Litt).

The primers for the study’s PCR reaction were synthesized according to the published sequence (Itokawa et al., 2003), and contained an additional seven-nucleotide fragment (*gtttctt*), which reduced the heterogeneity of the amplification products (see Table 1; Innocenti & Imle, 2005). This allowed the identification of the major and minor peaks clearly to identify the alleles. In addition, the forward primer was synthesized in two variants, with and without the chromophore FAM group linked to the 5'-terminus. The non-modified FWD primer was used for optimization of PCR conditions, and 5'-FAM-modified FWD primer for genotyping analysis of human genomic DNA.

To establish a robust method of (GT)n genotyping, we performed optimization of PCR reaction. The PCR Optimizer kit used in our study allowed rapid selection of PCR conditions in a series of parallel experiments. In this protocol, the thermal cycling conditions are not variable, and we used the standard conditions for all our reactions as indicated in the above section. All optimization reactions were performed using genomic DNA isolated from SW48 (297) human carcinoma cells, which were prepared for this work and kindly provided by Manali Phadke (Pharmacogenomics Lab, TU School of Pharmacy).

Specifically, a panel of buffers with varying concentration of $MgCl_2$ and pH, different concentrations of template (DNA), and primers were tested in three rounds of optimization. During the first round, a set of reaction buffers with $MgCl_2$ concentrations 1.5 to 3.5 mM and pH 8.5 to 10 were tested for maximum yield of the amplification product. The results of this experiment are shown in Figure 6. The buffers A and D provided the highest yield of the band with mobility corresponding to the expected size (223 bp) of the amplification product. This was a valid experiment since the bands were seen at the expected 223 bp length, and there was no target found in the negative control lane.

In the second round of optimization, reaction was performed in the presence of varying amount of template (DNA) in the range 22 to 700 ng per reaction. Figure 7 demonstrates that in the presence of buffers A and D (3.5 mM $MgCl_2$, pH 8.5) 22 to 44 ng of human genomic DNA produced amplification product in the quantity sufficient for further analysis. Because buffer D provided the strongest and high selectivity of amplification, buffer D was routinely used for genetic analyses (1.5 mM $MgCl_2$, pH 8.5). The DNA titration experiment also showed the same validity results (target band at correct length and no containments in the negative control lane).

Finally, the varying concentrations of primers in the range of five to 300 ng per reaction were used, to reduce the level of non-specific amplification products. The results of this experiment are shown in Figure 8 and demonstrated the same validity as the previous PCR optimization experiments. We selected the primer concentration 19 ng per reaction for all subsequent reactions, as under these conditions the accumulation of non-specific products was minimal.

Validation of PCR product

To confirm the identity of the amplified fragment, we performed cloning and sequence verification of the generated PCR product. To this end, PCR products were prepared using genomic DNA from SW48(297) cell line, and from one sample of human genomic DNA. The amplification products were used to prepare recombinant plasmids by inserting into pCR-TOPO cloning vector. This procedure was performed by Dr. Krynetskiy (Pharmacogenomics Lab, Temple University School of Pharmacy).

Next, the recombinant constructs were used for transformation of bacterial strain TOP10, and subsequent isolation of individual clones. About ten clones were screened for the presence of inserts, and plasmid preparations were then analyzed by sequencing. Sequence analysis of the clones prepared from SW48(297) and a volunteer verified that PCR reaction under optimized conditions indeed produced the amplification product encompassing the promoter region of *GRIN2A* gene.

This sequence analysis provided an exact number of (GT)n repeats in the promoter region of SW48(297) demonstrated in Figure 9 and human genomic DNA shown in Figure 10. Therefore, using two genomic DNA, we generated molecular markers for exact evaluation of the number of GT repeats in our samples. The calculated number of GT repeats correlated with the sequence analysis. This enabled a valid calculation of the GT repeats in other samples. Briefly, outside the GT repeat motif there were 167 bp of flanking sequences. The electropherogram generated by Peak Scanner software estimated the total number of the bp in the target sequence. Therefore, the GT repeat was calculated by the (entire target sequence -167 bp)/2[(GT)n dinucleotide repeat]. Therefore, for all future experiments we used SW48(297) genomic DNA as a positive control with known number of GT repeats.

Capillary Electrophoresis Analysis of the PCR Products

FAM-labeled PCR products were analyzed by capillary electrophoresis in an outsource facility (Genewiz, South Plainfield, NJ). The representative electropherogram are shown in Figure 11 and Figure 12. Fragment length polymorphism analysis (FLP) was performed by measuring the migration time of a PCR product, and extrapolation to the known fragments in the DNA standard ladder. As expected, the PCR products were not homogenous because of a well-known phenomenon of slippage of taq polymerase on a monotonous template (Inonocenti & Imele, 2005). The presence of several peaks on the electropherogram did not impede the analysis of homozygous genotypes because the strongest signal clearly identified the major product, and was used for calculating the number of GT repeats. But, for the heterozygotes the presence of several peaks could mask additional alleles with a different number of GT repeats.

To solve this ambiguity, a pedigree analysis was performed from six members of a three-generation family with homo-and heterozygous genotypes at the *GRIN2A* promoter region. Six samples of saliva were collected from six volunteers (four females and two males), and genotyped according the established protocol. The results of genotyping experiments, as well as a pedigree chart are shown on Figure 13. The pedigree chart analysis confirmed that the protocol established in this work accurately detected the presence of allelic variants both in homo- and heterozygous individuals, and that the corresponding alleles are inherited according to the Mendel's laws. Therefore, the pedigree analysis validated the protocol for (GT)n genotyping both in homo- and heterozygotes.

Procedures

Potential participants were referred to the Center due to a suspected concussion. The Center's clinical coordinator (Ms. Jacqueline Phillips) explained the consent and assent forms and discussed the study as well as answered the patient's questions. If the patient decided to participate he or she signed the appropriate IRB forms. The clinical coordinator then evaluated the patient using the standardized concussion battery assessment protocol, and the patient provided a saliva sample. The concussion battery assessment was always preformed in the same manner first a health history, followed up with the vestibular ocular exam, BESS test, and ended with the ImPACT exam. A physician then evaluated and determined appropriate follow-up care and appointments for the patient. Data from the initial evaluation and follow-up appointments were transferred to a spreadsheet and participants were assigned an identification number for confidentiality purposes. The preserved saliva was stored at room temperature, and DNA was extracted and genotyped as described previously.

Data Analysis

Data were analyzed using descriptive and inferential statistics. For aim one the association between (GT)n VNTR (long or short alleles groups) and duration of recovery (normal versus prolonged) was assessed using Chi-squares or Fisher's exact tests. A univariate regression analysis for binary outcomes was used to estimate the extent to which this VNTR and non-genetic factors contributed to prolonged recovery variability in this study. This was followed by multivariate and stepwise regressions for binary outcomes. Non-genetic factors included in the regression were age, sex, race, concussion history, migraine history, and history of dizziness at time of injury. Differences in concussion severity between long or short alleles utilizing the dominant, recessive, and codominant allele models were analyzed using one-way ANOVAs (vestibular ocular score, BESS score & ImPACT scores). Pearson correlations were used to determine the relationship between the descriptive and concussion severity data. To determine the inclusion of the covariates a Pearson correlation coefficient of $r > 0.60$ was used. No covariates met this threshold. SPSS version 21.0 (SPSS IBM Inc. Armonk, NY) was used for all analyses, and the alpha level was set at $p \leq .05$.

Results

Statistical tables are presented in Appendix J. Raw data are presented in Appendix K. Participant descriptive data by recovery group are presented in Table 3. Participant descriptive data by VNTR dominant, recessive, and codominant models are shown in Table 4, Table 5, and Table 6, respectively. There were no significant associations in descriptive data between recovery groups or among dominant, recessive, or codominant model groups.

Association Between Recovery and Long or Short Alleles

Figure 14 shows the allele distribution pattern of the current population of (GT) allele frequency. The mean repeat number of (GT)n was 24.8, and the repeat number ranged from 19 to 36. The cut point classification gave rise to three groups: 1. short allele genotype (47%), 2. long allele genotype (29%), 3. short/long genotype (24%). To determine differences between long and short alleles three genotypes within *GRIN2A* groups were classified into

Table 3. Participants' Descriptive Data by Recovery Group

Variables	Long Recovery	Normal Recovery
Sex n (%)		
Male	18 (35)	20 (39)
Female	10 (20)	3 (6)
Race		
White	15 (29)	7 (14)
African American	3 (6)	2 (4)
Latino	2 (4)	0 (0)
Other	1 (2)	2 (4)
Migraine		
No Hx	20 (39)	6 (12)
Hx	8 (16)	17 (33)
ADHD		
No Hx	26 (51)	6 (12)
Hx	2 (4)	17 (33)
Dizziness		
No HX	17 (33)	11 (22)
Hx	10 (20)	12 (24)
Age M (SD)	19.6 (8.5)	17.6 (3.3)
Prv Conc	1.4 (2.1)	0.6 (0.97)

Note. M (mean), SD (standard deviation), n (number), Hx (history), ADHD (attention deficit disorder), Dizziness (reported acute symptom of dizziness immediately following concussion) Prv Conc (number of previous concussions).

Table 4. Participants' Descriptive Data in Dominant Model

Variables	LL	SS + LS
Sex n (%)		
Male	11 (22)	27 (53)
Female	3 (6)	10 (20)
Race		
White	5 (10)	17 (33)
African American	0 (0)	5 (10)
Latino	1 (2)	1 (2)
Other	2 (4)	1 (2)
Migraine		
No Hx	10 (20)	10 (20)
Hx	4 (8)	27 (53)
ADHD		
No Hx	13 (25)	32 (63)
Hx	1 (2)	5 (10)
Dizziness		
No HX	6 (12)	14 (27)
Hx	8 (16)	22 (43)
Age M (SD)	19.3 (7.5)	18.5 (6.4)
Prv Conc	1.3 (1.4)	1.0 (0.9)

Note. M (mean), SD (standard deviation), n (number), LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), Hx (history), ADHD (attention deficit disorder), Dizziness (reported acute symptom of dizziness immediately following concussion) Prv Conc (number of previous concussions).

Table 5. Participants' Descriptive Data in Recessive Model

Variables	LL +LS	SS
Sex n (%)		
Male	32 (63)	7 (14)
Female	7 (14)	2 (4)
Race		
White	21 (41)	1 (2)
African American	2 (4)	3 (6)
Latino	1 (2)	1 (2)
Other	2 (4)	1 (2)
Migraine		
No Hx	31 (61)	6 (12)
Hx	11 (22)	3 (6)
ADHD		
No Hx	36 (71)	9 (18)
Hx	6 (12)	0 (0)
Dizziness		
No HX	22 (43)	6 (2)
Hx	19 (37)	5 (10)
Age M (SD)	18.9 (7.2)	17.6 (2.6)
Prv Conc	1.2 (1.8)	0.4 (1.0)

Note. M (mean), SD (standard deviation), n (number), LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), Hx (history), ADHD (attention deficit disorder), Dizziness (reported acute symptom of dizziness immediately following concussion) Prv Conc (number of previous concussions).

Table 6. Participants' Descriptive Data in Codominant

Variables	LL	SS	LS
Sex n (%)			
Male	12 (24)	7 (14)	19 (37)
Female	2 (4)	3 (6)	8 (16)
Race			
White	5 (10)	2 (4)	15 (29)
AA	0 (0)	2 (4)	2 (4)
Latino	1 (2)	1 (2)	0 (0)
Other	2 (4)	1 (2)	0 (0)
Migraine			
No Hx	10 (20)	6 (12)	21 (41)
Hx	4 (8)	4 (8)	6 (12)
ADHD			
No Hx	13 (25)	10 (20)	22 (43)
Hx	1 (2)	0 (0)	5 (10)
Dizziness			
No HX	5 (10)	7 (33)	17 (33)
Hx	9 (18)	3 (6)	10 (20)
Age M (SD)	20.6 (5.1)	18.0 (3.9)	18.0 (8.2)
Prv Conc	1.4 (1.1)	1.0 (2.0)	0.9 (1.8)

Note. M (mean), SD (standard deviation), n (number), LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), Hx (history), ADHD (attention deficit disorder), Dizziness (reported acute symptom of dizziness immediately following concussion) Prv Conc (number of previous concussions).

two subgroups short allele carriers (n = 46) and long allele carriers (n = 56). Chi-square data are presented in Table 7. A 2 (group) x 2 (allele) chi-square was used to analyze association between normal or prolonged recovery and long or short allele. The chi-square revealed no significant association ($p = 0.094$).

Association Between Recovery and Dominant, Recessive, and Codominant Model

Three Fisher's exact test statistics are presented in Table 8 to analyze the association between long or normal recovery between the dominant, recessive and codominant allele models. There was a significant association found in the dominant model ($p = 0.036$). Specifically, the long allele was underrepresented within the normal recovery group. No significant associations existed between recessive model ($p = 0.152$) or codominant model ($p = 0.280$).

Association Between Risk Factors of Prolonged Recovery and Long or Short Alleles

Normal or prolonged recovery data were used as outcomes in univariate analysis seen in Table 9. Each predictor was analyzed using univariate logistic regression appropriate for binary outcomes.

Table 7. Association Between Allele Frequency and Recovery

Recovery	LA	SA	Total	X^2	*p*
Normal	21	25	26	2.80	0.094
Prolonged	34	22	56		
Total	55	47	102		

Note. LA (long allele), SA (short allele), X^2 (Chi-square), *p* (alpha level), *df* = 1.

Table 8. Association Between Recovery and Model

Model	Normal	Prolonged	X^2	*p*
Dominant			4.37	0.036*
LL	3	11		
SS + LS	20	17		
Recessive			2.05	0.152
LL + LS	17	6		
SS	27	3		
Codominant			2.54	0.280
LL	4	10		
SS	6	4		
LS	13	14		

Note. LL (homozygous long alleles), SS (homozygous short alleles), LS (heterozygous long allele and short allele) X^2 (Chi-square), *p* (alpha level), *df* = 1. *significance at $p \leq .05$.

Table 9. Univariate Regression Analysis of Risk Factors for Prolonged Recovery

Parameter	Wald X^2	*p*	Odds Ratio	95% CI
Age	1.07	0.300	1.06	0.95- 1.18
Sex	3.18	0.075	0.27	0.06- 1.13
Race	2.69	0.100	2.64	0.83- 8.39
Migraine	0.04	0.843	1.13	0.33- 3.92
ADHD	1.21	0.272	0.37	0.06- 2.21
N_Prev	2.22	0.135	1.43	0.89- 2.30
Ac Dizz	1.14	0.284	0.54	0.17- 1.67
Dominant	4.01	0.045*	4.31	1.03-18.04
Recessive	1.94	0.163	2.91	0.65-13.40
Codominant	1.04	0.307	0.71	0.36- 1.37

Note. Hx (history), ADHD (attention deficit hyperactivity disorder), Ac Dizz (reported acute symptom of dizziness immediately following concussion) N_Prev (number of previous concussions), Est (estimate), Std Error (standard error), X^2 (chi-square), *p* (alpha level), CI (confidence interval). *significance at $p \leq .05$.

There was an association found between the dominant allele group (LL versus SS + LS; $p = 0.045$) and recovery, where the chance of prolonged recovery was 4.3 times greater for those carrying the long allele. Sex was trending towards significance ($p = 0.075$), where males had a higher chance of prolonged recovery. Table 10 and Table 11 present the

Table 10. Multivariate Analysis Summary for Risk Factors of Prolonged Recovery

Parameter	dF	Est	Std Error	Wald X^2	*p*	Pt Est	95% CI
Age	1	0.03	0.06	0.15	0.696	1.03	0.91-1.16
Sex	1	-1.59	1.00	2.54	0.111	0.20	0.03-1.44
Race	1	1.45	0.89	2.64	0.104	4.25	0.74-24.32
Migraine	1	-0.52	0.85	0.37	0.542	0.60	0.11-3.15
ADHD	1	0.33	1.23	0.07	0.790	1.39	0.12-15.48
N_Prev	1	0.30	0.25	1.32	0.250	1.34	0.81-2.21
Ac Dizz	1	-1.94	0.58	4.34	0.036*	0.54	0.17-1.67
Dominant	1	2.55	1.02	6.29	0.012*	0.60	0.27-1.31

Note. Hx (history), ADHD (attention deficit hyperactivity disorder), Ac Dizz (reported acute symptom of dizziness immediately following concussion) N_Prev (number of previous concussions), Est (estimate), Std Error (standard error), X^2 (chi-square), *p* (alpha level), CI (confidence interval). *significance at p ≤ .05.

Table 11. Stepwise Selection Summary for Risk Factors of Prolonged Recovery

Parameter	Est	Std Error	Wald X^2	*p*	95% CI
Sex	-1.61	0.77	4.31	0.037*	0.04-0.91
Dominant	1.74	0.76	5.15	0.023*	1.27-25.56

Note. Est (estimate), Std Error (standard error), X^2 (chi-square), *p* (alpha level), CI (confidence interval). *significance at p ≤ .05.

Multivariate analysis with follow up stepwise regression, respectively, revealed two significant variables the dominant model (p = 0.012) and sex (p = 0.036). Univariate regression for recessive (p = 0.163) and codominant (p = 0.307) models yielded no significant variables therefore there was no follow up multivariate or stepwise regressions.

Means and standard deviations from initial concussion assessment scores between recovery groups are presented in Table 12. Two independent t-tests were used to analyze differences in VOR score and BESS score between long or normal recovery. A one-way (recovery) ANOVA was used to analyze differences in ImPACT scores. No significant differences for the main effects were found.

Vestibular Ocular Score Difference by Models Between Recovery Groups

Means and standard deviations for vestibular ocular score within dominant, recessive, and codominant allele groups are presented in Table 13. Three one-way (allele model) ANOVAs were used to analyze differences in vestibular ocular score. No significant main effects were identified within the dominant (p = 0.138), recessive (p = 0.149), or codominant (p = 0.560).

Table 12. Concussion Assessment Differences in Scores Between Recovery

Variables	Long Recovery M	Long Recovery SD	Normal Recovery M	Normal Recovery SD	*p*
VOR	0.82 ±	0.95	0.83 ±	0.89	0.787
BESS	17.71 ±	8.48	17.13 ±	7.84	0.594
Verbal %	54.89 ±	36.72	55.86 ±	35.52	0.897
Visual %	44.48 ±	27.26	52.91 ±	30.19	0.217
Motor Speed	48.77 ±	28.24	45.91 ±	34.83	0.752
Reaction Time	40.41 ±	28.98	43.41 ±	31.91	0.715
Sign/Symptom	22.41 ±	24.69	19.23 ±	18.67	0.526

Note. Long Recovery n = 27, Normal Recovery n = 22.VOR T-Test $F(2, 49) = .074$, , BESS T-Test $F(2, 49) = 0.28$, Verbal % ANOVA $F(2, 49) = 0.017$, Visual % ANOVA $F(2, 49) = 1.562$, Motor Speed ANOVA $F(10, 84) = 0.101$, Reaction Time ANOVA $F(2, 49) = 0.135$, Sign/Symptom ANOVA $F(2, 49) = 0.409$.

BESS Score Difference by Models Between Recovery Groups

Means and standard deviations for BESS score between dominant, recessive, and codominant allele models are presented in Table 14. Three one-way (allele model) ANOVAs were used to analyze the difference in BESS score. No significant main effects were identified within the dominant ($p = 0.416$), recessive ($p = 0.241$), or codominant ($p = 0.557$).

Table 13. Vestibular Ocular Score by Models

Model	VOR Score M ± SD	F	*p*
Dominant		0.033	0.138
LL	0.79 ± 0.89		
SS + LS	0.84 ± 0.93		
Recessive		2.145	0.149
LL + LS	0.74 ± 0.89		
SS	1.22 ± 0.97		
Codominant		0.560	0.560
LL	0.71 ± 0.73		
SS	1.10 ± 0.99		
LS	0.78 ± 0.97		

Note. LL (homozygous long allele), SS (homozygous short allele), LS (heterozygous long allele and short allele). M (means), SD (standard deviations), *p* (alpha level).

Table 14. BESS Score by Models

Model	BESS Score M ± SD	F	*p*
Dominant		0.674	0.416
LL	15.93 ± 6.02		
SS + LS	18.03 ± 8.79		
Recessive		0.241	0.351
LL + LS	17.19 ± 7.78		
SS	18.67 ± 10.03		
Codominant		0.557	0.526
LL	15.50 ± 5.76		
SS	18.50 ± 9.47		
LS	18.07 ± 8.74		

Note. LL (homozygous long allele), SS (homozygous short allele), LS (heterozygous long allele and short allele). M (means), SD (standard deviations), *p* (alpha level).

ImPACT Scores Difference by Models Between Recovery Groups

Means and standard deviations for ImPACT scores between dominant, recessive, and codominant allele models are presented in Table 15, Table 16, and Table 17, respectively. Three one-way (allele model) ANOVAs were used to analyze differences in ImPACT scores. No significant main effects were identified within verbal percent scores, visual percent scores, motor speed scores, reaction time, or signs and symptoms within the dominant (p = 0.649, p = 0.534, p = 0.722, p = 0.792, p = 0.684), recessive (p = 0.185, p = 0.942, p = 0.166, p = 0.911, p = 0.321), or codominant (p = 0.571, p = 0.970, p = 0.641, p = 0.970, p = 0.597) models, respectively.

Discussion

This is the first study to investigate genetic association to a concussion utilizing the known pathophysiology events to create a better understanding of the recovery process. The purpose of this research was to determine if the variation of (GT)n within *GRIN2A* is associated with concussion recovery as well as concussion severity scores. The hypothesis was that individuals carrying the long allele will have better concussion outcomes compared to those carrying the short allele.

Table 15. ImPACT Scores within Dominant Model

Variables	LL M ± SD	SS + LS M ± SD	*p*
Verbal	59.21 ± 35.75	53.77 ± 36.23	0.649
Visual	53.36 ± 20.41	46.23 ± 31.35	0.534
Motor	50.03 ± 26.53	46.47 ± 33.01	0.722
Reaction Time	39.61 ± 28.07	42.31 ± 33.68	0.792
S/S	18.50 ± 20.78	21.97 ± 22.73	0.684

Note. LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), S/S (signs and symptoms). M (means), SD (standard deviations), *p* (alpha level), Verbal % ANOVA $F(1, 49) = 0.219$, Visual % ANOVA $F(1, 49) = 0.392$, Motor Speed ANOVA $F(1, 49) = 0.128$, Reaction Time ANOVA $F(1, 49) = 0.070$, Sign/Symptom ANOVA $F(1, 49) = 0.168$.

However, it was found the chance of prolonged recovery was four times greater for those carrying the long allele genotype compared to carrying the short allele genotype or heterozygous genotype. Clinically, this could mean that those carrying the long allele genotype may be predisposed to prolonged recovery following a concussion injury. The definition of prolonged recovery varies in the literature as it has be defined as an injury persisting over ten, 14, or 21 days (Harmon et al., 2013; Lau et al., 2011; Iverson, et al., 2003).

Table 16. ImPACT Scores within Recessive Model

Variables	LL + LS M	SD	SS M	SD	*p*
Verbal	58.50 ±	35.55	41.22 ±	35.47	0.185
Visual	48.20 ±	27.87	48.56 ±	33.60	0.942
Motor	50.42 ±	30.67	34.44 ±	31.17	0.166
Reaction Time	41.78 ±	31.62	40.44 ±	35.12	0.911
S/S	19.58 ±	19.23	27.22 ±	32.50	0.321

Note*.* LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), S/S (signs and symptoms). M (means), SD (standard deviations), *p* (alpha level), Verbal % ANOVA $F(1, 48) = 1.808$, Visual % ANOVA $F(1, 48) = 0.005$, Motor Speed ANOVA $F(1, 48) = 1.983$, Reaction Time ANOVA $F(1, 48) = 0.013$, Sign/Symptom ANOVA $F(1, 48) = 1.006$.

Fifty-five percent of this concussed population took > 20 days to recover, and 45% recovered in ≤ 20 days. Compared to previous research, where 80% typically recover in ≤ 10 days (Harmon et al.; Lau et al.; McCrory et al., 2013) the majority of the current studies' population took > 20 days to recover. This may be due to the current population being recruited from a concussion center compared to being treated daily in the athletic training room. Athletes with no access to a certified athletic

Table 17. ImPACT Scores within Codominant Model

Variables	LL M	LL SD	SS M	SS SD	LS M	LS SD	*p*
Verbal	57.14	± 34.52	44.80	± 35.30	58.52	± 37.32	0.571
Visual	50.14	± 19.94	47.20	± 31.96	47.64	± 32.21	0.970
Motor	48.14	± 28.45	39.30	± 33.16	50.39	± 32.28	0.641
Reaction	39.71	± 27.91	42.40	± 33.68	42.32	± 34.43	0.970
S/S	17.86	± 20.74	26.80	± 30.66	20.40	± 19.10	0.597

Note. LL (homozygous and heterozygous long allele), SS (homozygous and heterozygous short allele), LS (heterozygous long allele and short allele), Reaction (reaction time), S/S (signs and symptoms). M (means), SD (standard deviations), *p* (alpha level), Verbal % ANOVA $F(2, 48) = 0.568$, Visual % ANOVA $F(2, 48) = 0.031$, Motor Speed ANOVA $F(2, 48) = 0.449$, Reaction Time ANOVA $F(2, 48) = 0.031$, Sign/Symptom ANOVA $F(2, 48) = 0.521$.

trainer, or that have a problem recovering after a concussion may be more likely to seek attention at a concussion center.

Researchers have reported several risk factors associated to prolonged recovery, which contribute to a greater risk of sustaining a concussion. For example, females report more concussions than their male counterparts, and females tending to report more s/s at the time of injury (Dvorak, McCrory & Kirkendall, 2007; Harmon et al., 2013). This increased risk has been attributed to decreased head-neck strength, higher estrogen levels, and females tend to be more honest about their injuries (Covassin et al., 2006; Gioia, Collins & Isquith, 2008; Tierney et al., 2005). Our findings disagree with previous research. In the current study the stepwise regression indicated a trend toward significance with the association of sex and recovery, where males were more likely to carry the long allele and have prolonged recovery.

Age has also been reported as a contributing factor to prolonged recovery (Harmon et al., 2013). Youth athletes are more at risk due to their developing brain, musculature, and skull sutures (Giza & Hovda, 2001; Guskiewicz & Valovich McLeod, 2011; Zuckerman et al., 2012). Though, no association was found between age and prolonged recovery in this study. This may be due to this population's youngest participant was 12 years old, and the bulk of the current population was high school and college age athletes.

Additionally, there have been several specific symptoms that were found to be contributing risk factors for prolonged recovery (Harmon et al., 2013; McCrory et al., 2013). Dizziness, specifically at the time of injury was a significant predictor within high school football athletes that take more than 21 days to recover (Lau et al., 2009; Lau et al., 2011). This research found a trend toward significance agreeing with previous research, where athletes who reported dizziness at the time of injury were over four times more likely to have prolonged recovery. A history of migraines has also been associated with prolonged recovery (Harmon et al.; Lau et al.), though the current study did not support this finding.

Furthermore, athletes that have had previous concussions were reported to have a two to five times greater risk of sustaining a subsequent concussion (Bruce & Echemendia, 2004; Colvin et al., 2009; Guskiewicz et al., 2003; Iverson et al., 2004; Schultz et al., 2004). There has also been an association found with prolonged recovery time and previous history of concussion (Slobounov et al., 2007); however, some research is conflicting in this area of research (Lau et al., 2009). The current study supports that there is no association between previous concussion history and prolonged recovery. Although research is not in agreement with all the risk factors contributing to prolonged recovery, there is an agreement that certain athlete may be more vulnerable for a more severe injury or longer recovery. Controlling for extrinsic factors (e.g., location of injury, sport), researchers can view variable clinical outcomes (Broglio et al., 2010; McCrory, Johnston, Mohtadi & Meeuwise, 2001), which allows researchers to observe the athlete's intrinsic characteristics that may be important factors in determining their risk and ability to recover following a concussion injury (Ommaya, 1995). One possible varying intrinsic factor is a person's genotype.

In the current study, the long allele carriers were four times more likely to have prolonged recovery following a concussion. This may be due to decreased transcription of *GRIN2A* to produce the NR2A subunit within NMDA channel. This suggests that the NR2A subunit is necessary for NMDA functioning following a concussive event. *GRIN2A* was chosen in the study because of its role in producing the NMDA NR2A subunit that glutamate attaches to allow Ca^{2+} into the cell following a concussion (Giza & Hovda, 2001). Similar to this study's findings, previous research has demonstrated that polymorphisms in this gene may be a contributing risk factor for poor outcomes (e.g., schizophrenia, bipolar disorder; Itokawaet al., 2003; Itokawa, Yamada, Yoshitsugu, et al., 2003; Iwayama-Shigeno et al., 2005). It has also been attributed to decreased hippocampal and amygdala regions (Inoue et al., 2010).

Following a brain injury, NR2A protein levels become naturally down regulated, which was not attributed to decreases in transcription levels (Giza, Santa Maria & Hovda, 2006; Kumar, Zou, Yuan, Long & Yang, 2002). Clinically, this change in NR2A/NR2B ratio could lead to developmental deficits and impaired neuron plasticity like those seen following a concussion injury (Giza et al., 2006). The decrease of the NR2A subunit protein could be attributed to enzymatic degradation from calpain-like protease (Kumar et al., 1997). Calpain has been shown to increase after a concussion significantly, which degrades glutamate receptors by cleaving the C-terminus off the glutamate receptor subunits (Bi & Baudry, 2000; Kampfl et al., 1997). However, using NR2A or NR2A and NR2B knockout mice, researchers found less brain ischemia in mice with no NR2A receptor. Authors suggested that NR2A might mediate glutamate neurotoxicity due to the decreased amount of blood volume, which could be due to the fact that it produced less NMDA channel activity (Morikawa et al., 1998). This suggests that the decrease of NR2A within the channel leads to a protective effect from brain injury.

The (GT)n VNTR in the promoter region has been associated with altered expression level of *GRIN2A* (Itokawa et al., 2003; Iwayama-Shigeno et al., 2005). The length of (GT)n repeat modulates *GRIN2A* expression level, with longer alleles (≥ 25 repeats) typically associated with poorer outcomes (Inoue et al. 2010; Itokawa et al.; Iwayama-Shigeno et al.). Subsequently, the (GT)n repeat is hypothesized to be associated with hypofunction of the NMDA receptor due to the decrease in NR2A transcription (Itokawa et al.). The theory behind this association was that hypofunction of the NMDA is the leading hypothesis contributing to schizophrenia and bipolar disease. However, none of the previous (GT)n VNTR studies determined if the transcription levels correlated to the protein levels produced were altered. This suggests that the NMDA channel plays an important role in neuronal recovery, where decreased levels of NR2A may be thought to have a protective effect against glutamate excitotoxicity.

It was hypothesized if there are less glutamate binding sites following a concussion pathophysiology event, then there is less Ca^{2+} allowed into the cell, thus less risk of excitotoxicity. However, we were not able to identify this protective association. This could be attributed to the need for very high levels of extracellular levels glutamate required in order to produce excitotoxicity (Obrenovitch, Urenjak, Zilkah, 2000). Researchers also suggested that glutamate levels within the synaptic cleft cannot be clearly detected and therefore cannot be associated with extracelluar glutamate levels (Obrenovitch et al., 2000). This was established when researchers utilized glutamate injections within brain tissue and found that daily doses did not promote spreading depression (Obrenovitch et al.). Researchers attributed the ability of the neuron to protect itself from excitotoxicity due to effective uptake systems to overcome high local concentrations of glutamate (Obrenovitch et al.). For instance, it has been hypothesized that during events that cause brain ischemia (e.g., concussion) there is a down regulation of GLUR2 that renders the AMPA receptors permeable to Ca^{2+} that can consequently cause glutamate excitotoxicity (Gorter, 1997; Pellegrini-Giampetro, 1992). Therefore, hypofunction of the NMDA channel may not contribute to protective outcomes following brain injury. Neuron pathology is likely a result from multifactorial processes such as a reduction of ion flux to cope with mitochondrial damage, decreased metabolism. Altered NR2A subunits due to the long allele VNTR may yield further ion transport reduction, which may contribute to prolonged recovery. Therefore, if an athlete carries the long allele genotype within the *GRIN2A* promoter and sustained a concussion, the NR2A levels may be lower than that of a carrier of the short allele genotype or heterozygous genotype. Therefore, the promoter (GT)n VNTR could contribute to the time it takes an athlete to recover following a concussion.

Promoter repeat polymorphisms are critical to study because they have the potential to form, facilitate, and recruit various DNA secondary structures affecting the regulation of gene expression, which could ultimately result in disease (Kouzines & Levens, 2007; Sawaya et al., 2013). It has been demonstrated that repeat polymorphisms are common and highly conserved in the regulatory regions and function to facilitate the recruitment of transcription factors (Daidone et al., 2007; Sawaya et al.). Though this function is not fully understood, many pathophysiological conditions support the notion that repeat polymorphisms within the promoter region alter gene transcription (Daidone et al.; Itokawa et al., 2003; Rich & Zhang, 2003). The promoter (GT)n motif has also been shown to alter transcription in a length dependent way in other genes in addition to *GRIN2A*.

Similar to the *GRIN2A,* there are several genes that contain the (GT)n VNTR, where those carrying the short (GT)n allele (< 25 repeats) had higher levels of expression compared to those carrying the long (GT)n allele (≥ 25 repeats). Within *HO-1,* those with the short allele had an increased risk to rheumatoid arthritis (OR = 0.80, 95% CI = 0.70-0.90, $p = 0.019$; Rueda, 2007). The (GT)n repeat within *HMOX1* promoter has also been shown to alter transcription activity, where those carrying the long allele (≥ 25 repeats) had an increased risk for type II diabetes compared to those carrying the short allele (< 25 repeats; OR = 1.25, 95% CI 1.02-1.24, $p = 0.02$; Bao et al., 2010). Conversely, researchers found that those carrying the short allele (> 20 GT repeats; 13.8 ± 5.10 *u*M) in the *HMOX-1* contributed to increased levels of bilirubin compared to the long allele (< 29 GT repeats; 9.18 ± 3.73 *u*M, $p < 0.001$; D'Silva, Borse, Colah, Ghosh & Mukherjee, 2011). Increases in bilirubin have been shown to contribute to cardiovascular disease (D'Silva et al., 2011). Thus, VNTR is a general biological phenomenon, and has been established as a potential marker for association studies. Concussions are also caused by biochemical imbalances that are provoked by an increase in glutamate.

The method used to analyze VNTR in this study is applicable to other genes that also contain dinucleotide (GT)n repeat polymorphisms. Appendix I contains a summary of the known genes containing polymorphic (GT)n sequences. Importantly, the functional significance of (GT)n polymorphism on gene expression remains to be elucidated, as activity of some genes have not clearly associated the length of (GT)n repeat to the disease. This work contributes to further characterization of gene expression regulated by (GT)n-containing promoters. In addition to the (GT)n repeat within *GRIN2A,* previous research examined genotype in other genes as a factor in brain injury.

In each of the previous studies the combination of genetic and environmental factors leads to poor outcome. For example, several studies examined the association of concussion and genetic polymorphisms such as polymorphisms within *APOE* (Kristman et al., 2008; Terrell et al., 2008; Terrell et al., 2012; Tierney et al., 2010). A study in college athletes who self-reported a concussion history that carried all three APOE rare alleles were ten times more likely to report a concussion (Tierney et al., 2008). A larger prospective cohort study found no association between the APOE rare alleles and concussion risk; however, there was a trend toward significance (*p* = 0.09; Terrell et al.). Genetic polymorphisms could also increase susceptibility of a concussion. Researchers used a case-control study in collegiate athletes to find an association between a SNP (single nucleotide polymorphism) within *NEFH* that was associated with diminished neuron cell structure stability and diagnosed concussion history (McDevitt et al., 2010). However, no association between concussion susceptibility and genotype was found (McDevitt et al.).

Within the present study, there was a standardized concussion assessment protocol implemented. Instructions were given to the athlete for the progression to RTP. Concussed athletes were tested for a concussion using the vestibular ocular, balance test, and neurocognitive tests. There were no significant differences found in concussion severity scores between groups. Both allele and recovery group initial evaluation assessment scores were poor, lower than normative baseline scores, suggesting this was an effective detection method for diagnosing a concussion.

This was the first study to analyze VNTR between concussion recovery and concussion severity. It should be noted that there were several limitations in this study.

First, the number of participants was small and this research should continue to expand to include a larger cohort. Additionally, the athletes were not seen at the time of injury, which could have affected severity scores. Also, due to scheduling conflicts some athletes may have been delayed in their full RTP. This could affect the normal versus prolonged recovery groups, which was a factor in determining normal recovery ≥ 20 days to ensure normal recovery participants would be included in this group. Lastly, only one gene and one polymorphism were examined; however, concussions are multifactorial injuries and most likely influenced by several genes and numerous polymorphisms. One of the primary study outcomes was that a valid methodology to determine associations of promoter (GT) repeat polymorphisms was determined. This methodology could be implemented in other studies that involve genes with promoter (GT)n repeats that could be associated with concussion recovery.

Decisions on Aims and Hypothesis

Based on the results of this study, the following decisions on the hypotheses were made:

Aim 1

There was a statistically significant association found between the dominant (LL versus SS +LS) model and recovery. These results suggest that carriers of the long allele (≥ 25 GT repeats) are at an increased risk of prolonged recovery following a concussion injury. Hypothesis 1 was rejected.

Aim 2

No differences were detected between those carrying the long or short (GT)n repeat allele in concussion severity scores. Hypothesis 2 was accepted.

Conclusion

This was a novel study that investigated the association between VNTR and concussion recovery and severity. Individual structural differences in neuronal proteins, such as channel proteins that allow ion flow, could influence the way an individual's brain responds to an applied force and may represent a predisposing or protective factor to concussions. Concussion injury and demographic data was collected for 51 participants and followed the participants' recovery to full return to play following a concussion injury. A standardized methodology was established for DNA collection, estimation, and genotyping protocol of VNTR within the *GRIN2A* for 96 samples. Accuracy of genotyping method was demonstrated. Finally, statistical analysis demonstrated an association found between the dominant allele group (LL versus SS + LS) and recovery, where the chance of prolonged recovery was 4.3 times greater for those carrying the long allele genotype. Sex and reporting dizziness at the time of injury were also trending towards significance, where males and reporting dizziness at the time of injury had a higher chance of prolonged recovery following a concussion injury.

Recommendations for Future Research

A large prospective study representative of the entire athletic population is necessary for further research. Future studies should try and control for concussion history, athletic exposure, age, and other potential predisposing risk factors for concussions to determine if there are genetic predispositions that could increase ones risk of concussion injury, severity of injury, or affect the recovery from a concussion. Lastly, future research should consider the GT repeat polymorphisms in addition to other variations in other genes involved in the pathophysiology following a concussion in addition to the *GRIN2A*. Based on the results of the present work, the prospective genotyping of athletes before the sport season could help improve monitoring and concussion management of athletes who experience concussion injuries.

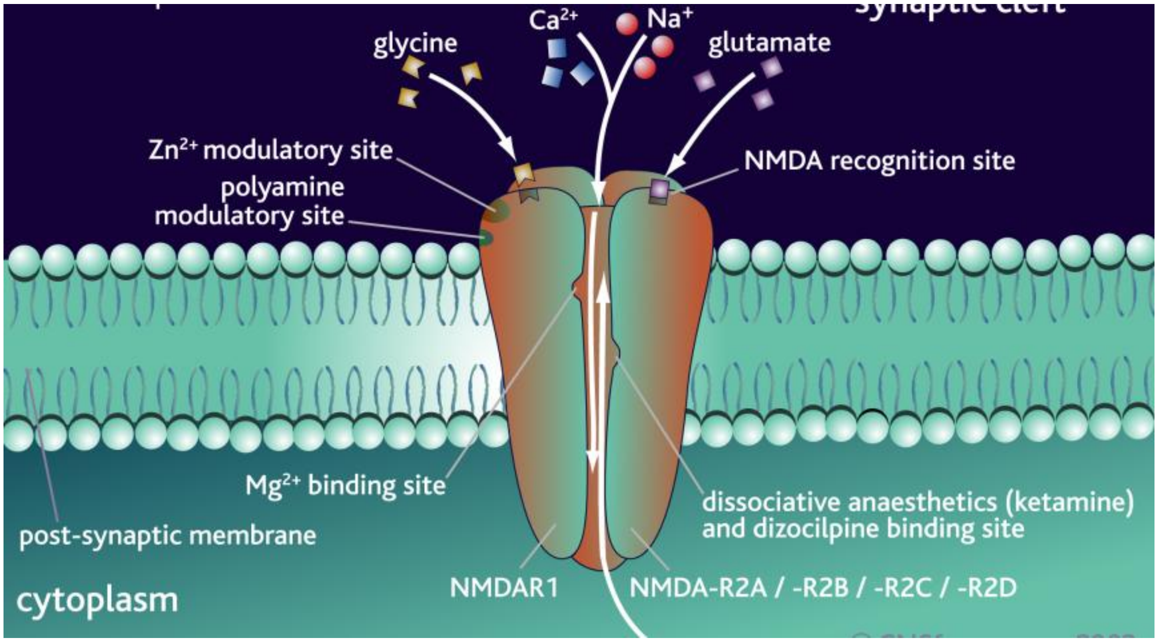

Figure 1. Schematic of NMDA Channel (Rang, Dale & Ritter, 2001).

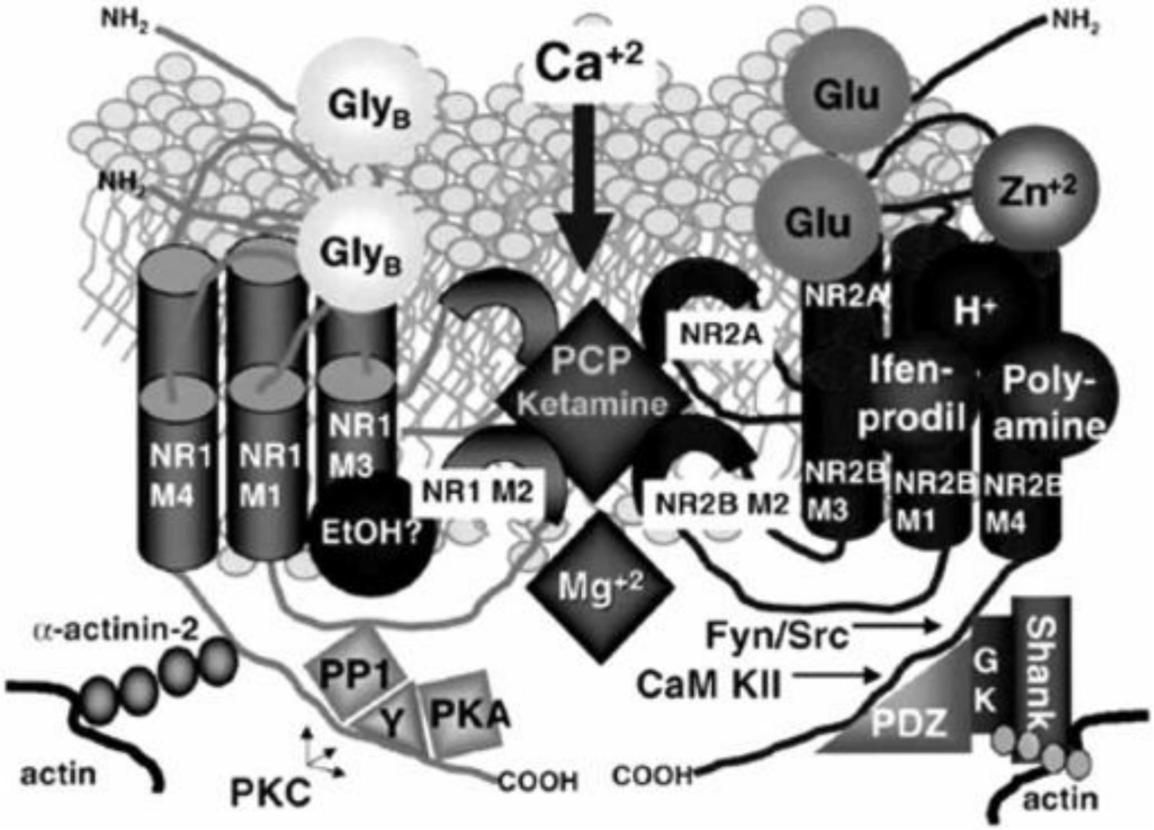

Figure 2. NR2A & NR2B NMDA Subunits (openi.nlm.nih.gov)

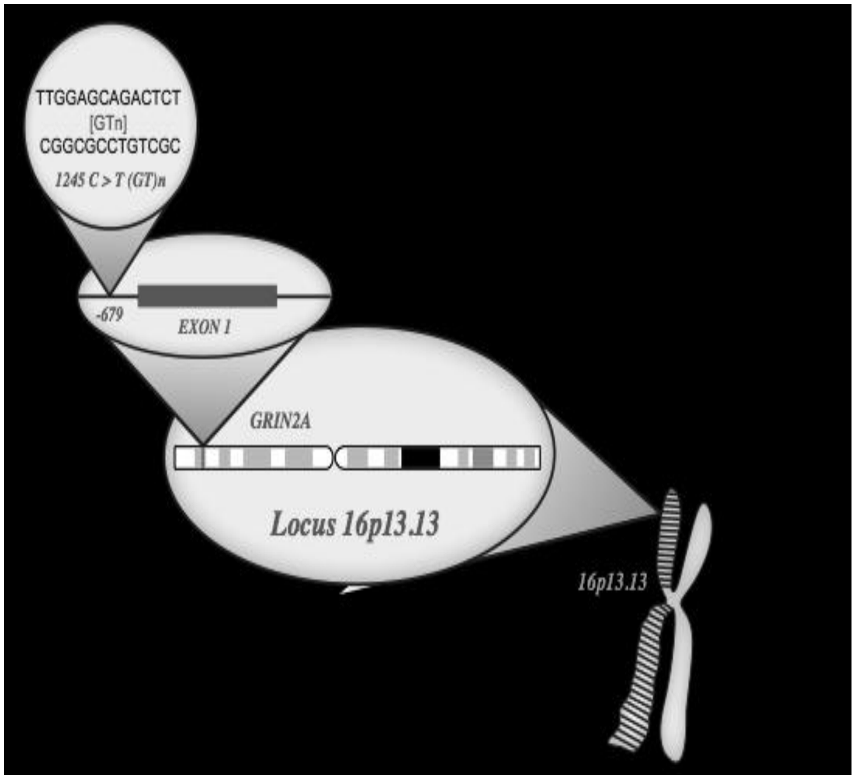

Figure 3. Diagram of GT(n) Tract within *GRIN2A*

1 <u>GAAGGAAGCATGTGGGAAATGCAG</u>ATGTCTTTGCTTTTAGGATTCTGGCT CCCTGAGATC

61 AGAGGAGTGGGTTCCCGTACGGGGGTGGCGAGGCACAGGGAGAAGCATTGGAGCAGACTC

121 T**GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG T**CGGCGCGCCTGTGCGTGG<u>A</u>

181 <u>GGGGGATAACTGTACCCAGC</u>

Figure 4. *GRIN2A* Promoter Fragment Located at Chromosome 16. The sequence corresponds to positions 10217386 – 10217585 of contig GRCh37.p10 (NT_010393.16). Primers used for PCR analysis are underlined. Polymorphic GT repeat region is shown in bold font.

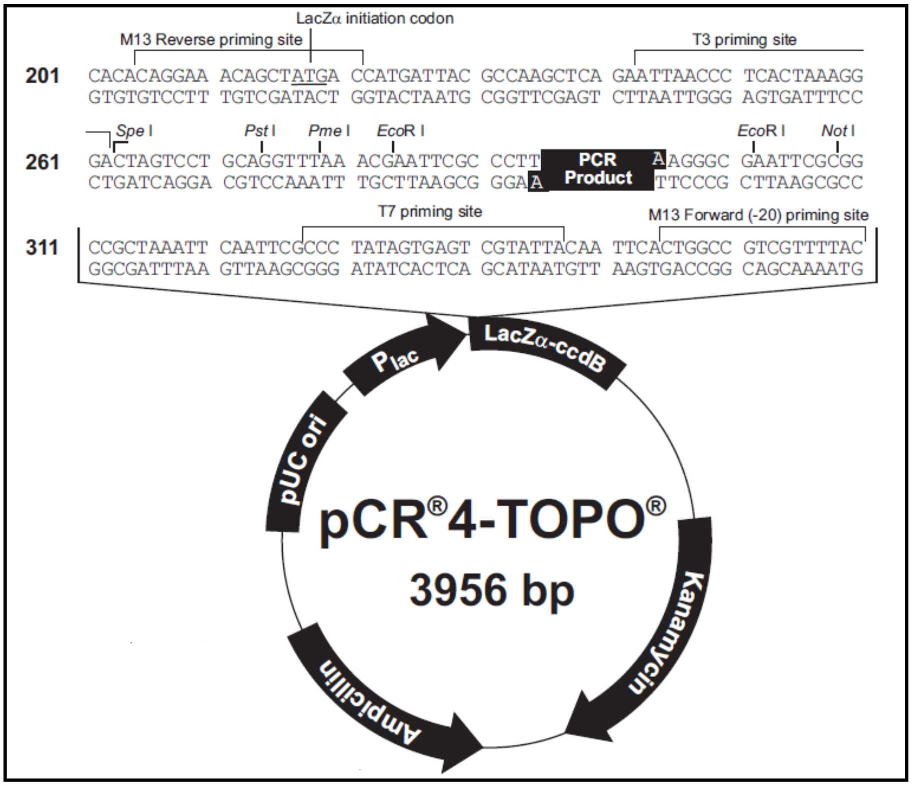

Figure 5. pCR4-TOPO Plasmid Map Used for Cloning of PCR Product from Genomic DNA (Invitrogen, Grand Island, NY). The cloning site for the PCR product is highlighted in black. (tools.invitrogen.com/ content/sfs/vectors/pcr4topo_map.pdf)

223 bp

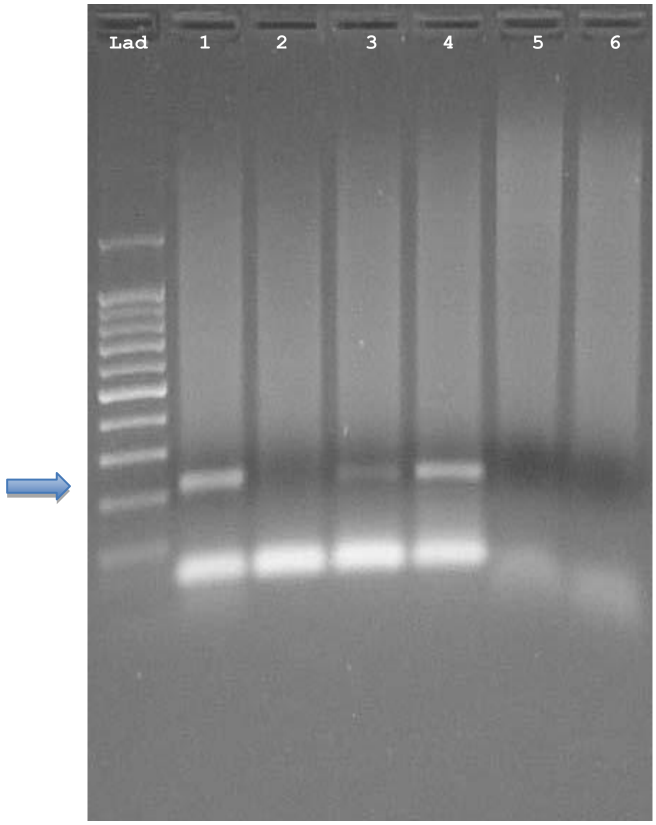

Figure 6. Optimization of Buffer for PCR Amplification of (-975 to -776) Promoter Region in *GRIN2A*. Lane 1: Buffer A; lane 2: Buffer B; lane 3: Buffer C; lane 4: Buffer D; lane 5: Buffer E; lane 6: Buffer F (PCR optimization kit, Invitrogen, Grand Island, NY). M-DNA ladder (100 bp). The amplified fragment of 223 bp is indicated with an arrow.

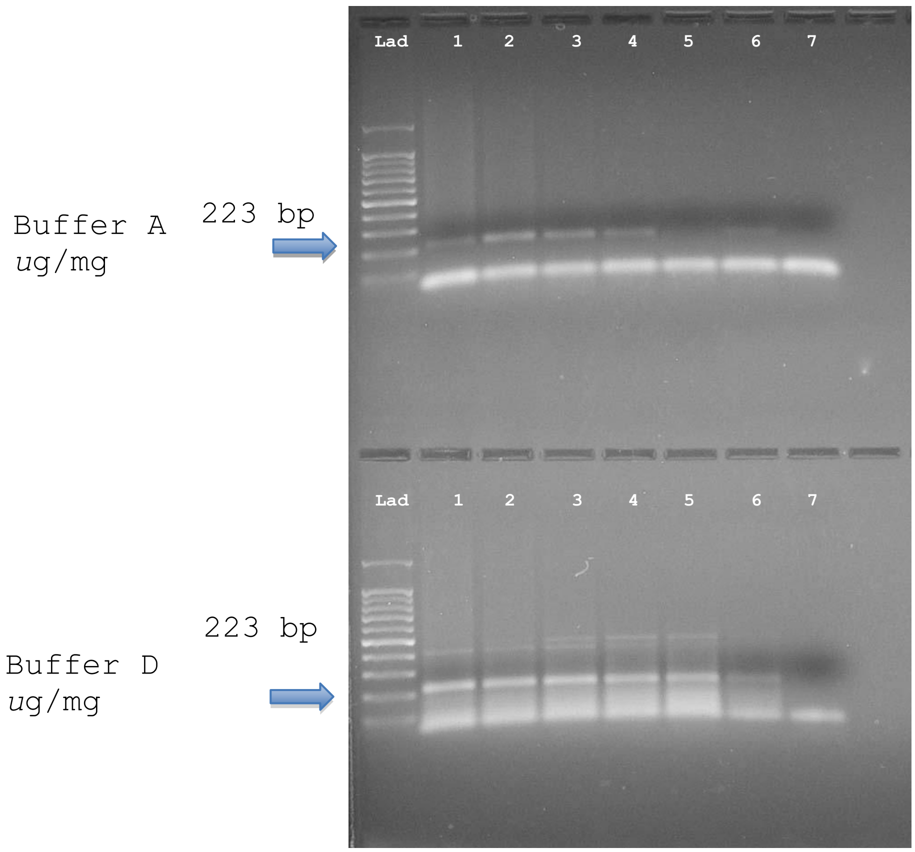

Figure 7. Optimization of PCR Reaction in Buffer A (upper panel) and Buffer D (lower panel), in the Presence of Varying Amounts of Template (PCR optimization kit, Invitrogen, Grand Island, NY). Lane 1: DNA 700 ng per reaction, lane 2: 350 ng DNA per reaction, lane 3: 175 ng DNA per reaction, lane 4: 88 ng DNA per reaction, lane 5: 44 ng DNA per reaction, lane 6; 22 ng DNA per reaction, and lane 7: contained no DNA template. M-DNA ladder (100 bp). The target segment of 223 bp is indicated with an arrow.

223 bp

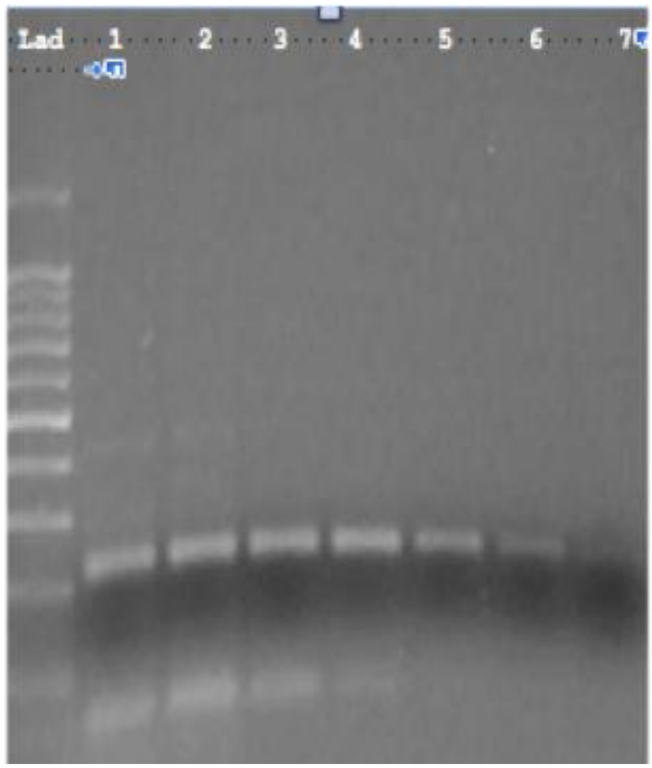

Figure 8. PCR Optimization with FAM Forward Primer Concentration (PCR optimization kit, Invitrogen, Grand Island NY). Lane 1: 300 ng of primers per reaction, Lane 2: 150 ng of primers per reaction, Lane 3: 75 ng of primers per reaction, Lane 4: 37.50 ng of primers per reaction, Lane 5: 18.75 ng of primers per reaction, Lane 6: 9.38 ng of primers per reaction, and Lane 7: 4.67 ng of primers per reaction. M-DNA ladder (100 bp). The target segment of 223 bp is indicated with an arrow.

Range 1: 53 to 250 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
351 bits(190)	2e-101	197/200(99%)	2/200(1%)	Plus/Plus

```
Query  1    GAAGGAAGCATGTGGGAAATGCAGATGTCTTTGCTTTTAGGATTCTGGCTCCCTGAGATC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  53   GAAGGAAGCATGTGGGAAATGCAGATGTCTTTGCTTTTAGGATTCTGGCTCTCTGAGATC  112

Query  61   AGAGGAGTGGGTTCCCGTACGGGGGTGGCGAGGCACAGGGAGAAGCATTGGAGCAGACTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  113  AGAGGAGTGGGTTCCCGTACGGGGGTGGCGAGGCACAGGGAGAAGCATTGGAGCAGACTC  172

Query  121  tgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtCGGCGCGCCTGTGCGTGGA  180
            |||||||||||||||||||||||||||||||||||||||  |||||||||||||||||||
Sbjct  173  TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT--CGGCGCGCCTGTGCGTGGA  230

Query  181  GGGGGATAACTGTACCCAGC  200
            ||||||||||||||||||||
Sbjct  231  GGGGGATAACTGTACCCAGC  250
```

Figure 9. Alignment of the PCR Fragments from SW48(297). The upper line, following the cloning in pCR4-TOPO and sequence analysis, with the reference sequence NT_010393.16 in NCBI database.

```
Query  1         GAAGGAAGCATGTGGGAAATGCAGATGTCTTTGCTTTTAGGATTCTGGCTCCCTGAGATC  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10217585  GAAGGAAGCATGTGGGAAATGCAGATGTCTTTGCTTTTAGGATTCTGGCTCCCTGAGATC  10217526

Query  61        AGAGGAGTGGGTTCCCGTACGGGGGTGGCGAGGCACAGGGAGAAGCATTGGAGCAGActc  120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10217525  AGAGGAGTGGGTTCCCGTACGGGGGTGGCGAGGCACAGGGAGAAGCATTGGAGCAGACTC  10217466

Query  121       tgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtcggcgcgcc  180
                 |||||||||||||||||||||||||||||||||||||||||          |||||||||
Sbjct  10217465  TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT----------CGGCGCGCC  10217416

Query  181       tgtgcgtgGAGGGGGATAACTGTACCCAGC  210
                 ||||||||||||||||||||||||||||||
Sbjct  10217415  TGTGCGTGGAGGGGGATAACTGTACCCAGC  10217386
```

Figure 10. Alignment of the PCR Fragment from Human Genomic DNA (sample 11015). The upper line, following cloning and sequence analysis. Allelic variant with 25 GT-repeats is shown.

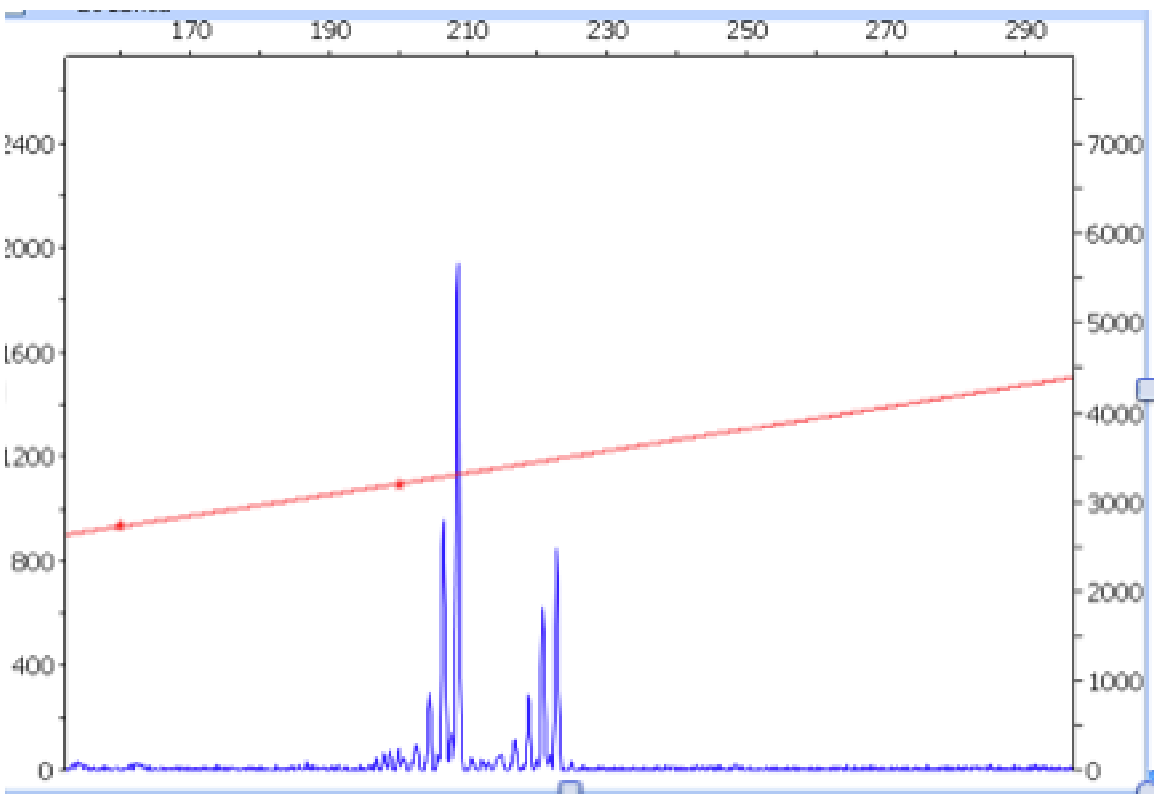

Figure 11. Representative Heterozygous Electropherogram. Fragment length polymorphism (FLP) analysis of the reaction mixture following PCR amplification of the human genomic DNA. The heterozygote carrier of two alleles (GT 22/GT 28) is shown. The calibration curve is the line with asterics, which indicate known lengths. The length of the fragments is indicated in bp above the electrophoregram profile. Y axis, relative fluorescence units (RFU).

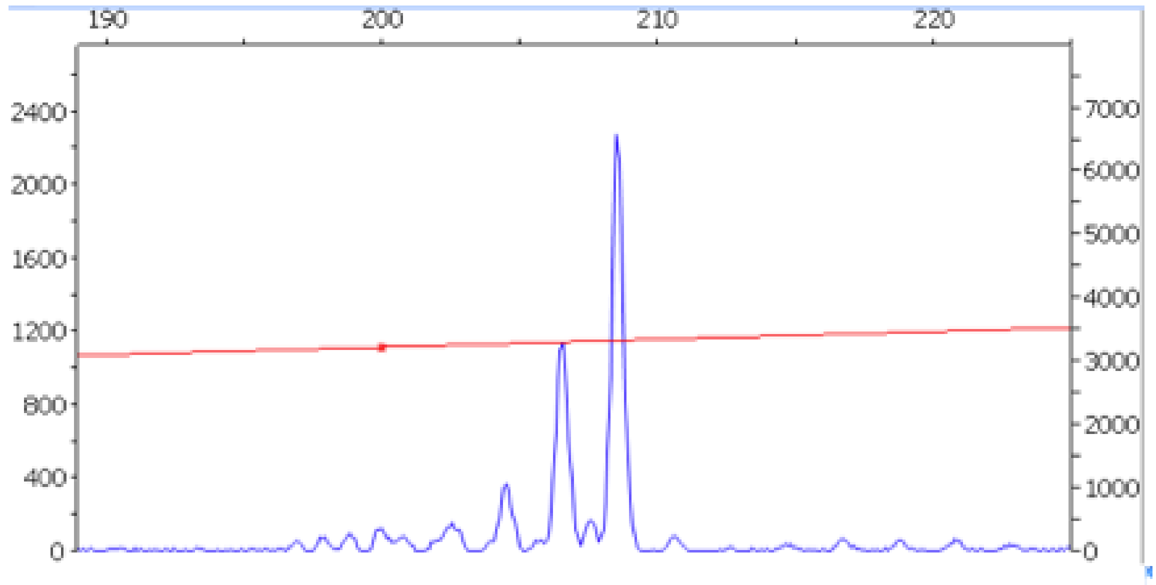

Figure 12. Representative Homozygous Electropherogram. FLP analysis of the reaction mixture following PCR amplification of the human genomic DNA. The homozygote with 21 GT repeats is shown. The calibration curve is the line with asterics, which indicate known lengths. The length of the fragments is indicated in bp above the electrophoregram profile. Y axis, relative fluorescence units (RFU).

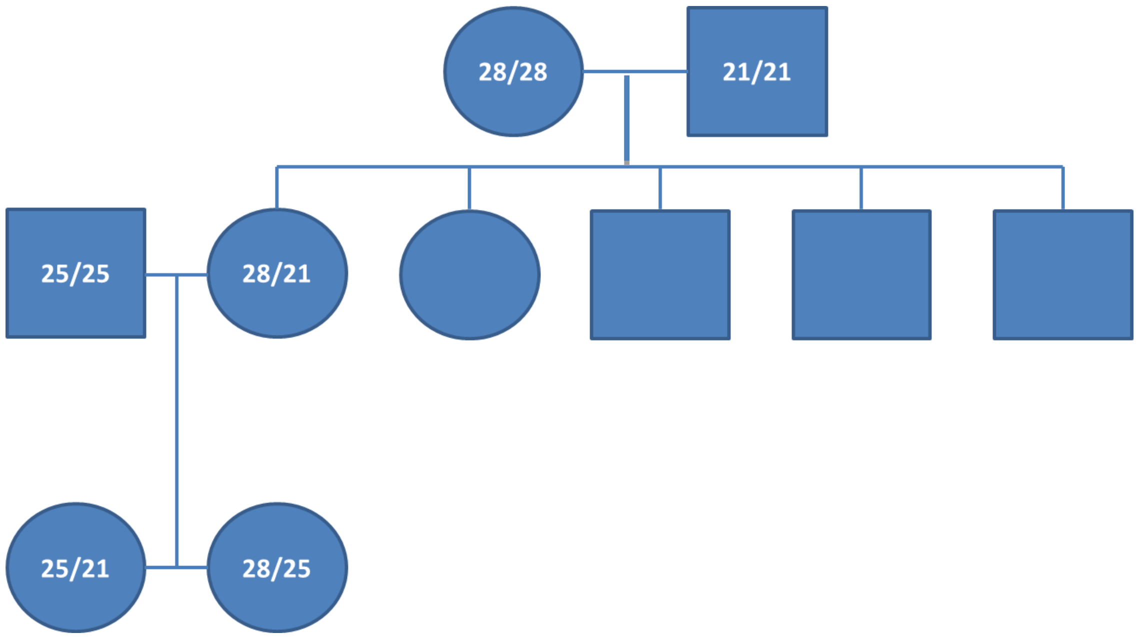

Figure 13. Pedigree Analysis of (GT)n Repeat Alleles in a Three Generation Family. Six members were genotyped in respect to the number of GT repeats in the GRIN2A promoter region. The number of repeats is indicated for each genotyped individual. ■, male. •, female

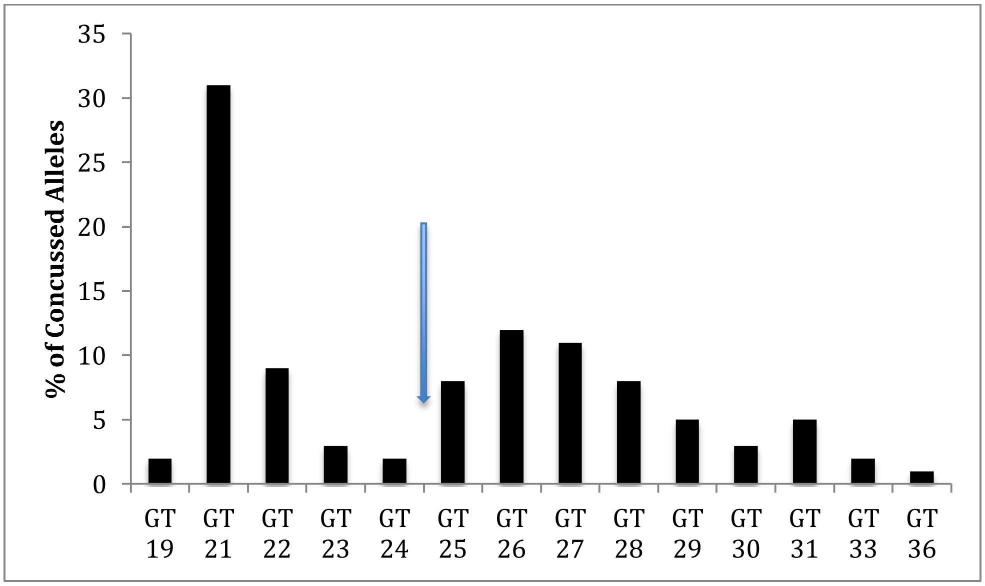

Figure 14. (GT)n Allele Frequency within the Concussed Participants. Arrow denotes long allele (to right) and short allele (to left).

CHAPTER 2

REVIEW OF LITERATURE

Concussions

Concussion is defined as a traumatically induced transient disturbance of the brain brought on by complex pathophysiological processes (Harmon et al., 2013). There are several common constructs that also help to define concussions. A concussive event may be caused by a direct blow to the head, neck, face, or body that results in an impulsive force on the brain within the cranium (McCrory et al., 2008). Concussions typically result in a rapid onset of short-lived impairments from neurologic dysfunction that resolves spontaneously (McCrory et al.). Athletes may present with or without the loss of consciousness along with other concussion signs and symptoms (s/s), which will vary in resolution time (McCrory et al.). Concussions may result in neuropathological changes but the acute s/s largely reflect functional disturbance rather than structural injury (McCrory et al.). Finally, abnormalities resulting from a concussion cannot be seen on standard structural neuroimaging (e.g., MRI, CT; McCrory et al.).

Concussions affect about 1.6 million to 3.8 million athletes yearly (Langlois, Rutland-Brown & Wald, 2006).

Although most concussions are considered “mild injuries,” more than $17 billion in treatment costs are incurred annually in the United States (Gerberding et al., 2003). Concussions represent 5% to 9% of all collegiate athletic injuries (Hootman, Dick & Agel, 2007). In recent epidemiologic study researchers found that concussions accounted for nearly 15% (1,056) of all sport-related injuries in high school athletes (Meehan, d’Hemecourt, Collins, Comstock, 2011). Overwhelmingly though, high school athletes fail to either recognize the mechanisms and/or the seriousness of their concussions, and tend not to report them (Broglio et al., 2010; McCrea, Hammeke, Olsen, Leo & Guskiewicz, 2004). Concussions are important health problems that may result in death, disability (Cassidy, Carroll, Cote, Holm & Nygren, 2004), and financial burden (Gerberding & Ginder, 2003).

Concussions most frequently occur following an event that involves an acceleration-deceleration mechanism, such as a blow to the head, or the head striking an object. This type of injury could lead to an alteration in mental status and one or more of the following symptoms: headache, nausea, vomiting, dizziness, balance problems, feeling “slowed down,” fatigue, difficulty sleeping, drowsiness, sensitivity to light or noise, loss of consciousness, blurred vision, difficulty remembering, and difficulty concentrating (Guskiewicz et al., 2004).

The most common s/s that have been reported within the high school population are headache, dizziness, difficulty concentrating, and disorientation (Meehan et al., 2011). High school athletes have been shown to display a decline in memory for at least seven days post injury (Lovell et al., 2003). A concussion injury may also cause short-term altered awareness or consciousness (e.g., sensation of being dazed or stunned, sensation of fogginess, seizure, or amnesic period; Omalu et al., 2005) if continued or repeated, and has been shown to lead to adverse long-term outcomes (e.g., emotional problems and depression; Guskiewicz et al., 2007; Macciocchi, Barth & Littlefield, 1998). Repetitive or multiple concussions are associated with more severe concussions resulting in longer recovery time and chronic diseases (Barkhoudarian, Hovda & Giza, 2011). Among retired professional soccer players, repeated heading might lead to subtle, chronic head injuries (e.g., central cerebral atrophy) and mild to moderate neuropsychological impairments (e.g., deficits in attention, concentration, memory, and judgment; Tysvaer & Lochen, 1991).

Athletes respond differently to concussions not only in the s/s they display, but also in number of days to recover. Eighty to 90% of athletes are returned to play (RTP) within seven to 10 days (McCrory et al., 2013). There are athletes that have rapid recovery and are symptom free within seven days (Lau et al., 2011). Conversely, some athletes have prolonged recovery lasting 21 or more days (Lau et al.). Professional football athletes were shown on average to RTP within 14 days (Pellman & Viano, 2006), though, 2% of this population took over 14 days to recover (Pellman & Viano).

The definition of prolonged recovery is conflicting in the research, and has been defined as an injury persisting over ten, 14, or 21 days (Harmon et al., 2013; Lau et al., 2011; Iverson et al., 2003;). There are several proposed risk factors that could contribute to prolonged recovery. Several risk factors for prolonged recovery that contribute to a greater risk of sustaining a concussion are specific populations (e.g., children; McCrory et al 2013), playing specific sports (e.g., football; Pellman et al., 2004), persistent concussive s/s, (e.g., dizziness; Bruce & Echemdia, 2004; Lau et al), and history of previous concussion (Colvin et al., 2009; Schultz et al., 2004).

Sex has also been associated with increased risk of concussion injuries. Specifically, females report more concussions than their male counterparts within similar sports (Dvorak, McCrory, & Kirkendall, 2007; Harmon et al., 2013). Also females tend to report more s/s at the time of injury (Dvorak et al., 2007; Harmon et al.). These risk factors have been contributed to decreased head-neck strength, higher estrogen levels, and due to the fact that females tend to be more honest about their injuries (Covassin et al., 2006; Gioia, Collins & Isquith, 2008; Tierney et al., 2005). However, there is no conclusive research that sex could be a modifying factor of concussion risk (McCrory et al., 2013). Age has also been noted as a contributing factor to prolonged recovery (Harmon et al., 2013). Youth athletes are more at-risk due to their developing brain, musculature, and skull sutures (Giza & Hovda, 2001; Guskiewicz & Valovich McLeod, 2011; Zuckerman et al., 2012).

Certain sports and positions have been associated with a greater risk of concussion. The most common mechanism of a concussion is player-to-player contact; therefore, sports that have more player-to-player contact have a higher risk of concussion (e.g., football, rugby, soccer; Harmon et al., 2013; Marar et al., 2012). Soccer players at both collegiate and high school levels reported that the most common mechanism of a concussion injury was player-to-player contact (Agel et al., 2007; Yard et al., 2008). Professional football studies have shown that quarterbacks, wide receivers, running backs, and defensive backs have three times greater risk of concussion than linemen, and kickoff plays resulted in four times greater risk of concussion compared to rushing and passing plays (Pellman et al., 2004). High school football studies revealed that defensive linebackers and offensive running backs were most commonly concussed compared to the athletes playing other positions (Harmon et al.).

Athletes that have a concussion history are reported to have a two to five times greater risk of sustaining a subsequent concussion (Bruce & Echemendia, 2004; Colvin et al., 2009; Guskiewicz et al., 2003; Iverson et al., 2004; Schultz et al., 2004). There has also been an association found with prolonged recovery and having a history of concussion (Slobounov et al., 2007); however, there are conflicting reports in this area of research (Lau et al., 2009). Furthermore, athletes with a concussion history have been associated to reporting more s/s at baseline compared to those with no concussion history (McLeod & Leach, 2012; Schatz et al., 2011).

A greater number, severity, and duration of s/s after a concussion were found to be predictors of prolonged recovery (Bruce & Echemendia, 2004; Harmon et al., 2013). It has been shown that athletes presenting with four or more concussions are more likely to take greater than seven days to recover (Christman, Rivarra, Schiff, Zhou, & Comstock, 2013). One study within rugby players found that athletes that reported three or more s/s, or headaches lasting more than 60 hours were associated with longer recovery (Makdissi et al., 2006). Additionally, several specific symptoms have been defined as contributing risk factors for prolonged recovery (Harmon et al.; McCrory et al., 2013). Dizziness reported at the time of injury was found to be a predictor of prolonged recovery within high school football athletes (Lau et al., 2009; Lau et al., 2011). A history of migraines has also been associated with prolonged recovery (Harmon et al.; Lau et al., 2009). Reporting several cognitive s/s at the time of injury have also been associated with prolonged recovery (Lau et al., 2009; Slobounov et al., 2007). Furthermore, athletes that reported feeling drowsy, nauseated, or feeling confused along with concentration difficulties at the time of injury were also more prone to take more than seven days to recover (Christman et al., 2013). Additionally, sensitivity to both light and noise were associated with almost a 3-fold increased risk for concussive symptoms lasting more than seven days (Christman et al.). Loss of consciousness that is longer than one minute in duration is also a predictor of prolonged recovery (McCrory et al.). Therefore, it is imperative that the s/s be accurately reported and documented as part of the concussion evaluation and RTP protocol.

Athletes report different s/s following a concussion injury. Researchers suggest that s/s evaluation alone is not sensitive enough for return to play decisions and propose several different tests (e.g., neurocognitive assessment, vestibular ocular exam, cranial nerve) be used to ensure appropriate RTP decisions (Lau et al., 2011). Sensitivity scores for each concussion evaluation measurement is not high (e.g., BESS sensitivity = 34%, ImPACT sensitivity = 65%); however, when using a multi tool approach consisting of a computerized neurocognitive exam, postural control exam, and a paper and pencil test sensitivity exceeded 90% (Broglio et al., 2007). Computer neurocognitive exams (e.g., ImPACT) have been used to help evaluate concussion and determine RTP. Researchers using this tool found most concussions, regardless of mechanism or magnitude of the injury, healed within seven to 14 days in both college and high school athlete populations (McClincy, Lovell, Pardini, Collins & Spore, 2005). High school athletes have been shown to display concussion s/s for at least seven days post-concussion injury, though, self-reported s/s resolved around four days post-concussion injury (Lovell et al., 2003). Since athletes present with concussion injuries differently a standardized concussion protocol must be implemented for valid concussion diagnosis and progression to RTP measures are followed.

Athletes seem to react differently to similar head impacts, which may be due to patients experiencing symptoms at lower thresholds than others (Guzkiewicz et al., 2004; Schnebel, Gwin, Anderson & Gatlin, 2007). For example, researchers recorded linear acceleration for impacts of Division I collegiate football players and reported a range of 81.9 g to 145.7 g that elicited a concussion (Schnebel, et al., 2007). Some players received an impact of 98 g or higher once every 70 impacts and did not develop a concussion (Schnebel, et al.). It was also found that resulting s/s can differ depending on the force to the head, degree of metabolic dysfunction, tissue damage, and number of previous head injuries, which contribute to the amount of time that the athlete takes to recover (Guskiewicz et al., 2004). Researchers have tried to determine a force threshold for diagnosing a concussion, but one study further complicates this, because researchers found that a concussion can result not only from a single impact but also from mild repetitive impacts (Beckwith et al., 2013). Additionally, this study found that regardless of when the athletes were diagnosed with the concussion (i.e., within several days or immediately after the head impact) the timing did not affect their recovery time, which suggests that multiple mild head impacts are just as severe as a single impact (Beckwith et al.). Some researchers now believe certain athletes may be more vulnerable to a concussion or more severe concussions due to extrinsic factors (e.g., concussion history), intrinsic factors (e.g., genetics), or both (McDevitt et al., 2010; Tierney et al., 2009).

Concussion threshold has yet to be established because the accelerations required to cause injury are dependent on these intrinsic and extrinsic factors (Tierney et al., 2009). Extrinsic factors include impact magnitude, direction, and location (Broglio et al., 2010; Ommaya & Gennarelli, 1974; Pellman, Viano, Tucker, & Casson, 2003). Intrinsic factors include head position upon impact (Ommaya, 1995), number and severity of prior impacts, and other individual characteristics of the athlete (e.g., genetics; Guskiewicz et al., 2007). Controlling extrinsic factors researchers can view variable clinical outcomes (Broglio et al., 2010; McCrory, Johnston, Mohtadi, & Meeuwise, 2001), allowing researchers to observe the athlete's intrinsic characteristics that may be important factors in determining their susceptibility to concussions (Guskiewicz et al., 2007; Ommaya, 1995). One possible varying intrinsic factor is a person's genotype.

An individual's genotype determines the structure and function of their body's proteins and, ultimately, how their body will respond to stress (e.g., head impact). For example, a commonly occurring variation (e.g., repeat polymorphism) existing in a gene promoter region, where transcription for a protein begins could cause neuronal protection against injury. A variation within the promoter region may alter the amount of protein levels, which could possibly alter the function of the protein as a result. Individual structural differences in neuronal proteins, such as channel proteins that allow ion flow, could influence the way an individual's brain responds to an applied force and may represent a predisposing or protective factor to concussions. Much research has been performed to diagnose and identify s/s of concussions, but no well-controlled study has explored the reasons why some athletes take a longer time to recovery from a concussion than others.

Pathomechanics & Pathophysiology

A mechanical insult resulting in acceleration and deceleration of the skull can initiate a complex cascade of neurochemical events (Barkhoudarian et al., 2011). This occurs when the head is moving quickly and then is abruptly halted by a blunt object (Gaetz, 2004). Researchers have reported that better outcomes come from sagittal plane (front to back) impacts and poorer outcomes come from a coronal plane (side to side) movement, while rotational injuries had the worst outcomes (Gaetz). Stress waves are generated by the collision and cause nervous tissue strain

(Davis, 2000). Mild forces will cause stress closer to the surface of the brain and as the force increases the deeper the damage will be within the brain (Gaetz). The Ommaya-Gennarelli model demonstrated that the depth of the lesion is positively correlated with level of consciousness (Ommaya & Gennarelli, 1974). Several researchers reported concussions can develop with as little as to 60 g to 80 g to as high as 127.8 g ± 31.8 g (Schnebel et al., 2007; Zhang, Yang, & King, 2004). As the acceleration and deceleration forces increase, structural and/or chemical damage occurs on a continuum (Gaetz). The mild forces interrupt the superficial aspect of the brain tissue projecting inward with more devastating as forces (Gaetz).

This head acceleration is directly related to brain injury severity (Genneralli et al., 1982) and causes a neurometabolic cascade (Giza & Hovda, 2001). Damage will cause stress to the nodal region of the neuron. If the neuron is strained 5% to 10% beyond its resting length, it will lead to several short-lived (two to three minutes) of ionic imbalances, where an action potential will fail to fire (Gennarelli, 1996). If the strain is 15% to 20% past its resting length, this structural change causes a functional ionic imbalance within the neuron. Specifically, there is an increase in intracellular Ca^{2+} and an efflux of K^{+}. Over 20% beyond the neuron's resting length results in axonatomy, or complete tear of the neuron (Gennarelli). Most concussion injuries are caused when the neuron is stretched beyond the 15% range, typically by the brain shaking within the skull (Gaetz, 2004).

Primary or focal injury is caused by the acceleration-deceleration or rotational brain injury; however, secondary injury is what continues the injury process. When there is a focal injury, that area of the brain receives significantly less blood flow and causes the neuron to become ischemic (Bullock et al., 1991). The indiscriminate release of excitatory amino acids such as glutamate can also lead to excitotoxicity, playing a specific role in secondary ischemic damage (Choi, 1988). This is why a concussion is a process and not an event. There are two main theories concerning neuropathology of concussion injuries. The first is termed diffuse axonal injury, where neuropathologic brain injury is caused by sudden deceleration strain on the neuron by a shearing force. These shearing forces disrupt small blood vessels and axons causing swelling, hemorrhage, and decreased connectivity (Polvishock & Becker, 1985). The second is referred to as the neurometabolic cascade. This occurs when the neuron is stretched beyond 15% to 20% resting length, which initiates action potentials and an indiscriminate release of neurotransmitters (Giza & Hovda, 2001).

Persistent action potentials results in an ionic imbalance, which causes a release of an excitatory amino acid, glutamate, to bind to its receptor on the N-methyl-D-aspartate channel (NMDA) that leads to further neuronal depolarization along with the efflux of potassium (K^+; Giza & Hovda, 2001). K^+ is typically picked up by glial cell, but such a massive efflux cannot be efficiently maintained (Giza & Hovda). Extracellular increases in K^+ can continue for up to several hours (Giza & Hovda). The ionic imbalance causes the sodium-potassium pump to work harder trying to restore homeostasis (Giza & Hovda). The sodium-potassium pump needs adenosine triphosphate (ATP) to function triggering the need for more ATP (Giza & Hovda; Saladin, 2007), placing the brain in a hypermetabolic state (Giza & Hovda). Due to the already diminished blood flow, where hypoxic levels can be sustained up to 30 minutes following a concussive injury, there is also causes a lower concentration of glucose and oxygen to produce ATP (Giza & Hovda). Day four-post concussion has shown that cerebral blood flow decreases from 50 mL per 100 g per minute to 32.3 mL per 100 g per minute (Martin et al., 1997). This energy crisis state leaves the brain vulnerable, decreasing its ability to respond adequately to a second head impact (Giza & Hovda).

Within this period of elevated glucose utilization, the neurons goes into a state of depressed metabolism (Giza & Hovda, 2001). Persistent increases in intracellular Ca^{2+} worsen the condition by impairing mitochondria metabolism (Giza & Hovda). Even at rest oxidative metabolism is running at near maximum capacity so the slightest disruption can cause problems (Giza & Hovda). Due to low glucose and oxidative metabolism, there is a production of lactic acid (Giza & Hovda). Pre-injury levels of lactic acid are about 0.150 mM, and after a concussive event lactic acid increases to about 0.315 mM (116.6% increase above resting levels; Kawamata, Katayama, Hovda, Yoshino, & Becker, 1995). Lactic acid also causes inflammation, altered neurotransmission, and increases in blood brain barrier permeability.

The accumulation of lactic acid causes a decrease of magnesium levels (Mg^{+}; Giza & Hovda). Mg^{+} is necessary for maintaining resting membrane potential and is the ‘plug’ to halt Ca^{2+} from entering through NMDA channel. Decreases of Mg^{+} levels can sustained for up to four days. Increased intracellular Ca^{2+} ions are typically picked up by the mitochondria; however, mitochondria become swollen and dysfunctional because of the massive Ca^{2+} influx and cannot fulfill their function. Therefore, this pushes the neuron further into an energy crisis and severely decreases ATP output (Giza & Hovda). This causes further depolarization and further release of glutamate to allow Ca^{2+} into the cell. Increased intracellular Ca^{2+} also signals calpain, an enzyme involved in disrupting neurofilament arrangement, (slices off neruofilament side arms) decreasing neural connectivity (Giza & Hovda). Increased intracellular Ca^{2+} also initiates glutamate excitotoxicity (Giza & Hovda).

Glutamate

Glutamate is the most abundant excitatory amino acid in the brain and serves numerous roles (Saladin, 2007). The immediate precursor of the inhibitory neurotransmitter γ-aminiobutric acid (GABA; Bak, Schousboe, & Waagepetersen, 2006), glutamate is an essential part of metabolism, a building block for many proteins, and is a potent neurotoxin (Bak et al., 2006). It is a nonessential amino acid that is not able to cross the blood brain barrier so it must be produced in the neuron from precursors (Saladin). Surrounding glial cells (i.e., astrocytes) maintain specific glutamate level by taking up excess glutamate (Danbolt, 2001). This shuttling system is known as the glutamate/GABA-glutamine cycle (Bak et al.). Assisted by the enzyme glutamine synthase, this system converts glutamate into the non-active form glutamine (Danbolt). There are five isoforms that have a high affinity for glutamate and are found throughout the brain to assist in the removal of glutamate from the neuron (Bak et al.) EAAT1 and EAAT2 are the most predominant within the brain (Danbolt). Glutamine is then released into the extracellular space where it is then transported from glial cells to the neurons (Danbolt). Finally, glutamine is converted into glutamate via phosphate-activated glutamine dehydrogenase (Wieonska, Stachowicz, Nowak, & Pilc, 2011). If an inhibitory action is needed, then glutamate will be further converted into GABA by decarboxylation catalyzed by glutamic acid decarboxylase (Liang et al., 2006). Glutamate is synthesized from the astrocytes surrounding the neuron, which is then packaged into vesicles by the transporter VGLUT, and released during an action potential (Coyle, 2006). Glutamate acts in three areas: 1. the presynaptic neuron 2. postsynaptic neuron and 3. between the glial cells (Machado-Viera et al., 2009).

Glutamate binds to one of three ionotropic glutamate receptors (e.g., NMDA; Choi, 1987). This allows the post-synaptic membrane channel to open and cause an influx of Ca^{2+} (Choi). Activation of the NMDA channels results in a significant flux of Ca^{2+} into the cell, which during a pathologic event (e.g., concussion) accumulates in the mitochondria and causes glucose and oxidative dysfunction. At rest, glutamate has been measured in rats to be approximately 3.62 *u*M, while five minutes after a concussion injury, glutamate increased to 4.60 *u*M (Katayma, Becker, Tamura, & Hovda, 1990). During a concussion, glutamate can be increased 10% to 15% beyond resting levels and may remain elevated for up to four days (Giza & Hovda, 2001).

Calcium

For normal cell function and resting membrane potential, there must be constant regulation of low intracellular Ca^{2+} between 50 nm to 300 nm (Choi, 1987). Following a brain injury, the influx of Ca^{2+} may be exacerbated by the amount of glutamate released and creates a positive feedback loop (Choi, 1988; Faden, Demediuk, Panter, & Vink, 1989). After a concussion, Ca^{2+} can be elevated as high as 12% to 64% beyond resting levels for two to four days; however, Ca^{2+} can continue to be elevated for up to 14 days (Giza & Hovda, 2001). In experimental models, Ca^{2+} was shown to be elevated hours after a concussive event and persisted for two to four days post-injury (Giza & Hovda). Cellular uptake mechanisms (e.g., taken up by mitochondria) at this point are unable to purge the excess glutamate due to the hypoxic condition (Choi). Glutamate enhances the ionic flux that promotes cytocellular edema (Katamaya et al., 1990; Strong et al., 2002), depletes energy stores (Anderson & Marmarou, 1992), and degrades structural proteins and the cell membrane (Choi).

Some researchers suggested that a concussive event does lead to a massive flux of ions, but not enough to produce cell death (Yoshino et al., 1991). However, this ionic flow does result in dysfunction within specific regions of the brain (e.g., hippocampus, cerebrum; Fineman et al., 1993). In a study with Ca^{2+} 45 tracer’s, researchers were able to follow the flow of Ca^{2+} and determined that there was no significant tissue death, but there was a marked increases in Ca^{2+} remaining up to four days that resulted in ischemia (Fineman et al., 1993). Researchers suggested that, though the first insult did not generate cell death, a second insult would result in cell death (Fineman et al., 1993). Thus, it is vital to maintain low intracellular Ca^{2+} concentrations for the brain to be able to respond efficiently following brain injury.

There are four main ways Ca^{2+} enters the cells: the Na^{+}/Ca^{2+} exchange, voltage-gated Ca^{2+} channels, release of Ca^{2+} from intracellular stores, and entry though the NMDA channel, which is the predominant route (Nicholls et al., 2001). Studies involving blocking the NMDA channel suggest that routes of Ca^{2+} other than NMDA channels are neither sufficient nor necessary for glutamate-induced excitotoxicity (Choi, 1988). Excitotoxicity is a term used to describe excitatory amino acid-mediated degeneration of neurons (Olney, 1978). NMDA is one of the three ionotropic glutamate receptors (Arundine & Tymianski, 2003) and may play a role in brain’s response to a head impact.

NMDA Channel

Sustaining a concussion can alter the activation of NMDA receptors. NMDA receptors are necessary for synaptic plasticity, specifically long-term potentiation (Jablonska et al., 1995). The primary function of the NMDA channel is to act as synaptic connectivity between two neurons as well as trigger postsynaptic potentials and dendritic spikes, where action potentials are formed (Coyle, 2006). The regulated release of glutamate from one neuron to another is the basis of synaptic transmission, which occur at ~ 10^{14} synapses in the human brain (Kandel, Schwartz & Jessel, 1981).

Due to the NMDA's channel specific functions, it has an intricate structure. The NMDA channel contains both ionotropic and a voltage-gated mechanism for channel function. An ionotropic glutamate receptor is one where a ligand attaches to the receptor to cause a conformational change in the protein shape, which will then allow a biochemical cascade (Nicholls et al., 2001). Due to this structure, the NMDA channel needs two signals to activate (i.e., membrane voltage change & neurotransmitter binding). Thus, NMDA has two specific areas of sites (Machado-Vieira et al., 2012). The first site area is located on the outside of NMDA channel and allows for the ligand gating mechanism to function (Machado-Vieira et al.). Specifically, there is the D-serine site where glycine binds and the homocysteine site where glutamate binds (Machado-Vieira et al.). The second area is located within the NMDA channel’s pore for the voltage-gated mechanism. The pore contains three sites: the phencyclindine site, zinc site, and the magnesium site (Machado-Vieira et al.). The zinc site and phencyclindine sites inhibits the current while the magnesium site physically blocks the pore of the channel (Machado-Vieira et al.).

A voltage change (i.e., depolarization) releases the Mg^{2+} plug and glutamate along with glycine binding allowing for cation influx (i.e., Ca^{2+} Na^{+}; Barkoudarian et al., 2011). Two of the NMDA subunits (NR1) are bound with glycine, and two of the subunits (NR2) are glutamate-binding sites. The subunits are situated so the NR1 subunits are diagonal to the NR2 subunits (Rambhadran, Gonzalez & Jayaraman, 2009). The functional diversity of the NMDA receptors is embedded in their heteromers, whereas non-NMDA receptors like AMPA can form functional channels with glutamate alone. NR1 is required for channel function, and NR2 affects channel biophysical and pharmacological characteristics (Lynch & Guttmann, 2001).

The rate at which the NMDA receptors depolarize and repolarize upon application and removal of their agonists (e.g., glutamate), respectively, are slower than the non-NMDA receptors (e.g., AMPA; Forsythe & Westbrook, 1988;

Vicini et al., 1998). It is this slower rate of deactivation that measures the duration of the excitatory postsynaptic potential, which is the measure of strength of synaptic signaling (Lester, Clements, Westbrook & Jahr, 1990). NMDA is composed of four subunits producing a ligand gated cation (e.g., Ca^{2+}) channel (Mosley et al., 2009). Crystal structures of the NR2-NR1 heterodimer complex structure suggest its function for opening the ion-channel with ligand application (Furukawa, Singh, Mancusso & Gouaux, 2005). Analysis of the NR1-NR2 heterodimer complex along with biochemical and patch clamp experiments confirms that the NR1-NR2A heterodimer is the functional unit in the four-subunit NMDA channel (Furukawa et al., 2005). This NR1-NR2A heterodimer crystalizes as a single complex with three subsites (i.e., I, II, & III). This arrangement of subunit interaction is responsible for the conservation of the ligand-binding core within the NMDA receptors, NMDA modulation, and its ability to have a 'clam shell' in closer proximity compared to non-NMDA channels (e.g., glycine-glutamate complex is approximately 32 angstroms & GLUR2-glutamate complex is approximately 38 angstroms; Furukawa et al.).

With regard to its function for long-term potentiation, of the neuron NMDA channels are associated with learning and memory (Barkhoudarian, Hovda, & Giza, 2011). Thus, dysfunction of the NMDA channel has been implicated in several cognitive brain diseases (e.g., Parkinson's disease, Huntington's disease, & schizophrenia; Clements & Westbrook, 1991). These types of receptors mediate excitatory synaptic transmission in the central nervous system (Mosley et al., 2009). In homeostatic conditions, the NMDA subunits have roles in learning, synaptic communication, and memory (Wang, Hu & Tsien, 2006). Under abnormal conditions such as during a concussion, NMDA receptors are being over stimulated from an increase of glutamate within the synaptic cleft (Seripa et al., 2008). This will then lead to an increase in postsynaptic neurons being stimulated (Seripa et al.). Within a study utilizing calciseptine (i.e., a reagent that block L-type Ca^{2+} channels) researchers demonstrated that it blocked excitotoxicity producing a neuroprotective effect *in vitro* and *in vivo* (deWeille, Schwietz, Maes, Tartar, & Lazdunski, 1991). Though, there may be several key players to produce glutamate excitotoxicity, ionotropic receptors NMDA is recognized as the major source (Choi, 1987). It has also been noted that glutamate excitotoxicity is primarily dependent on the influx of Ca^{2+} when glutamate attaches to the NR2 receptors (Choi).

NR2A NMDA Subunit

Each NR2 subunit has four membrane domains, an extracellular amino terminal, and an intracellular C-terminal tail (Hollmann & Heinemann, 1994). The main NR2 subunits are called NR2A and NR2B (Morikawa, 1998), and are encoded by the *GRIN2A* and *GRIN2B* genes, respectively (Wu et al., 2010). The crystal structure of NR2A reveals specific recognition sites and binding mechanisms for glutamate (Furkukawa, Singh, Mancusso & Gouaux, 2005). Compared to non-NMDA receptors, NR2A has a negatively charged residue (D731), which binds to a positively charged aspartate group negating the need for a salt bridge like in non-NMDA glutamate receptors. Glutamate binds to NR2A by displacing water molecule W2 (Furkukawa et al., 2005). Another feature that is distinct to NR2A compared to non-NMDA glutamate receptors is its Van der Waals contact between y-carboxylate group and the Y730 residue, conserved in NR2 subunits, (Furkukawa et al.). This contact may partially account for its high affinity for glutamate (Furkukawa et al.). NR2A remains open for a shorter time compared to NR2B subunits (Scheetz & Constantine-Paton, 1994). One main difference between NR2 subunits and NR1 subunits is that NR2 may independently bind to PDZ1 domain of PSD-95, mediates the linkage and proximity for synaptic organization, (Niethammer, Kim & Sheng, 1996). This scaffolding activates the nitride oxide synthase, produces nitric oxide for cell signaling, by Ca^{2+} entry through NMDA rather than other channels (Garthwaite, Charles & Chess-Williams, 1988).

Intracellular Ca^{2+} activates several signaling pathways with different potential consequences (e.g., cell death, decreasing/increasing synaptic strength; Hardingham & Bading, 2003). The channel will close by a physiological concentration of Mg^{2+} acting as 'plug' and will only again permit flow of Ca^{2+} ions into the cell upon depolarization of the cell, which will remove the Mg^{2+} 'plug' (Rambhadran, Gonzalez, & Jayaraman, 2009). If there is excessive activation of the NMDA receptors, this can lead to neuronal death and has been proposed to occur in conditions in which glutamate levels are elevated (e.g., concussion; Lipton, 1993).

Using NR2A or NR2A and NR2B knockout mice, researchers found less brain ischemia in mice with no NR2A receptor.

Authors suggested that NR2A may mediate glutamate excitotoxicity due to the decreased amount of blood volume, which could be due to the fact that it produced less NMDA channel activity (Morikawa et al., 1998). Less functional NMDA channels equate to less Ca^{2+} entering the cell causing excitotoxicity. Clinically, athletes react differently to similar concussive forces; one of the reasons could be a genetic polymorphism within the NMDA channel.

Using a fluid percussion study with rats, researchers found that the number of NR2A subunits both bilaterally and ipsilateraly are down regulated in the cerebral cortex following a head injury (Giza et al., 2006). These NR2A levels were reduced for up to four days, while NR2B and NR1 subunits were not significantly affected (Giza et al.). However, these changes were only seen on a protein level and not within the mRNA transcription levels (Giza, et al.). This suggests there were no changes in number of NMDA receptors (Giza et al.). Clinically, this change in NR2A/NR2B ratio could lead to developmental deficits and impaired neuron plasticity (Giza et al., 2006).

Utilizing rat hippocampal brain tissue, researchers demonstrated that following a head injury (i.e., 6 hours, 12 hours) there was a significant decrease of the NR2A and

NR2B subunits within the contralateral and ipsilateral sides (Kumar, Zou, Yuan, Long, & Yang, 2002). However, there was no corresponding change of the mRNA subunits, which suggests that the decrease in subunit protein levels were not due to down regulation of gene expression post injury (Kumar et al., 2002). The decrease of subunit protein could be attributed to enzymatic degradation from calpain-like protease (Kumar et al.). Calpain has been shown to increase significantly after a concussion (Kampfl et al., 1997), which could degrade glutamate receptors by cleaving the C-terminus of the glutamate receptor subunits (Bi & Baudry, 2000). These researchers discussed the possibility that this transient decrease of NR2B subunit may interrupt temporary memory impairment following a TBI event, but would not contribute to any long-term memory affects (Kumar et al.). This suggests that the subunit is important for NMDA channel function, specifically for channel conformation to allow Ca2+ to flow into the cell. If the NR2A subunit is not produced it could generate a hypofunction effect within the NMDA channel, which could result in variable recovery from a concussion. One way the NR2A subunit would not be transcribed is by a genetic variation within the promoter region.

GRIN2A

There are two main genes encoding the NMDA receptor subunits. The *GRIN1* gene produces NR1 subunits. The GRIN2A and GRIN2B genes produce the NR2A and NR2B subunits, respectively (Anderson, Stenqvist, Attersand & von Euler, 2001). Expression of the NR2A subunit occurs predominantly in the cerebral cortex (Mohrmann, Hatt & Gottmann, 2000). Expression begins around puberty and maintains expression throughout adult life (Watanabe, Inour, Sakimura & Mishina, 1993). *GRIN2A* is located at locus 16p13.13 (Kebir, Tabbane, Sengupta & Joober, 2008). This gene contains 14 exons and 13 introns, and is about 3,387 nucleotides long (Itokawa et al., 2003).

Researchers utilizing *GRIN2A* knockout mice suggested mice lacking the NR2A subunit were less prone to cerebral injury after focal brain ischemia (Morikawa et al., 1998). Western blot analysis depicted that there was no compensatory NR2B subunit produced in the loss of NR2A and the NMDA channel activity in the hippocampus was 50% less than that of the wild type mice activity (Morikawa et al.). This demonstrates that a decrease in the functional NMDA channels made the mouse more resistant towards glutamate-induced excitotoxicity (Morikawa et al.). Wu et al. (2010)

examined several polymorphisms within the NR1 and NR2 subunit encoding genes *GRIN1* and *GRIN2*, respectively, and found that individuals carrying those polymorphisms have a reduced risk of Parkinson’s disease, due to the hypofunction effect of the NMDA channel. This disease is a neurodegenerative disease, and glutamate-mediated hyper-neurotransmission is associated with many neurodegenerative diseases (Wu et al.). There is one repeat polymorphism in particular [e.g., (GT)n] that has been suggested to decrease the amount of transcription, thus diminished production of the NR2A subunit. If there are less functional NMDA channels, this could lead to hypofunction of the NMDA channel resulting in a protective effect against brain injuries (e.g., concussions).

GRIN2A (GT)n Repeat Polymorphism

Researchers have studied the frequency of repeat polymorphisms within the genome and found that there are more repeat polymorphisms within the promoter regions compared to other regions of the gene, and those polymorphisms that lie in the promoter region near the transcription start site are highly conserved (Sawaya et al., 2013). They also suggest that many promoter repeat polymorphisms have the potential to affect phenotype by generating mutations in regulatory elements, which could ultimately result in disease (Sawaya et al.).

Approximately 3% of the human genome is made up of repeat polymorphisms, and the GT motif is an important polymorphism (Warren et al., 2008). The *GRIN2A* (GT)n polymorphism lies within the 5' upstream regulatory region in the promoter region (i.e., region that is responsible for transcriptional regulation; Wu et al., 2010). The (GT)n is a repeat polymorphism or a variable nucleotide tandem repeat (VNTR; i.e., a repeating sequence of DNA), where n is the number of times the nucleotides guanine (G) and thymine (T) repeat themselves in the sequence (Itokawa et al., 2003). The (GT)n VNTR is found in *GRIN2A* at position 16p13.13, which codes for the sequence that develops the production of the NR2A subunit within the NMDA channel. The short arm of chromosome 16 has shown modest linkages for mood disorders (e.g., bipolar, schizophrenia; Itokawa et al.). Several compelling candidate genes have come from this area due to its relevance to the "hypoglutametergic hypothesis" for mood disorders, specifically in *GRIN2A* (Itokawa, Yamada, Iwayama-Shigeno et al., 2003; Itokawa, Yamada, Yoshitsugu, et al., 2003). It has been shown that blocking NMDA receptor produces behavior that is similar to cognitive problems similar to those seen in schizophrenia (Gilmour et al., 2012). Cognitive problems are also associated with a concussion injury (Guskiewicz et al., 2007), so this VNTR may also have an association with concussion severity and recovery. These genes affect glutamatergic neurotransmission or impairing the NMDA receptors producing negative symptoms in schizophrenic patients (Coyle, 2006). One of these VNTR that reduces glutamate binding is the (GT)n repeat within *GRIN2A*.

Several researchers investigated the (GT)n VNTR. Investigators used an *in vitro* promoter assay to demonstrate that longer alleles of (GT)n VNTR expression of *GRIN2A* (Itokawa et al., 2003). It was found that the longer the repeats, the more transcriptional activity is repressed, where this reduction would cause a diminution in promoter activity (Itokawa et al.). This could mean that there are less glutamate binding sites available within the cerebral cortex of the brain (Itokawa et al.). This glutamate dysfunction has been associated with several diseases (e.g., schizophrenia, alcoholism; Blecha et al., 2011; Harrison & West, 2006). If this decrease in *GRIN2A* transcription translated into less protein being produced, that could alter the channel activity. For example, if less of that protein is being produced, than less glutamate can bind to allow Ca^{2+} into the cell. Shang et al. (2011) showed a VNTR within an exon region caused alternative splicing, where shorter repeats increased the ratio of alternatively spliced compared to regularly spliced product. The alternatively spliced mRNA was not able to produce the same amount of protein the regularly spliced product produced (Shang et al., 2011). However, there is no research on promoter (GT)n repeat polymorphism affecting protein levels, or on protein channel activity levels within *GRIN2A.* There has been some research that associates this decrease in transcription to several phenotypes like levels of bipolar disease and susceptibility to schizophrenia (Itokawa, Yamada, Iwayama-Shigeno et al., 2003; Itokawa, Yamada, Yoshitsugu, et al., 2003)

In a Japanese population case-control study investigating promoter VNTR, researchers were able to demonstrate a significant difference between those with a long allele (≥ 25 repeats) compared to those with the short allele (< 25 repeats; Iwayama-Shiegeno et al., 2005). Those with the long allele were more susceptible to schizophrenia (Iwayama-Shiengo et al.). Inoue et al. (2010) examined the promoter VNTR and found that those carrying longer repeat had significantly less volume within the hippocampal and amygdala brain regions compared to those carrying the short allele. The (GT)n repeat has been shown to have similar effect within other genes.

Within the *HO-1* promoter, there was a difference seen between those carrying the long versus short (GT)n repeats and promoter expression activity (Rueda et al., 2007). Those with the short allele had higher levels of expression with an increased risk for rheumatoid arthritis compared to those carrying the long allele (Rueda et al., 2007). The (GT)n repeat within *HMOX1* promoter has also been shown to alter transcription activity (Bao et al., 2010). Those carrying the long allele had an increased risk for type II diabetes compared to those carrying the short allele due to the increased *HMOX1* promoter activity in those carrying the short allele (Bao et al.). Thus, the (GT)n repeat is a genetic biological phenomenon, and has been established as a potential marker for association studies. Concussions are also caused by a biochemical imbalance that is provoked by an increase in glutamate levels.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are DNA sequence or allelic variations that occur when a single nucleotide (adenine, thymine, cytosine, or guanine) is altered in the genome (Saladin, 2007). A SNP is a single nucleotide that has a variant allele in greater than 1% of the population (Roth, 2007). SNPs are distributed randomly through the genome. This results in thousands of genetic variations that may contribute to the structural diversity of human proteins (Venter et al., 2001). In a large sample of aligned human sequences there was approximately 1 SNP for every 500 to 1,000 bases (Sherry, Ward & Sirotikin, 1999).

SNPs may fall within exons, introns, or in the intergenic regions (Gelehrter et al., 1998). Only small portions of SNPs, approximately 1%, have the ability to impact the functions of proteins. The rest are silent SNPs and do not affect the protein’s structure or function (Gelehrter et al., 1998). There are several classes of SNPs (e.g., substitution, deletion, insertion, repeat) based on the type of alteration. The simplest class of polymorphisms is a substitution, where a single base mutation substitutes one nucleotide for another (Palmer & Cookson, 2001). For instance, there is an adenine substituted for a thymine. In addition to the SNPs that directly alter the protein structure and/or function (e.g., insertion, missense, nonsense), there are also SNPs that occur outside the exon area, in the promoter, intergenic and intron regions (Roth, 2007).

A SNP in the promoter region of a gene does not affect the amino acid sequence directly, however, it can alter the level, location, or timing of transcription (i.e., gene expression), with relative risk of phenotype ranging from low to high depending on the region (Roth, 2007). Due to the promoter function, a SNP within the promoter region could have a significant impact on the protein. A SNP in the promoter region can inactivate the transcription control element involved in that gene's transcription (Barnes & Gray, 2003), as well as affect a binding site, which could potentially affect another gene or could have no direction function at all (Barnes & Gray). Therefore, identification of the binding site affected by the SNP is not enough to estimate the functional consequence (Barnes & Gray).

SNPs in regulatory regions are difficult to assess because it is challenging to locate (Barnes & Gray, 2003). Once it is determined the SNP lies within the promoter region it is possible to evaluate the effect of the SNP on the regulatory unit (Barnes & Gray). In addition, it is necessary to ascertain whether or not affected element is part of the transcriptional module or not (Barnes & Gray). SNPs within genes associated with ionotropic glutamate channels in the brain may alter the protein channels, creating an environment that could be detrimental if one sustained a head impact. SNPs can cause variations within genes, which alter protein function and result in reduced integrity of the channel.

Repeat Polymorphism

Another type of genetic variation is known as a repeat polymorphism or VNTR (Roth, 2007). Repeat polymorphisms are repetitive stretches of short DNA sequences (Roth). VNTRs are sequences that consist of two nucleotides (i.e., dinucleotide) that are consecutively repeated (e.g., GTGTGTGTGT). VNTR DNA make up about 3% of the human genome (Lander et al., 2001). They vary throughout the genome as well as among individuals in the number of repeats at that specific site (Klug & Cummings, 2003). Dinucleotide repeats are more frequent than mononucleotide and trinucleotide repeats (Ellegren, 2004). The nomenclature for a microsatellite begins with four main pieces of information. First 'D' means that it involves DNA (Knight, 2009). The next number refers to its chromosome, and 'S' indicates that it is short tandem repeat type of VNTR (Knight). Finally, it ends with a four digit unique identifier (Knight).

Genomic repeats form from the processes known as replication DNA slippage within the cell cycle (i.e., outside of the S period) in which DNA mismatch repair is inactive (Hawk et al., 2005). Other processes that can form repeat polymorphisms are copy-choice recombination, indel slippage, and recombination events (Richard & Paques, 2000; Leclercq, Rivals & Jarne, 2010; Viguera, Caceill & Ehrlich, 2001). DNA slippage involves three main steps (Viguera et al., 2001). First, DNA polymerase pauses during the replication process due to a barrier on the lagging strand (Viguera et al.). It has been found that DNA pausing can arise from a GC-rich environment, or commonly pauses at the base of the hairpin structure (Viguera et al.). This first step was confirmed based on the correlation of replication pausing and frequent rearrangements in repeated sequences (Viguera et al.). Secondly, when DNA polymerase pauses it briefly dissociates from 3’ end of DNA, which is partly due to a trapping agent that prevents further extension at the pause site (Viguera et al.). When DNA polymerase disassociates it must be replaced by a specialized polymerase, which can lead to the addition of nucleotides (Viguera et al.). Finally, DNA polymerase re-anneals, where only the tip of the new strand dissociates and re-anneals with the complimentary base sequence (Viguera et al.).

There are several factors that may stimulate slippage that would produce repeat polymorphisms, such as single-stranded DNA binding protein or specific point mutations within the polymerase exonuclease domain (Canceill, Viguera & Ehrlich, 1999). DNA polymerase slippage is correlated to the number of mismatches, where the more mismatches equals the number of mismatches on the primer strand (Sia et al., 1997). It has also been noted that chromosomal regions rich in guanine and cytosine affect base substitutions (Brown & Jiricny, 1989). There is also a correlation between distance between mismatch and rate, where mismatch repair efficiency increases as the distance between the mismatch and the nick (directs strand specific removal) decreases (Marsischky & Kolodner, 1999). Additionally, mutation rate increases with longer repeat polymorphisms; however, this has recently been shown to plateau at a certain length (Leclerq, Rivals & Jarne, 2010).

The (GT)n repeat is one of the more common repeat polymorphisms (Tautz & Renz, 1984). The number of sequence repeats differs among individuals (Roth, 2007). The number of repeats is used to differentiate the disease causing from the non-disease causing allele, thus making the length (i.e., number of times the sequence is repeated) the genotype (Roth). This can allow many different genotypes depending on the number of repeat in the individual (Roth). The VNTR also differs in rate of frameshift mutations, which was probably due to DNA polymerase slippage (Hawk, Stefanovic, Boyer, Petes & Farber, 2005). Hawk et al. (2005) found that during replication of the (GT)n segment the primer and template DNA strands dissociated and re-associated out of alignment. When the displaced repeat is on the primer strand and there is a failure to correct this misalignment, and leads to an addition (Hawk et al.). Conversely, if the mismatch appeared on the template strand the result would be a deletion (Hawk et al.). Additions are more common than deletions because the template strand have a better substrate for mismatch repair than those displaced on the primer strand (Sia, Jinks-Robertson & Petes, 1997).

Due to the variable length of the DNA sequence, typical DNA sequencing methods (e.g., florescent tags, restriction fragment length polymorphisms) cannot be used for sequencing a repeat polymorphism because they work on one specific point, as would be the case in a SNP (Roth, 2007). Instead, the entire target DNA segment must be sequenced. The common method in sequencing repeat polymorphisms is using the Sanger sequencing method or chain termination sequencing (Roth). Another technique used is capillary electrophoresis followed by fluorescent-based fragment size analysis. The primary steps in a fragment size analysis (e.g., VNTR genotyping) begins with the generation of the fragment with target polymorphism, separation of the fragments using capillary electrophoresis, determination of fragment size, and assignment of alleles (e.g., (GT)n repeat length; Innocenti & Imele, 2005). The fluorescent reagents utilized are designed to emit specific wavelengths of light when excited (Innocenti & Imele). Lasers are used to induce fluorescent light excitation and detection of the emitted light (Innocenti & Imele). These florescent tags are attached to the 5′ end of the forward primer. These primers are then used during PCR and will generate copies of the fluorescent tags incorporated into the targeted gene sequence, which will be detected by the capillary electrophoresis system (Innocenti & Imele). Capillary electrophoresis also incorporates electrosmosis, or the migration of fluid to positive end of detector. This reaction involves electrophoretic migrations, where the negative ions move, because DNA is a negative material due the utilization of buffers, to the positive end (Innocenti & Imele,). It is run within a capillary tube instead of on an agarose gel (Innocenti & Imele). The end product is a production of an electropherogram, which is similar to the chromatogram except that there are multiple paths on a chromatogram and only one flow path on electropherogram (Innocenti & Imele). This eliminates the steps of running it on a gel and wavelength detector (Innocenti & Imele) Utilizing the electropherogram as well as computer software (e.g., genemapper, or peak scanner software) the genotype can be determined by the height of the peak correlating to the nucleotide length (Innocenti & Imele). Dinucleotide polymorphisms like the (GT)n are typed using PCR along with unique primers that flank the repeat and then genotype can be determined with capillary electrophoresis; however sometimes 'shadow bands arise' due to several different reasons (e.g., incomplete denaturation of PCR product, slipped strand mispairing, or due to annealing of truncated products; Hauge & Litt, 1993). Researchers demonstrated by direct sequencing that different genotype length depend solely on the variations of the dinucleotide length, and rule out PCR recombination and support slipped strand mispairing for the generation of 'shadow bands' (Hauge & Litt).

Repeat polymorphisms have been used traditionally as markers because they can cause more variation (e.g., more possible alleles) than a SNP (Roth, 2007). However, most repeat polymorphisms occur in the intronic region of DNA, but can still be correlated to the phenotype (e.g., longer return to play time; Roth). In exonic DNA microsatellites can cause frameshift mutations disrupting codon reading (Metzgar et al., 2000). The most frequent dinucleotide repeat (i.e., CA, AC, TG or GT) are found on average once every 36 kb (Ellegren, 2004). SNPs may cause dinucleotide repeats, where altering one sequence would cause a run of three dinucleotide repeats. Additionally, an insertion or deletion may generate a new dinucleotide repeat, and these small repeats serve for expansion of the genome (Knight, 2009). Many neurodegenerative diseases are associated with length modifications of repeat polymorphisms (Wells, 1996).

A variable dinucleotide repeat (GT)n within *GRIN2A* promoter region (the region involved with gene expression) has been shown to regulate the gene expression level in a length dependent manner, where ≤ 25 is the long allele, and ≥ 24 is the short allele. Genetic variants within the promoter region have functional effects such as affecting the level, location, or timing of gene expression (Roth, 2007). For example, polymorphism in the promoter may alter the sequence of a transcription factor binding signal which may prevent the transcription factor from binding to the promoter and could alter the amount of mRNA and protein that are available to the cell (Roth).

If the promoter was not working efficiently, less of that protein would be produced, in this case less NR2A subunits within the NMDA channels. This could alter the amount of glutamate receptors available and therefore cause hypofunction of the NMDA channel resulting in a decrease in excitotoxicity. Genetic variation within the NR2A subunit of NMDA receptor may be a factor in regulating glutamate binding and Ca^{2+} influx, and ultimately, concussion recovery as well as concussion severity. Clinically, due to a potential decrease of Ca^{2+} entering the cell this variant could cause a protective effect in concussed athletes against more severe concussions.

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Appendix A

Informed Consent, Assent & HIPAA Form

College of Health Professions
TEMPLE UNIVERSITY®

Department of Kinesiology
Pearson Hall (048-00)
Philadelphia, PA 19122

phone 215-204-8707
fax 215-204-4414

CONSENT FORM

TITLE: The Temple University Concussion Registry
PROTOCOL NO.:
PARTICIPANT: ________________________________ **Number:** ________
SPONSOR: Temple University
INVESTIGATOR: Ryan Tierney, PhD, ATC, Jacqueline Phillips, MS, ATC
Department of Kinesiology, Temple University
Joseph Torg, MD; Department of Orthopaedics, Temple University

SITE(S):

Temple University Hospital	Temple Orthopaedics & Sports Medicine Satellite Office	Temple Orthopaedics & Sports Medicine Satellite Office
3509 N. Broad Street 5th Floor Boyer Pavilion Philadelphia, PA 19140 **215-707-2111**	414 Commerce Drive Fort Washington, PA 19034 **215-642-0700**	One Greentree Centre Suite 104 Marlton, NJ 08053 **856-596-0906**

STUDY-RELATED
PHONE NUMBER(S): Ryan Tierney, PhD, ATC: 215-204-4001 (office); 302-465-6836 (24 hour)
Jacqueline Phillips, Study Coordinator: 215 204-1963
Joseph Torg, MD, 215 707-1321

Temple University is not receiving monetary compensation for performing this research study. If you are a legal guardian providing consent for a minor, then below the term 'you' refers to 'your child.'

This consent form may contain words that you do not understand. Please ask the research team to explain any words or information that you do not clearly understand.

PURPOSE OF THE STUDY

You are being asked to allow us to store information measured during your concussion evaluation and treatment at Temple University. We are collecting this information to learn more about sport concussion.

PROCEDURES

Following signing of consent forms, you will proceed with the normal concussion evaluation which will be explained by the clinical coordinator. There is no difference in treatment based on your decision to participate. Your demographic and concussion evaluation information will be stored anonymously for future use.

During the evaluation we will ask you about the concussion event and your personal history. Personal history questions ask about your or your family history of headaches, migraines, ADHD, learning disability, depression, anxiety, and any other psychological or developmental issues. We will also assess how your brain is functioning by having you, for example, make facial expressions (e.g., smile), follow an object with your eyes, turn your head quickly side to side or up and down while focusing on a object at eye level, and balancing for 20 seconds while standing with your feet in a different positions and your eyes closed. Finally, we will also assess your brain function by having you take a computer test. The test will last for around 30 minutes and will assess, for example, your memory and reaction time by having you click on certain shapes or objects under various situations. Some questions we ask you may be personal, such as do you have any history of depression.

RISKS AND DISCOMFORTS

There are no short or long-term physical risks associated with this research. Some questions we ask you may be personal and make you uncomfortable. For example, do you have any history of depression? There is always a risk of losing data confidentiality; however, to reduce this risk we are storing all data anonymously in secure locations.

BENEFITS

You will not benefit directly from this study. However data from this research will be used to learn more about head injury in sports.

COSTS

There are no costs associated with participating in this project.

PAYMENT FOR PARTICIPATION

You will not be compensated for participating in this project.

CONFIDENTIALTY

All documents and information pertaining to this research study will be kept confidential in accordance with all applicable federal, state, and local laws and regulations. You understand that medical records and data generated by the study may be reviewed by Temple University's Institutional Review Board and the Office for Human Research Protections (OHRP) to assure proper conduct of the study and compliance with federal regulations. You understand that the results of this study may be published. If any data is published, you will not be identified by name.

COMPENSATION FOR INJURY

If you are injured as a result of the project or a procedure required to be done only as part of the project, your hospital and medical care will be provided at Temple University Hospital at no cost to you. Other financial compensation (such as lost wages or pain and suffering) for such injuries is not routinely available. By signing this consent form you are not waiving any of the legal rights that you otherwise would have as a participant in a research study.

VOLUNTARY PARTICIPATION AND WITHDRAWAL

Your participation in this project is entirely voluntary, and refusal to participate will involve no penalty or loss of benefits to you. You may discontinue your participation at any time without penalty or loss of benefits. Your participation in this project may be stopped at any time by the study investigator or sponsor without your consent.

REASONS FOR REMOVAL FROM THE PROJECT

The investigator may remove your data from the project if, for example, you fail to come to scheduled appointments.

OTHER INFORMATION

You understand you have no right to any commercial products derived from this study.

Information gained in this study is of no value to you or your doctor at this time.

QUESTIONS

If I have any questions about my rights as a research subject, I may contact the Institutional Review Board Coordinator at (215) 707-3390. The IRB Coordinator may also be reached by email: IRB@temple.edu or regular mail: Institutional Review Board Coordinator
Temple University Research Administration
Student Faculty Conference Center
3340 North Broad Street – Suite 304
Philadelphia, PA 19140

If you have any research-related and injury-related questions, you may contact Ryan T. Tierney, PhD, ATC: 215-204-4001 (office); 302-465-6836 (24 hour)

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions. If you agree to participate in this study, you will receive a signed and dated copy of this consent form for your records.

CONSENT

I have read this consent form and the study has been explained to me. All my questions about the study and my participation in it have been answered. I freely consent to participate in this research study. By signing this consent form I have not waived any of the legal rights that I otherwise would have as a subject in a research study.

The Temple University Concussion Registry

____
Subject Name

____________________________________ ________________
Signature of Subject Date

____________________________________ ________________
Signature of Person Conducting Informed Consent Discussion Date

____________________________________ ________________
Signature of Principal Investigator Date

College of Health Professions
TEMPLE UNIVERSITY®

Department of Kinesiology
Pearson Hall (048-00)
Philadelphia, PA 19122

phone 215-204-8707
fax 215-204-4414

ASSENT FORM

Study Title: The Temple Concussion Registry

What is this project about?

Professors from Temple University are doing a research study. The purpose of this study is to collect information about how sports-related concussions occur and why some people may take longer to recover.

Why are you being asked to be involved in this project?

You are being asked because you are a patient at Temple's concussion center.

What will happen to you if you are in this study?

If you and your legal guardians agree you can help, then you will go through the normal concussion evaluation. The study coordinator (the person who gave you this paperwork) will explain what happens during the evaluation. You will not be treated different based on your decision to help with the project. If you help, the only difference is that the information we collect will be stored in a computer file so we can use it to answer questions about concussions.

Will any parts of the study hurt?

Because you may have a concussion, part of the evaluation may make your concussion symptoms worse. For example, you may feel dizzier when asked to turn your head during the evaluation.

What if you have questions?

Please do not hesitate to ask the researchers any questions you may have about the study at any time. You can also ask your parents to ask questions for you.

What are your choices?

With your parent(s) consent, you can choose whether to participate in this study.

SIGNATURES

I have explained the study to ______________________(*print name of minor here*) in language he/she can understand, and the minor has agreed to be in the study.

____________________________________ ________________
Signature of Minor Date

____________________________________ ________________
Signature of Person Conducting Assent Discussion Date

____
Name of Person Conducting Assent Discussion (*print*)

Authorization to use and disclose personal health information for research at Temple University, Temple University Health System Affiliates, and Temple University Clinical Faculty Practice Plan

Information that will be collected from you and disclosed

During the course of this research study, which is described by title in the attached consent form and study-specific document, **certain** personal health information **will be collected and disclosed to** recipients **identified in this document. It is important for you to know that your personal health information may identify you by name, address, telephone number, photograph, social security number, health plan number, and date of birth, dates relating to various tests and procedures, or other personally identifiable information. This information may be obtained from your medical records, physical examinations and procedures: (a) to determine if you are eligible to participate in the research study or (b) created as a result of your participation in the research study.**

How your information will be used and to whom it will be disclosed

By signing this authorization form, you give Temple University, Temple University Health System affiliates, and Temple University Clinical Faculty Practice Plan, Temple University Institutional Review Board, and the investigator(s) named in the attached study-specific document, permission to use your personal health information and to disclose this information to the following recipients (as applicable): sponsor; sponsor's agents; governmental entities overseeing research in the United States and abroad, which may include in the United States, the Food and Drug Administration and the Department of Health and Human Services. It is important for you to know that the recipients, and their agents or representatives, will take all reasonable efforts to maintain your personal health information in confidence, and to use appropriate safeguards to prevent further use or disclosure by those not authorized to use or disclose your personal health information. However, once your health information is disclosed to the recipients, then your personal health information may no longer be protected by federal privacy laws and regulations and there is a potential for re-disclosure of this information. However, the laws of the Commonwealth of Pennsylvania or your state of residence may provide further privacy protection.

How you can access your information

You should know that you have the right to see and receive a copy of your personal health information that was collected from you during the research study for as long as this information is maintained by Temple University and the principal investigator. However, while the research study is in progress, you will not be able to access your personal health information in order to preserve the integrity of the research. You will be able to access this information when the study is completed. There may be associated charges for copying these materials.

How to revoke your authorization

You should also know that you can revoke your authorization to disclose your personal health information at any time by sending a written notice to the principal investigator and Temple University at the address listed in the attached study-

specific document. Should you decide to revoke your authorization, Temple University and the principal investigator will stop collecting your study-related health information. In addition, Temple University and the principal investigator will stop using and disclosing your personal health information, except to the extent such information was collected prior to your revocation. For instance, Temple University, principal investigator, recipients, and their agents or representatives may use the information obtained before you revoked your authorization in order to preserve the scientific integrity of the research study.

Important notices

You will receive a signed copy of this authorization to acknowledge your approval for Temple University and the principal investigator to the release your personal health information. If you do not sign this authorization or if you revoke this authorization, the principal investigator and Temple University may decide not to permit you to participate in or to continue to participate in the research study identified in the attached study-specific document.

Version #2: 05/21/2010 (second)

STUDY-SPECIFIC DOCUMENT

1. RESEARCH STUDY: The Temple University Concussion Registry

2. PRINCIPAL INVESTIGATOR: Ryan Tierney, PhD, ATC 120 Pearson Hall, 048-00. Department of Kinesiology Temple University Philadelphia, PA 19122

3. EXPIRATION DATE: ______ This Authorization does not expire.

4. OTHER INFORMATION: Personal and concussion event history. Concussion evaluation information from tests used to assess brain injury.

____

Signature of Patient Date

____

Printed Name of Patient

____

Signature of Personal Representative of the Patient Date

____

Printed Name of Personal Representative of the Patient and Relationship to Patient

____

Signature of Person Collecting Authorization Date

____

Printed Name of Person Collecting Authorization

Version #2: 05/21/2010 (second)

Appendix B

Genotyping Consent, Assent & GINA Forms

College of Health Professions
TEMPLE UNIVERSITY®

Department of Kinesiology
Pearson Hall (048-00)
Philadelphia, PA 19122

phone 215-204-8707
fax 215-204-4414

CONSENT FORM

TITLE: The Temple University Concussion Registry - Genotype
PROTOCOL NO.:
PARTICIPANT: ___________________________________ **Number:** ________
SPONSOR: Temple University
INVESTIGATOR: Ryan Tierney, PhD, ATC, Jacqueline Phillips, MS, ATC

Department of Kinesiology, Temple University
Joseph Torg, MD
Department of Orthopaedics, Temple University
Evgeny Krynetskiy, School of Pharmacy, Temple University

SITE(S):

Temple University Hospital	**Temple Orthopaedics & Sports Medicine Satellite Office**	**Temple Orthopaedics & Sports Medicine Satellite Office**
3509 N. Broad Street 5th Floor Boyer Pavilion Philadelphia, PA 19140 **215-707-2111**	414 Commerce Drive Fort Washington, PA 19034 **215-642-0700**	One Greentree Centre Suite 104 Marlton, NJ 08053 **856-596-0906**

STUDY-RELATED
PHONE NUMBER(S): Ryan Tierney, PhD, ATC: 215-204-4001 (office); 302-465-6836 (24 hour)
Jacqueline Phillips, Study Coordinator: 215 204-1963
Joseph Torg, MD, 215 707-1321

Temple University is not receiving monetary compensation for performing this research study. If you are a legal guardian providing consent for a minor, then below the term 'you' refers to 'your child.'

This consent form may contain words that you do not understand. Please ask the research team to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

PURPOSE OF THE STUDY
You are being asked to allow us to store information measured during your concussion evaluation and treatment at Temple University. We are collecting this information to learn more about sport concussion.

PROCEDURES
Following signing of consent forms, you will proceed with the normal concussion evaluation which will be explained by the clinical coordinator. There is no difference in treatment based on your decision to participate. Your demographic and concussion evaluation information will be stored anonymously for future use. To assess your genotype, we will ask you to spit into a tube enough to reach a fill line (2 milliliters). If necessary, we can provide sugar packets to help you create enough saliva.

RISKS AND DISCOMFORTS
This research involves sampling your DNA and the risk of loss of confidentiality can not be completely guaranteed. To reduce these risks your DNA will be stored in a secure location accessed only by research team members and their agents.

BENEFITS
You will not benefit directly from this study. However data from this research will be used to learn more about head injury in sports.

COSTS
There are no costs associated with participating in this project.

PAYMENT FOR PARTICIPATION
You will not be compensated for participating in this project.

CONFIDENTIALTY
All documents and information pertaining to this research study will be kept confidential in accordance with all applicable federal, state, and local laws and regulations. You understand that medical records and data generated by the study may be reviewed by Temple University's Institutional Review Board and the Office for Human Research Protections (OHRP) to assure proper conduct of the study and compliance with federal regulations. You understand that the results of this study may be published. If any data is published, you will not be identified by name.

COMPENSATION FOR INJURY
If you are injured as a result of the project or a procedure required to be done only as part of the project, your hospital and medical care will be provided at Temple University Hospital at no cost to you. Other financial compensation (such as lost wages or pain and suffering) for such injuries is not routinely available. By signing this consent form you are not waiving any of the legal rights that you otherwise would have as a participant in a research study.

VOLUNTARY PARTICIPATION AND WITHDRAWAL
Your participation in this project is entirely voluntary, and refusal to participate will involve no penalty or loss of benefits to you. You may discontinue your participation at any time without penalty or loss of benefits. Your participation in this project may be stopped at any time by the study investigator or the sponsor without your consent.

REASONS FOR REMOVAL FROM THE PROJECT
The investigator may remove your data from the project if, for example, you fail to come to scheduled appointments.

OTHER INFORMATION
You understand you have no right to any commercial products derived from this study. Information gained in this study is of no value to you or your doctor at this time.
QUESTIONS
If I have any questions about my rights as a research subject, I may contact the Institutional Review Board Coordinator at (215) 707-3390. The IRB Coordinator may also be reached by email: IRB@temple.edu or regular mail: Institutional Review Board Coordinator
Temple University Research Administration
Student Faculty Conference Center
3340 North Broad Street – Suite 304
Philadelphia, PA 19140

If you have any research-related and injury-related questions, you may contact Ryan T. Tierney, PhD, ATC: 215-204-4001 (office); 302-465-6836 (24 hour)

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions. If you agree to participate in this study, you will receive a signed and dated copy of this consent form for your records.

CONSENT

I have read this consent form and the study has been explained to me. All my questions about the study and my participation in it have been answered. I freely consent to participate in this research study. By signing this consent form I have not waived any of the legal rights that I otherwise would have as a subject in a research study.

The Temple University Concussion Registry- Genotype

____
Subject Name

______________________________________ __________________
Signature of Subject or Legal Guardian Date

______________________________________ __________________
Signature of Person Conducting Informed Consent Discussion Date

______________________________________ __________________
Signature of Principal Investigator Date

College of Health Professions
TEMPLE UNIVERSITY®

Department of Kinesiology
Pearson Hall (048-00)
Philadelphia, PA 19122
phone 215-204-8707
fax 215-204-4414

ASSENT FORM

Study Title: The Temple Concussion Registry - Genotype

What is this project about?
Professors from Temple University are doing a research study. A research study is when people collect a lot of information to learn more about something. The purpose of this study is to collect information about how genetics may influence how sports-related concussions occur and why some people may take longer to recover.

Why are you being asked to be involved in this project?
You are being asked to participate because you are a patient at Temple's concussion center.

What will happen to you if you are in this study?
After the concussion evaluation you will spit into a small plastic tube so we can find identify genes that may be important to our concussion research. Your saliva will only be used for concussion research. There is no difference in how you will be treated based on your decision to help with the project.

Will any parts of the study hurt?
No.

What if you have questions?
Please do not hesitate to ask the researchers any questions you may have about the study at any time. You can also ask your parents to ask questions for you.

What are your choices?
With your parent(s) consent, you can choose whether to participate in this study.

SIGNATURES

I have explained the study to ______________________(*print name of minor here*) in language he/she can understand, and the minor has agreed to be in the study.

___________________________________ ________________
Signature of Minor Date

___________________________________ ________________
Signature of Person Conducting Assent Discussion Date

____
Name of Person Conducting Assent Discussion (*print*)

Genetic Information Nondiscrimination Act Notice
Temple University Institutional Review Board

Genetic Information and why it is special

During the course of this research study, which is described by title in the study-specific document **attached, your** genetic information **will be collected, stored and/or analyzed as described in the Informed Consent Document. It is important for you to know the specific risks of genetics research and how you are being protected from these risks. Genetic information is different from other health information because it could be used to predict future health problems of you and/or your relatives. This information may be of interest to health insurance companies, life insurance companies, and disability insurance companies or your employer who could possibly use this information against you. For that reason, genetic information is considered separate from Personal Health Information which is defined on a separate HIPAA Authorization document.**

How your information will be used and your rights

You should know that you have consented for your genetic information to be used in the way and for the purpose described in the attached study-specific document. Storing your specimens containing the genetic information and use in future research may be considered. You should know that current and future research is overseen by a committee called the Institutional Review Board (IRB) which works to protect the rights and welfare of research participants. The IRB may require that you be re-contacted and ask for your consent to use your specimens in a specific research study. Refusal to consent at that time will not impact on your care or result in the loss of any benefits to which are you are entitled. Also, any blood, body fluids or tissue specimens obtained for this genetics study may contribute to significant scientific or medical breakthroughs. By signing this document and participating in this study, you waive the right to seek compensation for developments made using your genetic information.

Protection of your genetic information

By signing this authorization form, you give Temple University, Temple University Hospital and Temple University Clinical Faculty Practice Plan, the principal investigator and/or any sponsors named in the attached study-specific document permission to collect, store and/or analyze your genetic information. It is important for you to know that the recipients, and their agents or representatives, will take all reasonable efforts to maintain your genetic information in confidence, and to use appropriate safeguards to prevent further use or disclosure by those not authorized to use or disclose your genetic information. To further protect you, the GINA **law was passed to keep your genetic information from being used against you. GINA stands for the** Genetic Information Nondiscrimination Act **which bans health insurance providers and most employers from requiring or requesting your genetic information. The law prohibits health insurers from using genetic information to decide coverage, rates or preexisting conditions. Also, employers with more than 15 employees cannot hire fire or make promotion decisions based on your genetic information.**

Limitations of GINA

While GINA does help to protect you, you should know that certain information from your specimens is not protected by GINA. This includes blood counts, cholesterol tests, and liver-function tests. GINA also does not protect against analysis of proteins in your body that are related to a disease, disorder or condition that a doctor could find and use to diagnose a condition. Your health insurance company may increase your premium based on a blood test that shows that you have a preexisting disease. GINA will not protect your genetic information from being used against you by life insurance, disability insurance and

long-term care insurance companies. Also, GINA does not apply to employers with fewer than 15 employees. Individuals who are or will be working for such employers are not covered by GINA protections. These limitations may affect your risk of participating in this research.

The status of GINA and protection of your genetic information

GINA protects you as described above as of May 21, 2009 for discriminatory practice in health coverage and as of November 21, 2009 for employers with more than 15 employees.

Version: May 2009

STUDY-SPECIFIC DOCUMENT

1. RESEARCH STUDY**:The Temple University Concussion Registry**

2. PRINCIPAL INVESTIGATOR:

Ryan Tierney, PhD, ATC

120 Pearson Hall, 048-00,

Department of Kinesiology, Temple University

Philadelphia, PA 19122

3. RECIPIENTS**: Sponsor: Temple University**

Sponsor Agents: N/A

Temple University: Temple University Institutional Review Board

Other(s):OHRP- Office for Human Research Protections

4.EXPIRATION DATE: **None**

Signature of Patient Date

Printed Name of Patient

Signature of Personal Representative of the Patient Date

Printed Name of Personal Representative of the Patient and Relationship to Patient

Signature of Person Collecting Authorization Date

Printed Name of Person Collecting Authorization

Version: May 2009

Appendix C

Concussion Center Evaluation Form

ID #:

Concussion Evaluation Form

Patient Name: ______________________________
DOB: ______________ Gender: M F
Date: ______________ Race: ______________
Date of Injury:__________

A. Background:

Reporter: ___Patient ___Parent ___ATC ___ Other: ________________
Method of Referral: ___ ATC ___Resident/Physician ___ Other (Specify):______________________________
School: ______________ Does the school have an ATC: ______________________________
Sport: ______________ Position: ________________ Yrs. Experience with sport: __________
Helmet type: ______________ Bladders or Padding: ________ Last Time Fitted: ______________
Average Academic Reports: ___A's ____ B's ____ C's ___ D's ____ F's
Medications: ____
Supplements: ____

Was the Athlete Wearing a mouth guard at the time of incident? Yes ____ No ____
Any viral illnesses in past month/fever: Yes ____ No ____
Has been on a normal sleep schedule: Yes ____ No ____
Any history of anticoagulation disorders: Yes ____ No ____
Does athlete know what a concussion is? Yes ____ No ____

Concussion History? Y ___ N ___	**Headache History? Y___ N ___**	√	**Developmental History**	√	**Psychiatric History**	√
Previous # 1 2 3 4 5 6+	Prior Treatment for Headache		Learning disabilities		Anxiety	
Longest Symptom Duration Days ___ Weeks ___ Months ___ Years ___ Dates Of Previous Concussions _____________________	History of migraine headache ___ Personal ___ Family ______________		Attention-Deficit/ Hyperactivity disorder		Depression	
			Other __________		Other _________	

B. History & Subjective Questioning:

Notes:

C. Injury Characteristics

Location of Injury for Weather Purposes: _______________

Date of Injury:______ Game or Practice: _______Time into Game or Practice: (i.e., 1st quarter) ____________

Did athlete return to play immediately after incident: Y___ N___

Injury Description

____

____

MOI: ___ Head on Collision ___ Impact with ground __ Hit with Stick ___Other _________________________________

Location of Impact: ___Frontal ___Lft Temporal ___Rt Temporal ___Lft Parietal___Rt Parietal ___Occipital ___Neck ___Indirect Force

2. Immediate S & S:

____

____

2a. Duration: ________________

Evaluated at Emergency Room: ____ Yes _____ No **Cat Scan**: ___ Yes ___ No ___ Other Imaging

3. Current S & S:

____

____

Notes:

D. Objective Screening

Ocular Motor	Yes	No	Bess Test	Firm Surface	Foam Surface	OTHER	Yes
Abnormal pursuits (asymmetry)			Double Leg Stance			Blood Pressure	
Abnormal saccades			Single Leg Stance			Collect Blood	
Observable Nystagmus			Tandem Stance			Collect Saliva	
Abnormal Convergence			**Surface Total**				
Eye Grounds							
Blurry/Dizzy/Nausea w/ VOR			**Normal**	3.37±3.10	8.65±5.13		

ImPACT Assessment

Composite Scores – In percentile

	Baseline	PI-1	PI-2	PI-3	PI-4
Verbal					
Visual					
Vis. Motor Speed					

Rxn Time					
Impulse Control					
Total Symptoms					
CEI					

Notes:

F. Follow-Up Action Plan:

Follow Up: ___No Follow up Needed ___ Physician Monitoring: Date of Next Follow Up ___________

Academics: ___Full Return to Academics ___Limited Participation in School No School Until _____

Return to Play: ___Full Return to Play ___No Return to Play until Follow up ___Graduated Return to Play

Medications: ___None ___Extra Strength Tylenol ___Other________________

Referrals: ___Neurology ___Psychiatrist ___Physical Therapist ___ Psychologist ___Other _____________________

Other Recommendations:

___ Increase Physical Activity
___ Limit any Physical Acitivity
___ Limit any Mental Stimulation (i.e. watching television)
___ MRI
___ Other _________________________________

Appendix D

Concussion Center Reevaluation Form

ID #: ______
Visit: ______

Concussion Re-Evaluation Form

Patient Name: ______________________________
Date: ________________ Date of Last Visit: __________
DOB: ________________ Date of Injury: ____________

A. Injury Characteristics

1. S & S experienced since last visit:

____
____
____
____
____

2. Restrictions since last visit.:

___ Academic Restrictions - ___ No Exams ___ No return to school for ____ days
___ No Return to Play
___ Graduated Return to Play
___ No contact Return to Play
___ Full Return To Play

3. Referred to other physician ___ Yes ___ No

If yes, what kind of physician

____

Notes:

C. Objective Screening

Ocular Motor	**Yes**	**No**	**Bess Test**	**Firm Surface**	**Foam Surface**	**OTHER**	**Yes**
Abnormal pursuits (asymmetry)			Double Leg Stance			Blood Pressure	
Abnormal saccades			Single Leg Stance			Collect Blood	
Abnormal convergence			Tandem Stance			Collect Saliva	
Observable Nystagmus			**Surface Total**				
Eye Grounds							
Blurry/Dizzy/Nausea w/ VOR			**Normal**	3.37±3.10	8.65±5.13		

D. ImPACT Assessment

Composite Scores – In percentile

	Baseline	**PI-1**	**PI-2**	**PI-3**	**PI-4**
Verbal					
Visual					
Vis. Motor Speed					
Rxn Time					
Impulse Control					
Total Symptoms					
CEI					

F. Follow-Up Action Plan:
Follow Up: ___No Follow up Needed ___ Physician Monitoring: Date of Next Follow Up __________
Academics: ___Full Return to Academics ___Limited Participation in School No School Until _____
Return to Play: ___Full Return to Play ___No Return to Play until Follow up
___Graduated Return to Play
Medications: ___None ___Extra Strength Tylenol
___Other________________
Referrals: ___Neurology ___Psychiatrist ___Physical Therapist
___ Psychologist
___Other ____________________
Other Recommendations:
___ Increase Physical Activity
___ Limit any Physical Acitivity
___ Limit any Mental Stimulation (i.e. watching television)
___ Other ____________________________________

Appendix E

DNA Collection, Extraction, & Genotyping Protocols

Oragene®·DNA Self Collection Kit Instructions

1. Spit into the Oragene®·DNA Self Collection Tube until liquid saliva reaches the black line.
2. Screw the big blue cap onto the funnel to mix the Oragene®·DNA solution with saliva.
3. Unscrew the funnel from the Oragene®·DNA Self Collection Tube.
4. Screw the small grey cap onto the Oragene®·DNA Self Collection Tube.
5. Invert the Oragene®·DNA Self Collection Tube 5 times.

DNA Extraction From Salvia

1. Check if the label attached to the closed Oragene container (ID number) corresponds to the ID number attached to the data collection sheet
2. Place data collection sheet into file cabinet
3. Label 2 1.5ml microcentrifuge eppendorf tubes and 3 1.5ml sample tubes of EZ1 Bio-Robot DNA extraction system with same ID number
4. Incubate Oragene container for 1 hour at 50C (air incubator)
5. In the hood: Open container and transfer 700uL of sample into 1.5 microcentrifuge eppendorf tube labeled with corresponding ID number. Measure the volume of the remaining saliva in the Oragene container, and include in the final spreadsheet.
6. Add 28uL of Oragene Purifier to the sample and mix gently by inversion 2-3 min using the rotating shaker. Incubate on ice for 10 min.
7. Centrifuge for 3 min at 13000 rpm at room temperature.
8. Carefully transfer the clear supernatant into a new 1.5 microcentrifuge tube labeled with the corresponding ID number; discarded the pellet into the biohazard container.
9. Transfer 200ul of the supernatant to each of the 3 1.5ml labeled tubes of EZ1 Bio Robot DNA extraction system (3 labeled tubes per Patients' sample).
10. Arrange tips, tip holders, elution tubes, and cartridges into the corresponding slot of the Bio-Robot EZ1 station and start extraction. Select number 1 tissue protocol. Sample and Elution volume is 200ul
    a. Load 1.5ml elution tubes in 1st row. Then press any button.
    b. Load same number of tip holders in 2nd row. Then press any button.
    c. Place 200ul sample in the sample tubes in 4th row. Press any button to continue.
11. Extraction process takes about 20 min and results in the DNA eluted in a final volume of 200uL in specified tubes (labeled with corresponding sample number).
12. Store DNA solution at +4C.
13. Proceed with DNA estimation by Fluorimetic assay to determine concentration of DNA.

14. Fill out DNA collection form including numbers of tube from same sample. DNA concentration, number of rack and number of box, date of DNA extraction.

Preparation of primers for PCR

1. Write down the labeling o the vials to the lab notebook
2. Label eppendorf microtubes according to the vials
3. Always use pipettors, plasticware and reagents dedicated to PCR work; clean area and UV-irradiate items
4. Dissolve primers in 200ul of deionized water; vortex, spin in microfuge full speed for 10 seconds
5. Transfer 10ul to 990ul of deoinoized water in an eppendorf tube
6. Set parameters on SpectraMax2:
    a. UV spectra at 6 wavelengths
    b. 220nm to 320nm every 10nm
7. Use quartz cuvettes
8. Record spectra using water as a reference and save results
9. Calculated concentration of the oligo at 260nm minus 320nm (Background
    a. OD at 260nm and 320nm
10. Calculated concentration of initial stock (x100)
11. Use Molar concentration from the tech sheet
    a. dA 15400 ou/mmol
    b. dG 11700 ou/mmol
    c. dT 9300 ou/mmol
    d. dC 7300 ou/mmol
12. Dilute solutions to the required concentration 100 uM

#	Oligo	Mw	OD_260	OD_320	260-320	Stock_260	E_max	Conc	Final conc	Dilution	Water	Stock
			ou	ou	ou	ou		uM	uM		ul	ul
1	GTfwd	7540	0.872	0.038	0.834	83.4	265354	.31	40	7.86	87.3	12.7
2	FAM-GTfwd	8077.4	1.467	0.061	1.406	140.6	284008	.50	40	12.38	91.9	8.1
3	GTrev	8512.6	0.993	0.045	0.948	94.8	243032	.39	40	9.75	89.7	10.3

PCR Analysis

1. Prepare microtubes for PCR reaction and label with number and concentration
2. Prepare Master Mix in this order

	Per 1 tube	Per 8 tubes	Unit
Water	35.5	284ul	molecular
grade			
Primer GTfwd	1	8ul	40uM
Primer GTrev	1	8ul	40uM
Taq-Pol	0.25	2ul	5
units/ul			
dNTP 10 mM	1.25	10ul	10 mM each
dNTP			
5xBuffer	10	80ul	
Total	49	392	

3. Mix by vortexing, spin briefly
4. Store in -20 C or continue with PCR reaction
5. Dispense 45 ul Master Mix per tube
6. Dispense 5 ul DNA from tubes
7. Lock tubes and Proceed with PCR
8. User: ek1 Protocol PCR Optimizer

Conditions:	Temp oC	Duration min	# cycles
Step 1	94	2	1
Step 2	94	1	35
	55	2	
	72	1	
Step 3	72	7	1

Analysis of PCR products by gel electrophoresis

1. Rinse and assemble the electrophoretic tank with comb
2. Transfer 100ml 1XTBE buffer to a 100ml flask
3. Weigh 1.4g agarose for electrophoresis and add to the flask; mix well
4. Heat in microwave for 2 min (buffer should boil)
5. Check for complete melting of agarose
6. Let cool to about 50C (when you can hold it with your hand)
7. Add 10ul ethidium bromide to agarose and mix
8. Pour agarose into the electrophoretic tray
9. Let agarose solidify and wait an additional 20 min
10. Slowly remove the comb and inspect the wells
11. Assemble the electrophoretic device and fill with 1XTBE so that buffer covers the gel about ¼”
12. Load samples and the DNA ladder
    a. Sample preparation: Mix 20ul reaction mix and 5ul 6Xloading buffer
    b. Vortex spin and load into the wells

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
DNA ladder	A	B	C	D	F	G	N
5 ul	25 ul	25 ul	25 ul	25 ul	25 ul	25 ul	25 ul

13. Run electrophoresis at 100 mV until the tracking dye migrates approximately 3 cm to 4 cm
14. Inspect the gel in UV light (use eye protection)
15. Take picture of the gel, label lanes and document the experiment

PCR cleanup and submission to GENEWIZ for analysis

1. Prepare a bucket of regular ice
2. Prepare a strip of 8 tubes (Thermogrid Denville) and label the first and last tube on the side
3. Thaw the PCR reaction mixes, spin briefly and stick on ice.
4. Transfer 5ul of the reaction mixes to the strip of tubes and place on ice
5. Remove ExoSAP-IT reagent from (-20C) freezer and keep on ice at all times
6. Spin ExoSAP-IT briefly
7. Add 2 ul of ExoSAP-It to each tube and mix by pipetting up and down 5 times; avoid bubbling
8. Seal the tube with a strip of stoppers
9. Insert in the PCR machine
10. Start program: User: ek1 Program: gt-analysis- aprox 30 min
    a. Incubate at 37C for 15 min to degrade remaining primers and nucleotides
    b. Incubate at 80C for 15 min to inactivate ExoSAP-IT reagent
11. PCR product is now ready for DNA sequencing
12. Fill out form and submit to room 300 AHB building (Dr. Sapienza’s secretary)

Appendix F

Molecular Biology Primer

The cell is the basic functional unit of life. It contains many specific types of cellular machinery with specific functions; one in particular is the nucleus. In eukaryotes the nucleus is surrounded by a nuclear membrane that separates the nucleus from the other cell components. It is within the nucleus that the DNA (deoxyribose nucleic acid) is contained, or our genetic material. DNA provides the instructions or sequence for its production of proteins. Genes are regions of DNA that control hereditary characteristics and are the basic physical and functional unit of heredity (Gelehrter, Collins & Ginsburg, 1998). Genes can be as short as 1,000 base pairs to several hundred thousand base pairs long (Venter et al., 2001). The human genome is estimated to include 30,000 genes and 3.1 billion base pairs (Gelehrter et al., 1998).

Human DNA is condensed to form 46 chromosomes (23 from each parent), which contain's unique base pair sequences for that person. Each chromosome has a specific banding pattern that enables researchers to find specific locations among the chromosomes. A specific location among a chromosome is referred to as a locus. A locus has three main elements (Roth, 2007). The first number refers to the chromosome number and it can be a number between 1-22, or X (female) or Y (male). The letter refers to what arm of the chromosome it is on. The p stands for petit or "short arm", and q stands for queu or "long arm." The last number is the band and sub-band number (Roth). For example, the (GT)n polymorphism has a locus of 16p13.13, the 16^{th} chromosome on the short arm at band 13 and sub-band 13.

DNA is a double helix that is composed of a ladder of nucleotides (NT). It is composed of the sense "coding" strand and antisense "template" strand (Roth, 2007). NTs are made up of a phosphate group, sugar (deoxyribose) group, and one of four NT bases adenine (A), thymine (T), cytosine (C), and guanine (G). The A and G are purines, and A and T are pyrmidines. The purines are larger in structure where they have a double ring structure compared to the single ring structure of the pyrimdines (Hartel & Ruvolo, 2012). Each NT is bound to a complementary NT via a hydrogen bond. The law of complimentary base pairing governs the sequence binding enabling the ability to predict the base succession of one strand of DNA if we know the sequence of the complimentary strand of DNA (Saladin, 2007). This law states that an A NT binds to a T NT and vice versa, and there is a G NT binds to a C NT and vice versa. The C-G pairings are much stronger because they are bound with three hydrogen bonds compared to the two hydrogen bonds that hold A-T together. Once the strands are paired the DNA will begin to twist in a clockwise or right-handed motion forming its famous double helix structure (Saladin).

A protein is composed of a specific sequence of amino acids (AA). This AA sequence is determined by its sequence of NTs in the gene that codes for that protein. A gene is a sequence of NTs bound in a series of three NTs, which is called a triplet or codon. For example the codons GAA and GAG code for the amino acid, glutamate. Though there are only 20 AA there are 64 possible codon possibilities (Roth, 2007). Thus, each AA will have more than one triplet sequence coding. When many AA are bond together via a peptide bond they form a polypeptide chain, which can be used to make a functional protein. Protein coding begins at a specific triplet “TAC” so it is called the start codon. It continues coding for that protein until it reaches one of three stop codons: “ATT,” “ATC,” or “ACT.”

There are four main regions of a gene a promoter, exon, intron, and terminator. The promoter (the first 20-200 NTs) and terminator (at the end) are flanking regions that control gene expression (Roth, 2007). The promoter region facilitates transcription of exons. In between each flanking region are exons that are coding segments of DNA, and are interrupted by intervening spaces of introns whose segments of DNA are not translated into proteins (Gelehrter et al., 1998). Exons compose about 1.1% of the human genome (Venter et al., 2001). Exons are the transcribed regions of a gene that are present in mature mRNA (m stands for mature or messenger) and contain coding information, which controls protein structure (Gelehrter et al.). The intron section of the gene is initially transcribed into RNA but is then removed from the primary transcript by splicing together the exon sequences on either side of it (Gelehrter et al.). Intron regions comprise 75% the human genome (Venter et al.).

The first step to protein synthesis is transcription. Transcription is the process that creates a complimentary RNA copy from DNA. Transcription also regulates gene expression. A gene is up regulated if transcription is taking place. A gene is down regulated when there is no protein being produces thus no transcription is taking place. When there is the need for a protein to be produced the DNA unravels at a specific gene locus and transcription can begin. Transcription is composed of three stages: initiation, elongation, and termination.

Initiation of transcription begins in the promoter region and is located upstream to the initiation site (Hartl & Ruvolo, 2012). The strength of RNA polymerase binding depends greatly on the different promoters, which can lead to differences in the amount of gene expression. There are 2 main consensus or start sites (TTGACA or TATAAT; TATA box). In general, the closer the promoter sequences resemble the consensus sequences the stronger the promoter. Mutations such as repeat polymorphisms can change the NT sequence in the promoter and can alter the strength of the promoter. For example, if the repeat polymorphism causes the promoter sequence to have more resemblance it will increase the strength of the promoter region (Hartl & Ruvolo).

Promoters also have accessory proteins to activate transcription by RNA polymerase. These accessory proteins are called transcription factors. Transcription factors are a set of 26 different proteins needed for proper binding of RNA polymerase II (Hartl & Ruvolo, 2012). These are recruited by other proteins called enhancers or can be inhibited by silencers. Enhancers and silencers interact with the transcriptional activity to regulate transcription levels (Hartl & Ruvolo).

Transcription factors bind to the TATA box near the initiation site. Other enhancer and silencer proteins can also attach up stream to the start site to either enhance or silence the signal. When the transcription factors attach it initiates the signal for RNA polymerase. RNA polymerase is the enzyme that is attracted to the promoter, which will initiate transcription at the transcription start site (Hartl & Ruvolo, 2012). ATP hydrolysis adds the energy to allow RNA polymerase to being its function. There are many types of RNA polymerase. RNA polymerase I only produce that transcript that will be processed by ribosomal RNA. RNA polymerase II is responsible for transcribing the protein coding genes as well as those genes involved in RNA processing. RNA polymerase III is used in transcribing all transfer RNA genes as well as a portion of the ribosomal RNA (5s).

Transcription will occur downstream as the first DNA base is transcribed and is indicated by a + symbol (Hartl & Ruvolo, 2012). For example, +3 means that this NT is 3 NTs downstream from the initiation site. The transcription start site is the TAC codon. The active site is stabilized by a protein TFIIE so the enzyme DNA helicase can unwind the DNA strand (Hartl & Ruvolo).

Synthesis and elongation begins when DNA polymerase protein reads the DNA template strand creating a new RNA transcript chain. The RNA transcript NTs form phosphate bonds between the 3’ hydroxyl group and the 5’triphosphate of the next NT, and continues governed by the law of complementary base pairing (Hartl & Ruvolo, 2012). The chain is elongated at the 5’ end because NTs are being added to the 3’ end.

The third and final stage of transcription is chain termination, where RNA polymerase reaches a termination sequence. The three termination codons are ATT, ACT, and ATC. Once the termination sequence is reached, DNA can fold into a hairpin loop disrupting the RNA-DNA interaction and preventing further elongation (Hartl & Ruvolo, 2012).

That RNA molecule that was transcribed from the DNA template is called the primary transcript and contains introns and exons. Before the transcript can go to the ribosome for protein translation it must go through post-transcriptional modification. Post-transcriptional modification splices out the intron portions so it will only contain the protein coding exon regions. This process is also known as RNA splicing.

RNA splicing creates mRNA that moves to the ribosome for translation. Short NT sequences set up cues of where to cut for the splicing machinery. For example, splicing begins at the 5’ end of the first intron at the GU sequence. A protein complex called the splicesome will bind to the GU sequence and loop to the splicesome proteins (composed of 6 main SnRPPs) at the A branch site within the intron. The adenine NT within the A branch site will attack the 5’ splice site by cutting the sugar phosphate backbone of the RNA. The loop will covalently bond and be released and degraded. Then, the 3’ site of the first exon will attach to the 5’site of the second exon. Post-transcriptional events take place allowing the RNA transcript to have a poly adenlyaline tail and a methylated cap. After the intron has been removed the exon goes into further alternative splicing. There are about 25,000 genes, but there are 100,000 proteins. This is due to alternative splicing whereby the same genetic coding sequence can be used to generate multiple or unique proteins through shuffling or deletion of specific exons (Roth, 2007). The exons are removed similar to the excision of the intron (Roth). Once all the introns are removed the RNA strand is now a mature messenger RNA, and can be used for protein coding or translation.

Translation is the process of reading the mRNA transcript to produce a specific AA chain or polypeptide (Saladin, 2007). The ribosome is where protein synthesis will take place and align transfer RNA (tRNA). Initiation begins when ribosome recognizes the 5′ end (methylated cap) of the mRNA (Roth, 2007). The tRNA assists the ribosome by bringing the corresponding AA to the ribosome as the 3-letter sequence (codons) is being read. The tRNA is composed of a head, featuring an anticodon, and a tail, with a binding site for a specific amino acid. There are three main stages of translation: initiation, elongation, and termination.

First initiation takes place when a small ribosome subunit attaches to the mRNA methylated cap and goes to the initiation site AUG. The tRNA molecule will also attach the anticodon, which is complementary to the mRNA codon and represents the AA that the tRNA is carrying. The complementary AA for the codon AUG is methionine. That means each protein in the body begins their lives as a methionine AA (Roth, 2007). After that, another larger ribosomal subunit binds on top like a cap creating the ribosomal unit.

There are 2 sites within the ribosomal unit. The first is the P site, which is the first site for the tRNA carrying the anticodon complementary to mRNA AUG start codon, again which is carrying the AA methionine. The second site A is for the next tRNA carrying the next AA complementary to the anticodon. The elongation stage occurs when the AA on the tail of the tRNA at the P site will be transferred to the AA on the tail of the tRNA at the A site. Once the transfer happens the P site tRNA exits and the A site tRNA moves up to the P site and a new tRNA enters the A site. This process continues to move along the mRNA creating a polypeptide chain. The region between the start and stop codon is called the open reading frame where the ribosome will freely read mRNA sequence along this length until the stop codon (UGA, UAA, UAG) is reached. The final termination stage transpires when one of the stop codons is reached signaling that the coding is complete and the newly formed protein is released.

Translation will create an amino acid sequence that is an exact match for the codons corresponding to the original

DNA sequence. Post-transcriptional modifications may be necessary and can include the folding or shaping of the actual protein (assisted by other proteins called chaperones and other enzymes), in order to function properly. Completed proteins may be used in the cell, return to the nucleus, or be packaged and released for extra-cellular use.

A person's genetic makeup is called a genotype (Gelehrter et al., 1998). Genotypes consists of 2 sets of genes; one maternal and one paternal (Saladin, 2007). Genotypes are expressed as dominant or recessive traits. For example, genetic traits such as eye color are described as dominant or recessive. Dominant traits are controlled by one active gene in the pair (one maternal and one paternal) and recessive traits require both genes in the gene pair to collaborate for regulation of the trait (Saladin). A phenotype is the observed expression resulting from the interaction of the genotype with environmental factors (Gelehrter et al.). Every person receives 2 copies of chromosomes from each parent (except for X and Y chromosomes) and combines to form a genotype.

There can be three different combinations to form a person's genotype. An allele is one of several different forms of a gene. When the two alleles are the same allele they are known as homozygotes alleles. If the two alleles are different alleles they are called heterozygotes alleles. For example, the mother and father could both pass on A alleles (AA), or mother could give an A allele and father could give a G allele (AG). The third option is that both parents give the G allele (GG) so the three different potential genotypes at a particular sequence location would be AA, GG, or AG.

Humans have over 99.9% of our DNA sequence in common so it is that .01% that makes everyone unique. It is the way those genes are regulated and expressed that cause these differences or genetic factors (Roth, 2007). It is estimated that there is a sequence variation or genetic variation every 100 to 1,000 NTs (Roth). For example, everyone has almost the same 25,000 genes, but they do not all have the same sequence of NTs so the proteins produced from those genes are slightly different. Genes can be expressed many different ways depending on the way they are regulated.

Any sequence position where there is more than one NT found in the population is called a mutation (found in less than 1% of the population; Roth, 2007) or a polymorphism (found in greater than 1% of the population; Roth). Each variant found at that mutation or polymorphism site is referred to as an allele. The frequency of that allele can be found and reported as either the rare allele (found less frequently), or common allele (found more frequently; Roth).

There are two different ways these genetic variations arise. One is by evolution, which is the idea that the DNA sequence does change over time by environmental factors (mutagens; Roth, 2007). These changes become widespread and permanent in those individual populations over generations (Roth). The second way is by genetic variations become introduced are by mistakes done by cell machinery during DNA replication (Roth). Lastly, these changes occur normally during meiosis where groups of exons shuffle (Roth). Repeat polymorphisms are one way that can change gene expression in an individual.

Appendix G

Introduction to Single Nucleotide Polymorphisms

There are several types of SNP categories. A SNP classified as silent does not produce a change in the protein. For example, there is a change in the DNA sequence, however, it codes for the same AA (e.g., CCU, CCC both code for proline with a U/C SNP). A missense SNP occurs when the presence of an altered or variant allele in the coding sequence changes an amino acid in a protein. When the amino acid is changed to one with similar properties, it is known as conservative and has a low to very high relative risk of altering the phenotype, depending on the location of the SNP. That is, a SNP can cause a change from a glycine to an alanine and they are both non-polar AA structures with similar R groups. In contrast, when an amino acid is changed to one with different properties, it is known as a nonconservative missense SNP and has a moderate to very high relative risk of changing the phenotype (Roth, 2007). This occurs when a non-polar glycine with an R group of one H molecule changes to a tryptophan molecule with a double ring structure as its R group.

Insertion/deletion SNPs are the presence (i.e., insertion) or absence (i.e., deletion) of one or more DNA NTs. Insertion/deletion polymorphisms may affect greater than one to 100’s of NT base(s), from the addition or loss of a few NTs. This insertion/deletion can either be in-frame or cause a frameshift. Due to the fact that insertion/deletion SNPs can occur in both coding and noncoding regions and can change the amino acid sequence they can have different effects on the protein. In-frame insertion/deletion SNPs are divisible by three, and depending on the location, these SNPs have a low to very high relative risk of altering phenotype. Frameshift insertion/deletion SNPs can be more deleterious because the amount of NTs inserted or deleted are not divisible by three; therefore, there is a high potential for phenotype disruption (Roth, 2007).

When one of the altered alleles in a coding region incorrectly codes for one of the three stop codons (i.e., ATT, ACT, ATC), the result is a nonsense SNP. When this occurs, the coding for the protein may prematurely stop. The relative risk of phenotype change from a nonsense SNP is very high due to the possible altering of the protein structure and function. Exon SNPs are the most likely to have a functional effect (Roth, 2007).

In addition to the SNPs that directly alter the protein structure and/or function, there are also SNPs that occur outside the exon area, in the promoter, intergenic and intron regions. A SNP in the promoter region of a gene does not affect the amino acid sequence directly, however, it can alter the level, location, or timing of transcription (i.e., gene expression), with relative risk of phenotype ranging from low to high depending on the region. Similarly, intergenic SNPs occur within noncoding regions between genes and may affect gene expression with a relatively low risk of altering the proteins and phenotypes (Roth, 2007). SNPs within genes associated with ionotropic glutamate channels in the brain may alter the protein channel creating an atmosphere that could be detrimental if one sustained a head impact.

Appendix H

Basic Genetic Sequencing Introduction

DNA sequencing is the process of identifying specific NTs and their order within a DNA segment. This process relies on fluorescent tags (Roth, 2007). First, a PCR reaction must be done. The first PCR reaction is a standard PCR reaction where a DNA sequence of interest is amplified. The second PCR reaction is called the sequencing reaction and is performed with only one primer (versus having a forward and reverse primer in typical PCR reaction), the PCR product itself, and dideoxynucleotides (Roth). Dideoxynucleotides are fluorescent tags that prevent the elongation because they lack the hydroxyl end on their deoxyribose sugar. Thus, a phosphodiester bond cannot be formed with another incoming NT. The tagged NTs are only added to a small fraction of the NT in the PCR reaction mix so the PCR reaction is rarely stopped (Roth).

The millions of PCR products produced will now have a tagged NT at the first base, another at the second, another probe will be tagged at the third base until the last base is tagged. These PCR products will be run on a standard agarose electrophoresis gel and researchers can verify the separation of the different band lengths. In electrophoresis gels the agarose gel slows down bigger fragments and allows smaller fragments to run quicker down the gel towards the positive end. This allows the NTs to be ordered from shortest (beginning of the sequence) to longest (the end of the sequence).

After that the gel can be read through a florescent reader identifying the tagged NTs by producing a electropherogram. An electropherogram is a graph showing the DNA sequence as a wave pattern. Each wave produced is a single NT with a specific color. It can also display SNPs as dashed lines (Roth, 2007).

Appendix I

(GT)n Repeats Polymorphism in Other Genes

Gene	**GT Allele**	**Author**
Heme Oxygenase 1 *HMOX-1*	Promoter GT repeats altered transcription activity, where those carrying the long allele (< 25 GT repeats) had an increased risk for type II diabetes compared to those carrying the short allele (> 24 GT repeats).	Bao et al., 2010
CD3 molecule, gamma, CD3-TCR complex *(CD3G)*	A GT box located 50 NT from initiator may function as a weak secondary promoter and may generate transcripts.	Badran et al., 2005
Scurfin (*FoxP3)*	GT 15 was significantly more frequent in those with Type 1 diabetes than controls.	Bassuny et al., 2003
Estrogen Receptor-Alpha *(ESR1)*	Carriers of the GT 17 or 18 allele had a decreased risk of breast cancer due to reduced expression of progesterone receptor.	Cai et al., 2003
von Willebrand factor (*VWF)*	There was no association between the long GT genotype (20-24) or short GT genotype (15-19) and VWF levels.	Daidone et al., 2009
von Willebrand factor *(VWF)*	Short GT alleles (under 20 repeats) were associated with greater risk of high VWF levels in response to excess cortisol.	Daidone et al., 2010
von Willebrand factor *(VWF)*	Patients with the short GT allele (15-19) had higher VWF levels.	Daidone et al., 2011
Relaxin (*H1 & H2*)	Transcription regulation may differ between H1 and H2 due to differences in GT repeats polymorphism length.	D'Elia et al., 2003
Alpha2(I) Procollagen (*COL1A2)*	Deletions may represent an unrecognized but rare event in the multistep process of carcinogenesis	Dietzsch et al., 2002
Heme Oxygenase 1 *(HMOX-1)*	Carrying the short allele (>20 GT repeats) contributed to increased levels of bilirubin compared to the long allele (< 29 GT repeat), which was found to be	D’Silva et al., 2011

	associated with cardiovascular disease	
Lymphotoxin Alpha (LTA)	Microsatellite markers may have considerable implications on multifactorial diseases	Epplen et al., 1997
(STAT6)	GT 13 was associated with atopic asthma; GT 16 showed decreased risk for atopic asthma	Gao et al., 2004
N-methyl-D-aspartate receptor 2A subunit *(GRIN2A)*	There was an overrepresentation of long GT alleles (25 or more) in patients with schizophrenia.	Iwayama-Shigeno et al., 2005
Lamin A (*LMNA)*	The GT motif is a determinate of lamin A promoter activity and binds to the Sp family of transcription factors.	Janaki Ramaiah et al., 2006
Methylthioadenosine Phosphorylase *(MTAP)*	There was no association found between GT 6 deletion and MTAP deficiency.	Kadariya et al., 2002
Tumor Necrosis Factor *(TNF)*	Those with a GT length of 111 bp showed an association to prolific diabetic retinopathy. Those with a GT length of 103 bp suggested a lower risk for developing retinopathy.	Kumaramanickavel et al., 2001
(TIGR/MYOC)	Long alleles were GT 15 & 16; short alleles were GT 13 & 14; no association to myopia	Leung et al., 2000
Neprilysin *(NEP)*	No significant difference in the distribution GT repeats between Alzheimer cases compared to controls	Lilius et al., 2003
Natural Resistance-Associated Macrophage Protein 1 *(NRAMP1)*	GT genotype 10/10 could result in impaired immunity related to NRAMP1 causing higher susceptibility to TB for HIV-positive individuals.	Ma et al., 2002
Class II transplantation antigens *(HLA-DRB1)*	Different GT alleles create different protein binding patterns.	Mäueler et al., 1999
Toll-like receptor 2 *(TLR2)*	The combination of the TLR2 GT genotypes of 20 or more along with NOD2	Nischalke et al., 2011

	variants may improve identification of spontaneous bacterial peritonitis.	
Solute Carrier Family 11 Member A1x *(SLC11A1)*	Presence of allele 2 may be weakly associated with decreased risk of autoimmune and inflammatory disease.	O'Brien et al., 2008
Heme Oxygenase-1 *(HO-1)*	Carrying the short (GT)n allele (less than 25 repeats) had higher levels of expression compared to those carrying the long (GT)n allele (25 or more repeats), where those with the short allele have an increased risk to rheumatoid arthritis.	Rueda, 2007
Cyclin-Dependent Kinase *(CDKN1A/p21,CDKN1C/p57, & CDKN2A/p16)*	Longer GT alleles (GT 12 & 13) were associated with decreased in vitro transcription, and could increase risk to atherosclerosis.	Rodríguez et al., 2007
150 Cucurbita Genes	GT/CA and CT/GA-rich motifs regulate expression within the vascular system.	Ruiz-Medrano et al., 2011
Scurfin (*FoxP3*)	There was no association between GT allele frequency and autoimmune diseases (i.e., lupus erythermatosus, rheumatoid arthritis, ulcerative colitis, Crohn's disease, & celiac disease)	Sánchez et al., 2005
Double Bromodomain containing gene *(BRD2)*	The long GT allele (GT 13) reduces TATA binding protein interfering with transcription, which results in a shorter transcript *in vitro.*	Shang et al., 2011
(GLC1A)	There was no difference in the allele distribution and provides no association with the risk of developing glaucoma.	Sjöstrand et al., 2002
Serotonin 5-HT2C Receptor *(5-HT2C)*	-995/-759 and -697 variants were higher in non-obese subjects	Yaun et al., 2000

Toll-like Receptor 2 *(TLR2)*	GT allele lengths were significantly different among racial groups (i.e., African American, Caucasian, & Korean) and longer and shorter repeats may have higher promoter activity compared to mid sized alleles.	Yim et al., 2004
Interferon alpha Receptor *(IFNAR1)*	GT 5/14 repeats polymorphism may increases risk for the development of depressive symptoms during IFN alpha therapy for hepatitis C.	Yoshida et al., 2005
Plant Transcription Factor	GT elements demonstrated complex regulatory features of plant transcription	Zhou et al., 1999

Appendix J

Statistical Tables

APPENDIX F-1. ANOVA Summary for Participants Between Recovery

Variable	SS	dF	MS	F	*p*
Age	52.65	1	52.65	1.20	0.280
Sex	0.65	1	0.65	3.52	0.067
Race	1.00	1	1.00	0.84	0.363
Migraine	0.01	1	0.01	0.04	0.847
ADHD	0.13	1	0.13	1.26	0.267
Dizziness	0.29	1	0.29	1.14	0.136
Prv Conc	7.77	1	7.77	2.77	0.108

APPENDIX F-2. ANOVA Summary for Participants Between Dominant Model

Variable	SS	dF	MS	F	*p*
Age	6.93	1	6.93	0.15	0.696
Sex	0.03	1	0.03	0.16	0.689
Race	0.51	1	0.51	0.43	0.517
Migraine	0.00	1	0.00	0.01	0.914
ADHD	0.04	1	0.04	0.39	0.538
Dizziness	0.34	1	0.34	1.35	0.252
Prv Conc	0.35	1	0.35	0.39	0.537

APPENDIX F-3. ANOVA Summary for Participants Between Recessive Model

Variable	SS	dF	MS	F	*p*
Age	13.97	1	13.97	0.31	0.579
Sex	0.01	1	0.01	0.06	0.809
Race	3.62	1	3.62	3.20	0.080
Migraine	0.04	1	0.04	0.18	0.671
ADHD	0.15	1	0.15	1.44	0.236
Dizziness	0.13	1	0.13	0.49	0.487
Prv Conc	3.87	1	3.87	1.30	0.260

APPENDIX F-4. ANOVA Summary for Participants Between Codominant Model

Variable	SS	dF	MS	F	*p*
Age	68.59	2	34.29	0.77	0.469
Sex	0.24	2	0.23	0.62	0.544
Race	5.14	2	2.57	0.56	0.112
Migraine	0.23	2	0.17	0.56	0.573
ADHD	0.29	2	0.15	1.40	0.257
Dizziness	0.90	2	1.31	1.98	0.150
Prv Conc	3.09	2	1.54	0.50	0.607

APPENDIX F-5. T-Test Summary for VOR Score Between Recovery Groups

Effect	*t*	dF	F	*p*
Recovery	-0.10	49	0.07	0.78

APPENDIX F-6. T-Test Summary for BESS Score Between Recovery Groups

Effect	*t*	dF	F	*p*
Recovery	0.25	49	0.29	0.594

APPENDIX F-7. ANOVAs Summary for ImPACT Scores Between Recovery Groups

Recovery	SS	dF	MS	F	*p*	Power
Verbal	21.92	1	21.92	0.02	0.90	0.51
Visual	1306.82	1	1306.82	1.56	0.21	0.17
Motor Speed	99.37	1	99.37	0.10	0.75	0.06
S/S	200.32	1	200.32	0.41	0.52	0.08
Reaction	140.31	1	140.31	0.14	0.72	0.35

APPENDIX F-8. Chi-Square Summary for Concussed and General Population Allele Frequencies

Variable	Total Concussed Alleles	Total Control Alleles	df	X^2	p
LA	55	54	1	0.95	0.330
SA	47	34	1		

APPENDIX F-9. Chi-Square Summary Between Alleles and Recovery

Recovery	LA	SA	Total	df	X^2	p
Normal	21	25	26	1	2.80	0.094
Prolonged	34	22	56	1		
Total	55	47	102			

APPENDIX F-10. Chi-Square Summary Between Dominant Model and Recovery

Recovery	LL	SS + LS	Total	df	X^2	*p*
Normal	3	20	28	1	4.37	0.036*
Prolonged	11	17	23	1		
Total	14	37	51			

Note: *significant at $p \leq .05$.

APPENDIX F-11. Fisher's Exact Test Between Recessive Model and Recovery

Recovery	LL	SS + LS	Total	df	X^2	p
Normal	17	6	28	1	2.05	0.152
Prolonged	27	3	23	1		
Total	42	9	51			

APPENDIX F-12. Fisher's Exact Test Summary Between Codominant Model and Recovery

Recovery	LL	SS	LS	Total	df	X^2	p
Normal	4	6	13	28	2	2.54	0.280
Prolonged	10	4	14	23	2		
Total	14	10	27	51			

APPENDIX F-13. ANOVA Summary for VOR Score Within Dominant Model

Model	SS	dF	MS	F	*p*	Power
Dominant	1.94	1	0.03	0.03	0.857	0.191

APPENDIX F-14. ANOVA Summary for VOR Score Within Recessive Model

Model	SS	dF	MS	F	*p*	Power
Recessive	1.74	1	1.74	2.15	0.149	0.087

APPENDIX F-15. ANOVA Summary for VOR Score Within Codominant Model

Model	SS	dF	MS	F	*p*	Power
Codominant	0.99	2	0.99	0.59	0.560	0.082

APPENDIX F-16. ANOVA Summary for BESS Score Within Dominant Model

Model	SS	dF	MS	F	*p*	Power
Dominant	44.73	1	44.73	0.67	0.416	0.486

APPENDIX F-17. ANOVA Summary for BESS score within Recessive Model

Model	SS	dF	MS	F	*p*	Power
Recessive	16.15	1	16.15	0.24	0.626	0.517

APPENDIX F-18. ANOVA Summary for BESS Score Within Codominant Model

Model	SS	dF	MS	F	*P*	Power
Codominant	74.78	2	37.39	0.56	0.557	0.404

APPENDIX F-19. ANOVAs Summary for ImPACT scores within Dominant Model

Scores	SS	dF	MS	F	*P*	Power
Verbal	268.25	1	268.25	0.21	0.649	0.075
Visual	336.04	1	336.04	0.39	0.534	0.120
Motor Speed	126.28	1	126.28	0.13	0.722	0.064
S/S	82.51	1	82.51	0.17	0.684	0.077
Reaction	72.91	1	72.91	0.07	0.792	0.068

APPENDIX F-20. ANOVAs Summary for ImPACT Scores Within Recessive Model

Scores	SS	dF	MS	F	*P*	Power
Verbal	2237.43	1	2237.43	1.81	0.185	0.252
Visual	4.56	1	4.56	0.01	0.942	0.050
Motor Speed	1875.37	1	1875.37	1.98	0.166	0.281
S/S	487.16	1	487.16	1.01	0.321	0.151
Reaction	13.10	1	13.10	0.01	0.911	0.082

APPENDIX F-21. ANOVAs Summary for ImPACT Scores Within Codominant Model

Scores	SS	dF	MS	F	*P*	Power
Verbal	1454.07	1	727.03	0.57	0.571	0.252
Visual	54.22	1	27.11	0.03	0.970	0.050
Motor Speed	887.55	1	443.78	0.45	0.641	0.281
S/S	514.85	1	257.43	0.52	0.597	0.151
Reaction	65.20	1	32.60	0.03	0.970	0.082

APPENDIX F-22. Univariate Analysis Summary for Risk Factors of Prolonged Recovery

Parameter	dF	Est	Std Error	Wald X^2	p	Odds Ratio	95% CI
Age	1	0.06	0.05	1.07	0.300	1.06	0.95-1.18
Sex	1	-1.31	0.73	3.18	0.075	0.27	0.06-1.13
Race	1	0.97	0.59	2.69	0.100	2.64	0.83-8.39
Migraine	1	0.13	0.63	0.04	0.843	1.133	0.33-3.92
ADHD	1	-1.01	0.92	1.21	0.272	0.37	0.06-2.21
N_Prev	1	0.36	0.24	2.22	0.135	1.432	0.89-2.30
Ac Dizz	1	-0.62	0.58	1.14	0.284	0.54	0.17-1.67
Dominant	1	1.46	0.73	4.01	0.045*	4.31	1.03-18.04
Recessive	1	1.08	0.77	1.94	0.163	2.91	0.65-13.40
Codominant	1	-0.34	0.34	1.04	0.307	0.71	0.36-1.37

Note: *significant at $p \leq .05$.

APPENDIX F-23. Multivariate Analysis Summary for Risk Factors of Prolonged Recovery

Parameter	dF	Est	Std Error	Wald X^2	p	Pt Est	95% CI
Age	1	0.03	0.06	0.15	0.696	1.03	0.91-1.16
Sex	1	-1.59	1.00	2.54	0.111	0.20	0.03-1.44
Race	1	1.45	0.89	2.64	0.104	4.25	0.74-24.32
Migraine	1	-0.52	0.85	0.37	0.542	0.60	0.11-3.15
ADHD	1	0.33	1.23	0.07	0.790	1.39	0.12-15.48
N_Prev	1	0.30	0.25	1.32	0.250	1.34	0.81-2.21
Ac Dizz	1	-1.94	0.58	4.34	0.036*	0.54	0.17-1.67
Dominant	1	2.55	1.02	6.29	0.012*	0.60	0.27-1.31

Note: *significant at $p \leq .05$.

APPENDIX F-24. Multivariate Stepwise Selection Summary for Risk Factors of Prolonged Recovery

Parameter	dF	Est	Std Error	Wald X^2	*p*	Pt Est	95% CI
Sex	1	-1.61	0.77	4.31	0.037*	0.20	0.04-0.91
Dominant	1	1.74	0.76	5.15	0.023*	5.70	1.27-25.56

Note: *significant at $p \leq .05$.

Appendix K

Raw Data

APPENDIX G-1. Raw Data from Concussion Assessment

Participant	Age	Sex	Race	NumPrev Concussions	Acute Dizziness
1	22	1	2	0	0
2	18	1	1	1	1
3	21	2	1	2	1
4	20	2	1	2	0
5	16	2	1	0	0
6	18	1	1	1	1
7	18	1	1	0	0
8	17	1	0	0	0
9	21	2	0	0	0
11	13	1	0	0	0
12	22	2	0	0	0
13		2	0	0	0
14	20	1	0	3	0
15	34	1	0	0	0
16	19	1	0	0	0
17	17	2	1	0	0
18	21	1	2	1	0
19	53	2	1	2	1
20	18	1	1	1	1
21	18	1	4	1	1
22	16	2	1	1	1
23	17	1	1	0	0
24	17	1	1	0	0
25	21	1	0	1	0
26	20	1	1	3	0

APPENDIX G-1. (continued)

Participant	Age	Sex	Race	NumPrev Concussions	Acute Dizziness
27	16	1	1	0	1
28	18	1	0	0	0
29	20	1	0	1	1
30	21	1	1	1	0
31	17	1	0	1	0
32	15	1	1	0	0
33	18	1	2	0	1
34	14	1	2	0	1
35	14	1	1	0	1
36	18	1	0	0	0
37	18	1	0	2	1
38	18	1	4	0	0
39	17	1	0	1	1
40	12	1	2	1	0
41	28	1	0	3	1
42	17	2	1	2	0
43	18	2	3	0	0
44	22	2	1	6	0
45	18	1	1	5	0
46	20	1	1	9	1
47	15	1	0	0	1
48	14	2	1	0	1
49	15	1	0	1	1
50	15	1	0	0	0

APPENDIX G-1. (continued)

Participant	Age	Sex	Race	NumPrev Concussions	Acute Dizziness
51	14	2	1	0	1
52	24	2	3	0	1

APPENDIX G-1. (continued)

Participant	Migraine Hx	ADHD Hx	Pursuits	Nystagmus/ Saccades	Convergence
1	1	2	0	0	0
2	2	1	0	0	0
3	2	2	0	0	0
4	2	2	0	0	0
5	1	2	0	0	0
6	2	2	0	0	0
7	2	2	0	0	0
8	2	2	0	0	0
9	2	2	0	0	0
11	2	2	0	0	0
12	2	2	0	0	1
13	2	2	0	1	1
14	2	2	0	1	0
15	2	2	0	0	1
16	2	2	0	0	0
17	2	2	0	0	1
18	2	2	0	0	0
19	2	2	0	0	0
20	2	2	0	0	0
21	2	1	0	0	0
22	2	2	0	1	0
23	2	1	0	0	0
24	2	2	0	0	0
25	2	2	0	0	0
26	1	2	0	0	0

APPENDIX G-1. (continued)

Participant	Migraine Hx	ADHD Hx	Pursuits	Nystagmus/ Saccades	Convergence
27	1	1	0	0	0
28	2	2	0	0	0
29	2	2	0	0	0
30	2	2	0	0	0
31	1	2	0	0	1
32	1	1	0	0	0
33	2	2	0	1	0
34	2	2	0	0	0
35	2	2	0	1	0
36	2	2	0	0	0
37	2	2	0	0	0
38	1	2	0	0	0
39	2	2	0	1	0
40	2	2	0	0	0
41	1	2	0	0	0
42	2	2	0	0	0
43	2	2	0	1	1
44	1	2	0	0	0
45	1	2	0	1	1
46	2	2	0	1	0
47	1	1	0	0	0
48	1	2	0	0	0
49	1	2	0	0	0
50	2	2	0	0	0

APPENDIX G-1. (continued)

Participant	Migraine Hx	ADHD Hx	Pursuits	Nystagmus/ Saccades	Convergence
51	2	2	0	0	0
52	1	2	0	0	0

APPENDIX G-1. (continued)

Participant	VOR	Vestibular Ocular Score	BESS	Verbal %	Visual %
1	1	2	37	1	1
2	1	1	22	99	76
3	2	0	17	59	52
4	1	1	15	96	74
5	1	1	12	64	95
6	2	0	18	88	80
7	2	0	17		
8	2	0	14	84	94
9	1	2	20	67	33
11	1	2	14	7	7
12	1	2	21	77	64
13	1	3	22	92	77
14	1	2	14	25	52
15	2	1	6	63	51
16	2	0	13	67	2
17	1	2	29	6	4
18		0	24	59	52
19	2	0	42	28	30
20	2	0	10	27	12
21	1	1	20	1	35
22	1	2	17	3	1
23	2	0	16	96	38
24	2	0	3	84	99
25	1	1	15	96	59
26	2	0	18	84	99

APPENDIX G-1. (continued)

Participant	VOR	Vestibular Ocular Score	BESS	Verbal %	Visual %
27	2	0	17	48	44
28	2	0	12	96	67
29	2	0	8	89	92
30	1	1	24	18	73
31	1	2	30	1	3
32	2	0	11	97	39
33	1	2	14	67	44
34	2	0	22	56	75
35	2	1	12	8	15
36	2	0	13	48	26
37	1	2	16	63	32
38	2	0	19	30	46
39	2	1	11	96	46
40	2	0	7	98	85
41	2	1	25	84	77
42	2	0	19	51	40
43	2	2	19	88	76
44	2	0	17	77	35
45	1	2	25	73	44
46	1	2	8	4	1
47	1	2	42	2	19
48	1	1	16	1	61
49	2	0	8	5	67
50	2	0	11	78	60

APPENDIX G-1. (continued)

Participant	VOR	Vestibular Ocular Score	BESS	Verbal %	Visual %
51	2	0	16	99	79
52	2	0	12	19	27

APPENDIX G-1. (continued)

Participant	Motor Speed	Signs & Symptoms	Reaction Time	Days to full Return	Long/Short Recovery
1	1	103	0.001	365	1
2	23	35	0.48	49	1
3	22	68	0.43	365	1
4	84	43	0.50	75	1
5			0.44	19	2
6	13	12	0.36	11	2
7				23	1
8	19	16	0.50	12	2
9	90	23	0.42	16	2
11	83	36	0.19	12	2
12	33	24	0.48	7	2
13	36	24	0.48	50	1
14	36	29	0.27	270	1
15	58	1	0.28	170	1
16	46	11	0.37	330	1
17	1	20	-0.12	45	1
18	21	2	0.38	150	1
19	83	15	0.16	365	1
20	87	0	-1.28	28	1
21	8	46	0.25	17	2
22	1	54	0.35	24	1
23	80	0	0.41	10	2
24	47	0	0.45	11	2
25	72	6	0.46	21	1
26	99	0	0.30	14	2

APPENDIX G-1. (continued)

Participant	Motor Speed	Signs & Symptoms	Reaction Time	Days to full Return	Long/Short Recovery
27	44	35	-0.31	14	2
28	82	0	0.38	9	2
29	47	0	0.38	21	1
30	85	19	0.18	90	1
31	2	18	0.12	17	2
32	27	9	0.33	36	1
33	14	43	0.20	18	2
34	1	10	0.32	17	2
35	88	44	0.26	7	2
36	61	0	0.33	7	2
37	10	15	-0.13	16	2
38	83	4	0.28	24	1
39	66	4	0.48	25	1
40	35	3	0.57	30	1
41	26	0	0.40	18	2
42	76	14	0.58	70	1
43	80	5	0.40	34	1
44	83	23	0.58	28	1
45	43	26	0.44	80	1
46	36	61	0.19	28	1
47	1	59	0.18	19	2
48	68	37	0.00	14	2
49	49	17	0.12	28	1
50	94	5	0.49	14	2

APPENDIX G-1. (continued)

Participant	Motor Speed	Signs & Symptoms	Reaction Time	Days to full Return	Long/Short Recovery
51	29	1	0.46	77	1
52	53	8	0.22	69	1

APPENDIX G-2. Participant (GT)n Genotype and Allele Data

Participant	Genotype	No Long Alleles	No Short Alleles Group	Dominant Alleles Group	Recessive Alleles Group	Codominant Alleles Group
1	21	0	2	2	2	2
2	21, 29	1	1	2	1	3
3	26	2	0	1	1	1
4	29	2	0	1	1	1
5	19, 26	1	1	2	1	3
6	29	2	0	1	1	1
7	29	2	0	1	1	3
8	23, 27	1	1	2	1	3
9	22, 27	1	1	2	1	3
11	21, 31	1	1	2	1	3
12	21, 26	1	1	2	1	3
13	27, 36	2	0	1	1	3
14	21	0	2	2	2	2
15	25	2	0	1	1	1
16	21, 27	1	1	2	1	3
17	21, 30	1	1	2	1	3
18	22, 25	1	1	2	1	3
19	21, 27	1	1	2	1	3
20	26	2	0	1	1	1
21	26, 31	2	0	1	1	1
22	21, 25	1	1	2	1	3
23	21, 29	1	1	2	1	3
24	23	0	2	2	2	2

APPENDIX G-2. (continued)

Participant	Genotype	No Long Alleles	No Short Alleles Group	Dominant Alleles Group	Recessive Alleles Group	Codominant Alleles Group
25	26	2	0	1	1	1
26	22, 28	1	1	2	1	3
27	22, 26	1	1	2	1	3
28	24, 28	1	1	2	1	3
29	19, 27	1	1	2	1	3
30	21, 30	1	1	2	1	3
31	21	0	2	2	2	2
32	22, 30	1	1	2	1	3
33	21	0	2	2	2	2
34	21	0	2	2	2	2
35	21, 28	1	1	2	1	3
36	22	0	2	2	2	2
37	28, 33	1	1	2	1	1
38	28	2	0	1	1	1
39	25	2	0	1	1	1
40	21, 31	1	1	2	1	3
41	28	2	0	1	1	1
42	21, 26	1	1	2	1	3
43	21	0	2	2	2	2
44	21, 24	0	2	2	2	2
45	27, 30	2	0	1	1	1
46	22, 26	1	1	2	1	3
47	21, 25	1	1	2	1	3
48	21	0	2	2	2	2

APPENDIX G-2. (continued)

Participant	Genotype	No Long Alleles	No Short Alleles Group	Dominant Alleles Group	Recessive Alleles Group	Codominant Alleles Group
49	27, 33	2	0	1	1	1
50	21, 25	1	1	2	1	3
51	21, 27	1	1	2	1	3
52	27	2	0	1	1	1

APPENDIX G-3. Control (GT)n Genotype and Allele Data

Participant	Genotype	No Long Alleles	No Short Alleles
1	26	2	0
2	21, 31	1	1
3	21	0	2
4	21,31	1	1
5	26	2	0
6	21, 28	1	1
7	21,	0	2
8	26	2	0
9	21, 29	1	1
10	25	2	0
11	25	2	0
12	23, 27	1	1
13	21	0	2
14	22, 30	1	1
15	24, 27	1	1
16	22, 31	1	1
17	20, 25	1	1
18	21, 29	1	1
19	21, 29	1	1
20	25	2	0
21	29	2	0
22	22, 28	1	1
23	23, 29	1	1
24	28, 33	2	0

APPENDIX G-3. (continued)

Participant	Genotype	No. Long Alleles	No. Short Alleles
25	21	1	1
26	21, 27	1	1
27	27	2	0
28	21, 25	1	1
29	21, 28	1	1
30	22, 28	1	1
31	24	0	2
32	19, 33	1	1
33	22, 29	1	1
34	21	0	2
35	28, 31	2	0
36	27	2	0
37	21, 31	1	1
38	28	2	0
39	26, 31	2	0
40	27	2	0
41	25	2	0
42	25, 27	2	0
43	25	2	0
44	26	2	0
45	20	0	2