EXPLORING THE GENETICS OF MALE HOMOSEXUALITY: FINE MAPPING OF 7Q36 AND EXPRESSION PROFILING OF THE HYPOTHALAMUS

This paper was written by a student who had BioSci 199 research experience, although in a different field. While the topic is sensitive and controversial, the hypothesis, background research, experimental approach and description of the methods are excellent. This paper received 10/10.

Abstract

Homosexuality is an intriguing feature of the animal kingdom due to its social stigma, diverse cultural history, and evolutionary incongruity. To biologists, homosexuality is a phenotype, like diabetes or dyslexia, although its genetic factors have entertained only a few researchers. This project will examine male sexual orientation from two directions. First, we will conduct a case-only study to create a high resolution map of a candidate gene locus on chromosome 7. Second, we will use microarray technology to spotlight the differences in the hypothalamus of post-mortem brain tissue collected from heterosexual and homosexual men. These two methods are united in the sense that both seek to unearth the genes underlying the homosexual phenotype. Our objective is to narrow the susceptibility locus to a small number of genes, while concurrently identify genes differentially expressed in the hypothalamus.

Specific Aims

The long term objective of this project is to establish a definitive genetic component of homosexuality. The goal of this immediate project is to combine genetic and genomic techniques to identify the genes underlying homosexuality. The implications of this study will further our understanding of the intricacies of complex diseases and resolve certain paradoxical facets of evolution (e.g., survival without reproductive fitness).

★ The **first aim** is to perform fine mapping of a candidate locus, 7q36. This moderately high susceptibility region was previously identified by Mustanski and DuPree (2005) in the first genome-wide scan of homosexuality. Our **hypothesis** is that a denser linkage map will increase resolution and return a high mlod score. Hence, instead of weighing the functions of hundreds of potential genes, the narrowed locus will reduce the number of candidates to a handful. The genes that we identify can be analyzed in future studies with resequencing arrays and functional analyses.

★ The **second aim** is to use laser capture microdissection followed by microarray analysis to study three cell clusters of the hypothalamus. The hypothalamus, which secretes a variety of neurohormones, has been connected to numerous disparities between men and women and also between homosexuals and heterosexuals. Our **hypothesis** is that the expression profile of post-mortem brain tissue will differ depending on the individual's sexual orientation.

The integration of these two aims satisfies the recent fusion of genomics and genetics into "genetical genomics," a rather clumsy term coined by Jansen and Nap in 2001. This marriage of techniques is somewhat analogous to the benefits of mapping and sequencing a genome at the same time (Blumberg 2007). Genetical genomics is similarly beneficial because genes can be identified in chorus, both functionally (with expression profiling) and proximally (with linkage studies).

Why Study Homosexuality?

An Evolutionary Paradox: Homosexuality is a paradox for evolutionary biologists in that it violates a fundamental attribute of natural selection: maximize reproduction (Kirkpatrick 2000). Several theories have been proposed, two of which are acknowledged here. The *kin-selection hypothesis* states that homosexuals forgo having children so that they can take care of their relatives' offspring. The *balanced polymorphism hypothesis* proposes that homosexuality is maintained because it "co-occurs" with another trait that possesses positive selection (Hutchinson 1959).

All theories have flaws, however, efforts towards solving this quandary are not made in vain. Indeed, several other aspects of evolution are contradictory. For example, language acquisition and communication, abilities unique to human beings, have proven difficult to explain from a Darwinian perspective—cost/ benefit analyses yield perplexing results (Komarova 2006). Also, certain mental disorders like schizophrenia are too widespread to be attributable to a mere accident. Figuring out one mystery like homosexuality might facilitate the resolution of others.

An Archetypal Complex Trait: Another reason to study homosexuality is that both biological and environmental factors play a role. It is amusing to watch anthropologists discount theories of genetic basis and biologists discount theories of societal influence. Homosexuality is the quintessential example of a complex trait, and thus both nature and nurture arguments are compelling. Thus, if the details of homosexuality are sorted out, diseases like hypertension, Alzheimer's disease, and diabetes will grow closer to a cure.

Eliminating Social Stigma: One might also argue that answering the "born gay" question will eliminate the societal pressure and familial humiliation often associated with homosexuality. Such speculation is not relevant for this discussion, nevertheless, the idea is interesting.

Background & Preceding Research

Linkage Analysis of the 7q36 Chromosomal Region: In 2005, Mustanski and DuPree found that the 7q36 region near the microsatellite marker, D7S798, returned a moderately robust mlod score of 3.45. Their low-resolution (10 cM) map corresponds to approximately 100-300 genes, a number too large to begin sequencing or exploring the functional identity of each individual gene (Glazier 2002). By increasing the density of markers, high resolution mapping promises to reduce the

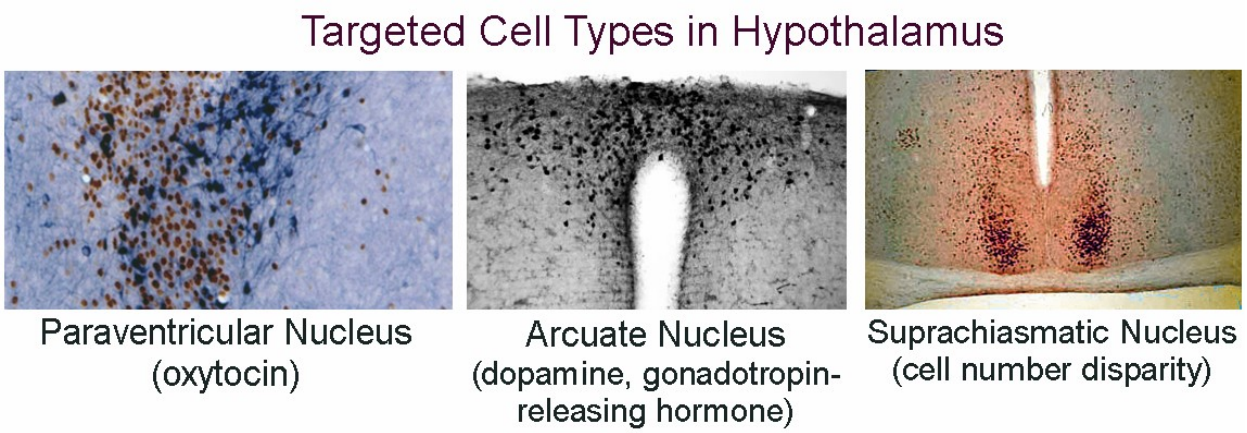
candidate gene interval. However, complex traits are tricky, and there is not a single mathematical procedure to tackle the multifarious inheritance patterns. Complex traits are generally polygenic (they do not segregate perfectly with a single locus) and environmental factors cloud the level of their genetic contribution. Thus, we must deal with problems like incomplete penetrance, where the gene is not consistently associated with the phenotype, or phenocopy where an environmentally acquired condition mimics the phenotype of a gene (Kruglyak & Lander 1995).

Two main techniques for the fine-mapping of loci have been developed: the Haseman-Elston method, and the variance components method. The former is a nonparametric procedure that incorporates two parameters: *trait values*, a continuous measure of the phenotype contributed by each sibling pair, and *identity-by-descent* (*IBD*), the property that both siblings inherited the genotype from the same ancestor. If a particular marker is linked to the trait, the level of IBD sharing will be high and the corresponding difference in trait values will be low. A negative linear regression curve is observed and a simple t test can evaluate linkage.

The variance components method has much greater statistical power, although it must assume that the trait is normally distributed. This approach does not restrict evaluation of linkage to sib pairs only (and waste pedigree information like the Haseman-Elston method). Variance components partitions the total variance into categories like genotype, environment, and most importantly, the QTL (quantitative trait locus). The statistical significance of the QTL component is calculated, and thus its overall contribution to the phenotype is obtained (Pratt et al. 2000). Because we do not know, *a priori*, the shape of our sampling distribution, the choice of using Haseman-Elston or the variants components method will be decided after the subjects have been selected.

Expression Profiling of the Hypothalamus: In 1976, Günter Dörner proposed that the hypothalamus of homosexual men would be characterized by female patterns and differentiation. This assumption lead to the misconception that "gay men have female brains." Follow-up studies revealed that the neuroanatomy of the hypothalamus in homosexual and heterosexual men is indeed different. However, the existence of sexually dimorphic brain regions *does not imply* that identical differences are observed in homosexual and heterosexual individuals. Hence, researchers have taken the view that homosexuals do not have a female brain, but rather, a third brain entirely.

In comparing the expression profiles of heterosexual and homosexual hypothalamic brain tissue, we will target three specific regions with laser capture microdissection: paraventricular nuclei (PVN), arcuate nuclei (ARN), and suprachiasmatic nuclei (SCN). The SCN will be studied with regard to a neuroanatomical discovery of Swaab & Hofman in 1990. These researchers found that the SCN showed no male/ female differentiation, yet homosexual men had twice as many vasopressin neurons compared to heterosexuals. Vasopressin hormone has been well-studied and implicated in the social and sexual behavior of animals. For example, Pitkow et al. (2001) showed that vasopressin assists in the bonding of two individuals in a monogamous relationship (i.e. the "pair-bond formation"). The PVN and ARN are also of interest due to their expression of oxytocin and gonadotropin-releasing hormone, respectively. Oxytocin is involved in sexual behavior, social bonding, and trust (Winslow & Insel 2002). Gonadotropin-releasing hormone stimulates the secretion of luteinizing hormone, which causes the Leydig cells of the testis to secrete testosterone (Veldhuis et al. 2001).

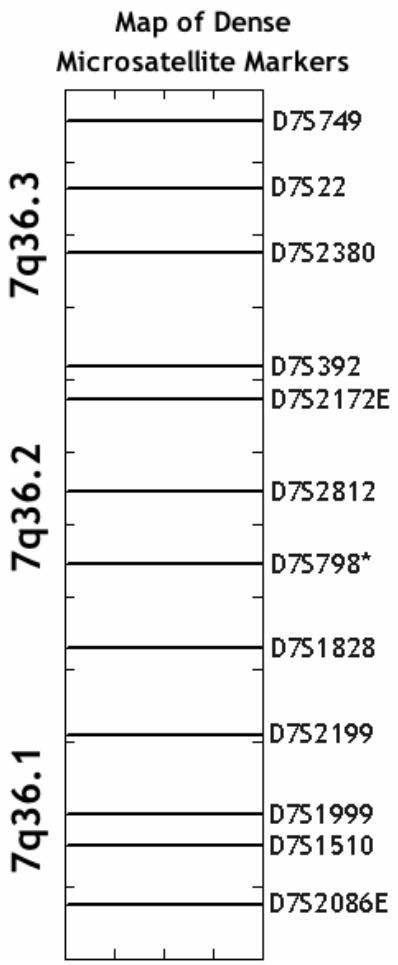


Laser capture microdissection (LCM) is a relatively new technology, but has proven effective for analyzing such complex, heterogeneous tissue as the brain. LCM is a "point and shoot" mechanism that allows efficient collection of pure cell populations (Curran et al. 2000). Thus, the PVN, ARN, and SCN (pictured above) can be targeted quite effectively. Although LCM is commonly used in cancer research due to the heterogeneity of tumors, the procedure has been realized in many applications. For example, Glasow et al. (1998) compared cortical and adrenomedullary cells and found differentially expressed mRNA for the prolactin receptor. LCM is suitable for this project and other neurological experiments because microarrays of brain tissue have been notoriously insensitive, due to the "averaging out" of multiple cell varieties (Mirnics 2001). Hence, LCM should enhance the downstream accuracy of our microarray analysis.

Experimental Approach: Linkage Analysis of 7q36

Sampling: An appropriate sample size required for 90% statistical power and $a = 0.05$ significance in linkage studies is equal to or greater than 300 families, each having at least two gay brothers (Liu 1998). We plan to obtain participants through magazine publications like OUT, Instinct, Genre, SCENE, or Passport. Once the families are recruited, they will sign an informed consent form, answer questions about their sexual orientation, and donate blood (i.e. their DNA). Most commonly, the Kinsey scales of sexual orientation are employed to quantitate the phenotype (Kinsey 1948). Although other measures exist, we will remain consistent with previous work and ascertain where upon the continuum (seven-point gradation) each individual lies.

Genotyping: Genotyping will be accommodated by the UCLA Sequencing and Genotyping Core. The Core is equipped with two ABI PRISM® 3700 DNA Sequencers. These are high-throughput machines that can analyze more than ninety samples at one time with unattended operation. DNA samples will be isolated and provided to the Core in 96 deep-well storage plates. Custom markers, positioned across the 7q36 region (spanning about 11,328,910 bases or 11cM) will be supplied for fine-mapping (see figure and table below).



Marker	Start/End	Marker	Start/End
D7S2086E	S: 147743589 E: 147743678	D7S2812	S: 153457036 E: 153457202
D7S1510	S: 148560324 E: 148560945	D7S2172E	S: 154731280 E: 154731478
D7S1999	S: 149008948 E: 149009130	D7S392	S: 155197883 E: 155198248
D7S2199	S: 150086535 E: 150086855	D7S2380	S: 156754649 E: 156754827
D7S1828	S: 151298780 E: 151299375	D7S22	S: 157636176 E: 157637406
D7S798*	S: 152442713 E: 152443013	D7S749	S: 158560592 E: 158560815

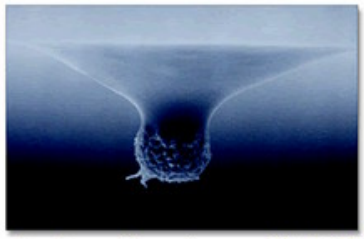
*Marker found by Mustanski & DuPree to have highest linkage to male homosexuality

Linkage Analysis: As mentioned above, accessing how the statistical analysis will be conducted is difficult without prior knowledge of our sample population. Nevertheless, we plan to use Kyazma® MapQTL® 5.0 in order to calculate mlod scores and create a linkage map. MapQTL requires the input of text files with quantitative trait data and marker information. MapQTL has been incorporated into numerous studies, and has proven very reliable.

Experimental Approach: Expression Profiling of the Hypothalamus

Sampling: The Orange County Coroner Division will be contacted to obtain ~26 brains via the Brain Donor Program at the University of California, Irvine. Informed consent from the next of kin will be acquired by the UCI IRB before the brain is received. Case (homosexual) and control (heterosexual) brain tissue will be matched according to age, ethnicity, post-mortem delay, and most importantly, cause of death. Past studies have included AIDS patients, but this creates confusion in the resultant data. Differences between case and control should be limited to sexual orientation and not confounded by the post-agonal effects of AIDS.

Brain Tissue Preparation: Sliced brain blocks will be preserved in an embedding medium like Tissue-Tek® OCT Compound and frozen on dry ice. The hypothalamus will be isolated and cut very thin (~6-8 μm) using the LEICA CM3050 cryostat apparatus. This allows for uniform slicing and consistent freezing so that the sample is not degraded. The slices will be mounted onto glass slides and stained with Nissl. The quality and quantity of the hypothalamic mRNA will be confirmed using the RNA 6000 Nano LabChip kit.



single cell capture with LCM

Laser Capture Microdissection (LCM): The LCM procedure can be briefly described as follows: The tissue harboring our cells of interest (PVN, ARN, or SCN) is placed under a microscope and covered with a thermoplastic film. An infrared laser beam is emitted onto the cells causing them to adhere to the film (see figure left). Using the PixCell® IIe LCM, neurons from each of the three hypothalamic regions will be "captured."

RNA Amplification: In 1999, Luo et al. designed a protocol to isolate mRNA from 1000 LCM-captured cells and then conduct a successful microarray experiment. We plan to incorporate the general flow of Luo's design, making technical modifications when necessary. Note that the specificity and accuracy gained by laser capture microdissection causes loss of RNA abundance. LCM produces an RNA input of about 50 ng, whereas bulk tissue delivers about 5 μg (Klur et al. 2004). The minimum amount of RNA required by Affymetrix is 7μg, although more is preferred. Therefore, time-consuming, cumbersome amplification procedures are unavoidable.

Total RNA from the samples will be extracted and purified using Stratgene's Micro RNA Isolation Kit as a starting point (modifications to the protocol are always expected). The next step is two-round amplification of RNA using RT-PCR followed by in vitro transcription. We prefer to proceed manually through the RT-PCR, however Ambion MessageAmp kits are often used. The first stand reaction will commence by mixing and incubating the RNA with T7-oligodT primer, RNasin® (RNase-inhibitor), and AMV RT provided by Seikagaku America. Second-strand synthesis should yield double stranded cDNA with $1x10^6$-fold amplification of the original RNA material. Then, by means of *in vitro* transcription, double-stranded cDNA will harvest antisense RNA. For this step, we plan to use AmpliScribe™ T7-Flash™ Transcription Kit which promises quick and high yields of RNA. This will conclude the first amplification round—repetition of RT-PCR and cDNA-to-aRNA transcription will provide enough product for microarray analysis.

Microarray Analysis: Homosexual and heterosexual RNA for each of the three cell types will be hybridized to Affymetrix microarrays (six chips total, part # 900468). Affymetrix is currently offering the GeneChip Human Genome U133 Set for analysis of 18,400 transcripts. Our method for hybridization, washing, and scanning will follow directly from the protocol defined by Affymetrix. Differentially expressed transcripts will be identified using Stratagene's ArrayAssist® Expression Software which is fully compatible with Affymetrix and supports several algorithms.

Conclusion

Homosexuality will never receive the same attention from the scientific community as Alzheimer's Disease or cancer. This is not my expectation because homosexuality, although debilitating in the face of discrimination, is not a life-threatening disease (unless we consider its correlation to HIV).

Nevertheless, I suspect that more attention would be granted to this complex trait if the religious component was drained. The equivalence relationship of homosexuality and sin that permeates society is irrational and propagates the high-controversy status of homosexuality. Minus such polemic elements, research in this arena might be better funded. Furthermore, examining the diverse inter-workings of sexual orientation is likely to unleash clues about evolution and complex diseases. Our project delivers special consideration to differential gene expression in homosexual men. We feel that this is the appropriate next, if not the first, step in the genetic/genomic study of homosexuality. However, the meshing of polygenic interactions and environmental influences will hold the most powerful answers in the end.

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