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ABSTRACT

The genetic variation A118G, which is located in one of the opioid receptors, has in various studies been connected to development of addiction to morphine analogues. As addiction is a complex issue with far-ranging effects in society and medicine this connection warrants research. This paper is about trying to confirm or disprove the aforementioned association through genetic analysis, using an analysis method called the Handy-Biostrand, of people accepting treatment for substance abuse and a control group recruited from the general population. In total 17 patients and 16 controls were genotyped for the A118G variation. The result demonstrated that no greater preference for either genotype AA or AG was found in either group.
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Introduction

Opium has had an important role in the history of the world, both as a medicinal herb and for the purpose of recreational drug use. The first records of this plant date back as far as the time of the Sumerians, roughly 3400 BC. It has been cultivated and grown throughout the centuries by almost all great civilizations up until today (Brownstein, 1993).

The active ingredient in the opium plant is morphine and, to a lesser extent, codeine. These chemical substances mainly affect a specific receptor found in the central and peripheral nervous systems, which can lead to feelings of euphoria and an absence of pain, but also respiratory depression, nausea and dysphoria. Regular use of these drugs can lead to dependence and sensitization (Freye and Latasch, 2003).

Structure and function

The receptors affected by morphine and its analogues are called opioid receptors and usually bind to neuropeptides called endogenous opioids. The three classic receptor-types are called mu(μ), kappa(κ) and delta(δ) receptors. The classic agonists to these receptors are divided into three groups, each with a preference for one of the receptor types (Purves et al., 2008). Dynorphin is a peptide that binds preferentially to the κ-receptor. It is found in all of CNS, but mainly in the midbrain area, pons, thalamus and spinal cord. It is associated with the stress response and the resulting dysphoria, as well as euphoria, dyspnea and psychomimetic effects (Bruchas et al., 2010, Trescot et al., 2008). Another peptide is called enkephalin and binds to the δ-receptors spread throughout the brain. It is well known for its role in mediating pain directly in the spine. The receptor with which most research has been devoted is the μ-receptor, which is where endorphins preferentially bind. This receptor can be found mainly in the intestine and the brain (Purves et al., 2008, Trescot et al., 2008).

The main function of opiates is mediated through an inhibitory effect on other neurons. When the receptors bind one of these neuropeptides a G-coupled protein (Galpα) dissociates from the receptor and is released into the cytosol of the neuron. This protein activate outward rectifying potassium channels, inhibit inward calcium channels and inhibits adenylylcyclase, which otherwise activates protein kinase A by increasing the cAMP level in the cell. This enzyme would then activate sodium channels that
decrease the membrane potential, as well as phosphorolyse several transcription factors that increase the production of neuropeptides, thereby making the nerve more excitable. This system also works as a negative feedback, mediating desensitization, as PKA assists Galpha binding to the potassium channel, so that the longer PKA is inactive, the harder it becomes to activate those ion channels. Thus the opiate-receptors inhibit neuronal activation, both by directly manipulating the membrane potential and counteracting increasing excitability induced by other signaling factors who work by activating PKA (Trescot et al., 2008, Yu, 1996).

**Opiates and addiction**

The euphoric effect of the opiates is believed to be mainly because of their role in the dopaminergic mesolimbic system, which is implicated in the brains reward system, as well as cognition, learning and emotion. The general structure considered most relevant in research of drug abuse consists of dopaminergic neurons in the medial forward bundle which connects the ventral tegmental area (VTA), in the brainstem, to the nucleus accumbens (NAcc) in the limbic forebrain (Iversen, 2010). These two areas have a role to play in what is called the ‘limbic loop’, which is a system modulating neuronal processing unconnected to motor programs, as in emotive responses, cognition, memory, learning and the reward system (Purves et al., 2008). The nucleus accumbens accepts signals from various cortical structures and, if the stimulation is great enough, disinhibit mediodorsal nucleus by inhibiting the ventral pallidum and substantia nigra pars reticulata, two structures which otherwise inhibit the stimulating effect. The VTA affect the system by modulating the ventral striatum with dopamine, which heightens the excitability of the neurons, and so enables a greater inhibitory signal towards the pallidum. The result of this signaling is believed to be the reward system.

All drugs currently abused somehow affect the VTA and NAcc structures, either by inducing dopamine-like effects or mimicking the body’s own endogenous cannabinoid or opiate receptors. In the case of the opiates the general area of effect lies in the inhibitory neurons in the ventral tegmentum. Here the opiates bind to the µ-receptor and thereby lessens their excitability, making them more unlikely to inhibit the dopaminergic neurons which project towards the nucleus accumbens. These inhibitory neurons are
otherwise tonically active, helping to create the baseline of dopamine emission in the brain (Koob and Volkow, 2010, Nestler, 2005).

An interesting point about drug use and the development of addiction lies in the fact that many of the brain areas associated with memory has been shown to relate to the nucleus accumbens and VTA-system. This seems to mean that to use these drugs would lead to the formation of emotionally intense memories, which would then lead to a psychological craving for the drug, as well as the physical one (Koob and Volkow, 2010).

**Opiates and pain relief**

The other major role opiates play is in the pain relief system, where it is involved in both peripheral and central pain relief. The structure of the central pain modulating system relies on an area in the midbrain called the periaqueductal gray, which is a region of grey matter very close to the brainstem. This structure receives signals from the somatic sensory complex, hypothalamus and the amygdala, after which, should the signals be strong enough, it activates several separate areas in the brain implicated in descending pain control. These structures are called the parabrachial nucleus, the rostral ventromedial medulla, the locus coeruleus and the raphe nuclei. These areas project downwards and activate inhibitory interneurons in the dorsal horn of the spine. These intermediate neurons inhibit the postsynaptic projection of the ascending pain-nerve by secreting enkephalin, which lowers the excitability of the presynaptic painfiber. Various animal studies have confirmed that opiates have an effect, not only on the neurons sensitive to endogenous opiates in the spine, but also have an effect in the periductal grey area, as well as raphe nuclei and other sources of descending projections (Purves et al., 2008).

**A118G Polymorphism**

This project revolves around a specific mutation; a single nucleotide polymorphism (SNP) called A118G in the μ-receptor gene OPRM1.

A SNP is a genomic variation of a single nucleotide where both possible variants are present in more than 1% of the population.
SNPs can cause downregulation of proteins as well as different forms of protein malformations, as they can exchange one aminoacid for another, which may have drastic consequences on the function of the protein (Goodman et al., 2006).

The A118G polymorphism in this project lies in exon 1 in the gene on chromosome number 6 and leads to a substitution of an amino acid, asparagine for aspartic acid in the receptor on amino acid position 40 (N40D). The amino acid in question lies in the extracellular segment of the opiate receptors’ structure. As this mutation directly affects the ligand-binding part of the protein configuration of the major target for morphine, heroin and endogenous opioids, it isn’t improbable that it somehow affects the response one has to these substances.

This polymorphism has been linked in various studies to an increased risk for addiction, by way of frequency comparison (Bart et al., 2004, Szeto et al., 2001), as well as several studies showing that there isn’t any real difference between any one of the three genotypes in regards as to frequency in addicted or non-addicted people (Befort, et al., 2001, Crowley et al., 2003, Franke et al., 2001, Gelernter et al., 1999). On one hand there have been studies that demonstrated a higher binding affinity for endorphin to the mu receptor in the N40D variation (Bond et al. 1998), but others were unable to replicate the result with transfected cells (Beyer, et al., 2004). There has been at least one study that showed an expression imbalance of the μ-receptor in cells transfected with the OPRM1 gene carrying the 118G variation, which may be relevant to heroin abuse (Zhang et al., 2005).

**Aims of the study**

The purpose of this project was to examine the frequency of the different genotypes and compare them to each other, trying to see if there is a connection between the G-variation and the development of substance abuse. For this, samples supplied by the Uppsala methadone program were used, which has been treating people with opioid dependence since 1966 and control samples recruited from the general population.

The methadone program is used, not only for people with heroin addiction, but also for certain chronic pain patients that have, as a result of the pain medication they depend on, developed a dependence on this (Rhodin et al., 2006).
Methods and Materials

Sample Collection
For this project blood samples from the national Swedish methadone program were used. These samples were collected over a period of time in 2005 and stored at -20°C, until further analysis. The samples were subdivided into 5 pain patients and 12 heroin abusers, as well as 16 control samples. These patients range in age from 24 to 63 and are mostly female, in the ratio of 22 women to 11 men. Of these patients all but two are Swedish, the ones who aren’t come from Finland and Iran.

DNA extraction
DNA was purified from 200 μl of whole blood by the Magtration System 12GC (Precision System Science, Chiba, Japan) using the Magtration-MagaZorb DNA Common Kit. This kit lysates the cells in the blood and extracts the DNA, washing away the cellular debris and thereby enabling PCR.

A118G SNP Genotyping
The Handy Bio-Strand was used for the SNP analysis, a kit that enables genotype identification (Ginya et al., 2007). This kit requires about 50 ng/μl of the small DNA-segment that is analyzed for SNP-variation, which is tested by competitive fluorescent probe hybridization. To achieve this purity and concentration, a nested-PCR reaction was used, and, after that, the ChargeSwitch PCR Cleanup Kit from Invitrogen. These procedures give a solution with a high concentration of DNA, without unincorporated dNTP:s, polymerases or salts.

PCR
Nested PCR is a specific kind of PCR, where one first runs a reaction on template DNA with one set of primers, and then another reaction with a new set of primers within the sequence that was cloned up in the first PCR. This reaction will mainly use the cloned segment as a template, and thereby quickly produce high quantities of DNA.

The primers used for the first PCR were OPRM1-NP-F(oward) and OPRM1-NP-R(everse) and have the sequences CTGACGCTCCTCTCTGTCTCA (forward) and
CAACATTGAGCTTGGGAGT (reverse). These form a PCR mixture when they were added in such a way that there were 0.2 μM of each primer, 0.2 mM of the dNTP mixture, 2.5 mM of MgCl₂, 1xLA buffer and 0.05 U/μl of LA Taq polymerase. The template DNA in this solution was 1 ng/μl. This mixture was run on a heating block for 3 minutes in 95°C, then in 30 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C on the Mastercycler EP gradient S (Eppendorf, Germany) for 1 min. The resulting fragment should theoretically be 532 bp long.

The second PCR's primers are called OPRM1-For1 and OPRM1-Back1 and their sequences are GAAAAGTCTCGGTGCTCCTG and GCACACGATGGAGTAGAGGG, respectively. This is the set that is “nested” within the previous pair of primers.

2 μl of each of the samples were added to 48 μl of mastermix. In these 50 μl of PCR-mixture the concentration was 0.20 μM for each of the primers, 0.20 mM for the dNTP, 2.5 mM for MgCl₂, 1xReaction Buffer and 0.025 U/μl of HotGoldStar polymerase. The concentration of the DNA was unknown. This solution was run on the same cycle as the first PCR, using the same cycler.

After PCR 2 the samples were resolved on a 2% ethidium bromide gel to test if the DNA fragment had been properly magnified.

After the second PCR the resulting DNA-solution was purified from the proteins, enzymes and salt by using the Invitrogen PCR Cleanup Kit (Invitrogen, Carlsbad, CA), adapted for the Magtration robot system.

All successfully magnified DNA-samples were tested on NanoDrop (Long Beach, USA) to see what concentrations of DNA were achieved. A small volume of each sample were then diluted until 9 μl of solution contained about 500 ng DNA, after which 1 μl of a solution of NaOH with a concentration of 10 M was added. The function of the NaOH at this stage was to denaturate the DNA in preparation for the hybridization.
5 μl of each of the 33 samples were then set to a 384 multimeterplate in doubles, along with a positive control and a landmark of a diluted Cy5 oligonucleotide, to allow the recognition of separate samples. These samples were then taken by the disposal multispotting unit and set to the thread on a HandyBiostrand Cassette. The thread the DNA samples were stamped on was then rolled onto a Handy Biostrand core pin, and then put into a UV-light emitting device that fixed the DNA onto the strand itself. As the test is for different variations of a SNP, one has to run two separate strands with complementary probes to be able to properly see all different genotype variations.

**Probes**

The pin was then used as a substrate for hybridization with fluorescent probes called HUMOR-A-2 and HUMOR-G-3 with the respective sequences of Cy5-ATGGCAACCTG and Cy5-ATGGCGACCTG. To minimize the risk of false positives competitive probes were also added. These probes are called HUMOR-NL-A2 and HUMOR-NL-G3, and have the respective sequences of ATGGCAACCTG and ATGGCGACCTG. They are, besides the absence of the fluorescent molecule Cy5, identical to the probes. In the hybridization process the probe and the competing segment that isn’t identical was added. To HUMOR-A-2, HUMOR-NL-G3 was added and vice versa.

**Hybridization**

The hybridization reaction was done on the Magtration System using the Hybri-Machine protocol. The pins were washed with a pre-hybridization solution containing 2xSSC (1xSSC is 0.15 M NaCl and 15 mM sodium citrate), 0.1% SDS and 200 μg/ml salmon sperm DNA (to cover the thread and thereby prevent nonspecific hybridization). After that the hybridization solution was applied. This solution contained 2xSSC, 0.1% SDS, 200 μg/ml salmon sperm DNA and 10 nM of the fluorescent probe as well as 10 nM of the non-fluorescent competing probe. Once the hybridization reaction had taken place, the spool was washed in 3 separate solutions of SSC and SDS to remove the probes that bound weakly or onto the strand instead of the DNA segments. The first washing buffer contains 2xSSC, the second 1xSSC and the third 0.1xSSC and they all had a SDS
solution of 0.1%. After the wash, the spool was suspended in 2xSSC without any SDS, run through the Handy Bio-Strand Scanner and analyzed using the software HySoft.

**Statistical methods**

To analyze the data, the accepted method of statistical interpretation requires the calculation of the Odds Ratio (OR) of the samples. By comparing the odds of exposure to one of the genotypes between the control group and the methadone patients one gets an estimate on the relevance of the genotype for addiction. This is done by first setting up a contingency table:

<table>
<thead>
<tr>
<th></th>
<th>AG</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methadone Patients</strong></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>(C)</td>
<td>(D)</td>
</tr>
</tbody>
</table>

This is then filled with the numbers of patients carrying the genotype in that particular group. To calculate the OR the formula \( OR = \frac{A}{B} \frac{C}{D} = \frac{AD}{CB} \) was used, which resulted in the comparative odds ratio for genotype AG in the methadone patients group. If the odds ratio is 1, there is no difference between the two factors in the different groups. If the OR is higher than 1, then this genotype have a higher odds of being found in the addiction group than the control group. Should the OR be less than 1, then that means there is a higher chance of the AG genotype being found in the control group. This method of data analysis is, however, only showing a general trend. To see if this result is relevant the P-value was analyzed using the software Prism, which uses the exact Fisher Method for analyzing the P-value.

The P-value determines the strength of the statistical evidence by calculating the chance of getting the same result, assuming the variation of the samples follows a normal variation interval.

**Results**

The total number of samples analyzed were 33, 16 control samples and 17 patient samples. When the project started, there were 73 samples, numbering from 7 to 80. Of these there were some that had to be excluded, first number 62 which was ignored.
because it had to little concentration of DNA for the first PCR reaction, 7-33 because there wouldn’t be time to do all of the samples, 34, 44 and 50 to 54 because the second PCR failed. There was no sample 70. To fit the rest of the samples onto a single BioStrand spool, samples 38 and 46-49 were disregarded. This resulted in 33 samples, which is the maximum amount one can load on a single BioStrand assay.

In Figure 1 an example of an electrophoresis gel is shown. The gel demonstrates a successful second PCR with a product size of 302 bp.
The Biostrand Hybridization assay resulted in figures 2 and 3.

Figure 2: Biostrand spool after hybridization with HUMOR–A–2 + G3.

Figure 3: Biostrand spool after hybridization with HUMOR–G–3 + A2.
By running the figures shown in figure 2 and 3 through the HySoft program the information was quantified and interpreted. Once the program was adjusted and calibrated, the following information was received, as shown in table 1 and figure 4.

<table>
<thead>
<tr>
<th>Samples</th>
<th>A</th>
<th>G</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>741</td>
<td>8488</td>
<td>GG</td>
</tr>
<tr>
<td>T</td>
<td>17288</td>
<td>3455</td>
<td>AA</td>
</tr>
<tr>
<td>C+T</td>
<td>7891</td>
<td>7257</td>
<td>AG</td>
</tr>
<tr>
<td>35</td>
<td>2771</td>
<td>645</td>
<td>AA</td>
</tr>
<tr>
<td>36</td>
<td>2616.5</td>
<td>572.5</td>
<td>AA</td>
</tr>
<tr>
<td>37</td>
<td>2885.5</td>
<td>661</td>
<td>AA</td>
</tr>
<tr>
<td>39</td>
<td>2985.5</td>
<td>723</td>
<td>AA</td>
</tr>
<tr>
<td>40</td>
<td>2691.5</td>
<td>608</td>
<td>AA</td>
</tr>
<tr>
<td>41</td>
<td>2952.5</td>
<td>619</td>
<td>AA</td>
</tr>
<tr>
<td>42</td>
<td>2908</td>
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<td>AA</td>
</tr>
<tr>
<td>43</td>
<td>1619.5</td>
<td>2618.5</td>
<td>AG</td>
</tr>
<tr>
<td>45</td>
<td>1533.5</td>
<td>2418</td>
<td>AG</td>
</tr>
<tr>
<td>55</td>
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<td>56</td>
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<td>666</td>
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<td>61</td>
<td>2728</td>
<td>712.5</td>
<td>AA</td>
</tr>
<tr>
<td>63</td>
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<td>640</td>
<td>AA</td>
</tr>
<tr>
<td>64</td>
<td>1521</td>
<td>429</td>
<td>AA</td>
</tr>
<tr>
<td>65</td>
<td>1806</td>
<td>421</td>
<td>AA</td>
</tr>
<tr>
<td>66</td>
<td>2773</td>
<td>650.5</td>
<td>AA</td>
</tr>
<tr>
<td>67</td>
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<td>2349.5</td>
<td>581</td>
<td>AA</td>
</tr>
<tr>
<td>71</td>
<td>2082</td>
<td>473.5</td>
<td>AA</td>
</tr>
<tr>
<td>72</td>
<td>2603.5</td>
<td>609</td>
<td>AA</td>
</tr>
<tr>
<td>73</td>
<td>2772.5</td>
<td>644</td>
<td>AA</td>
</tr>
<tr>
<td>74</td>
<td>1493.5</td>
<td>2442</td>
<td>AG</td>
</tr>
<tr>
<td>75</td>
<td>2858</td>
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<td>77</td>
<td>2816</td>
<td>637.5</td>
<td>AA</td>
</tr>
<tr>
<td>78</td>
<td>2029</td>
<td>476.5</td>
<td>AA</td>
</tr>
<tr>
<td>79</td>
<td>1091</td>
<td>1839</td>
<td>AG</td>
</tr>
<tr>
<td>80</td>
<td>1424</td>
<td>2127.5</td>
<td>AG</td>
</tr>
</tbody>
</table>

Table 1: Flourescence intensity and SNP analysis of samples.
From the results in table 1 and figure 4 one can see that out of the 16 control samples there were 13 who carried the AA genotype and 3 who carried the AG genotype. Of the 12 patients admitted to the methadone program for the purpose of treating heroin addiction, there were 9 who carried the AA genotype and 3 who carried the AG genotype. Finally, in the group of 5 patients who were admitted to the methadone program for their mismanagement of their persistent pain, there were 4 who carried the AA genotype and 1 who carried the AG genotype.

As the pain patients and addiction patients are both in methadone treatment for addiction, they are both compared to the control group in regards to the relative frequency of the different genotypes.

As can be seen in figure 5, the relative frequencies are fairly similar. In the methadone patients group there is a slightly higher rate of the genotype AG. The third possible genotype, GG, was not found in either group.

<table>
<thead>
<tr>
<th></th>
<th>AG</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odds Ratio</td>
<td>1,333</td>
<td>0,75</td>
</tr>
</tbody>
</table>

Table 2: Resulting OR values for the two genotypes.
Table 2 demonstrates that the odds ratio is a little bit higher for the AG genotype; there is a slightly higher chance of finding the AG genotype in the group of patients with known issues of drug abuse. Using the software Prism the P-value is calculated as 1.000, signifying that the results couldn’t be classified as statistically relevant.

**Discussion**

In this project we obtained SNP frequencies from patients and controls using the Handy Bio Strand method and analyzed the relative frequencies. We found that there was a slightly increased chance of finding the AG genotype in the patient group, but that there wasn’t any statistical strength to the findings.

Figure 1 demonstrates an example of the electrophoresis gel, which was run to determine if the PCR were successful. The right size for the sequence we were trying to clone was 302 bp long, and should be the only band on the gel, which the gel shows we succeeded in achieving.

Figure 2 and 3 show how a biostrand tip looks after hybridization, indicating the success of both the securing of the DNA to the nylon strand as well as the binding of the fluorescent probes.

Figure 4 depicts the fluorescence of each spot of DNA on the tip and demonstrates how the program analyzes which spot carries which genotype.

The various studies that support a connection, such as Bart et al, and the many studies that indicate there is no connection, for example Crowley et al, made it hard to form a hypothesis on what result would be achieved in this project (Bart et al., 2004, Crowley et al., 2003).

The reasons for the lack of a discernible affinity for either of the genotypes in either of the groups could be manifold, the simplest being that there simply weren’t enough samples to accurately define a confidence interval. This seems, at the very least, not unlikely, as with only 16 control samples and 17 substance abuse positive samples, there would have to be a very clear connection between any genotype and substance abuse to be able to draw any sort of reasonable conclusion.
Franke et al have also shown the same lack of a connection, with a pool of 287 heroin addicts and a control group of 365 people, which confirm the conclusion in this study (Franke et al., 2001). The possibility still remains that the effect of the variation is so subtle that only a truly massive sample pool will be able to prove its effect, although that puts into question if the SNP has any real relevance at all. To confuse the matter further, Szeto et al also have a large sample pool of 200 addicts, although only 97 controls, but show very clear results of a connection between the variation and substance abuse. This study indicate that the 118G SNP have a relatively large influence on people’s sensitivity towards opiate addiction, which contradicts Franke et al’s results as well as this study’s. Demonstrating a propensity toward addiction, with a relatively large sample pool, suggests that perhaps the result of this study would have been different with a larger collection of samples (Szeto et al., 2001).

Recently, however, the general consensus seems to be that there really isn’t a connection between any of the genotype variants and susceptibility to addiction, as suggested by, among others, Gelernter et al (Gelernter et al., 1999). The lack of reproducible positive results with different cohorts in the available studies suggests that if there were a connection between the G-variant in the receptor and the development of substance abuse, it may be a weak one, possibly attenuated by other mutations or external factors, such as the social circumstances of the individual.

Considering that opiates have such a complex and potent effect on some of the most crucial neurological systems there could be many genetic variations that, together, add up to a greater or lesser sensitivity to opiate addiction. To study this, a genome-wide association study may be more useful than analysis of a single SNP. There should also be more effort directed toward finding, or co-analyzing, other genetic variants with a more indirect connection towards substance dependence, for example the genetic variation connected to risk taking personality traits in the gene monoaminoxidase A(MAOA). Naturally, a larger sample size may catch a connection this study missed, but as there have been several studies done on the A118G variation, and there still haven’t been any clear, undisputed connection made to addiction, there might be better variations to look at.
To summarize, our results indicate that there is no connection between the A118G SNP and a predisposition for substance abuse.
References


