A KCNJ6 (Kir3.2, GIRK2) gene polymorphism modulates opioid effects on analgesia and addiction but not on pupil size

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Abstract

Aim: KCNJ6 coding for potassium inwardly-rectifying channels (Kir3.2, GIRK2) is important for opioid receptor transmission. The KCNJ6 rs2070995 AA genotype has been associated with increased opioid analgesic requirements in Japanese. We analyzed its consequences for other opioid effects.

Methods: Genotyping was done in 85 methadone substituted former heroin addicts, 352 opioid treated chronic pain patients, and in 51 healthy volunteers where miotic effects of levomethadone had been measured. Expression of Kir3.2 in the Edinger-Westphal nucleus of rat brains was analyzed by means of immunohistochemistry.

Results: Average daily methadone substitution doses during the first therapy year were larger in the AA genotype (n = 4, 119.7 ± 49.6 mg/d) than in other rs2070995 genotypes (77.5 ± 26.2 mg/d, p = 0.003) whereas AA carriers lacked opioid withdrawal symptoms. A similar tendency toward less opioid effectiveness was observed toward higher opioid dosing demands for analgesia in the AA genotype (n = 17, opioid dose 2.03 ± 0.45 log mg oral morphine equivalents per day, controls: 1.81 ± 0.52 log mg OME/d, p = 0.093). In contrast, no pharmacogenetic effects were observed on miotic opioid effects. This could be traced back to the absence of Kir3.2 from the Edinger-Westphal nucleus in rat brains, a key cerebral structure governing pupil constriction.

Conclusions: The association of the KCNJ6 rs2070995 AA genotype with increased opioid requirements extends from analgesia to opiate substitution therapy. Opioid induced miosis is exempted for molecular histological reasons.

Key words

Opioid pharmacodynamics, addiction, pain
Introduction

Pain as a multi-factorial symptom [1] is modulated by several genes of which so far 296 have been identified in mice (PainGenes Database, http://www.jbldesign.com/jmogil/enter.html [2], January 15, 2009). This has been translated to common human pain only for less than a 10th of these genes. Information from additional genes may improve the utility of genotyping information. One gene listed in the PainGenes database as associated with altered opioid antinociception is KCNJ6. It codes for potassium inwardly-rectifying channels, subfamily J, member 6, (Kir3.2, GIRK2). This G protein-coupled channel is important for opioid receptor transmission and is involved in opioid effects [3] on postsynaptic inhibition [3] and mediating a significant component of analgesia [4, 5].

Genetic variants in KCNJ6 have recently been shown to increase opioid requirements in Japanese patients after abdominal surgery [6]. Considering interethnic differences in opioid effects and pharmacogenetics and in light of a generally poor reproducibility of genetic modulations, new associations are increasingly required to be shown in at least two independent cohorts. To support a functional association of the KCNJ6 rs2070995 G>A single nucleotide polymorphism (SNP) with decreased opioid effects [6], we have analyzed this pharmacogenetic modulation of opioid effects in data available from three independent cohorts of methadone substituted former heroin addicts [7], chronic pain patients in tertiary outpatient care [8] and healthy volunteers [9].

Methods

Assessments were on available data acquired following the Declaration of Helsinki on Biomedical Research Involving Human Subjects. The University of Frankfurt Medical Faculty Ethics Review Board (and local boards in cohort two [8]) approved the study protocols, and written informed consent was obtained from all subjects before the assessments.
Study cohorts

The first available cohort [7] consisted of a random sample of 59 unrelated caucasian men and 26 women (aged 22 to 58 years, mean age ± standard deviation of 35.4±8.5 years, body weight 38 to 103 kg, mean 65.9±12.5 kg) who were on methadone substitution therapy at a drug users outpatient center (“Malteser Drogenambulanz Schielestraße”, Frankfurt am Main, Germany) because of heroin addiction and fulfilled the criteria of DSM-IV-TR® for opioid-related disorders, codes 305.50 “Abuse” and 304.00 “Dependence”. At the start of therapy racemic methadone hydrochloride oral solution (6-dimethylamino-4,4-diphenyl-heptan-3-one hydrochloride, Methaddict®, Addicare Arzneimittel GmbH, Emmerich, Germany) was administered once daily at a dose of 30, 40 or 50 mg, selected by the investigator to meet the individual patients requirements. The dosage was then gradually adapted according to the clinical picture and therapy success, including the assessment of withdrawal symptoms such as sweating or sleepiness. The maximum dose was not limited by any guideline but adapted according to the particular patient’s needs. Daily methadone doses were available for the predefined observation period of 12 months following initiation of substitution therapy.

The second available cohort was a random sample of 156 men and 196 women, aged 58.5 ± 14.6 years, treated with opioids for 1 – 600 months (63.4 ± 92.4 months) for pain at three different University outpatient centers of tertiary care [8]. Reasons for opioid treatment were cancer pain (n = 47), craniofacial pain (n = 10), inflammatory pain (n = 19), mechanical low back pain (n = 87), musculoskeletal pain (n = 45), neuropathic pain (n = 109), somatoform pain disorders (n = 5) and other kinds of chronic pain, e.g., posttraumatic or chronic post surgery pain (n = 30). Opioids administered were tilidine or tramadol (n = 811 each), morphine (n = 74), fentanyl (n = 55), buprenorphine

1 Because some patients received two opioids, the sum of the patients numbers assigned to the particular opioids exceeds the total sum of patients included in cohort 3.
(\(n = 41\)), oxycodone (\(n = 38\)), hydromorphone (\(n = 14\)), dihydrocodeine (\(n = 2\)), and levomethadone or piritramide (\(n = 1\) each). In this cross-sectional assessment opioid doses and current 24-h pain intensity according to an 11-point numerical rating scale ranging from 0, “no pain”, to 10, “maximum pain”, were recorded once at enrolment.

The third cohort was available from a single-occasion open-label study [9] in a random sample of 25 healthy men and 26 women (aged 21 to 46 years, mean age ± standard deviation of 27.2±5.3 years, body weight 51 to 100 kg, mean 69.6±12 kg) who had received a single oral dose of 0.075 mg/kg levomethadone solution (L-Polamidon\(^\text{®}\)-Tropfen, Aventis Pharma, Bad Soden, Germany). As a reliable and sensitive parameter to quantify central nervous opioid effects, the pupil diameter was assessed by means of a pupillograph (“CIP”, Amtech GmbH, Weinheim, Germany). After two initial baseline measurements pupil size was measured with the start of medication at 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 7, 8 and 9 h. Measurements took place in a room without daylight, with light of 13.6 Lux effused by two computer screens [9].

**Genetic Analyses**

The main aim of this study was the verification of a recently proposed association of the \(KCNJ6\) variant rs2070995G>A with reduced opioid effects, which had been concluded from increased post-operative opioid dosing requirements [6]. To diagnose this single nucleotide polymorphism (SNP), genomic DNA was extracted from venous blood on a BioRobot EZ1 workstation applying the blood and body fluid spin protocol provided in the EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany). The \(KCNJ6\) polymorphism was screened for by means of a newly developed and validated Pyrosequencing\(^\text{TM}\) assay on a PSQ 96 MA System (Qiagen, Hilden, Germany), with assay details given in the online materials. Since a haplotype composed of \(KCNJ6\) rs2070995/rs6517442 had been suggested to be functional [6] but contained two SNPs only very weakly linked (\(r^2 = 0.02\), \(D' = 0.037\) [6], rs6517442A>G localized in the 5’ untranslated region and rs2070995G>A localized in exon 3), and
considering the known difficulties of haplotype estimation in the presence of low linkage disequilibrium [10], we analyzed the whole KCNJ6 gene locus. We therefore added the frequent (minor allele frequency ≥5%) variant rs702859T>C in the coding region (exon 4) and the HapMap tagging SNPs rs2409943A>G, rs11908866A>G, rs2835959A>T, rs2835885A>C, rs1787394T>G and rs10483038A>G (http://snp.cshl.org/cgi-perl/gbrowse/hapmap27_B36/ with data source “Hapmap Data Rel 27 Phase II+III, Feb09, on NCBI B36 assembly, dbSNP b126”). This SNP selection approach finally produced a set of nine frequent genetic variants in the KCNJ6 gene (Figure 3).

Statistics

KCNJ6 genetics

The correspondence between the observed number of homozygous and heterozygous individuals and the numbers expected from the Hardy-Weinberg equilibrium was checked by means of $\chi^2$ goodness-of-fit tests. SNPs with a minor allelic frequency of ≥5% were included in in-silico haplotype analysis using the PHASE computer software (version 2.1.1 for Linux [11]). Linkage disequilibrium (parameters D’ and $r^2$) between SNPs found at allelic frequencies ≥5% was analyzed using the Haploview software (version 4.2 [12]). Cohorts were compared with respect to allelic frequencies employing $\chi^2$ statistics (basic allele test, i.e., allelic frequency comparisons) with the SVS computer software (version 7.1.1 for Linux, Golden Helix, Inc., Bozeman, USA).

Genotype-phenotype associations

Since the KCNJ6 rs2070995 G>A SNP was reported to be functional only in homozygous carriers of the minor A allele [6], in this verificatory assessment genotype comparisons were done for AA versus AG/GG groups. This focused on the SNP and not the also reportedly functional haplotype because molecular consequences had been shown only for the SNP. Pharmacogenetic association
analysis was done at an exploratory level for the haplotype, however, as a separate analysis needed for discussion and not as part of the main analysis.

**Methadone substitution therapy**

Parameters of methadone substitution therapy analyzed for genetic influences were (i) the average daily dose during the first year of treatment, (ii) the maximum daily methadone dose, averaged per month, during the first year of treatment and (iii) the time in months when that maximum dose was reached, and (iv) the occurrence of withdrawal symptoms. Methadone doses were compared between homozygous carriers of the rs2070995 A allele and the other patients by means of t-tests (PASW Statistics version 18 for Linux, SPSS Inc., Chicago, USA). Effect sizes were calculated using Cohen’s d, which quantifies the standardized difference in parameter means between the group of interest, rs2070995 AA and the rest of the subjects, AG and GG. The time to reach the maximum daily dose was analyzed by means of Cox regression, with stepwise forward inclusion of candidate predictors consisting of the rs2070995 AA genotype, body weight, age and gender. The occurrence of withdrawal symptoms (0 = no, 1 = yes) was compared between genotype groups by means of $\chi^2$ statistics.

**Analgesic effects of opioids**

Parameters of opioid pain therapy analyzed for genetic influences were (i) the daily opioid dose and (ii) the actual 24-h pain score. Since several different opioids had been administered, doses were converted to oral morphine equivalents (OME) [8, 13]. Association analysis of the *KCNJ6* rs2070995 SNP with the opioid dose and the 24-h pain score was done by t-tests as above.

**Miotic effects of levomethadone**

Parameters of pupil size assessment analyzed for genetic influences were (i) the maximum miotic effects calculated as percent changes from baseline and (ii) the areas under the percent-change in pupil diameter from baseline versus time curve (AUC) calculated using the linear trapezoidal rule.
Genotype comparisons of maximum miosis and AUCs were done by means of t-tests (rs2070995 AA versus AG/GG).

*Immunohistochemistry*

Since pupil size data suggested that rs2070995 has no effect on levomethadone induced miosis, we investigated whether Kir3.2 is absent from the Edinger-Westphal nucleus, as it has been shown for Kir2.3 [14]. The Edinger-Westphal nucleus signals parasympathetic information to the ciliary ganglion, thus representing the output nucleus for constriction of the eye’s sphincter pupillae muscle leading to miosis. Expression analysis was done in rat brains. All animals were handled following the German animal protection laws and were approved by the governmental authorities. Adult Wistar rats (Charles River, Sulzfeld, Germany) were deeply anesthetized and fixed via transcardial perfusion with 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. Brains were dissected, cryoprotected in 0.8 M sucrose in PBS and shock-frozen as previously described [15]. Free-floating 20 µm coronal cryostat sections were preincubated in 10% normal goat serum (NGS) containing 0.3% Triton X-100 for 30 min at room temperature, incubated with anti-Kir3.2 primary antibody (diluted in 0.3% Triton X-100 in 10% NGS) for 36 hours at 4°C, and biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Peterborough, UK, 1:2000 in 0.2% bovine serum albumin in PBS) for 20 hours at 4°C. Antibodies were detected according to the manufacturer protocol using the Vectastain Elite ABC kit (Vector Laboratories) and visualized with nickel-enhanced 3,3’-diaminobenzidine tetrahydrochloride reaction [15]. Production and extensive characterization of the polyclonal monospecific affinity-purified anti-Kir3.2 antibody was described previously [16].
Results

Genotype-phenotype associations

Methadone substitution therapy

Both the average and the maximum daily methadone doses during the first year of substitution therapy were significantly larger in homozygous carriers of the *KCNJ6* rs2070995 A allele (n = 4, average dose: 119.7 ± 49.6 mg/d, maximum dose: 132.8 ± 54.7 mg/d) than in the other rs2070995 genotypes (average dose: 77.5 ± 26.2 mg/d, p = 0.003, Cohen’s d = 1.4; maximum dose: 91.2 ± 30.7 mg/d, p = 0.013, Cohen’s d = 1.2) (Figure 1). The time needed to reach the maximum methadone dose was not associated with the *KCNJ6* genotype (Cox regression: p = 0.48). However, homozygous carriers of the rs2070995 A allele had no withdrawal effects while those were diagnosed in 55 of the 81 patients not homozygous for this variant allele ($\chi^2$ test: p = 0.006).

Analgesic effects of opioids

The opioid doses followed more a log-normal than a normal distribution. Opioid dosing demands in homozygous carriers of the *KCNJ6* rs2070995 A allele (n = 17, opioid dose 2.03 ± 0.45 log mg OME/d) tended to be higher than those of the other chronic pain patients (1.81 ± 0.52 log mg OME/d, p = 0.093, Cohen’s d = 0.42) (Figure 1). This achieved similar pain scores of 3.2 ± 2.7 and 3.8 ± 2.5 of the 11-point rating scale (p = 0.405). The distribution of *KCNJ6* rs2070995 AA carriers differed significantly neither among pain diagnoses ($\chi^2$ test: p = 0.275) nor among opioids ($\chi^2$ tests: p always >0.05).

Miotic effects of levomethadone

Neither the maximum miotic effects nor the areas under the miosis versus time curves were significantly smaller in homozygous carriers of the *KCNJ6* rs2070995 A allele (n = 5, maximum miosis: -
42.6 ± 7.8%, AUC: -231.2 ± 49.1%-h) than in the heterozygous and wild-type genotypes (maximum miosis: -41.9 ± 9.8%, p = 0.88, Cohen’s d = -0.073, AUC: -242.3 ± 80.6%-h, p = 0.76, Cohen’s d = 0.14) (Figure 1).

**Absence of Kir3.2 from the Edinger-Westphal nucleus**

Survey micrographs of the rat midbrain at the level of superior colliculus demonstrate the specific Kir3.2 protein expression with highest levels in the substantia nigra pars compacta (Figure 2 A). The dark neurons of the Edinger-Westphal nucleus are clearly detected in a cresyl violet-stained section below the ventral margin of the periaqueductal gray (Figure 2 B). Edinger-Westphal neurons in an adjacent section are devoid of Kir3.2 immunoreactivity (Figure 2 C).

**KCNJ6 gene locus**

All observed distributions of homozygous or heterozygous carriers of a SNP corresponded to the Hardy-Weinberg equilibrium (χ²-test: p>0.05 for the nine KCNJ6 SNPs diagnosed in healthy volunteers and drug addicts, and p>0.05 for rs2070995 in the pain patients). The distribution of KCNJ6 variant alleles was similar between the cohort of healthy volunteers and the opiate addicts (genotype association tests using χ² statistics: p>0.09 at an uncorrected α-level for all KCNJ6 variants, Table 1). In-silico haplotype analysis included all nine KCNJ6 genetic variants. One haploblock with high linkage was identified including the SNPs rs2835885A>C, rs1787394T>G, rs10483038A>G and rs702859T>C localized in intron 3 and exon 4, respectively (Figure 3). The linkage between the other five SNPs reaching from SNP rs6517442A>G in the 5’ UTR up to SNP rs2070995G>A localized in exon 3 was low. Especially, the linkage between the two variants rs6517442A>G and rs2070995G>A reported to compose a functional haplotype [6] was very low with linkage disequilibrium values of $r^2=0.01$ and $D’=0.13$. 
Discussion

In an independent cohort we could repeat the finding that the KCNJ6 rs2070995 polymorphism increases opioid dosing demands in homozygous carriers of the minor A allele [6]. The result extends the association of the KCNJ6 rs2070995 AA genotype from increased postoperative opioid requirements to increased dosing requirements during opioid substitution therapy. According to Cohen’s d of >0.8 the effects for opioid dosing for drug substitution are large. However, a comparison between analgesia and substitution therapy is not valid because of the different data qualities and study designs. The effects fit to the decreased opioid effects due to lower expression of an important postsynaptic component of the opioid receptor signaling cascade. In addition, the effects fit to reported importance of Kir3.2 for CNS effects of alcohol [17, 18] and for schizophrenia [19] that partly involve similar molecular systems as addiction. In addition to the decreased methadone effects for heroin substitution, the KCNJ6 variant decreased the incidence of opioid withdrawal effects. This provides a clinical correlate for the attenuated withdrawal effects in GIRK2/3 knockout mice [20].

In a further clinical sample, a statistical tendency of p < 0.1 was obtained for an effect for opioid analgesic therapy that pointed in the expected direction. The non-significance with two-sided testing may be attributed to the cross-sectional nature of the sample not ideally suited for genetic assessments. Furthermore, the inclusion of patients receiving various opioid analgesics required opioid conversions, which are estimates derived from several studies including meta-analyses resulting in several equally performing conversion systems [21]. However, such a sample reflects the clinical utility of a genotype in everyday practice and provides therefore valuable information supporting the functional association. According to the accepted interpretation of Cohen’s d with a value of d = 0.2 as indicative of a small effect, 0.5 of a medium and 0.8 of a large effect size [22], the effect sizes for opioid demands for analgesia are small (Cohen’s d = 0.42) although slightly larger than in the original paper, where an effect size of 0.25 for postoperative opioid demands was found [6].
Again, the cross-sectional setting of our cohort 2 obtained from outpatients treatment of all kinds of pain was expected to have resulted in noisier data and therefore the significance level of $p = 0.093$ can be taken as supportive evidence for a pharmacogenetic effect. Nevertheless, when considering that the same data set has been searched for the effects of several other genotypes [8], the result would not withhold conservative statistical criteria even when the unidirectional hypothesis allows for single-sided testing and thus a significance was possible. Without the previous finding [6], the result could hardly be used as a proof for the genetic effect.

The present positive associations were seen only when contrasting homozygous carriers of the \textit{KCNJ6} rs2070995 A allele with pooled heterozygous and non-carriers. A gene dose effect or a significant difference between carriers and non-carriers, i.e., pooling heterozygous and homozygous carriers, was not seen (details not shown). This verifies the previous report of a molecular and clinical effect of the variant only when homozygously present [6].

In contrast to drug substitution and analgesia, the \textit{KCNJ6} variant did not significantly modulate the miotic opioid effects of levomethadone. The difference in the maximum miotic effects was even in the wrong direction, i.e., toward increased opioid effects in homozygous carriers of the rs2070995 A allele contrasting to reduced effects in analgesia or opioid substitution therapy. At least the AUC difference was in the expected direction toward smaller opioid effects. With lack of statistical significance for an expected effect, the first possible reason is insufficient sample size. This would have been surprising because pupil size data usually are less noisy than pain data, especially clinical ones, and would also contrast to the results with a positive control genotype (\textit{OPRM1} 118A$\rightarrow$G, rs1799971) from a previous assessment of this data set [9]. However, a sample size calculation to obtain the presently observed difference in pupil size AUCs statistically significant at a power of 0.8 resulted in 231 cases per group. Hardy-Weinberg equilibrium assumed, 231 AA carriers would be found in a random sample of 4367 subjects based on the present minor allele frequency. In contrast, the pupil size data allowed to detect the effect of the \textit{OPRM1} rs1799971A$\rightarrow$G in this data set.
[9] with the same number of homozygous carriers as presently found for KCNJ6 rs2070995. When taking only homozygous carriers of the OPRM1 variant, the difference between -110.9±49.1 %·h in OPRM1 rs1799971 GG carriers and -247.2±73.4 %·h in OPRM1 rs1799971 AA/AG carriers was significant at p = 0.003.

Thus, the lacking effect on miosis seemed statistically sound despite the small sample size of homozygous carriers. From this, we abductively reasoned that a likely cause may be the absence of Kir3.2 from the Edinger-Westphal nucleus. This conclusion was subsequently verified by our finding that the neurons of the Edinger-Westphal nucleus indeed lack expression of the Kir3.2 channel protein. For brain structures involved in drug dependence, the positive finding with the drug addicts’ data (cohort 1) can be taken as a strong indication that Kir3.2 is expressed in relevant structures of addictive behaviors. This is supported by the finding of Kir3.2 in neurons of the mesolimbic system [23].

The haplotype rs6517442G/rs2070995A had previously been reported to be also associated with decreased opioid effects [6]. However, the structure of the human KCNJ6 gene judged by the present selection of nine variants showed low linkage between the two variants rs6517442 and rs2070995 suggested to form a functional haplotype. This raises question about the combination of SNPs being a relevant haplotype and raises doubt about the existence of this haplotype, considering the known difficulties of haplotype estimation in the presence of low linkage disequilibrium, stressing the importance of careful consideration of confidence measures when using estimated haplotype frequencies and individual assignments in biomedical research [10]. An exploratory association analysis for this haplotype was negative in all data sets (details not shown). We therefore propose to not further stress the functional importance of that “haplotype”.

In conclusion, we could reproduce the association of the KCNJ6 rs2070995 AA genotype with increased opioid requirements. The modulation of opioid effects was extended to methadone substi-
tuation therapy and consists in increased dosing requirements and reduced withdrawal effects. In addition, based on absent effects on levomethadone induced miosis in a small sample of volunteers and subsequently verified immunohistochemically, we could show that KCNJ6 genetics does not affect pupillary effects of opioids. This emphasizes that opioid effects may be differentially modulated by genetic variants.

References


Table 1: Minor allele frequency of *KCNJ6* SNPs and haplotypes in two independent cohorts (methadone substituted drug addicts and healthy volunteers) and significance of the differences in allelic frequency. For the *KCNJ6* rs2070995 SNP in the focus on this paper, a comparison with healthy volunteers was additionally done for a cohort of 352 opioid treated chronic pain patients. The other *KCNJ6* SNPs were only analyzed for gene locus and haplotype re-evaluation, which did not require all cohorts, and are grayed out.

<table>
<thead>
<tr>
<th><em>KCNJ6</em> variant</th>
<th>Chronic pain patients (n=352)</th>
<th>Minor allele frequency</th>
<th>Significance of the difference “addicts versus control” (uncorrected)</th>
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<td>rs6517442A&gt;G</td>
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*Haplotype composed of positions *KCNJ6* rs2835885A>C, rs1787394T>G, rs10483038A>G, rs702859T>C that were linked in a haploblock (Figure 3). Minor alleles underlined.
Figure 1: The influence of the *KCNJ6* rs2070995 G>A polymorphism on opioid effects (means, indicated as open circles, and 95% confidence ranges) **Left**: Average methadone maintenance dose during the first year of substitution therapy in 85 heroin users (AA genotype: n=4, **: p < 0.01). **Middle**: Daily opioid doses in 352 chronic pain patients treated for pain of various reasons in tertiary outpatient care (AA genotype: n=17, (*): 0.05 < p < 0.1). **Right**: Maximum percent decrease in pupil size from baseline following oral administration of 0.075 mg levomethadone per kg body weight to 51 healthy volunteers (AA genotype: n=5).
Figure 2: Protein distribution of the Kir3.2 channel in a rat midbrain section shows highest signal in the substantia nigra pars compacta (A). The dark neurons of the Edinger-Westphal nucleus (B, thin lines) are easily detected in an adjacent section stained with cresyl violet (B, area boxed in A). These neurons do not display Kir3.2 immunoreactivity when the corresponding area (boxed in A) is inspected at higher magnification (C). Aq, cerebral aqueduct; EW, Edinger-Westphal nucleus, PG, periaqueductal gray; SC, superior colliculus; SN, substantia nigra. Scale bars represent 2 mm in (A) and 200 µm in (B) and (C).
Figure 3: **Top:** Distribution of nine genetic variants in the *KCNJ6* gene. The SNPs are given in reference to the ATG start codon. Nucleotide 1 is the A of the ATG start codon, nucleotides localized in the 5'-UTR have negative numbers. Intronic nucleotides are numbered in relation to the nearest exonic nucleotide e.g., dbSNP rs2835959A>T in intron 2 is localized 52461 nucleotides downstream to the last nucleotide of exon 2 (c.25+52461) and dbSNP rs10483038A>G in intron 3 is localized 26985 upstream from the first nucleotide of exon 4 (c.947-26985).

**Bottom:** Results of linkage analysis using the solid spine of LD method implemented in Haploview. Linkage disequilibrium is quantified by values of \( r^2 \) (displayed as percentage according to the default of the Haploview software) indicated in each box within the triangular plot, whereas values of \( D' \) are indicated color-coded (red representing high \( D' \), blue indicating low \( D' \)).