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COMPLETE KNOCKOUT OF THE NOCICEPTIN/ORPHANIN FQ RECEPTOR IN THE RAT DOES NOT INDUCE COMPENSATORY CHANGES IN μ, δ AND κ OPIOID RECEPTORS

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Abstract—The nociceptin/orphanin FQ (N/OFQ) opioid peptide receptor (NOPr) is a new member of the opioid receptor family consisting of μ, δ and κ opioid receptors. The anti-opioid properties of its endogenous ligand, N/OFQ provide the receptor interesting potentials in symptoms and processes related to drug addiction, learning and memory, anxiety and depression, and nociception. Using target-selected N-ethyl-N-nitrosourea (ENU)-driven mutagenesis we recently generated a rat model bearing a premature stop codon in the opioid-like receptor (oprl1) gene, and here we describe the primary characterization of this novel model. Data revealed that [^{3}H]N/OFQ binding to brain slices was completely absent in rats homozygous for the premature stop codon (oprl1$^{-/-}$). Heterozygous rats displayed an intermediate level of NOPr binding. Oprl1 receptor transcript levels, as determined by Northern blot analysis, were reduced by approximately 50% in oprl1$^{-/-}$ rats compared to wild-type controls (oprl1$^{+/+}$), and no alternative spliced transcripts were observed. Quantitative autoradiographic mapping of μ, δ and κ opioid receptors using [^{3}H]DAMGO, [^{3}H]deltorphin and [^{3}H]CI-977, respectively, did not show any changes in opioid receptor binding. In conclusion, we present a novel mutant rat lacking NOPr without compensatory changes in μ, δ and κ opioid receptors. We anticipate that this mutant rat will have heuristic value to further understand the function of NOPr.

Key words: drug addiction, autoradiography, alternative splicing, ENU.

The gene encoding the nociceptin/orphanin FQ (N/OFQ) opioid peptide receptor (NOPr), officially termed the opioid-like receptor (oprl1) gene (HUGO gene nomenclature, www.genenames.org), has recently been cloned in several species, including the rat (Bunzow et al., 1994), and is a new member of the opioid receptor seven-transmembrane receptor family consisting of the μ, δ and κ opioid receptors. The endogenous ligand for NOPr is the hepatadecapeptide N/OFQ, named after two independent discoveries of the peptide. Meunier and colleagues (1995) introduced the peptide as nociceptin because of its pronociceptive effects. Reinscheid and coworkers (1995) introduced the peptide as orphanin FQ based on the amino acid composition of the peptide. N/OFQ binding to NOPr results into the activation of inhibitory G proteins that inhibit adenylate cyclase, inhibit N-type Ca^{2+} channels, and activate inward rectifying K^+ channels (Meunier et al., 1995; Reinscheid et al., 1995; Chan et al., 1998). N/OFQ is described as an anti-opioid peptide, as it is able to reverse or prevent morphine antinociception (Mogil et al., 1996; Morgan et al., 1997). Further, N/OFQ has acute inhibitory effects on spontaneous locomotor activity (Reinscheid et al., 1995; Rizzi et al., 2001), reduces cocaine-stimulated (Lutfy et al., 2002), reduces rotarod performance (Marti et al., 2004), blocks ethanol-induced conditioned place preference (Kuzmin et al., 2003; Sakoori and Murphy, 2008), reduces alcohol intake (Ciccocioppo et al., 1999, 2000, 2003), interferes with learning and memory functions (Sandin et al., 1997; Noda et al., 2000; Higgins et al., 2002), and has anxiolytic and antistress effects (e.g. Jenck et al., 2000; Vitale et al., 2006; Gavioli and Calo, 2006; Rodi et al., 2008; but see Fernandez et al., 2004; Green et al., 2007).

Using target-selected N-ethyl-N-nitrosourea (ENU)-driven mutagenesis we recently generated a knockout rat bearing a premature stop codon in the oprl1 gene (Smits et al., 2006, 2008; van Boxtel et al., 2008b). A premature stop codon theoretically results into a knockout of the targeted gene, as we previously have shown for the 5-HT transporter knockout rat (Homberg et al., 2007), melanin concentrating hormone knockout rat (Mul, unpublished observations) and mismatch repair protein knockout rat (van Boxtel et al., 2008a). Because the rat has several species-specific advantages, including optimal usage in cognitive behavioral studies and the easier application of invasive techniques, an opioid-like receptor gene knockout (oprl1$^{-/-}$) rat would be a valuable complementary model to existing oprl1$^{-/-}$ mice. These mice have revealed several interesting functions of NOPr. oprl1$^{-/-}$ Mouse phenotypes include reduced ethanol consumption and preference, slightly increased ethanol-induced place preference (Sakoori and Murphy, 2008), increased cocaine-induced conditioned place preference Marquez et al. (2008), increased anxiety-

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Abbreviations: ENU, N-ethyl-N-nitrosourea; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N/OFQ, nociceptin/orphanin FQ; NOPr, nociceptin/orphanin FQ opioid peptide receptor; N.S., non-significant; oprl1, opioid-like receptor gene; oprl1$^{-/-}$, oprl1 knockout.

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related behavior in the elevated plus maze test and anxiolytic-like behavior in the novelty-suppressed feeding test (Gavioli et al., 2007), an antidepressant-like profile (Gavioli et al., 2003), increased learning and memory (Manabe et al., 1998), and improved rotarod performance (Marti et al., 2004). Furthermore, oprl1$^{-/-}$ mice have proven valuable as control for the specificity of NOPr ligands.

The first aim of the present study was to establish the molecular knockout phenotype of NOPr in our rat model, for which we applied [^{3}H]nociceptin autoradiography. The rat oprl1$^{-/-}$ gene consists of four exons (Ensembl Genome Browser: ENSRNOG00000016768), and the open reading frame consists of 1283 bp. Primer extension analysis of the gene revealed two major transcription initiation sites: one in the 5′ flanking region and the other in intron 1. Several alternatively spliced transcripts of the oprl1$^{-/-}$ gene have been reported (Currò et al., 2001). Four splice variants deleted for exon 1 are expressed only in the brain. In addition, five isoforms containing exon 1 are expressed in various tissues, such as brain, testes, and gastrointestinal tract (Currò et al., 2001). Although no alternative splice sites have been reported for exon 3, in which the premature stop codon is located in the oprl1$^{-/-}$ rat and where several of the seven transmembrane domains are encoded, it could be that alternative transcripts are maintained in the knockout rat. As it is unclear whether truncated proteins resulting from alternative splice events have biological relevance, an animal model expressing a selection of alternative splice forms could be valuable to obtain insight into the function of the truncated proteins. Using Northern blot analysis and probes directed to exons 1, 3 and 4, the second aim was to study and quantify oprl1 transcript levels in our rat model.

NOPr is highly homologous to the classic μ, δ and κ opioid receptors with around 80% homology in the 2nd, 3rd and 7th membrane spanning domains. Despite this structural homology, NOPr does not bind opioid receptor ligands (Bunzow et al., 1994; Wick et al., 2004). Yet, some studies have pointed out that NOPr and μ opioid receptors do interact. For example, i.c.v. administration of N/OFQ attenuates the development of morphine-induced place preference (Murphy et al., 1999; Ciccocioppo et al., 2000). This raises the possibility that compensatory adaptations occur in μ, δ and/or κ opioid receptors. Although limited, changes in μ, δ and κ opioid receptor binding potential indeed have been reported in oprl1$^{-/-}$ mice (Clarke et al., 2001). Therefore, the final aim of this study was to determine whether compensatory adaptations have taken place in μ, δ and/or κ opioid receptor expression in the oprl1$^{-/-}$ knockout rat model.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Animal Care Committee of the Royal Dutch Academy of Sciences and conducted according to the Dutch law for animal experiments. All efforts were made to minimize the amount of animals. NOPr knockout rat (OPRL1^{1Hubr}) was generated by target-selected ENU-induced mutagenesis in a Brown Norway background (for detailed description, see Smits et al., 2006). The animals were outcrossed for two generations on a Brown Norway background. Because Brown Norway rats breed poorly, they were subsequently backcrossed on a Wistar (Wistar/Crl) background for four generations. Backcrossings were performed to eliminate possible additional mutations induced by ENU-mutagenesis. Heterozygous oprl1$^{+/-}$ rats were crossed to generate the experimental animals. Under the used mutagenesis conditions the mean mutation frequency was roughly one in 1.0–1.2 million base pairs (about 1 cM). Although the chance for the occurrence of a very tightly linked mutation with a phenotypic effect is very small, this possibility should be taken into account in the design and interpretation of experiments. To control for this possibility as much as possible, we compared littermates. At the age of 3 weeks ear cuts were taken under anesthesia and used for genotyping. Genotypes were reconfirmed after experimental procedures were completed. Animals were housed under standard conditions in groups of two to four per cage per gender under controlled experimental conditions (12-h light/dark cycle, 21±1 °C, 60% relative humidity, food and water *ad libitum*). Only male rats, aged 12–20 weeks, were used.

Genotyping

Genotyping was performed as described earlier (Smits et al., 2006). Genomic DNA was isolated from ear cuts that were sampled in a 96 deep well block (2.5 ml Riplate, Ritter, Schmidlin, Neuheim, Switzerland) and lysed overnight at 55 °C in 300 μl lysis buffer (100 mM Tris–HCl pH 8.5, 200 mM NaCl, 0.2% SDS, 5 mM EDTA and 100 μg/ml freshly added proteinase K). Tissue debris was centrifuged down for 15 min at 15,000×*g* and supernatant was transferred to fresh tubes. DNA was precipitated by adding an equal volume of isopropanol, mixing and centrifugation at 15,000×*g* at room temperature. The supernatant was removed by gently inverting the block and the pellets were washed with 70% ethanol, and dissolved in 400 μl water. A fragment of the oprl1 gene, containing the ENU-induced mutation was amplified using gene-specific primers (forward: position 3522, GGGTTGAGAGATTCAAAGACC; reverse: position 3826, GATAGCCACATAGCGGTCTAC; see Fig. 1 for location) and a touchdown PCR cycling program (92 °C for 60 s; 12 cycles of 92 °C for 20 s, 65 °C for 20 s with a decrement of 0.6 °C per cycle, 72 °C for 30 s, followed by 20 cycles of 92 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s; 72 °C for 180 s; GeneAmp9700, Applied Biosystems). PCR reactions contained 5 μl DNA, 0.2 μM forward primer, 0.2 μM reverse primer, 400 μM of each dNTP, 25 mM Tricine, 7% glycerol (w/v), 1.6% DMSO (w/v), 2 mM $MgCl_2$, 85 mM ammonium acetate (pH 8.7), and 0.2 U Taq polymerase in a total volume of 10 μl. The samples were checked on a 1% agarose gel containing ethidium bromide for the presence of the proper PCR fragment. The PCR reactions were diluted with 25 μl water, mixed by pipetting, and 1 μl was used as template for the sequencing reactions. Sequencing reactions, containing 1 μl BigDYE (v3.1: Applied

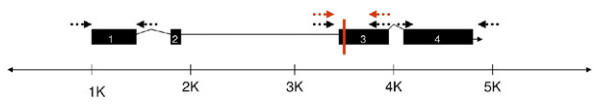

Fig. 1. Schematic overview of NOPr transcript. The premature stop codon in located in exon 3 (red bar). The arrows indicate the primers that were used for genotyping (red arrows), and the generation of probes for Northern blotting (black arrows). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.