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# NEONATAL OPIOID WITHDRAWAL SYNDROME
# NEONATÁLNÍ OPIOIDNÍ ABSTINENČNÍ SYNDROM

Bachelor's thesis

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# Outline

## 1. Abstract

Neonatal opioid withdrawal syndrome (NAS) is a consequence of opiate use during pregnancy. The severity of NAS outcomes depends not only on the type of opioid administration, but also on gestation age and other substances used, such as tobacco or antibiotics. The single nucleotide polymorphisms (SNPs) in genes for opioid receptors, dopamine, and methadone metabolisms and the epigenetic markers such as methylation of μ-opioid receptor genes are related to different NAS symptoms and changes in length of hospital stay and pharmacological treatment. This work includes a description of the molecular principles of opioid dependence, genetic predispositions, risks of opioid exposure during pregnancy and modern methods of treatment of neonatal opioid withdrawal syndrome.

Keywords: Neonatal opioid withdrawal syndrome, drugs in pregnancy, opioid dependence, opioids, morphine

**Abstrakt**

Neonatální opioidní abstinenční syndrom (NAS) je důsledkem užívání opiátů během těhotenství. Závažnost projevů NAS zaleží nejen na typu používané opioidní látky, ale i na gestačním věku a dalších používaných látkách, jako jsou tabák anebo antibiotika. Jednonukleotidové polymorfismy (SNP) v genech pro opioidní receptory, metabolismus dopaminu anebo metadonu a epigenetické markery, jako je methylace v genech pro μ-opioidní receptor, souvisí s různými příznaky NAS a změnami délky pobytu v nemocnici a farmakologické léčby. Tato práce zahrnuje popis molekulárních principů vzniku závislosti na opiátech, genetické predispozice, rizik užívání těchto látek v období gravidity a moderní metody léčby vzniklého neonatálního opioidního abstinenčního syndromu.

Klíčová slova: Neonatální opioidní abstinenční syndrom, drogy v těhotenství, závislost na opiátech, opioidy, morfin

## 2. List of abbreviations

| | |
|---|---|
| ABCB1 | ATP binding cassette subfamily B sember 1 |
| AC | Adenylyl cyclase |
| BDNF | Brain-derived neurotrophic factor gene |
| cAMP | Cyclic adenosine monophosphate |
| CeA | Central nucleus of the amygdala |
| COMT | Catechol-O-methyl transferase |
| CREB | cAMP response element-binding protein |
| CYP2B6 | Cytochrome P450 2B6 |
| CYP2D6 | Cytochrome P450 2D6 |
| CYP2E1 | Cytochrome P450 2E1 |
| DAMGO | D-Ala(2), N-Me-Phe(4), Gly(5)-ol |
| DOR | δ-opioid receptor |
| ERK | Extracellular signal-regulated kinase |
| GABA | γ-Aminobutyric acid |
| GRK | G-protein-coupled receptor kinase |
| GTP | Guanosine-5'-triphosphate |
| HDAC | Histone deacetylase |
| KOR | κ-opioid receptor |
| LC | Locus coeruleus |
| MAPK | Mitogen-activated protein kinase |
| MMT | Methadone maintenance therapy |
| MOR | μ-opioid receptor |
| NA | Nucleus accumbens |
| NAS | Neonatal abstinence syndrome |
| NMDA | N-methyl-D-aspartate receptor |
| NRM | Nucleus raphe magnus |
| OPRD1 | δ-Opioid receptor gene |

| | |
|---|---|
| OPRK1 | κ-Opioid receptor gene |
| OPRM1 | μ-Opioid receptor gene |
| OR | Opioid receptor |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| POMC | Proopiomelanocortin |
| RP | R-adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium |
| VTA | Ventral tegmental area |

## 3. Introduction

The relevance of the topic of this work is related to a significant increase in opioid use over the past decade. For the year 2016, 26.8 million people were reported to suffer from opioid use disorder globally (Vos et al., 2017). Opioid administration contributes not only to risks of morbidity or mortality, but it could also affect fetus during pregnancy. In utero opioid exposure results in neonatal abstinence syndrome (NAS) and for the year 2017 the incidence rate of NAS (in the United States) was 7.3 per 1000 in-hospital births and the rate of maternal opioid-related diagnoses was 8.2 per 1000 delivery hospitalization in contrast with the year 2010 with 4.0 for NAS rate and with 3.5 for maternal opioid-related diagnoses rate (Hirai et al., 2021).

The purpose of this work is to process data of research focused on molecular mechanisms of opioid dependence, epigenetic changes and to describe NAS as a complex consequence of many different factors, composed of genomic variations in several genes and epigenetic markers, which could affect the scale of NAS severity. Furthermore, the pharmacological management will be considered from the side of pharmacological and non-pharmacological approach.

## 4. Opiates

### 4.1 A brief history of opiates

The opium poppy is classified as *Papaver somniferum*, the genus is taken from Latin noun *papāver* means poppy and *somniferum* as a species was created by meaning "sleep inducing". Swedish naturalist Carl Linnaeus was the first who classified this plant in his publication Genera Plantarum (1753). In fact, opium is a dried poppy extract, which contains several alkaloids (Tab. 1).

| | |
|---|---|
| Phenanthrene alkaloids: | |
| Morphine | 9–17% W/W |
| Codeine | 0.3–4 |
| Thebaine | 0.5–1 |
| Benzylisoquinoline alkaloids | |
| Papaverine | 0.5–1 |
| Narcotine | 6.0 |
| Narceine | 0.3 |
| Noscapine | 2–8% |

**Table 1.** Major constituents of opium, adopted from: (Budd, 1981).

The first mention appeared in low Mesopotamia (now the Arab Kingdom of Iraq). The Sumerians learned to cultivate poppies and isolate opium from seed capsules 5000 B.C. and called this plant the flower of joy ("Hul Gil") (Tompkins, 1953).

Then it spread to pharaonic Egypt with their famous poppy fields and opium as a treatment to calm children`s cries and some intestinal pains caused by parasites and later it appeared in ancient Greece. Hippocrates wrote about poppy juice as a useful narcotic to help with convulsions, also as a hypnotic, cathartic, and styptic drug (Kritikos & Papadaki, 1967).

After some traveling through Persia, India, and China, opium became a taboo in the 14$^{th}$ century in Europe with the arrival of the Holy Inquisition and was related to the Devil because of its East genesis. Mentions about opium disappeared for two hundred years but then during the Renaissance Paracelsus reintroduced it as a treatment called *the stone of immortality* or laudanum, a mixture which contained opium and strong alcohol like rum or whiskey (Aragón-Poce et al., 2002). This blend served for surgery preparations.

The first who started using opium to treat pain as postoperative analgesia was English surgeon, J. Moore (1749 – 1834). He noted that opium is highly expedient to abate the smarting of the wound after the operation is over and to induce sleep (Hamilton & Baskett, 2000).

In the year 1805, Friedrich Sertürner isolated the main active ingredient of opium. He discovered a new acid in opium, "mekonsaure" or "mohnsaure" (Hamilton & Baskett, 2000), but it was inactive. Then he found a water-insoluble crystalline substance which had an "almost alkaline-like character" and named it in honor of the Greek god of dreams (Morpheus), "morphium". It was used in an oral form, morphine acetate, which was a difficult and expensive salt to prepare.

With the invention of the hypodermic syringe and needle in the 1850s, morphine became widespread. It played a big role in treating soldiers during wars (Brook et al., 2017). Alas, it has been shown that morphine has the same abusive potential as opium and can cause withdrawal syndrome. Realization of this serious drawback led to the preparation and testing of various morphine derivatives such as methylmorphine (codeine) and diacetylmorphine (heroin) in the 19$^{th}$ century.

The new substance heroin, which was introduced as a pharmacological product in 1898, was presented by Bayer as a new drug that would be of immense value for the treatment of severe respiratory disease since it not only suppressed cough but could even assist in clearing the lungs of excess phlegm and other matter (Sneader, 1998). However, it turned out that repeated heroin administration leads to addiction with a very high tolerance and euphoric effect, which was the cause of heroin abuse.

In the year 1946, I.G. Farbendustrie invented oral methadone. Initially, it was not provided as an analgesic treatment (Payte, 1991), but it proved to be a good substitution for morphine and heroin addicts with withdrawal syndromes. By the 1960s, methadone maintenance treatment (MMT) was introduced as a treatment for opioid dependence. Buprenorphine was discovered in 1966 and found to be beneficial for the treatment of opioid dependence, similarly as methadone (Campbell & Lovell, 2012)

The first case of the neonatal abstinent syndrome (NAS) in infants and its treatment was reported in 1875 and was called “congenital morphinism” and later it was termed as “congenital neonatal addiction” (Jones & Fielder, 2015). Nowadays neonatal withdrawal is widespread and has a serious epidemic status. For example, the population-based study in Washington State reported that the proportion of diagnosed neonates with opioid withdrawal syndrome (exposed prenatally to opioids) increased from 26.4% in 2000 to 41.7% in 2008 (Creanga et al., 2012).

### 4.2 Endogenous opioid system

#### 4.2.1 Opioid peptides

The first step of understanding endogenous opioid system was the realization that all these opiate derivates drugs must bind to some specific sites with specific affinity or receptors on nerve cells. It was later revealed that all these narcotics mostly have a dual action, some of them antagonize and others work as agonists. Gyang and Kosterlitz tested morphine-like analgesics on guinea-pig ileum by responses and highlighted morphine, levorphanol or phenazocine as "narcotic agonists" and nalorphine, cyclazocine, N-methylallylnormorphine as "narcotic antagonists" (Gyang & Kosterlitz, 1966).

***Enkephalins.*** Two pentapeptides were identified with potent opiate agonist activity in 1975 by Hughes and his colleagues. They isolated a substance from the pig brain, which was named enkephalin. In this research, they showed that the spectrum of the derivative of a mixture of the two synthetic peptides is

identical to that of natural enkephalin and contains two pentapeptides **H-Tyr-Gly-Gly-Phe-Met-OH** (methionine-enkephalin) and **H-Tyr-Gly-Gly-Phe-Leu-OH** (leucin-enkephalin) (Hughes et al., 1975). This trial helps to realize, that these natural peptides may work as neurotransmitters to transmit signals from neurons to target cells or as neuromodulators, because of their inhibitory (agonist) activity at opiate receptor sites. Further, discovered β-lipotropin could be a precursor for these peptides.

***Endorphins.*** In 1976 β-endorphin was isolated from human pituitary glands. The proof that β-endorphin has opiate activity was provided by blocking its effect by opiate antagonist naloxone. Also, β-endorphin had a similar sequence of the COOH-terminal 31 amino acids of human β-lipotropin (C. H. Li et al., 1976). It has been established that β-endorphin is a product of β-lipotropin. Later it showed that β-endorphin contains a sequence of the first 16 amino acids which is similar to α-endorphin. In the same year, R. Guillemin and collaborators isolated and described α-endorphin and γ-endorphin from an extract of porcine hypothalamus-neurohypophysis and their morphine-like activity was established by reversing their effects with naloxone. The tentative primary structure of α-endorphin is **H-Tyr-GlyGly-Phe-Met-Thr-Ser-Glx-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH** and for γ-endorphin it is **H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-SerGln-Thr-Pro-Leu-Val-Thr-Leu-OH** (Ling et al., 1976). Interestingly, the first five amino acids in α-endorphin have the same sequence for methionine-enkephalin, which suggests that the protein β-lipotropin is a precursor for endorphins and methionine-enkephalin.

In fact, there is a protein that is a common precursor for corticotropin and opioid peptides derived from β-lipotropin, named pro-opiocortin. The identification of this molecule was based on a peak of opioid activity that was eluted at the position of the nonapeptide β-LPH (61-69), which was also the same fragment obtained by trypsin digestion of β-lipotropin or β-endorphin (Rubinstein et al., 1978). In the same study, leucin-enkephalin was described as a β-lipotropin derivative.

***Dynorphins.*** Porcine dynorphin A was isolated from the pituitary and described as a potent opioid peptide, which had a sequence of Leu-enkephalin by terminal -COOH extension **-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH** (Goldstein et al., 1979). Dynorphin showed great efficiency, which was more potent than leucin-enkephalin by about 700 times. This high potency could be ascribed to the structure of dynorphin, which includes leucin-enkephalin as an enhancer. A new peptide α-neo-endorphin was isolated from the porcine hypothalamus (Kangawa et al., 1979) and its structure is -**Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-** (Kangawa et al., 1981). Then, rimorphin (dynorphin B) was purified from bovine posterior pituitary glands with $NH_2$-Tyr-Gly-Gly-Phe-LeuArg-Arg-Gln-Phe-Lys-Val-Val-Thr-COOH sequence (Kilpatrick et al., 1982).

It was proved by immunocytochemical methods that α-neo-endorphin, A dynorphin and B dynorphin occur within the same cells of several hypothalamic and caudal brainstem nuclei, what summed up the presence of pro-dynorphin as a precursor for these three peptides (Watson et al., 1983).

### 4.2.2 Opioid receptors

Opioid receptors belong to the G protein-coupled receptors and are known to participate in modulating pain reception. The identification of three different syndromes induced by morphine congeners postulated the existence of three distinguishable receptors. Morphine was established as an agonist for the μ-opioid receptor, activation of which is characterized by miosis, bradycardia, hypothermia, and a general depression of the nociceptive responses; ketocyclazocine as an agonist for the κ-opioid receptor produces constriction of pupils, depresses the flexor reflex. and produces sedation but does not markedly alter pulse rate or the skin twitch reflex; and SKF-10,047 as an agonist for the σ-opioid receptor causes mydriasis, tachypnea, tachycardia. and mania (Martin et al., 1976). One year later, the fourth type of opioid receptor was proposed, the δ-opioid receptor, which was found in the mouse vas deferens (Lord et al., 1977). Later, after purification and molecular cloning of $\sigma_1$-receptor, it was clear, that there is no homology with opioid receptors, but with fungal proteins involved in sterol synthesis (Hanner et al., 1996). Nowadays there are three main groups of opioid receptors:

***MORs (μ-opioid receptor)*** have an affinity to endogenous enkephalins (Robson & Kosterlitz, 1979) and high affinity to β-endorphin (Waterfield et al., 1979). Their location was found in the neocortex, amygdala, hippocampus, nucleus accumbens, thalamus, superior and inferior colliculi, and raphe nuclei (Mansour et al., 1987) and it was also detected in layers of the dorsal horn of the spinal cord (Besse et al., 1990). They play a role in supraspinal and spinal analgesia, braking gastrointestinal transit (Porreca et al., 1984), in bradycardia and hypothermia (Martin et al., 1976), and can modulate striatal dopaminergic transmission (Wood et al., 1980).

***DOR (δ-opioid receptor)*** binds to endogenous Leu-enkephalin (Robson & Kosterlitz, 1979) and Met-enkephalin (Waterfield et al., 1979). Their sites were identified in the anterior cingulate cortex, neocortex, amygdala, olfactory tubercle, nucleus accumbens, and caudate putamen (Mansour et al., 1987) and throughout the spinal cord gray matter, with the highest densities in the superficial dorsal horn (Arvidsson et al., 1995). DOR like all opioid receptors has a supraspinal and spinal analgetic effect (Porreca et al., 1984) and can affect serotoninergic and noradrenergic neurons by modulating the release of neurotransmitters (Arvidsson et al., 1995).

***KOR (κ-opioid receptor)*** relates to dynorphins as a specific endogenous ligand (Chavkin et al., 1982). Receptors are located in the amygdala, olfactory tubercle, nucleus accumbens, caudate putamen, medial preoptic area, and hypothalamus (Mansour et al., 1987). They control supraspinal and spinal analgesia and gastrointestinal transit by slowing it (Porreca et al., 1984), suppress the flexor reflex, and produce sedation (Martin et al., 1976).

## 5. Mechanisms of opioid addiction

"*Tolerance* is characterized by a decrease of the effect on the same drug dose; *dependence* refers to the development of an altered physiologic state which requires continued administration of a drug to prevent the appearance of *withdrawal*" (Fraser, 1957).

### 5.1 Receptor downregulation

Receptor downregulation describes a long-term exposure to agonists accompanied by a reduction in the density of opioid receptors as a part of receptor desensitization, which includes receptor internalization and recycling (Blanchard et al., 1982). However, the tolerance phenomenon could occur without downregulation (Polastron et al., 1994). It was shown that chronic morphine treatment can produce such adaptive changes as downregulation or even upregulation (increasing in receptor density) or stay without changes in the density of opioid receptors (Fábián et al., 2002), suggesting that downregulation is not necessary in opioid-induced tolerance.

### 5.2 Receptor desensitization

Receptor desensitization, as a mechanism of opioid tolerance, includes a decrease of signaling and reduced response after prolonged exposure to agonists, which is characterized by internalization of the receptors and reduction of cellular response (Trapaidze et al., 1996).

There are two types of desensitization, the first one, called homologous, desensitizes only agonist-activated receptors. The study showed that neurons desensitized to Met-enkephalin have no sensitivity to morphine, when cells desensitized to morphine stay sensitive to the inhibitory action of the opioid peptides (Williams & Zieglga¨nsberger, 1981). The second type is heterologous desensitization, which means that agonist-activated and non-activated receptors with the same signaling pathways are desensitized (Attali et al., 1989); for example, MOR and $\alpha_2$-adrenoceptor can be desensitized via G-protein-coupled receptor kinase (GRK)-mediated mechanism in the locus coeruleus (Fiorillo & Williams, 1996).

Receptor desensitization (Fig. 1) is based on the phosphorylation of the C-terminal amino acid residues (Ser/Thr). It was observed that [D-Ala(2), N-Me-Phe(4), Gly(5)-ol]-enkephalin (DAMGO)-induced receptor desensitization requires GRKs 2 and 3 (Doll et al., 2012). Morphine-induced MOR phosphorylation is PKC-dependent (Chu et al., 2010). Morphine could also induce Ser phosphorylation by GRK5 (Doll et al., 2012). Then phosphorylated DAMGO-stimulated receptor binds to β-arrestin and uncouples from the G-protein, whereas morphine-activated MOR does not (Whistler & von Zastrow, 1998).

In the first case, where it came to G-protein uncoupling, receptors are subjected to:

1) internalization (endocytosis) (Kramer & Simon, 2000), the process of incorporation of receptor molecules into clathrin-coated vesicles in cytoplasmic membrane and moving them to the cytosol;
2) dephosphorylation by phosphatases (Doll et al., 2012);
3) recycling to the plasma membrane which requires β-arrestins, GRK, and dynamin (Tanowitz & von Zastrow, 2003);
4) resensitization (restoration of its functional activity) (Qiu et al., 2003).

In the case of morphine-activated receptors, there is no binding to β-arrestin. Apparently, this phenomenon is a consequence of the inability of the drug to initiate receptor phosphorylation or stimulate β-arrestin translocation (Zhang et al., 1998). In the same study by overexpressing of GRK2 was shown that morphine induces MOR phosphorylation accompanied by the rescue of β-arrestin translocation and receptor internalization. It is assumed that the development of tolerance is associated with the low ability of MOR to internalize (Narita et al., 2006). Furthermore, it was observed that tolerance could be reversed by PKC inhibition (Smith et al., 1999).

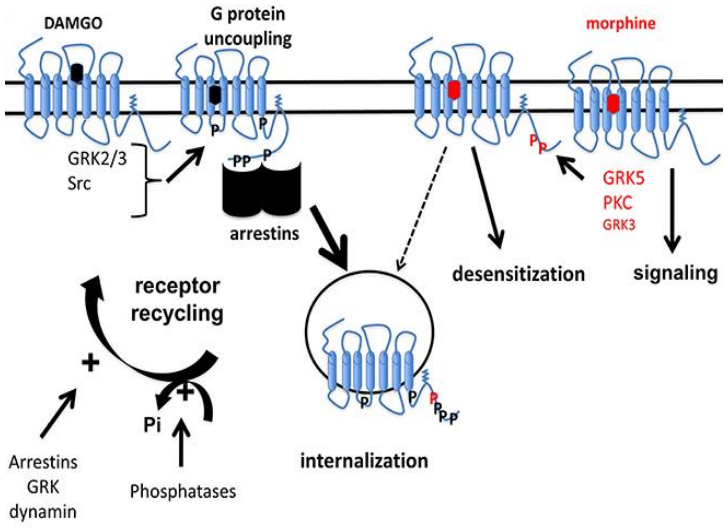


**Figure 1.** DAMGO binding **(left)** DAMGO binds to MOR and induces its phosphorylation by GRK2/3 kinases, which leads to binding of β-arrestins to MOR; then, G protein uncouples from MOR. This induces internalization of MOR accompanied by receptor dephosphorylation by phosphatases; then receptor can be recycled. MOR resensitization requires β-arrestins, GRK, and dynamin. Morphine binding **(right)** Morphine binds to MOR leading to Ser phosphorylation by GRK5; morphine exposure can also induce PKC-dependent phosphorylation of the receptor; morphine has a low ability to internalize MOR and in the most cases MOR is desensitized. Adopted from: (Allouche et al., 2014)

### 5.3 Superactivation of the cAMP pathway

Recently, the "RAVE' (for relative activity versus endocytosis) theory was based on the supposition that the ability of opioid agonists to induce receptor internalization and cyclic adenosine monophosphate (cAMP) superactivation are correlated (Whistler et al., 1999). In fact, cAMP superactivation is mediated by opioid receptor location at lipid rafts, which was proved after long-term MOR activation in an EcR293 cell model (Zhao et al., 2006).

In acute opioid exposure, opioid receptors send a signal via $G_{i/o}$ proteins, where the Gα subunit dissociated from the βγ subunits inhibits AC (Wong et al., 1991). This inhibition produces suppression of cAMP, which leads to reduced activity of PKA (Hayes & Mayer, 1981).

Modulation of the second messenger transmission system in chronic opiate administration is related to the development of tolerance and withdrawal. Another mechanism of opioid tolerance describes superactivation of the cAMP pathway (Fig. 2), which involves that chronic $G_{i/o}$-coupled receptor activation leads to supersensitization of AC (Nakagawa et al., 1998) and increasing PKA via high levels of cAMP accumulation (Nevo et al., 1998). The Gα subunit could also activate mitogen-activated protein kinases (MAPK) and phospholipase C (PLC) (Tsu & Wong, 1996), which leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) with calcium increasing. The βγ-subunits block the calcium channels and reduce the concentration of calcium (Moises et al., 1994), whereas potassium channel is activated and produces cell hyperpolarization (North et al., 1987). Also, levels of cAMP play a major role in producing inflammatory pain and hyperalgesia (Kress et al., 1996).

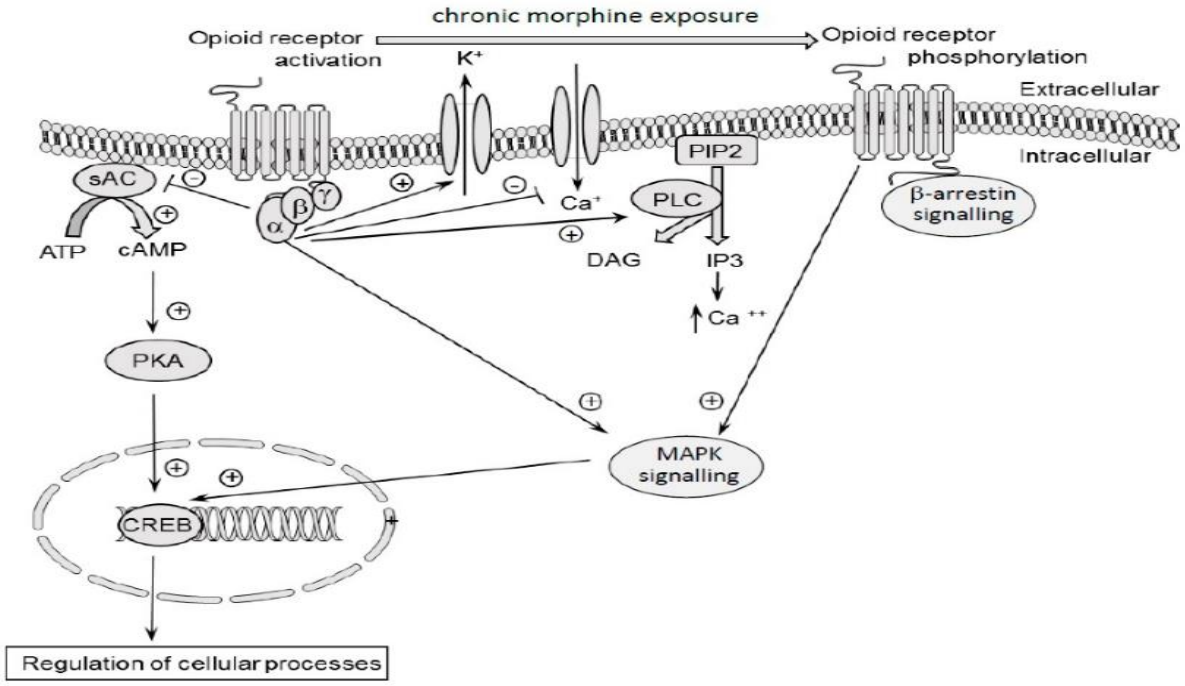


**Figure 2**. Superactivation of cAMP pathway: Opioid receptor activation occurs via binding of opioid ligand. Then Gα subunit dissociates from Gβγ dimer and activates AC. AC activation leads to increase

of cAMP, which activates PKA. CREB, located in the nucleus and regulates the transcription of genes, could be activated via PKA-dependent phosphorylation or MAPK signaling. Adopted from: (Listos et al., 2019)

**AC isoforms**

Adenylyl cyclase is a membrane-bound enzyme that converts ATP to cAMP. There are nine AC isoforms with different expressing profiles in different tissues. Several AC isoforms (I, V, VI, and VIII) are inhibited during acute morphine exposure and are activated during chronic morphine treatment. On the other hand, other isoforms of AC (II, IV, and VII) can be superactivated by acute MOR exposure or superinhibited during prolonged opioid agonist treatment (Nevo et al., 1998). Chronic morphine treatment enhances the phosphorylation of Gβ by PKC, which is associated with AC II stimulation via Gβγ (Chakrabarti & Gintzler, 2003). The acute increase in AC-II by Gα subunits is connected to Gβγ regulation, which was proved by PTX treatment and Gβγ scavengers, and may be the tolerance-producing mechanism (Schallmach et al., 2006). The upregulation of mRNA levels of AC isoform type VI and VIII and increase in AC V/VI levels were found in the nucleus raphe magnus (NRM) during morphine withdrawal (Bie, 2005). These neurons occupying the NRM and containing MOR are responsible for opioid modulation of pain. However, other data suggest that high levels of AC V isoform in the striatum play an important role in tolerance, physical dependence, and withdrawal, which was tested in mice lacking AC V (Kim et al., 2006). The behavioral effects of selective MOR and DOR agonists were reduced in knock-out mice, whereas KOR was unaffected.

### 5.3.1 Molecular changes in brain regions

In several brain regions, changes were detected in the G-protein-regulated cAMP system during chronic morphine treatment. For example, in the locus coeruleus chronic morphine increased levels of AC, cAMP-dependent protein kinase (PKA), and $G_i\alpha$ and $G_o\alpha$ subunits; the nucleus accumbens showed an increase in AC and PKA activity, but a decrease in levels of $G_i\alpha$ subunits; in the amygdala, there were increased levels of AC, PKA, and $G_i\alpha$ and $G_o\alpha$ subunits (Terwilliger et al., 1991). Similar changes were observed after abused drug administration, such as cocaine, which could support canonical aspects of tolerance and dependence mechanism by which several opioid-sensitive neurons adapt to chronic drug administrations.

***The locus coeruleus*** (LC) plays the main role in withdrawal development and some studies reported that LC noradrenergic neuron hyperactivity was induced by opiate withdrawal (Rasmussen & Aghajanian, 1989). This hyperactivity is mediated by endogenous excitatory amino acid (EAA) input (Akaoka & Aston-Jones, 1991), such as aspartate and glutamate efflux (Aghajanian et al., 1994). It was established that the withdrawal hyperactivity of LC neurons is PKA-dependent, what means that

specific inhibitors of PKA could suppress this withdrawal-induced activation (Ivanov & Aston-Jones, 2001).

***The ventral tegmental area*** (VTA) is a part of the mesolimbic dopamine system, which manages reward-related behaviour. The VTA consists of the large number of dopaminergic neurons and has projections to the amygdala and NA, which play a pivotal role in drug reward behaviour. Acute morphine injection into the VTA caused an increase of extracellular dopamine levels in the NA (Leone et al., 1991), what made clear that opioid receptors are involved in the VTA and mesolimbic dopamine pathway. It was shown that opioids hyperpolarized (by increased membrane potassium conductivity) secondary GABA-containing interneurons via activation of MOR (Johnson & North, 1992). Acute opioid receptor activation in the VTA mediates dopamine increase by reducing the GABA-mediated inhibition. During the withdrawal phase caused by chronic morphine treatment and subsequent cessation of the drug, GABA release was increased as a result of cAMP upregulation in the VTA (Bonci & Williams, 1997). In wild-type mouse, the increase in cAMP-depended GABA release was correlated with withdrawal behaviour outputs, whereas RMOR mouse (for Recycling MOR, in which genetic change in the receptor induces morphine-mediated desensitization and endocytosis of this receptor in the VTA GABA interneurons) did not have this GABA release adaptation and withdrawal syndrome after chronic morphine treatment was not manifested (Madhavan et al., 2010).

***The amygdala*** is associated with modulation of memory storage related to affective or emotional arousal (Packard & Cahill, 2001), processing positive emotions and negative ones, such as fear and anxiety (Baxter & Murray, 2002) and drug-seeking behaviour (Fuchs et al., 2005). The basolateral nucleus of the amygdala was observed and determined as a center for opiate withdrawal memories and it plays a role in the reconsolidation process of appetitive and aversive drug memories (Hellemans et al., 2006). The central nucleus of the amygdala (CeA) modulates withdrawal and anxiety-like behavior from acute morphine treatment through GABA receptors and MOR (Cabral et al., 2009). MOR agonists hyperpolarize CeA neurons by mediating the opening of $K^+$ channels (Zhu & Pan, 2004), regulate glutamate synaptic transmission through the phospholipase A(2) signaling pathway (Zhu & Pan, 2005) and inhibit GABAergic neurotransmission (Finnegan et al., 2006). Acute morphine administration in withdrawn rats increased basal level of miniature inhibitory postsynaptic currents (mIPSCs), currents that occur due to the spontaneous release of GABA neurotransmitters by presynaptic terminals in CeA neurons. This increase was suppressed by cAMP inhibitor (RP) and $G_{i/o}\alpha$ inhibitor (PTX), suggesting that the cAMP signaling pathway mediates the increased GABAergic transmission due to withdrawal (Bajo et al., 2014).

### 5.4 cAMP response element-binding protein

The cAMP second messenger cascade may induce gene transcription via activation of PKA (Fig. 2), which participates in phosphorylation of the cAMP response element-binding protein (CREB) transcription factor at serine-133 (Delghandi et al., 2005). CREB binds to CREs in the promoter regions of these genes and modifies their transcription (Simpson & McGinty, 1995). This activation could also be implemented through mitogen-activated protein kinases (MAPK) (Fig. 3) (Duraffourd et al., 2014) or calmodulin-dependent protein kinase (CaMK) (Takeda et al., 2007).

Upregulation of CREB phosphorylation could change gene expression which leads to opioid addiction. (Guitart et al., 1992). In the LC by prolonging morphine exposure, high levels of CREB immunoreactivity and CRE binding were found (Widnell et al., 1994). Infusions of CREB antisense oligonucleotide showed a reduction of AC type VII upregulation and tyrosine hydroxylase, but not PKA and $G_i\alpha$, what all together blocked the development of physical dependence to opiate. Knock-out in CREB leads to decrease levels in LC excitability, which involves high activity of the cAMP-CREB signaling pathway (Cao et al., 2010). By viral vectors encoding genes for caCREBGFP as a constitutively active CREB mutant, dnCREBGFP as a dominant-negative CREB mutant and CREBGFP for wild-type CREB (where GFP is green fluorescence protein for tagging) was shown that in vivo overexpression of CREBGFP in the LC exacerbated morphine withdrawal behaviors, whereas expression of dnCREBGFP had the opposite consequences; in vitro observation of caCREBGFP expression at the LC neurons is faster and had higher depolarized resting potential of the membrane (Han, 2006).

It was shown that overexpression of CREB in the NA leads to the symptoms of depression (Newton et al., 2002). The high CRE binding activity and CREB protein were detected in the NA and were compared to levels in the VTA. The increase of phosphorylated CREB in NA was presented in both wild-type and CREBalphaDelta (mice with a 90% reduction in CREB) mouse, whereas high levels of phosphor-CREB in the VTA was seen only in wild-type mice (Walters et al., 2003). To identify the role of CREB in drug reward, GFP-tagged CREB and mCREB (a dominant-negative form) were used. During increasing of CREB-GFP, the upregulation of expression levels of tyrosine hydroxylase and the AMPA glutamate receptor subunit GluR1 (genes, which contribute to drug reward in VTA) were detected, whereas during mCREB-GFP expression these genes were downregulated (Olson, 2005), what establish, that CREB increasing regulates drug reward in the VTA.

The low levels of PKA and pCREB are related to reduced withdrawal, which were found in the nucleus of the amygdala in opiate-treated rats (Van Bockstaele et al., 2006). The incubation of drug craving, by using conditioned place preference procedure, was associated with increased levels of extracellular signal-regulated kinase (ERK) phosphorylation and (CREB) phosphorylation in CeA, but not in the BLA (Li et al., 2008). The next study found that the conditioned place aversion behavior reduced

phosphorylation of NMDA receptor-dependent ERK and CREB in the BLA and dorsal hippocampus, but not in the CeA, what was initiated by NMDA receptor antagonist and MAPK kinase inhibitor injections. One more thing was suggested, the dorsal hippocampus trough in/direct pathway via NMDA receptors modulates the activity of ERK and CREB phosphorylation in BLA, because intra-dorsal hippocampus injection of AP-5 inhibitor before extinction training decreased ERK and CREB phosphorylation in BLA, whereas intra-BLA injection of the same inhibitor had no efficacy to ERK and CREB phosphorylation in the dorsal hippocampus (Wang et al., 2015).

In summary, CREB activity plays a pivotal role in dug-caused behavior and neural plasticity adaptations in several brain regions and the upregulation of CREB phosphorylation leads to dependence and withdrawal induced by opiates.

### 5.5 Epigenetic regulation

#### 5.5.1 OPRM1 methylation

Epigenetic regulation does not involve only changes in nucleotide sequences, but also changes in genes expression by adding methyl groups to the C5 position of the cytosine to form 5-methylcytosine. Most DNA methylation on cytosines occurs before guanine nucleotide CpG rich islands (Gruenbaum et al., 1981). This methylation modification could cause changes in gene transcriptions and leads to heritable phenotypes (Hwang et al., 2007).

It was found that silenced the OPRM1 gene could be activated through the decreased levels of methyl-CpG-binding protein 2 (MeCP2) expression, which suggests that DNA methylation is linked to the MeCP2-mediated chromatin structure of the OPRM1 gene (Hwang et al., 2007). OPRM1 was activated during P19 cells differentiation, when the transcription factor Sp1 and the chromatin remodeling factors Brg1 bound to the OPRM1 promoter. DNA methylation on the OPRM gene promoter could be reversed by the demethylation agent 5-Aza-2 -deoxycytidine (5-Aza-C) (Hwang et al., 2009). The role of MAPK and mitogen- and stress-activated protein kinase 1 (MSK1) in epigenetic activation of MOR was also indicated. It was observed that CpG methylation reduced MSK1 mediated increase in OPRM1 promoter activity and that MAPK inhibition reduced OPRM1 expression levels (Wagley et al., 2017).

The increased methylation at the OPRM1 22 CpG position between position -131 in the 5`UTR and position +185 in exon 1 was associated with chronic opioid exposure and could lead to phenotypic consequences for pain and opioid-induced hyperalgesia (Doehring et al., 2013). Methylation at a single OPRM1 promoter CpG site was detected in sperm of males with opioid dependence (Chorbov et al., 2011). Even short-term use of therapeutic opioids (≥ 90 morphine milligram equivalent) showed increased DNA methylation on 9 of 10 OPRM1 promoter sites (CpG1 to CpG9) and after fitting a regression model with adjustment for age and cell proportions, the leverage plots showed a significantly

higher average promoter methylation associated with higher morphine dose (Sandoval-Sierra et al., 2020). Hypermethylation in the OPRM1 promoter region is correlated with lower OPRM1 gene expression (Hwang et al., 2010), leading to OPRM1 silencing and reduced MOR levels.

### 5.5.2 Histone modifications

Histones are the class of nuclear proteins that are involved in the packing of DNA strands into chromatin and in the epigenetic regulation of nuclear processes. There are four types of histones: H2A, H2B, H3, and H4. Epigenetic modifications occur on the N-terminal tails of these proteins, which could lead to tightly packed silent heterochromatin or lightly packed active euchromatin.

Histone acetylation is one of the main epigenetic modifications involved in the remodulation of chromatin, which is regulated by histone acetyl transferases (HATs) promoting gene activation and by histone deacetylases (HDACs) to promoting gene repression. Several studies showed that histone acetylation is responsible for drug dependence and memory formation. Lysine acetylation caused the reduction of electrostatic tension between histones, thereby producing lightly packed active euchromatin. It was found that chronic administration of heroin increases H3 acetylation in NA (Sheng et al., 2011). In postmortem human striatum the hyperacetylation of lysine 27 of histone 3 promoted glutamatergic transcriptional changes associated with drug-seeking behavior and heroin self-administration, which could be blocked by bromodomain inhibitor JQ1 (the readout inhibitor of acetylated lysine); the same activities were found in rats (Egervari et al., 2017). Histone 3 lysine 9 acetylation (H3K9ac) was associated with BRG1 gene (main in transcriptional regulation of neuronal genes) expression in the medial prefrontal cortex, which was increased and correlated with heroin self-administration in rats, whereas there were no significant differences in the NA (Hong et al., 2019).

It was shown that conditioned place preference behavior could be reduced by histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA), in the BLA. This was accompanied by high levels of histone H3 lysine14 acetylation, upregulation of the BDNF and CREB (Wang et al., 2015). Hence, memory formation for opiate addiction could be regulated in the BLA by HDAC inhibition. Intracerebroventricular sodium butyrate injections (HDAC inhibitor) canceled the heroine self-administration behavior in trained rats, whereas the levels of H3K18, H4K5 were increased in the NA (Chen et al., 2016). The recent study indicated that morphine increased the production of IL-6, a pro-inflammatory mediator, and resulted in down-regulation of MOR, which could be blocked by HDAC inhibitors, leading to inhibition of IL-6 production and increased sensitivity of neurons concerning opioid agonists (Jindal et al., 2021).

### 5.6 Genetic association with opioid addiction

#### 5.6.1 Opioid receptor genes

***MOR*** is encoded by the OPRM1 gene, which is located on chromosome 6q24-q25 (Wand, 2002). Several SNPs, substitutions of a single nucleotide in DNA sequence, were detected in OPRM1 genes and were associated with opioid dependence. Five different variations in SNPs, the A118G, C17T, G24A, G779A and G942A (where the most prevalent polymorphisms were A118G and C17T) in exon 1, were found in 152 subjects with different ethnicity separated by heroin addicts in MMT and persons without any dependence (Bond et al., 1998). However, the association between C17T and opioid dependence was refuted (Kapur et al., 2007). For the A118G there were no significant differences between opioid-addicted and nonaddicted groups, whereas C17T SNP had a higher proportion of addicted individuals. Also, it was suggested that A118G polymorphism could decrease the protein expression and change agonist activity of β-endorphin for MOR activation, where the 118G allele is 3-time more potent than the 118A allele (Bond et al., 1998). Another study reported that individuals with the 118G allele had enhanced feelings of euphoria, stimulation/sedation, intoxication and reward caused by alcohol compared with 118A allele carriers (Ray & Hutchison, 2004). This data was reinforced by the observation which identified that persons with 118G allele produced more dopamine in the striatum during alcohol administration than OPRM1 118A allele carriers (Ramchandani et al., 2011). In Indian population, the presence of 118G allele in OPRM1 gene was associated with a risk factor of heroin and alcohol addiction, which may be regulated by PKA and ERK during opioid and alcohol exposure. Upon acute morphine administration to both wild-type and mutant hOPRM1 expressing Neuro 2A cells, the PKA activity was downregulated with induction of ERK levels, whereas chronic morphine treatment increased PKA activity and decreased ERK levels (Deb et al., 2010).

***DOR*** gene (OPRD1) is located on chromosome 1p36.1-p34.3 (Bergen et al., 2003). Eleven SNPs of OPRD1 gene were studied in European Americans with 620 cases of substance dependence (opioid, cocaine, and alcohol) and 443 controls. Only the G80T polymorphism in exon 1 showed a strong correlation with opioid dependence, where the minor G allele and G80T C/T heterozygote were significantly more frequent in opioid addicts (21.0% and 32.4%) than in controls (13.2% and 24.2%) (Zhang et al., 2008). Another two intronic SNPs, rs2236857 and rs2236855, of OPRD1 with GA haplotype consisting of the coupled minor alleles were strongly associated with heroin (Nelson et al., 2014).

***KOP*** is encoded by the OPRK1 gene with the 8q11.2 chromosome position (Yasuda et al., 1994). The frequency of 36G>T SNP (rs1051660) of the OPRK1 gene was studied on 106 heroin addicts (West European and Caucasian), where the presence of GT and TT genotypes was significantly higher in individuals with heroin dependence, whereas GG genotype was more frequent in the control group

(Gerra et al., 2007). However, OPRK1 contains mainly silent polymorphisms with no consequences on mRNA transcription and receptor structure (Gerra et al., 2007). Another SNP, rs6989250 C>G, was associated with stress-induced craving and higher cortisol levels to stress response activating by limbic and midbrain regions, and also with greater cocaine relapse risk, where the CG genotype was more frequent than CC one (Xu et al., 2013).

### 5.6.2 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor gene, BDNF, belongs to the neurotrophin family of growth factors. It is a protein encoded by BDNF gene located on chromosome 11p14 (Hanson et al., 1992). BDNF plays a role in the synaptic plasticity of neurons via affecting glutamate receptor activity by phosphorylating the receptor subunits (Singh et al., 2006) and also could modulate the function of other neurotransmitters. It was shown that high levels of BDNF in the NA induced the overexpression of the dopamine D3 receptor, which was similar to drug reward caused by opioids (Guillin et al., 2001). The site-specific deletion of the BDNF gene led to decreasing of BDNF expression in the dorsal hippocampus of adult mice, which was correlated with loss of aversive memory and reduced extinction of conditioned fear that is found in depression and anxiety disorders (Heldt et al., 2007).

BDNF contains an SNP in exon 2 at codon 66 (G196A, rs6265). The Val66Met polymorphism was associated with substance abuse cases, where the low frequency of 66Met allele was related to drug addiction (Cheng et al., 2005). In Chinese population, two SNPs, rs6265 and rs13306221, were strongly associated with heroin dependence, where the G allele (GG/GA genotypes) of rs13306221 was more common in individuals with heroin addiction (Jia et al., 2011). Nevertheless, it was detected that the BDNF Val66Met SNP does not affect expression levels of plasma BDNF, but low plasma BDNF concentration could be caused by long-term heroine dependency (Chen et al., 2015).

## 6. Neonatal opioid withdrawal syndrome

The drug delivery from mother to fetus occurs through the maternal placenta. Generally, this placental transfer involves two steps: 1) drug moves from the maternal circulation via placental villi of the syncytiotrophoblast layer; 2) enter to the fetal circulation through the fetal capillaries, which are in close contact with the syncytial membrane. All opioid drugs have low molecular weight and therefore easily cross the placenta and then distribute and accumulate in the tissues of the fetus. They disrupt the formation of metabolic processes in the fetus, have a damaging effect on the central nervous system. For example, heroin is metabolized to morphine in the placenta, which retains a variable amount of morphine (Kopecky et al., 1999), which could lead to oxidative stress of the syncytiotrophoblast and change placental barrier function due to toxic substances (Malek et al., 2009). The concentration of

EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), methadone metabolite, was higher in the placenta than in maternal plasma. A negative correlation was found between EDDP concentration in the placenta and head circumference and the EDDP concentration positively correlated with NAS signs (de Castro et al., 2011).

### 6.1 Pathophysiology of NAS

Opioid use during pregnancy could affect the fetus which leads to the development of neonatal opioid withdrawal syndrome. This is a complex process that includes not only prenatal opioid exposure, but many other factors, such as genetics, gestation age, tobacco smocking, type of opioid administration, and many more. A randomized controlled trial showed similar postnatal vulnerability and no significant differences between female and male neonates who were exposed to opioids during pregnancy (Unger et al., 2011). Other data indicated that preterm infants had shorter treatment and length of stay compared to infants born at term (Dysart et al., 2007). Furthermore, there were differences in the frequencies of NAS signs caused by buprenorphine and methadone. In methadone-exposed infants, undisturbed tremors and hyperactive Moro reflex were more frequent, whereas nasal stuffiness, sneezing, loose stools were more common in buprenorphine-exposed neonates. Also, it was observed that methadone-exposed infants required treatment significantly earlier versus buprenorphine ones, which make methadone-caused NAS more severe than buprenorphine-caused NAS (Gaalema et al., 2012).

In general, NAS is manifested in dysfunction of the central nervous system such as tremors, hypertonia, hyperreflexia, cry, and sleep disturbances, dysfunction of the autonomic nervous system such as hyperthermia, sweating, yawning, sneezing, and nasal stuffiness, dysfunction of the respiratory system, such as tachypnea, gastrointestinal dysfunction, such as poor feeding, loose stool, and poor weight gain and lower birth weight and microcephalia (Devlin & Davis, 2018). Also, it is necessary to consider the time intervals during which specific symptoms of NAS may develop. For example, the symptoms in infants with NAS with pharmacological treatment during the first year and unexposed infants were similar in neurodevelopment scores, but during the second year cognitive and language scores became worse in the NAS infant group (Benninger et al., 2020). Furthermore, prenatally opioid-exposed neonates showed a higher risk of infant mortality (death age <365 days) (Leyenaar et al., 2021). Interestingly, the risk group with no NAS diagnosed had higher mortality rates in comparison with the NAS diagnosed group. The severity of NAS is very variable and depends on genetic and epigenetic factors, described below.

### 6.2 Genetic factors

Just as polymorphisms in various genes appear in opioid-exposed adults, similar changes were detected in infants with NAS. In an attempt to determine the length of hospital stay, the genomic variations in several genes have been studied.

It was indicated that infants with the OPRM1 AG/GG genotype in the 118A>G SNP had a shorter length of hospital stay and were less treated than infants with AA genotype (Wachman et al., 2013). It means, if there is at least one copy of the G allele in 118A>G (rs1799971) SNP, the NAS outcomes will be less severe. However, as it was mentioned in the previous chapter, adults with G allele are associated with drug addiction, which may mean that infants with the same one could be more tolerant to withdrawal outcomes and have a predisposition to opioid addiction, because of increased of β-endorphin and decreased numbers of opioid receptor binding sites. The same study also suggested that maternal OPRM1 118>G AG/GG genotype could be correlated with less treatment of NAS (Wachman et al., 2013).

Another study discovered that the C allele of rs702764 in the OPRK1 gene contributes to worse NAS development requiring two medications ( Wachman et al., 2015). The SNP-SNP interaction between OPRM1 rs1799971 and OPRK1 rs702764 was associated with heroin addiction as well as alcohol addiction (Kumar et al., 2012).

The identified maternal OPRD1 rs204076 A allele leads to a longer hospital stay and increased need for treatment; PNOC rs732636 A allele has the same consequences (Wachman et al., 2015), where PNOC is a gene for prepronociceptin, a precursor for nociception, which binds to opioid receptor-like 1 receptor to drive nociception and locomotor activity. Maternal PNOC rs351776 A allele was associated with a two-fold increased risk for treatment in newborns with two medications and longer hospitalization (Wachman et al., 2017).

Another important genomic change was detected in the gene for catechol-O-methyltransferase, COMT, which is important in catecholamine degradation, such as dopamine. It was observed that COMT 158A>G (rs4680) GG genotype leads to less treatment and has a shorter length of hospital stay than AA infants, whereas AG genotype has no significant differences from the GG genotype (Wachman et al., 2013). Maternal COMT rs4680 G allele was found in neonates who required less treatment with two medications, whereas COMT rs740603 A allele led to reduced need (by 50%) for medications (Wachman et al., 2017). Because G allele of rs4680 leads to three- to-four-fold lower COMT enzyme activity (Rakvåg et al., 2005), the increased circulation of catecholamines may improve stress tolerance in infants with NAS.

Variations in infant CYP2B6, the gene that encodes a cytochrome P450 involved in the metabolization of methadone, have also been studied. It was shown that neonates with prenatal methadone exposure

with NAS expression were more likely to carry the homozygous (normal) allele at 516 and 785 sites of the CYP2B6 gene and there were no significant differences between treated and untreated infants (Mactier et al., 2017). Likewise, any associations with the ABCB1 (the gene for P-glycoprotein-1, which regulates pharmacokinetics) SNPs, were detected either (Elisha M. Wachman et al., 2013).

### 6.3 Epigenetic markers

Epigenetic modifications are known mechanisms that can cause changes in gene expression in infants with NAS. One of the main epigenetic mechanisms is methylation at CpG sites. For example, a more severe manifestation of NAS was associated with DNA hypermethylation at the -14, -10 and +84 CpG sites in the OPRM1 promoter region (Wachman et al., 2014). The possible molecular mechanism by which DNA methylation increases, is driven through G-coupled receptor-mediated induction of DNA methyltransferases (Doehring et al., 2013). It is hypothesized that high levels of DNA methylation could decrease OPRM1 gene expression, which leads to a reduction of levels of MOR (Wachman et al., 2014), but the mechanism is still unclear.

It is important to consider mother-infant dyads as a full model to personalize different possibilities of treatment due to differences in NAS severity. High levels of methylation at the -18, -14 and +23 CpG sites were correlated with higher rates of infant pharmacologic treatment and the hypermethylation at the maternal -169, 0152 and +84 sites were associated with longer infant stay in hospital (Wachman et al., 2018). These data suggest that infants at high risk should be treated more powerfully after birth. Recent studies suggested a role of placental DNA methylation and its dysregulation as the epigenetic adaptation, which leads to postpartum disorders; these dysregulations include CREB phosphorylation by melatonin, perturbed netrin signaling pathway and epigenetic changes in synaptogenesis encoding genes (Radhakrishna et al., 2021). Furthermore, not only increased methylation of OPRM1 genes was detected, the high DNA methylation of ABCB1 and CYP2D6 in newborns with prenatal methadon administrations compared to opioid-naïve newborns (McLaughlin et al., 2017).

Nevertheless, multiple factors could be associated with neonatal DNA hypermethylation. Prenatal administrations of antidepressants are associated with CYP2E1 methylation of DNA (Gurnot et al., 2015), tobacco smoking (Suter et al., 2011) and even antibiotics could cause lower birth weight via methylation of imprinted genes (Vidal et al., 2013). It is important to consider these lifestyle factors when thinking about the specific treatment of different NAS signs.

## 6.4 Methods of treatment

### 6.4.1 Nonpharmacological treatment

The nonpharmacologic approach should be based on a quiet and soothing environment and exclude environmental stimulations to minimize sleep disturbance, irritability and uncontrolled movements (Velez & Jansson, 2008), which altogether helps to reduce the severity of NAS outcomes (Patrick et al., 2016). Maternal care is an important part of nonpharmacologic treatment and should involve breastfeeding if there is no illicit drug use or HIV infection ("Committee Opinion No. 524," 2012). How it was showed maternal breast milk leads to a decrease in NAS severity and pharmacologic treatment (Abdel-Latif, 2006) and as a benefit is a physical contact, attachment and the possible compensation of low birth weight by optimization of cognitive potential (Vohr et al., 2006). Nonpharmacological approaches should be implemented along with pharmacological therapy during a hospital stay.

### 6.4.2 Pharmacological treatment

Pharmacological therapy is not uniform because of the differences in NAS protocols that were previously described. The treatment protocols should involve a rapid dose increasing, which is weight-based and symptom-based of NAS severity, to control NAS signs and direct future pharmacological strategies (Kraft et al., 2016). The first-line treatment by morphine and methadone exists for withdrawal symptoms in neonates, whereas second-line or adjunctive therapy by phenobarbital and/or clonidine involves more severe cases of NAS outcomes where first-line treatment failed (Wachman et al., 2015).

Morphine and methadone were compared in 41 methadone-exposed and 37 buprenorphine-exposed newborns. It was shown that methadone-treated infants had a shorter duration of treatment and length of stay than morphine-treated one (median 14 versus 21 days, respectively) (Brown et al., 2015). It was confirmed by a larger clinical trial that infants with methadone therapy had a shorter length of stay by 14% and shorter drug treatment by 16% (Davis et al., 2018).

Another possible treatment is based on using buprenorphine, which is a partial MOR agonist with high affinity and slow dissociation from the receptors blocking the effects of other opioids. Compared with methadone, partial effects of buprenorphine caused less symptoms of withdrawal syndrome, respiratory depression and arrhythmia (Kao et al., 2015). Sublingual buprenorphine administration led to a reduction of the duration of hospital stay from 42 to 32 days and it was more effective than morphine (Kraft et al., 2011).

Phenobarbital is common in adjunct therapy for multiple drug-exposed infants with opioid withdrawal to reduce hyperactivity, insomnia and controls irritability (Coyle et al., 2002). There were no significant

differences in length of treatment (8.5 +/- 5 days), hospital stay (12.5 +/- 5.5 days) and the necessity for adjunctive treatment between morphine-treated and phenobarbital-treated neonates with NAS (Nayeri et al., 2015). In contrast, the earlier study identified that morphine replacement treatment was more potent in NAS versus phenobarbital standard treatment, because of the shorter length of drug treatment (Jackson, 2004). Such discordant data could be explained by differences in drug types administered during pregnancy (in the previous study, neonates were also exposed to benzodiazepines, whereas recent study excluded this factor). In addition, breastfeeding of neonates was excluded in the present study, because of the possible effect on NAS symptoms. In the study of Jackson et al. the loading dose of phenobarbital was not used, which could turn in the favor of morphine replacement treatment. The loading dose is a high initial dose of a medicament, which quickly reaches the desired concentration of a drug in the body. The loading dose could be replaced by a lower maintenance dose. In the present study, for phenobarbital a loading dose was 20 mg/kg and the maintenance dose was 5 mg/kg/24 h (Nayeri et al., 2015), whereas in another study the dose of phenobarbital was 2 mg/kg (Jackson, 2004), what obviously could influence the differences in results.

Clonidine is an α2-adrenergic receptor agonist which suppresses noradrenergic activity when opioid exposure is terminated (Bada et al., 2015), producing a decrease in vasomotor tone and heart rate and is well tolerated in infants (≥35 gestation week) treated for NAS (Meddock & Bloemer, 2018). Clonidine as a single-drug therapy showed a shorter treatment duration (28 versus 39 median days) and higher neurobehavior scales than morphine treatment (Bada et al., 2015).

While some protocols advocate for clonidine or phenobarbital as a single-drug therapy, other studies support and compare them as adjunctive therapy after primary replacement morphine treatment. For example, phenobarbital had shorter treatment compared to clonidine, 12.5 versus 19.5 days; but longer post-discharge therapy (3.8 months) (Surran et al., 2013). Another study also showed that phenobarbital as adjunctive treatment, led to shorter length of morphine treatment (25.5 versus 34.4 days) compared with clonidine (Brusseau et al., 2020).

## 7. Conclusion

NAS represents a serious condition, firstly because of the dysfunction of the central and autonomic nervous systems, secondly because of unclear long-term neurodevelopmental impacts. The severity of NAS depends on several genomic variations and epigenetic markers. Maternal and infant OPRM1 118>G AG/GG genotype was correlated with less treatment of NAS. However, adults with G allele are associated with drug addiction, which may suggest that infants with the same allelle could be more tolerant to withdrawal and have a predisposition to opioid addiction, because of increased β-endorphin levels and decreased numbers of opioid receptor binding sites. Hypermethylation at the -14, -10 and +84 CpG sites in the OPRM1 promoter region was associated with more severe NAS manifestation. Such hypermethylation could lead to OPRM1 silencing and reduced level of MORs.

Nowadays, it is possible to treat NAS signs in newborns, but the prediction of NAS development is not fully understood, like its severity and future treatment possibilities. In view of variability in NAS signs and its severity, the pharmacological treatment does not have a generic approach. Future directions should be aimed at creating opportunities for personalized genomic medicine as an individualized therapeutic approach for this syndrome.

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