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Změny opoidní signalizace v průběhu ontogeneze

Changes in opioid signalisation during the ontogenesis

Bachelor's thesis

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Prohlášení:

Prohlašuji, že jsem tuto závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Táto práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, ……

Podpis: _____

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Abstrakt

Opioidné receptory interagujú s opioidnými zlúčeninami a vyvolávajú zníženie neuroexcitability. Aktivácia opioidných signalizačných dráh hrá nepostrádateľnú úlohu pri liečbe chronických a nádorových bolestí. Začiatok tejto práce zhrňuje všeobecné znalosti o opioidných receptoroch a o vzniku tolerancie. Nasledujúca časť sa zaoberá ontogenézou opioidných receptorov a ďalších komponent ich signalizácie, keďže sa zdá, že vek má veľký vplyv na molekuly v rámci samotnej signalizácie, ale i molekuly nepriamo s ňou súvisiace. Na záver tejto práce je poskytnutý súhrn informácií o vplyve morfia behom ontogenéze, keďže morfium je jedným z najčastejšie používaných opioidných zlúčenín používaných v klinickej praxi.

Kľúčové slová

Opioidné receptory, signalizácia, ontogenéza, závislosť, tolerancia, morfium

Abstract

Opioid receptors interact with opiate compounds, causing the inhibition of neuroexcitability. The activation of signaling pathway of opioid receptors plays crucial role in the treatment of chronic and cancer pain. Summary of the general knowledge about opioid receptors and about the development of tolerance is in the first part of this work. Next part of the thesis concerns on ontogenesis of opioid receptors and other components related to the opioid signaling pathways as age seems to have an immense influence on molecules within and related to the opioid signaling. Finally, last part of this work collects data about the influence of morphine during ontogenesis as morphine is one of the most used opiate compound used in clinical treatment.

Key words

Opioid receptor, signalization, ontogenesis, addiction, tolerance, morphine

Contents

Abbreviations

AC	adenylate cyclase
cAMP	cyclic adenosine 3',5'-monophosphate
CGRP	calcitonin-gene-related peptide
CNS	central nervous system
CREB	cAMP response element-binding protein
DALES	[D-Ala^2,Leu^5,Ser^6] enkephalin
DAMGO	[D-Ala^2, Me Phe^4,Glyol^5] enkephalin
Del-II	[D-Ala^2] deltrophin II
DOR	δ-opioid receptor
DPDPE	cyclic [D-Pen^2, D-Pen^5] enkephalin
DSLET	[D-Ser^2,Leu^5,Thr^6] enkephalin
GIRK	G-protein-gated inwardly rectifying potassium channels
GPCR	G-protein-coupled receptor
GRKs	G-protein-coupled receptor kinases
KOR	κ-opioid receptor
M6G	morphine-6β-glucuronide
MAPKs	mitogen-activated protein kinases
MOR	μ-opioid receptor
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthetase
NO	nitric oxide
OR	opioid receptor
PAG	periaqueductal gray
PKA	protein kinase A
PKC	protein kinase C
PLD2	phospholipase D2
PMA	phorbol-12-myristate-13-acetate
PNS	peripheral nervous system
RGS	regulator of G-protein signaling
SP-IR	substance P-like immunoreactivity
STATs	Signal Transducers and Activators of Transcription

1. Introduction

Opioid receptors (OR) belong to the family of the seven transmembrane-spanning G-protein-coupled receptors (GPCRs) that are activated both by endogenous opioid peptides and exogenous opiate compounds. Main physiological role of OR is to modulate the effects of neurotransmitters and hormones which leads into inhibition of neuroexcitability and releasing of neurotransmitters (Harrison, Kastin, and Zadina 1998; Minami and Satoh 1995). There are four basic types of OR; μ, δ, κ and ORL-1 (Minami and Satoh 1995; Mollereau et al. 1994). Each type of receptor and their subtypes bind increasingly selective ligands.

OR are very important from clinical point of view as many drugs used in medical care are targeting these receptors. Opioid drugs are used mainly for their analgesic effects, they relieve from acute, chronic or cancer pain. Problem is that side-effects, tolerance and dependence are developing, mainly with chronic treatment. There are age differences with development of side-effects or tolerance, suggesting that opioid signaling mechanisms change during ontogenesis. Thus the main aim of this work is to summarize current knowledge about changes in opioid signaling during ontogenesis in model organisms and human as well. It is possible that the age-dependent expression and function of molecules within and related to the opioid signaling pathways, along with cellular activity (such as internalization and desensitization), eventually lead to significant age-dependent changes in opioid analgesia and tolerance development (Zhao et al. 2012).

Also, because morphine is one of the most used opioid compounds in clinical practice, it is important to know what are the influences on brain development and generally on health during ontogenesis. Therefore the collection of data about the influence of morphine during ontogenesis is part of this work.

2. Opioid receptors

OR are part of the superfamily of GPCRs. Opioid ligands bind to membrane receptors. Guanosine triphosphate binding protein (G-protein) and effectors, such as adenylate cyclase (AC) and ion channels, are activated and the signal is therefore mediated inside the cell. The coupling of receptor to G-protein might be possible locus of specificity for different subtypes, but also can play a role in regulation during chronic ligand exposure (Harrison, Kastin, and Zadina 1998).

After finding OR differentially located in central nervous system (CNS) and in smaller amounts in peripheral nervous system (PNS) as well, it was proven that there are several types. Physiological study on dog with chronic spinal condition suggested three types- μ, κ and σ receptors. Each type was distinguishable by using a different agonist which caused three syndromes with different symptoms. Morphine was found to be the prototype agonist for μ-receptor, ketocyclazocine for κ-receptor and SKF-10,047 for σ-receptor (Martin et al. 1976). When σ-receptors were successfully cloned, it was shown they are single transmembrane-spanning protein with different ligands. Naloxolone could not block actions in which σ-binding sites took part. Therefore they are not a member of OR family (Monassier and Bousquet 2002). δ-receptor with high affinity to enkephalins was found in the mouse vas deferens (Minami and Satoh 1995). In 1996 a cDNA clone encoding hORL-1 (human Opioid Receptor-Like 1) was isolated. This receptor is related to OR from structural and functional point of view and is member of OR family, even though it appears not to be typical OR (Mollereau et al. 1994).

2.1. μ-opioid receptors

Beside basic types of OR, each type can be divided into several subtypes, these were proven by various pharmacological and physiological studies. Each single subtype binds increasingly selective ligands and is able to affect certain physiological parameter but not others (Harrison, Kastin, and Zadina 1998).

For μ opioid receptor (MOR) Wolozin and Pasternak (1981) suggested terms mu_1 and mu_2 sites, where mu_1 mediate opiate, enkephalin and β-endorphin analgesia. Some studies

showed obvious separation of mu_1 and mu_2 sites. For example, pretreatment with naloxonazine, potent antagonist, antagonizes morphine analgesia, but not respiration depression. Also naloxonazine does not block physical dependence, therefore the different receptor mechanisms mediate morphine analgesia and withdrawal symptoms of morphine dependence (Ling et al. 1984). After purification and partial amino acid sequencing (Eppler et al. 1993), a cloning of MOR brought an idea that different subtypes are the result of alternative splicing of one gene. MOR-1 has a total of 10 splicing variants in human, their differences in opioid ligand selectivity and affinity were only limited. Major differences appeared in efficacy and potency among these splicing variants (Pan et al. 2005). All variants contain exons 1, 2 and 3 which encode all seven transmembrane domains and the N-terminus, the difference comes in exons encoding the C-terminus. Variants do not differ in the binding cavity, but on the tip of C-terminus and they have unchanged affinity and selectivity for μ opioids (Xu et al. 2009).

In 1995, new MOR was isolated in a rat, called rMOR1B. Sequence of this receptor is almost identical to the rMOR1, it differs only at C-terminus, thus considered to be another splicing variant. Affinities to opioid compounds stay similar, but rMOR1B is much more resistant to agonist-induced desensitization, therefore alternative splicing of C-terminus may play a critical role in opiate tolerance (Zimprich, Simon, and Höllt 1995). Similarly, a splice variant of human MOR1, called MOR1A, has almost unchanged ligand affinity, but difference in C-terminus may modulate receptor's G-protein coupling or affect its cellular distribution (Bare, Mansson, and Yang 1994).

Compounds that were found to be highly selective for MOR include morphine, codeine, fentanyl, heroin, methadone, oxymorphone and oxycodone (Pasternak 2011). The MOR is predominantly responsible for the production of both analgesia and euphoria by the more common opioids (Taylor and Fleming 2001).

2.2. δ-opioid receptors

Similarly to MOR, there have been identified different subtypes of δ opioid receptors (DOR), called δ_1 receptor and δ_2 receptor. It is thought that δ_1 subtype is stimulated by cyclic

[D-Pen^2,D-Pen^5] enkephalin (DPDPE) and blocked by [Ala^2,Leu^5,Cys^6]enkephalin, whereas δ_2 subtype is stimulated by [D-Ser^2,Leu^5.Thr^6] enkephalin (DSLET) and [D-Ala^2] deltrophin II (Del-II) and blocked by naltrindole-5'-isothiocyanate (Quock et al. 1999; Vanderah et al. 1994).

Among suggested endogenous ligands for DOR are [Leu^5]enkephalin and [Met^5]enkephalin, respectively increasing and decreasing morphine antinociception (Quock et al. 1999). Therefore raising a hypothesis that MOR and DOR are interacting on functional and/or physical basis and DOR may exist within the μ-δ complex ($\delta_{complexed}$ (δ_{cx}) receptors), while others are not in this complex ($\delta_{non\text{-}complexed}$ (δ_{ncx}) receptors) (Heyman et al. 1989). Thus, enkephalins play a role in direct antinociception and may play a modulatory role as well. A study with [D-Ala^2,Leu^5,Ser^6]enkephalin (DALES) supported a hypothesis about δ subtypes as DALES produced antinociception, but failed to modulate morphine antinociception, which means it interacted with δ_{ncx} receptor (Mattia et al. 1991).

Beside the direct antinociception through DOR, their agonists show modulatory properties on MOR agonists, such as morphine (Heyman et al. 1989).

2.3. κ-opioid receptors

It has been established that ketocyclazocine-like opioids produce antinociception through κ opioid receptors (KOR) (Martin et al. 1976). KOR sites were shown in both rat and guinea pig brain through *in vitro* binding assays and quantitative receptor autoradiography. There were differences in these two species, showing that there are two different subtypes of KOR, termed κ_1 and κ_2, which have different affinities, different ligand selectivity profiles and even their neuroanatomical distribution is different (Zukin et al. 1988).

In 1993, rat KOR was cloned. When compared to the mouse KOR, it showed 97% similarity at the amino acid sequence and treatment with κ-selective agonist, bremazocine, indicated the KOR inhibitory coupling to AC (Chen et al. 1993).

2.4. ORL-1

DNA of the ORL-1 receptor structurally resembles other OR and receptor is negatively coupled with AC. The inhibition of AC is mediated by 'universal' opiate agonist, etorphine (Mollereau et al. 1994; Meunier et al. 1995). Interestingly, ORL-1 possesses a low level of specific naloxone and bremazocine binding (Bunzow et al. 1994).

ORL-1 is considered to be a non-opioid branch of OR family, as their agonists are inhibiting antinociception caused by other OR-selective ligands and may even cause hyperalgesia. ORL-1 mediates dual (opioid and anti-opioid) effects, which naloxone cannot reverse (Mogil et al. 1996).

3. Signaling

Opioid receptors belong to family of receptors that interact with the pertussis toxin-sensitive G-proteins of the G_i and G_o families (Connor and Christie 2006). However, they do not interact with them exclusively and it was shown that they interact with pertussis toxin-insensitive G_z protein as well, inhibiting AC and stimulating mitogen-activated protein kinases (MAPKs) (Tang and Hurley 1998).

OR signaling works on catalyzing ligand nucleotide exchange on G_i and G_o, causing inhibition of AC, inhibition of N-type calcium channels and activation of G-protein-gated inwardly rectifying potassium (GIRK) channels (Whistler et al. 1999). The most common action of OR includes inhibiting of AC, activation of K^+ conductance, inhibition of Ca^{2+} conductance, and an inhibition of transmitter release. Actions like activation of protein kinase C (PKC) or MAPK cascade, release of calcium from intracellular stores have been added to OR functions (Williams, Christie, and Manzoni 2001).

OR interacts not just with G-proteins, but with other signal molecules as well. An interaction with calmodulin (CaM) was reported and was suggested that CaM itself could be a second messenger (Wang et al. 1999). Another signaling pathway mediated by activated MOR includes STAT5A, which acts as the second messenger and alters transcription (Mazarakou and Georgoussi 2005).

4. Tolerance and dependence

The chronic use of opiates tends to induce tolerance and dependence easily due to adaptive changes in the response of subject to the agent. Tolerance is often defined as a decrease of sensitivity to an administered drug or a need of increasing amounts of the drug to achieve the original effect. On the other hand, dependence is a state when the drug is required to maintain normal physiological function and when it is removed a physical and/or psychological withdrawal syndrome is produced (Taylor and Fleming 2001; Harrison, Kastin, and Zadina 1998).

The existence of multiple forms of tolerance and dependence is suggesting a possibility that each component is regulated by a different cellular mechanism and tolerance/dependence is a complex behavior which is a result of multiple mechanisms (Taylor and Fleming 2001).

The expression of different types of tolerance occurs with different characteristics. The development of tolerance and dependence is induced by multiple mechanisms, such as receptor desensitization, receptor phosphorylation and uncoupling, up-regulation of cAMP pathway (which is long-term adaptation), and down-regulation of the Na^+,K^+ pumping. One type is desensitization; a decrease of efficiency of signalization, develops rapidly, and is due to uncoupling of the receptor from its affined G-protein (with or without internalization) and the initial key event is thought to be receptor phosphorylation. Mostly it is the result of exposure to high concentrations of agonist (Law and Loh 1999; Koch and Höllt 2008). Another form of tolerance is due to changes in AC cascade. Acute exposure inhibits cAMP pathway, while chronic exposure leads to up-regulation of cAMP pathway (higher concentration of AC, protein kinase A (PKA), and possibly other components of this pathway). Chronic administration of opiates alters cAMP response element-binding protein (CREB) phosphorylation or expression and CREB mediates up-regulation of the type VIII of AC, further contributing to cAMP pathway activation (Nestler and Aghajanian 1997; Cao et al. 2010). Third form is due to down-regulation of the Na^+,K^+ pumping, which causes reduction in its electrogenic contribution to membrane potential and partial depolarization of the cell membrane (Fleming 1999). Protein isoform, α_3 subunit, is down-regulated and thus, the activity of Na^+/K^+ ATPase decrease causing depolarization of the membrane (Li et al. 2010).

Involvement of different kinases for MOR phosphorylation seems to be agonist-dependent and it might be the explanation for differences in the induction of OR desensitization by various agonists (Koch et al. 2005).

The agonist-dependent phosphorylation increases affinity of OR to cytosolic β-arrestin proteins. Interaction of β-arrestins and OR ends in uncoupling of G-protein signaling and the involvement of endocytotic machinery leading to receptor internalization (Koch and Höllt 2008). A study where the agonist-induced internalization of the MOR was examined, showed that there are differences in the internalization of the MOR between various agonists. Etorphine caused internalization within 15 minutes after injection, while morphine, which is considered to be high affinity agonist of MOR, did not trigger detectable internalization, but it partially inhibited etorphine-induced MOR internalization. Naloxone, opiate antagonist, completely inhibited endocytosis (Sternini et al. 1996).

Studies indicate that highly addictive opiates such as morphine are failing in inducing the desensitization and endocytosis of receptors (Whistler et al. 1999). However, in cultured striatal neurons it was demonstrated that morphine-induced internalization exists, as well as [D-Ala^2,Me Phe^4,Glyol^5] enkephalin (DAMGO) induced internalization, and both of them require presence of β-arrestins. The reason why rapid morphine-induced endocytosis appears in striatal neurons in much greater amount than in other cell types might by that the major type of arrestin expressed in striatal cells is β-arrestin-2 (Haberstock-Debic et al. 2005). Surprisingly, it was shown that morphine induced internalization occurs in the dendrites but not in the soma of cultured nucleus accumbens neurons. This indicates that opiate drugs can have different effects on trafficking of OR present in distinct membrane compartments of the same neuron (Haberstock-Debic et al. 2003). Morphine has unique characteristics, as it causes sustained phosphorylation of Ser^{375}. Desensitized receptors like this are kept in membrane and cannot enter the recycling cycle and be internalized (Schulz et al. 2004). The expression level or subcellular localization of GPCR kinases and/or arrestins might designate destiny of morphine-activated receptors.

In 2003, evidence was provided that phospholipase D2 (PLD2) plays key role in agonist-induced endocytosis of MOR. Stimulating MOR with DAMGO showed an increasement in PDL2 activity, which was then blocked by antagonist naloxone. The DAMGO-mediated PLD2 activation was shown to be ADP-ribosylation factor dependent. Morphine failed to activate

PLD2, but when heterologous PLD2 was activated by phorbol-12-myristate-13-acetate (PMA), morphine could induce an internalization of MOR. These results suggest that both agonist-induced conformational change and stimulation of PLD2 were needed for inducing receptor endocytosis (Koch et al. 2003).

For a long time the idea was that phosphorylation/uncoupling and internalization are part of the process, when tolerance is developed as the number of OR is decreasing. However, multiple studies proved this idea incorrect. Internalization does not necessarily mean desensitization. A study with two isoforms of MOR showed they markedly differ in the rate of their desensitization. A shorter isoform, MOR1B desensitized at a slower rate, immunocytochemical analysis revealed that internalization proceeded at a much faster rate and therefore the rate of resensitization and recycling of receptors are accelerated. This provides an evidence that enhanced resensitization confers resistance to agonist-induced desensitization (Koch et al. 1998).

It was shown that mutation of single cytoplasmic domain (C-terminus) influence agonist selectivity of OR endocytosis (Whistler et al. 1999; T. Koch et al. 1998). The differences in endocytic regulation reflect differences between individual opiate drugs in their ability to promote the regulation of OR signaling. The different effects of antagonists on MOR trafficking suggest the presence of several mechanisms of responding to ligands (Sternini et al. 1996). It has been suggested that arrestin-mediated regulation of OR by endocytosis and reactivation of receptors may play a protective role by reducing the development of physiological drug tolerance. Morphine fails at promoting effectively arrestin-mediated regulation of MOR. The failure of morphine-activated receptors to uncouple from G-protein and endocytose may play an important role in inducing physiological tolerance (Whistler et al. 1999; Finn and Whistler 2001; He and Whistler 2005; He et al. 2002; Koch et al. 2005; Grecksch et al. 2006).

He et al. (2002) suggested that coapplication of DAMGO with morphine facilitates endocytosis of MOR. As a result, long-term treated rats with both drugs showed reduced tolerance. They proposed that endocytic properties are influenced by oligomerisation of MOR. And in 2005, He & Whistler published that cocktail consisting of morphine and small dose of methadone facilitates MOR endocytosis, provide full analgesia, but has no morphine dependence potency. However, study by Koch et al. (2005) investigated coapplication of clinically important

opioids (methadone was among them) with morphine. Their results showed an increase in receptor endocytosis when compared to application of morphine alone, but effect was smaller than that caused by clinically important drugs that induce receptor internalization alone. Other thing was, they failed to confirm results of He and his colleagues (2002) that DAMGO facilitates endocytosis of MOR. Thus, the reduction of tolerance by coapplication of low doses of DAMGO and avoiding a promotion of morphine dependence by cocktail of methadone and morphine is yet to be investigated more.

Morphine tolerance is associated with superactivation of cAMP, as written earlier, but it also includes redistribution of DOR. While MOR are found mainly on the surface of the cell, DOR are mainly intracellular type of receptor. Pretreatment with morphine induced increasing density of DOR on cell surface. Evidence showed that morphine pretreatment does not have a direct effect on DOR, but it is rather indirect acting through MOR. Quantitative analysis suggested that increasing density of DOR is not thanks to synthesis of receptor *de novo*, but it is recruitment of pre-existing reserve receptors (Cahill et al. 2001). Next study with other MOR-agonists, methadone, fentanyl and etorphine, showed similar results, i.e. increasing density of DOR on plasma membrane (Morinville et al. 2003).

The rewarding effect of morphine was believed to be result of cooperation of several OR and there are studies which suggest that development of tolerance is induced not just by MOR, but DOR are involved as well. Animals cotreated with morphine and naltrindole had attenuated development of antinociceptive tolerance and physical dependence, and had fewer withdrawal symptoms. Treatment with antisense oligodeoxynucleotide to the DOR blocked the development of tolerance and dependence (Fundytus et al. 1995; Kest et al. 1996; Hepburn et al. 1997). Moreover, tolerance did not develop in DOR gene knock-out mice (Zhu et al. 1999). On other hand prediction that highly MOR-selective agonist would induce less tolerance was not confirmed. On the contrary, there was a rapid onset of tolerance, though results may indicate that DOR still play a modulatory role in the maintenance of the tolerant state (Zhao et al. 2002). Other results indicate that DOR is not correlated with inducing of tolerance as the enhancement of DOR-mediated antinociception was no longer detectable after 24 hours after the last dose of the drug, from which we might assume that it is a reversible effect (Morinville et al. 2003).

Also study of mice lacking the MOR gene showed that the rewarding effect is abolished in MOR-deficient animals. And while morphine administration induced strong dose-dependent analgesia in wild type (mice with MOR), it had no effect on mutant type. Administration of naloxone failed to induce withdrawal symptoms in MOR-deficient mice, and there is no up-regulation of cyclase activity in striatum in the mutant type. All these results points to the fact that homozygous MOR-deficient mice do not develop physical dependence (Matthes et al. 1996).

Thus, the question of the role of DOR and its extent in development of tolerance and dependence is still open and yet to be studied more.

5. Ontogenesis in opioid receptors

The expression patterns of OR mRNAs are distinct at all ages. Though, first OR that appear in mouse embryo are KOR, detected in embryonic day 9.5 (E9.5) in gut epithelium. In the brain both MOR and KOR mRNAs are present in E11.5 already. Their adult expression patterns are established by E17.5. On the other hand DOR appear rather late (E19.5) and remains at low levels of expression. In contrast to this DOR is first OR expressed in dorsal root ganglion in E12.5 (Zhu et al. 1998). It was shown that MOR and KOR are present at spinal cord of rat pups at birth, while DOR appear in first 2 postnatal weeks. During the ontogenesis MOR is predominant OR in the spinal cord. Overall binding peaks at 7th postnatal day and decline to adulthood levels (Rahman et al. 1998). All 3 receptor types increase in density during postnatal development, different postnatal development of multiple OR appears. MOR initially declined in first few days, resulting in a 32% reduction by day 4. Rapid increase is following between days 7 and 14 and adult levels are reached by day 21. Sparse amounts of KOR are rising slightly, peaking at day 35 and declining to adult levels (Spain et al. 1985). MOR binding potential was found to increase with age in neocortical areas and in the putamen. Beside this, gender-by-age interactions were observed in the thalamus and amygdala. MOR binding potential decreases in postmenopausal women to levels below those of men. And while age-related increase in MOR density, but not affinity, were shown in one study (Zubieta et al. 1999). Another study shows no age influence on the density of MOR and reduced affinity of these receptors for DAMGO (Hoskins et al. 1998). Opioid drugs lose their efficacy with increasing age and it seems to be a result of several interacting changes due to aging.

Age-dependent alternations in both mechanical and thermal antinociception were observed. Antinociceptive effects of β-endorphin and morphine are mediated by activation of different pathways and neural mechanisms and they develop differently. Responses to mechanical responses were observed at 2-4 days old rat already. Inhibition develops progressively, but the one produced by β-endorphin reach maximum at 7 days, while morphine-induced at 28 days. Thermal responses developed later, at 7days old rats and reached maximum at 28 days (Tseng et al. 1995). These results suggest that two descending pain inhibitory systems activated by β-endorphin and morphine are differentially developed.

5.1. Effects of age on modulation of opioid receptors

Mechanisms of opioid tolerance and functioning of OR on molecular level are very complex. Many steps of molecular pathway require a modification of the expression and functions of signaling molecules. These modifications involve other receptors and proteins.

Binding of agonists to OR induces signaling transduction pathway activation and modulation of OR, i.e. phosphorylation and desensitisation. Continuous exposure to agonist leads to phosphorylation of OR by G-protein-coupled receptor kinases (GRKs), with GRK2 and GRK6 playing important role. Phosporylated receptor is then bound by β-arrestin and this leads to uncoupling of receptor from G-protein, causing desensitization and decreasing efficacy of opioid agonist. Rate and extent of desensitisation depends on cellular concentration of arrestins and GRKs, thus their age- and developmental-related changes influence OR functioning and tolerance development. Selective increase of arrestin 2 was demonstrated during neural differentiation in rat embryos (Gurevich et al. 2004). This steady increase continues in postnatal development, when arrestin 2 mRNA levels increased until 14th postnatal day and then decreased while arrestin 2 protein levels continued to rise (Gurevich et al. 2002). All GRKs subtypes were expressed in rat's fetal brain from embryonal week 12, but there was no change in their number with an age, beside increased expression of GRK5 which accompanied the increase in arrestin 2 (Gurevich et al. 2004). In human prefrontal cortex, the immunodensities of GRK2, GRK6 and β-arrestin 2 appear to decline significantly with aging (Ontl et al. 2004).

The phosphorylation of OR by GRKs and the binding of β-arrestin initiate the internalization of the ligand-bound receptors. A model by Koch and his colleagues suggests that internalization is preventing from development of tolerance. Morphine is inefficient in inducing internalization, but tolerance develops rapidly (Koch et al. 2005). Though evidence for morphine-induced rapid MOR endocytosis from striatal cells and periaqueductal gray (PAG) exists, suggesting that the process of internalization is specific for certain types of cells and tissues (Haberstock-Debic et al. 2005; Rodríguez-Muñoz et al. 2007). Endocytosis of OR is regulated by phosphorylation and association with β-arrestins (Whistler et al. 1999) and they endocytose via dynamine-dependent mechanism involving clathrine-coated pits. Age-related

decrease in the kinetics of cargo transit through clathrin-coated pits was observed (Blanpied et al. 2003). Though this study was not focused on endocytosis of OR, we might assume that clathrin dynamics change with age also in OR system.

5.2. Effects of age on other receptors and proteins influencing opioid signaling

There are molecules related to opioid signaling pathways, which negatively influence opioid action. Among various anti-opioid systems belong N-methyl-D-aspartate (NMDA) receptors, calcitonin-gene-related peptide (CGRP), substance P (SP) or regulator of G-protein signaling (RGS). These proteins play important role in opioid signaling and in the development of tolerance and aging has effects on all of them.

NMDA attenuates opioid receptor-mediated G-protein activation. MOR and NMDA receptors are colocalized in certain areas, mainly those related to rewarding behavior and antinociception- caudate nucleus and PAG. Thus MOR and NMDA receptors probably dually regulate the output of neurons (Wang et al. 1999; Commons et al. 1999). Colocalization of these receptors in nucleus accumbens suggests their dual involvement in the presynaptic release of neurotransmitters in this region (Gracy et al. 1997). Thus the differentially regulated expression and function of NMDA receptor during development and aging might play important role in this dual involvement. There is a significant decline in agonist binding to NMDA receptor in lateral prefrontal/frontal cortex during both development and aging, while decline in the medial cortex was less significant. Interestingly, ε2 subunit shows age-related decline in its expression, while ζ1 subunit shows sex-related expression and no influence of aging process on its expression (Ontl et al. 2004; Zhao et al. 2009).

It was shown that NMDA receptor antagonists are inhibiting the development of morphine tolerance in adult rats (Trujillo and Akil 1994; Herman et al. 1995). Next study specified the age at which inhibition appears as NMDA receptor antagonist does not inhibit or attenuate development of tolerance in neonatal rats, 7 days old. On the other hand, attenuation appeared in 14 days old rats and tolerance was significantly attenuated in 21 days old and older

rats, suggesting a transition age around second postnatal week for NMDA receptors to be effective in decreasing the development of tolerance (Zhu and Barr 2003).

In addition to NMDA receptors, there are some neuropeptides that are known to have anti-opioid effects, for example CGRP and SP. It was shown that age regulate their expression and function. *In vitro* study on cultured dorsal root ganglion neurons from 3 months and 10 months old rats showed an increase in the immunoreactivity of CGRP and SP. As MOR are co-localized with CGRP and SP-like immunoreactivity, it is likely that morphine acts through OR present in CGRP and SP. Age seems to play role in the opioid signaling as older animals were more sensitive to treatment with morphine. Smaller doses and shorter time was needed to induce increasing number of CGRP and SP neurons (Ma et al. 2000).

RGS proteins are negative regulators of G-protein-mediated opioid signaling and they play crucial role in opioid signaling mechanisms, facilitate OR desensitization and internalization. The interaction between RGS19 and DOR (Elenko et al. 2003) and between RGS20 and MOR was established (Garzón et al. 2004). Specific RGS proteins play important role, especially RGS2, RGS4, RGS9, RGS19 and RGS20. These proteins specifically interact with Gα subunit, where they facilitate switch of Gα from a GTP-active state to GDP-inactive state (for review see Xie and Palmer 2005; Ross and Wilkie 2000). Expression of RGS proteins was studied and was found age-dependent; the dependency is subtype-specific and also specific to the region of brain. Different subtypes of RGS proteins had onset of expression and its duration at different postnatal days (Ingi and Aoki 2002; Wilson et al. 2005; Gold et al. 1997). During embryonic and early postnatal development, two RGS9 transcripts are present in whole brain. After postnatal day 10, the expression of one transcript increases and concentrates in striatum, while the expression of other one decreases (Thomas, Danielson, and Sutcliffe 1998). RGS4 is expressed in different areas of brain in the developing postnatal brain (neocortex, hippocampus, cerebellum) and in the adult brain (neocortex, thalamus, cerebellum) of rats (Ingi and Aoki 2002). This different expressional onset might influence the OR in their signaling and may alter the effects of opioids.

The PAG is a major supraspinal site in opioid analgesic actions and a significant site of

cellular adaptations on chronic treatment with morphine (Bagley et al. 2005; Morgan et al. 1998). MOR-acting opioids are of limited efficacy in neuropathic pain and morphine analgesia is under negative functional regulation by the NMDA receptor-neural nitric oxide synthetase (nNOS) cascade (Rodríguez-Muñoz et al. 2008). PAG contains neuronal isoform of nNOS, which plays role in the development of antinociceptive tolerance to morphine as it generates NO that alternates MOR and its constituve activity (Kielstein et al. 2007). In the nNOS deficient mice less tolerance is exhibited (Heinzen and Pollack 2004). Alternation in the signaling and development of tolerance appears with increasing age. Chronic treatment with morphine has impact on PAG in both 7 days old rat pups and adult rats, but adaptive responses differ. In adults an up-regulation of genes associated with Fos and NADPH oxidase appeared (nitric oxide is implicated in oxidative stress), while in pups are changes in gene expression that alternates superoxide and peroxide metabolism (Bajic et al. 2012).

5.3. Age-dependent opioid analgesia, tolerance and dependence

Some patients require dose escalation of opioid drug. This escalation may develop due to several reasons, e.g. tolerance, addiction or progression of disease. Clinical data suggest that younger patients have a rapid onset of dose escalation. A study of terminally ill patients was done. Each decade after 40 years was less likely to require unusually high doses or doses over 120mg/day of oral morphine equivalent (Hall et al. 2003). Another study, confirming these results, showed that patients younger than 50 years escalated dose at twice the rate than older patients, mainly those over 60 years, which may mean older patients have reduced rate of development of tolerance (Buntin-Mushock et al. 2005). Intrathecal opioid dose escalation occurred more steeply in younger patients (Hayek et al. 2011).

Age influence on development of tolerance has been studied on model organism. Different age groups of rats (3 weeks, 3 months, 6 months and 1 year) were treated with morphine and examined. There was a 400% increase in the length of time during which tolerance was developed if 1 year rat is compared to 3 weeks old one. Thus the rate of tolerance

development is age dependent and tolerance occurs more rapidly in younger individuals than older ones. Plasma levels of morphine suggested that the molecular mechanism change with an age (Wang et al. 2005). Morphine was proven to be active in analgesia in rat pups 3 days old, it had increasing potency and reached plateau from postnatal day 9 until postnatal day 21. Tolerance was possible to induce with continous administration after 72 hours in first 2 postnatal weeks (Thornton et al. 1997). There are several studies on rats on dose alternations of opioids due to age influence and presence of inflammation, when epidural administration was used. A very low doses were effective at reversing inflammation-induced hypersensitivity, mainly at younger age. Low-doses effects were demonstrated *in vivo*, showing importance of development being taken into consideration when using local epidural anesthetics. Selective analgesic effect of lower doses decreases with increasing age of rat pups (Howard et al. 2001; Walker and Fitzgerald 2007; Walker et al. 2005). Another study reveals age-specific alternations in rat's nociception following different morphine schedules. Thus the morphine-induced changes in nociception depend on developmental phase in which exposure occurs and the dosing schedule. While intermittent administration may produce sensitization, continuous administration preferentially produces tolerance (Zissen et al. 2007).

Different studies show various results from model organisms, mainly rodents, but also primates (Young et al. 2005; Yon et al. 2005; Slikker et al. 2007). Their results point to neuroapoptosis caused by general anesthesia, the level of damage is dose and age dependent. Developing brain is most vulnerable at the peak of synaptogenesis, which means 7 day old rat pup. The least sensitive period is at the end of synaptogenesis- 14 days old (Yon et al. 2005). Neurogenesis event in whole brain of rat pup 7 days old (28 post-conceptional days) corresponds to 221 post-conceptional days in human and 14 days old rat pup (35 post-conceptional days) corresponds to 359 post-conceptional days in human. Model organisms mature and develop at different rate and age, therefore is important to extrapolate the timing of events of neurodevelopment from experimental species to human (Clancy et al. 2001; Workman et al. 2013; for review see Clancy et al. 2007).

5.4. Effects of age on opioid pharmacokinetics

Drug metabolism playes important role in administration of opioid drugs as well and it seems there are differences between pharmacokinatices between young and older individuals. The effect of old age on the antinociceptive response of morphine and morphine-6β-glucuronide (M6G) was observed. Morphine is metabolised into morphine-3β-glucuronide and M6G. While no difference in antinociceptive effect or concentration time-curve of morphine were found between young and old animals, the antinociceptive effect-time curve and plasma M6G concentration time-curve were greater in aged rats. This was probably due to diminished renal function, decreasing the clearence of M6G. Thus the aged rats have increased sensitivity to morphine probably due to elevated plasma M6G (Van Crugten et al. 1997). Morphine clereance in neonates is slower than in adults, though when it is standardised to 70-kg person, it shows that clereance is similar to adult within 6-12 months after birth (Bouwmeester et al. 2004). Different studies with various opioid drugs show different results in age-related changes of pharmacokinetics and pharmacodynamics, for example a study with short-acting opioid remifentanil increased sensitivity in elderly people and decreased clearance of drug (Minto et al. 1997). The EEG showed increased sensitivity of brain to opioids with increasing age, requiring a smaller dosage of drug to older patients (Minto, Schnider, and Shafer 1997). Effects of fentanyl on different age groups of foals were examined and difference in pharmacokinetics were observed in the earliest age stage after single dose of drug already (Knych et al. 2014).

On the other hand, methadone which is another long-lasting opioid drug, beside morphine that is widely used in health care showed no clearance maturation with an age. Neonatal clearance for the isomers is similar to those reported for adults and teenagers (Ward et al. 2014). Similar results showed studies with adolescents. Clearance rate in adolescents did not differ to the one of adults (Stemland et al. 2013; Sharma et al. 2013).

6. Influences of morphine during ontogenesis

There are studies showing that the relieving pain in infants and neonates plays an important role, as noxious stimuli which are not mitigated with analgesia might influence the development of brain in children, especially those born very preterm. Pain-related stress predicts lower cortical thickness, specifically in frontal, parietal and temporal regions, compared to healthy term born controls (Ranger et al. 2013). Neurodevelopment of preterm infants in relation to pain was examined and poorer cognition and motor function in the first 2 years of life were associated with a higher number of skin-breaking procedures during neonatal care (Grunau et al. 2009). Cortical responses to noxious stimuli have been recorded from post-conceptional age of 25 weeks and the magnitude of responses to stimuli increased with post-conceptional age (Slater et al. 2006). These negative influences of pain on developing brain suggest clinical usage of opioid drugs to relieve pain. One of the most used opioid compound is morphine, which is very effective, but it was shown that opioids in general,mainly morphine, influence neuronal cell death- apoptosis, which involves activation of a cascade of intracellular cysteine proteases. Caspase-3 plays a crucial role in the terminal or execution stage of apoptosis. Treatment of human fetal microglial cell, astrocyte and neuronal cell cultures with morphine proved that this opiate induces caspase-3-dependent apoptosis. Neurons were more sensitive than microglia, while astrocytes were resistant to morphine-induced apoptosis. Naloxone blocked apoptosis of these cell cultures, suggesting that opiate-receptor mechanism is involved (Hu et al. 2002). Similar results were presented with studies on rats, where morphine induced apoptosis of peritoneal macrophages and promoted accumulation of Bax protein; a death agonist from Bcl-2 family of apoptosis regulatory genes (Singhal et al. 1998). Study specifically oriented on nucleus accumbens and prefrontal cortex showed increase in apoptotic factors in these regions (Katebi et al. 2013). On mice macrophages was demonstrated that apoptosis may be mediated through TGF-β (Singhal et al. 2000). All these suggest that morphine can directly effect and modulate an immune system of drug addicted patients. There is also a difference between acute and chronic treatment with morphine. While acute treatment does not influence immunodensity of pro-apoptotic Fas receptor and anti-apoptotic Bcl-2 oncoprotein, there is a change in chronic treatment. The density of Fas receptor increases and the density of Bcl-2 decreases in cerebral

cortex. The chronic cotreatment of morphine and naloxone prevented any change in immunodensity of both Fas receptor and Bcl-2 (Boronat et al. 2001). Minocycline, second-generation tetracycline with known neuroprotective effects, was administered along with morphine to rats. It prevented morphine-induced apoptosis and increased number of anti-apoptotic agents, such as Bcl-2 and HSP70 in cerebral cortex and lumbar spinal cord. However, levels of caspase-3 stayed unchanged (Hassanzadeh et al. 2011). With these results we have to take into consideration the fact that morphine was administered when no noxious stimuli were present and therefore we cannot assess the negative effects of pain against negative effects of drug administration (Attarian et al. 2014). The effect of morphine administered to rat females on the developing fetal rat cerebrum was studied as well. Beside general decrease in fetal weight and crown-to-rump length, a reduced cortical thickness and number of neurons were observed in frontal cerebral cortex. This suggests that prenatal morphine administration has neurotoxic effects on both growth of fetus and neuronal proliferation and differentiation (Sadraie et al. 2008). Similar results are from evaluation of 18 months and 3 years old infants, who were exposed to morphine *in utero*. They were more likely to have neurodevelopmental impairment (Hunt et al. 2008). Even though not all of these studies provide results from infants, they still give a suggestion of direction for further investigation, that OR are not involved only in pain pathways.

On the contrary, the positive or no effect of morphine was observed as well. The effects of this opioid drug were studied when administered during brain growth spurt in rats. Both acute and chronic treatment did not alter the development of layer 5 pyramidal neurons. Only respiratory depression and mild acidosis were observed. Chronic exposure at early stages of brain growth caused higher proportions of spines with smaller head diameter (Massa et al. 2012). Neither lumbar intrathecal injections with morphine in early postnatal age in rat pups managed to detect an increase in neuronal injury or apoptosis. There were no long-term changes in hindlimb sensory thresholds or gait (Westin et al. 2010). Kim et al. (2001) demonstrated that high concentration of morphine has protective effects on primary rat neonatal astrocytes against NO-related free radicals, including NO and peroxynitrite, but does not have this effect on other cells. Naloxone completely blocked the protective effect. The antioxidant system, such as glutathione,

is required for protection of astrocytes through morphine. Pre-emptive neonatal morphine attenuates the consequences of neonatal injury, increases rate of recovery and reduces hyperalgesia after a subsequent inflammation in adulthood (Laprairie et al. 2008). Untreated pain in neonatal individuals may cause the damage to the immature brain and alter its development. However, morphine may have protective effects under conditions of pain and stress. Morphine seems to regulate some stress-related changes and has protective character only to certain limit. Opioids seem to have different effects in the presence and absence of pain (Dührsen et al. 2013). Comparable to these results, it was indicated that there is dose-dependent relationship between stress and neuronal damage and there are complex interactions of morphine with stress. Repeated stress in neonatal mice has a dose-dependent effect in hippocampal gene expresion, morphine alters subset of stress-related changes in gene expresion, which probably influences neurodevelopment (Juul et al. 2011).

Although, there is no evidence for long-term beneficial effects of treatment with morphine, there are several studies looking into negative long-term effects of pre-emptive morphine therapy in preterm neonates. One of them assesses children in the age of 5-7 years; ex-preterm infants were treated with either morphine or placebo. At age 5-7 were several physiological and psychological tests realized. It showed that morphine treated group has smaller circumference of head, smaller body weight, but height was same as in placebo group. Parents reported some social problems in morphine treated group and these infants needed longer time to make a choice in short-term memory tasks, but no effect was shown on IQ or academic results (Ferguson et al. 2012). Other studies among 5-year old ex-preterm infants showed that usage of morphine in low doses does not have long-term effects (de Graaf et al. 2011; Macgregor et al. 1998). But de Graaf with colleagues (2011) showed slightly worse results in the morphine group in visual analysis of intelligence test and suggested adverse effect on executive functions, i.e. management of cognitive processes. However, in follow-up study at the age of 8 and 9 in same group no such results were confirmed (de Graaf et al. 2013).

7. Conclusion

Opioid receptors are very important from clinical point of view as many drugs used in medical care are targeting these G-protein-coupled receptors. The impact of aging is apparent and significant in effects of opioids or in the development of tolerance and dependance as the complex regulation steps of signalisation changes over the lifespan.

One of the most used opioid drugs is morphine, which is targeting mainly MOR and develops rapid tolerance in chronic treatment. Both negative and positive effects of morphine were observed during ontogensis. Thus when using this drugs the complex of conditions such as age, stress and pain or duration of treatment should be taken into consideration.

Age-dependent changes may have important clinical implications on the effectiveness of opioid therapy and probably it should be considered to use some opioids in relation to age as most of them are dosed to weight of body.

Further study of changes of regulation steps and mainly how they interact with each other during ontogenesis may shed light onto signaling mechanisms and some negative effects of opioid usage.

8. References

Attarian, S., L. Tran, A. Moore, G. Stanton, E. Meyer, and Robert Moore. 2014. "The Neurodevelopmental Impact of Neonatal Morphine Administration." *Brain Sciences* 4 (2) (April 25): 321–334.

Bagley, Elena E, Billy C H Chieng, MacDonald J Christie, and Mark Connor. 2005. "Opioid Tolerance in Periaqueductal Gray Neurons Isolated from Mice Chronically Treated with Morphine." *British Journal of Pharmacology* 146 (1) (September): 68–76.

Bajic, D, C B Berde, and K G Commons. 2012. "Periaqueductal Gray Neuroplasticity Following Chronic Morphine Varies with Age: Role of Oxidative Stress." *Neuroscience* 226 (December 13): 165–177.

Bare, L a, E Mansson, and D Yang. 1994. "Expression of Two Variants of the Human Mu Opioid Receptor mRNA in SK-N-SH Cells and Human Brain." *FEBS Letters* 354 (2) (November 7): 213–216.

Blanpied, T, D B Scott, and M D Ehlers. 2003. "Age-Related Regulation of Dendritic Endocytosis Associated with Altered Clathrin Dynamics." *Neurobiology of Aging* 24 (8) (December): 1095–1104.

Boronat, M a, M J García-Fuster, and J a García-Sevilla. 2001. "Chronic Morphine Induces up-Regulation of the pro-Apoptotic Fas Receptor and down-Regulation of the Anti-Apoptotic Bcl-2 Oncoprotein in Rat Brain." *British Journal of Pharmacology* 134 (6) (November): 1263–1270.

Bouwmeester, N. J., B. J. Andersom, D. Tibboel, and N. H. G. Holford. 2004. "Developmental Pharmacokinetics of Morphine and Its Metabolites in Neonates, Infants and Young Children." *British Journal of Anaesthesia* 92 (2) (February 1): 208–217.

Buntin-Mushock, Chante, Lisa Phillip, Kumi Moriyama, and Pamela Pierce Palmer. 2005. "Age-Dependent Opioid Escalation in Chronic Pain Patients." *Anesthesia and Analgesia* 100 (6) (June): 1740–1745.

Bunzow, James R, Carmen Saez, Marty Mortrud, Claudia Bouvier, John T Williams, Malcolm Low, and David K Grandyap. 1994. "Molecular Cloning and Tissue Distribution of a Putative Member of the Rat Opioid Receptor Gene Family That Is Not a , U , 6 or K Opioid Receptor Type." *FEBS Letters* 347 (2-3): 284–288.

Cahill, C M, A Morinville, M C Lee, J P Vincent, B Collier, and A Beaudet. 2001. "Prolonged Morphine Treatment Targets Delta Opioid Receptors to Neuronal Plasma Membranes and Enhances Delta-Mediated Antinociception." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 21 (19) (October 1): 7598–7607.

Cao, Jun-Li, Vincent F Vialou, Mary Kay Lobo, Alfred J Robison, Rachael L Neve, Donald C Cooper, Eric J Nestler, and Ming-Hu Han. 2010. "Essential Role of the cAMP-cAMP Response-Element Binding Protein Pathway in Opiate-Induced Homeostatic Adaptations of Locus Coeruleus Neurons." *Proceedings of the National Academy of Sciences of the United States of America* 107 (39) (September 28): 17011–17016.

Chen, Yan, Anton Mestek, Jian Liu, and Lei Yu. 1993. "Molecular Cloning of a Rat K Opioid Receptor Reveals Sequence Similarities to the M and Δ Opioid Receptors." *Biochemical Journal* 628: 625–628.

Clancy, B, R B Darlington, and B L Finlay. 2001. "Translating Developmental Time across Mammalian Species." *Neuroscience* 105 (1) (January): 7–17.

Clancy, Barbara, Barbara L Finlay, Richard B Darlington, and K J S Anand. 2007. "Extrapolating Brain Development from Experimental Species to Humans." *Neurotoxicology* 28 (5) (September): 931–937.

Commons, K G, E J van Bockstaele, and D W Pfaff. 1999. "Frequent Colocalization of Mu Opioid and NMDA-Type Glutamate Receptors at Postsynaptic Sites in Periaqueductal Gray Neurons." *The Journal of Comparative Neurology* 408 (4) (June 14): 549–559.

Connor, Mark, and Macdonald J Christie. 2006. "BRIEF REVIEW OPIOID RECEPTOR SIGNALLING MECHANISMS." *Clinical and Experimental Pharmacology and Physiology* 26: 493–499.

De Graaf, Joke, Richard a van Lingen, Sinno H P Simons, Kanwaljeet J S Anand, Hugo J Duivenvoorden, Nynke Weisglas-Kuperus, Daniella W E Roofthooft, et al. 2011. "Long-Term Effects of Routine Morphine Infusion in Mechanically Ventilated Neonates on Children's Functioning: Five-Year Follow-up of a Randomized Controlled Trial." *Pain* 152 (6) (June): 1391–1397.

De Graaf, Joke, Richard A van Lingen, Abraham J Valkenburg, Nynke Weisglas-kuperus, Liesbeth Groot, Barbara Wijnberg-williams, Kanwaljeet J S Anand, Dick Tibboel, and Monique Van Dijk. 2013. "Does Neonatal Morphine Use Affect Neuropsychological Outcomes at 8 to 9 Years of Age ?" *Pain* 154 (3): 449–458.

Dührsen, Lasse, Sinno H P Simons, Mark Dzietko, Kerstin Genz, Ivo Bendix, Vinzenz Boos, Marco Sifringer, Dick Tibboel, and Ursula Felderhoff-Mueser. 2013. "Effects of Repetitive Exposure to Pain and Morphine Treatment on the Neonatal Rat Brain." *Neonatology* 103 (1) (January): 35–43.

Elenko, Eric, Thierry Fischer, Ingrid Niesman, Tim Harding, Tammie McQuistan, Mark Von Zastrow, and Marilyn G Farquhar. 2003. "Spatial Regulation of Galphai Protein Signaling in Clathrin-Coated Membrane Microdomains Containing GAIP." *Molecular Pharmacology*

64 (1) (July): 11–20.

Eppler, C M, J D Hulmes, J B Wang, B Johnson, M Corbett, D R Luthin, G R Uhl, and J Linden. 1993. "Purification and Partial Amino Acid Sequence of a Mu Opioid Receptor from Rat Brain." *The Journal of Biological Chemistry* 268 (35) (December 15): 26447–26451.

Ferguson, Sherry a, Wendy L Ward, Merle G Paule, R Whit Hall, and K J S Anand. 2012. "A Pilot Study of Preemptive Morphine Analgesia in Preterm Neonates: Effects on Head Circumference, Social Behavior, and Response Latencies in Early Childhood." *Neurotoxicology and Teratology* 34 (1): 47–55.

Finn, Andrew K, and Jennifer L Whistler. 2001. "Endocytosis of the Mu Opioid Receptor Reduces Tolerance and a Cellular Hallmark." *Neuron* 32: 829–839.

Fleming, W W. 1999. "Cellular Adaptation: Journey from Smooth Muscle Cells to Neurons." *The Journal of Pharmacology and Experimental Therapeutics* 291 (3) (December): 925–931.

Fundytus, M E, P W Schiller, M Shapiro, G Weltrowska, and T J Coderre. 1995. "Attenuation of Morphine Tolerance and Dependence with the Highly Selective Δ-Opioid Receptor Antagonist TIPP[ψ]." *European Journal of Pharmacology* 286 (1): 105–108.

Garzón, Javier, María Rodríguez-Muñoz, Almudena López-Fando, Antonio García-España, and Pilar Sánchez-Blázquez. 2004. "RGSZ1 and GAIP Regulate Mu- but Not Delta-Opioid Receptors in Mouse CNS: Role in Tachyphylaxis and Acute Tolerance." *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology* 29 (6) (June): 1091–1104.

Gold, S J, Y G Ni, H G Dohlman, and E J Nestler. 1997. "Regulators of G-Protein Signaling (RGS) Proteins: Region-Specific Expression of Nine Subtypes in Rat Brain." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 17 (20) (October 15): 8024–8037.

Gracy, K N, a L Svingos, and V M Pickel. 1997. "Dual Ultrastructural Localization of Mu-Opioid Receptors and NMDA-Type Glutamate Receptors in the Shell of the Rat Nucleus Accumbens." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 17 (12) (June 15): 4839–4848.

Grecksch, Gisela, Katharina Bartzsch, Antje Widera, Axel Becker, Volker Höllt, and Thomas Koch. 2006. "Development of Tolerance and Sensitization to Different Opioid Agonists in Rats." *Psychopharmacology* 186 (2) (June): 177–184.

Grunau, Ruth E, Michael F Whitfield, Julianne Petrie-Thomas, Anne R Synnes, Ivan L Cepeda, Adi Keidar, Marilyn Rogers, Margot Mackay, Philippa Hubber-Richard, and Debra

Johannesen. 2009. “Neonatal Pain, Parenting Stress and Interaction, in Relation to Cognitive and Motor Development at 8 and 18 Months in Preterm Infants.” *Pain* 143 (1-2) (May): 138–146.

Gurevich, E V, J L Benovic, and V V Gurevich. 2002. “Arrestin2 and arrestin3 Are Differentially Expressed in the Rat Brain during Postnatal Development.” *Neuroscience* 109 (3) (January): 421–436.

Gurevich, Eugenia V, Jeffrey L Benovic, and Vsevolod V Gurevich. 2004. “Arrestin2 Expression Selectively Increases during Neural Differentiation.” *Journal of Neurochemistry* 91: 1404–1416.

Haberstock-Debic, Helena, Kyung-Ah Kim, Y Joy Yu, and Mark von Zastrow. 2005. “Morphine Promotes Rapid, Arrestin-Dependent Endocytosis of Mu-Opioid Receptors in Striatal Neurons.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 25 (34) (August 24): 7847–7857.

Haberstock-Debic, Helena, Marc Wein, Michel Barrot, Eric E O Colago, Zia Rahman, Rachael L Neve, Virginia M Pickel, Eric J Nestler, Mark von Zastrow, and Adena L Svingos. 2003. “Morphine Acutely Regulates Opioid Receptor Trafficking Selectively in Dendrites of Nucleus Accumbens Neurons.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 23 (10) (May 15): 4324–4332.

Hall, Susannah, Rollin M Gallagher, Edward Gracely, Calvin Knowlton, and Douglas Wescules. 2003. “The Terminal Cancer Patient: Effects of Age, Gender, and Primary Tumor Site on Opioid Dose.” *Pain Medicine (Malden, Mass.)* 4 (2) (July): 125–134.

Harrison, L M, a J Kastin, and J E Zadina. 1998. “Opiate Tolerance and Dependence: Receptors, G-Proteins, and Antiopiates.” *Peptides* 19 (9) (January): 1603–1630.

Hassanzadeh, Kambiz, Bohlool Habibi-asl, Safar Farajnia, and Leila Roshangar. 2011. “Minocycline Prevents Morphine-Induced Apoptosis in Rat Cerebral Cortex and Lumbar Spinal Cord: A Possible Mechanism for Attenuating Morphine Tolerance.” *Neurotoxicity Research* 19 (4) (May): 649–659.

Hayek, Salim M, I Elias Veizi, Samer N Narouze, Nagy Mekhail, Opioid Dose Esca-, Noncancer Pain, and Chronic Back. 2011. “Age-Dependent Intrathecal Opioid Escalation in Chronic Noncancer Pain Patients.” *Pain Medicine* 12: 1179–1189.

He, Li, Jamie Fong, Mark von Zastrow, and Jennifer L Whistler. 2002. “Regulation of Opioid Receptor Trafficking and Morphine Tolerance by Receptor Oligomerization.” *Cell* 108 (2) (January 25): 271–282.

He, Li, and Jennifer L Whistler. 2005. “An Opiate Cocktail That Reduces Morphine Tolerance

and Dependence." *Current Biology : CB* 15 (11) (June 7): 1028–1033.

Heinzen, Erin L, and Gary M Pollack. 2004. "The Development of Morphine Antinociceptive Tolerance in Nitric Oxide Synthase-Deficient Mice." *Biochemical Pharmacology* 67: 735–741.

Hepburn, M J, P J Little, J Gingras, and C M Kuhn. 1997. "Differential Effects of Naltrindole on Morphine-Induced Tolerance and Physical Dependence in Rats." *The Journal of Pharmacology and Experimental Therapeutics* 281 (3) (June): 1350–1356.

Herman, Barbara H, Frank Vocci, and Peter Bridge. 1995. "The Effects of NMDA Receptor Antagonists and Nitric Oxide Synthase Inhibitors on Opioid Tolerance and Withdrawal." *Neuropsychopharmacology* 13 (4): 269–293.

Heyman, J S, Q Jiang, R B Rothman, H I Mosberg, and F Porreca. 1989. "Modulation of M-Mediated Antinociception by Δ Agonists : Characterization with Antagonists." *European Journal of Pharmacology* 169: 43–52.

Hoskins, D L, T L Gordon, and T Crisp. 1998. "The Effects of Aging on Mu and Delta Opioid Receptors in the Spinal Cord of Fischer-344 Rats." *Brain Research* 791 (1-2) (April 27): 299–302.

Howard, R F, D J Hatch, T J Cole, and M Fitzgerald. 2001. "Inflammatory Pain and Hypersensitivity Are Selectively Reversed by Epidural Bupivacaine and Are Developmentally Regulated." *Anesthesiology* 95 (2) (August): 421–427.

Hu, Shuxian, Wen S Sheng, James R Lokensgard, and Phillip K Peterson. 2002. "Morphine Induces Apoptosis of Human Microglia and Neurons." *Neuropharmacology* 42 (6) (May): 829–836.

Hunt, Rod W, Dimitra Tzioumi, Edith Collins, and Heather E Jeffery. 2008. "Adverse Neurodevelopmental Outcome of Infants Exposed to Opiate in-Utero." *Early Human Development* 84 (1) (January): 29–35.

Ingi, Tatsuya, and Yaeko Aoki. 2002. "Expression of RGS2 , RGS4 and RGS7 in the Developing Postnatal Brain." *European Journal of Neuroscience* 15: 929–936.

Juul, Sandra E, Richard P Beyer, Theo K Bammler, Federico M Farin, Christine A Gleason, and Occupational Health Sciences R P B. 2011. "Effects of Neonatal Stress and Morphine on Murne Hippocampal Gene Expression." *Pediatric Research* 69 (4): 285–292.

Katebi, Seyedeh-Najmeh, Yasaman Razavi, Shabnam Zeighamy Alamdary, Fariba Khodagholi, and Abbas Haghparast. 2013. "Morphine Could Increase Apoptotic Factors in the Nucleus Accumbens and Prefrontal Cortex of Rat Brain's Reward Circuitry." *Brain Research* 1540

(December 2): 1–8.

Kest, B, C E Lee, G L McLemore, and C E Inturrisi. 1996. “An Antisense Oligodeoxynucleotide to the Delta Opioid Receptor (DOR-1) Inhibits Morphine Tolerance and Acute Dependence in Mice.” *Brain Research Bulletin* 39 (3) (January): 185–188.

Kielstein, Anousheh, Dimitrios Tsikas, Gantt P Galloway, and John E Mendelson. 2007. “Asymmetric Dimethylarginine (ADMA)— A Modulator of Nociception in Opiate Tolerance and Addiction ?” *Nitric Oxide* 17: 55–59.

Kim, M S, Y P Cheong, H S So, K M Lee, T Y Kim, J Oh, Y T Chung, Y Son, B R Kim, and R Park. 2001. “Protective Effects of Morphine in Peroxynitrite-Induced Apoptosis of Primary Rat Neonatal Astrocytes: Potential Involvement of G Protein and Phosphatidylinositol 3-Kinase (PI3 Kinase).” *Biochemical Pharmacology* 61 (7) (April 1): 779–786.

Knych, H. K., E. P. Steffey, M. M. Mitchell, and H. C. Casbeer. 2014. “Effects of Age on the Pharmacokinetics and Selected Pharmacodynamics of Intravenously Administered Fentanyl in Foals.” *Equine Veterinary Journal* (May 7): 1–6.

Koch, T., Lars-Ove Brandenburg, Stefan Schulz, Yingjian Liang, Jochen Klein, and Volker Hollt. 2003. “ADP-Ribosylation Factor-Dependent Phospholipase D2 Activation Is Required for Agonist-Induced -Opioid Receptor Endocytosis *.” *The Journal of Biological Chemistry* 278 (11) (March 14): 9979–9985.

Koch, T., and V. Höllt. 2008. “Role of Receptor Internalization in Opioid Tolerance and Dependence.” *Pharmacology & Therapeutics* 117: 199–206.

Koch, T., S. Schulz, H. Schroder, R. Wolf, E. Raulf, and V. Hollt. 1998. “Carboxyl-Terminal Splicing of the Rat Opioid Receptor Modulates Agonist-Mediated Internalization and Receptor Resensitization.” *Journal of Biological Chemistry* 273 (22) (May 29): 13652–13657.

Koch, Thomas, Antje Widera, Katharina Bartzsch, Stefan Schulz, Lars-ove Brandenburg, Nicole Wundrack, Andrea Beyer, Gisela Grecksch, and H Volker. 2005. “Receptor Endocytosis Counteracts the Development of Opioid Tolerance.” *Molecular Pharmacology* 67 (1): 280–287.

Laprairie, Jamie L, Malcolm E Johns, and Anne Z Murphy. 2008. “Preemptive Morphine Analgesia Attenuates the Long-Term Consequences of Neonatal Inflammation in Male and Female Rats.” *Pediatric Research* 64 (6) (December): 625–630.

Law, P Y, and H H Loh. 1999. “Regulation of Opioid Receptor Activities.” *The Journal of Pharmacology and Experimental Therapeutics* 289 (2) (May): 607–624.

Li, Peng, Hercules T Maguma, Kathleen Thayne, Barbara Davis, and David a Taylor. 2010. "Correlation of the Time Course of Development and Decay of Tolerance to Morphine with Alterations in Sodium Pump Protein Isoform Abundance." *Biochemical Pharmacology* 79 (7) (April 1): 1015–1024.

Ling, G. S. F., J. M. MacLeod, S. Lee, S. H. Lockhart, and G W Pasternak. 1984. "Separation of Morphine Analgesia from Physical Dependence." *Science* 226 (4673): 462– 464.

Ma, W, W H Zheng, S Kar, and R Quirion. 2000. "Morphine Treatment Induced Calcitonin Gene-Related Peptide and Substance P Increases in Cultured Dorsal Root Ganglion Neurons." *Neuroscience* 99 (3) (January): 529–539.

Macgregor, Ruth, David Evans, David Sugden, Terence Gaussen, and Malcolm Levene. 1998. "Outcome at 5 – 6 Years of Prematurely Born Children Who Received Morphine as Neonates." *Arch Dis Child Fetal Neonatal Ed* 79: F40–F43.

Martin, WR, CG Eades, JA Thompson, RE Huppler, and PE Gilbert. 1976. "The Effects of Morphine- and Nalorphine- like Drugs in the Nondependent and Morphine-Dependent Chronic Spinal Dog." *The Journal of Pharmacology and Experimental Therapeutics* 197 (3): 517–532.

Massa, Horace, Claudia-Marvine Lacoh, and Laszlo Vutskits. 2012. "Effects of Morphine on the Differentiation and Survival of Developing Pyramidal Neurons during the Brain Growth Spurt." *Toxicological Sciences : An Official Journal of the Society of Toxicology* 130 (1) (November): 168–79.

Matthes, Hans W.D., Rafael Maldonado, Frederic Simonin, Olga Valverde, Susan Slowe, Ian Kitchen, Katia Befort, et al. 1996. "Loss of Morphine-Induced Analgesia, Reward Effect and Withdrawal Symptoms in Mice Lacking the Mu-Opioid-Receptor Gene." *Letters to Nature* 383: 819–823.

Mattia, A, T Vanderah, H I Mosberg, J R Omnaas, W D Bowen, and F Porreca. 1991. "Pharmacological Characterization of [D-Ala2 , LeuS , Ser 6] Enkephalin (DALES): Antinociceptive Actions at the 6non . Complexed-Opioid Receptor." *European Journal of Pharmacology* 192: 371–375.

Mazarakou, Georgia, and Zafiroula Georgoussi. 2005. "STAT5A Interacts with and Is Phosphorylated upon Activation of the Mu-Opioid Receptor." *Journal of Neurochemistry* 93 (4) (May): 918–31.

Meunier, J C, C Mollereau, L Toll, C Suaudeau, C Moisand, P Alvinerie, J L Butour, et al. 1995. "Isolation and Structure of the Endogenous Agonist of Opioid Receptor-like ORL1 Receptor." *Letters to Nature* 377: 532–535.

Minami, Masabumi, and Masamichi Satoh. 1995. “Molecular Biology of the Opioid Receptors: Structures, Functions and Distributions.” *Neuroscience Research* 0102 (95): 121–145.

Minto, C F, T W Schnider, T D Egan, E Youngs, H J M Lemmens, P L Gambus, V Billard, et al. 1997. “Influence of Age and Gender on the Pharmacokinetics and Pharmacodynamics of Remifentanil.” *Anesthesiology* 86: 10–23.

Minto, C F, T W Schnider, and S L Shafer. 1997. “Pharmacokinetics and Pharmacodynamics of Remifentanil.” *Anesthesiology* 86: 24–33.

Mogil, J S, J E Grisel, G Zhangs, J K Belknap, and D K Grandy. 1996. “Functional Antagonism of M-, Δ- and K-Opioid Antinociception by Orphanin fQ.” *Neuroscience Letters* 214: 131–134.

Mollereau, C, M Parmentier, P Mailleux, J L Butour, C Moisand, P Chalon, D Caput, G Vassart, and J C Meunier. 1994. “ORL1, a Novel Member of the Opioid Receptor Family. Cloning, Functional Expression and Localization.” *FEBS Letters* 341 (1) (March 14): 33–38.

Monassier, Laurent, and Pascal Bousquet. 2002. “Sigma Receptors: From Discovery to Highlights of Their Implications in the Cardiovascular System.” *Fundamental & Clinical Pharmacology* 16 (1) (February): 1–8.

Morgan, M M, P K Whitney, and M S Gold. 1998. “Immobility and Flight Associated with Antinociception Produced by Activation of the Ventral and Lateral/dorsal Regions of the Rat Periaqueductal Gray.” *Brain Research* 804 (1) (August 31): 159–166.

Morinville, Anne, Catherine M Cahill, M James Esdaile, Haneen Aibak, Brian Collier, Brigitte L Kieffer, and Alain Beaudet. 2003. “Regulation of -Opioid Receptor Trafficking via -Opioid Receptor Stimulation : Evidence from -Opioid Receptor Knock-Out Mice.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 23 (12): 4888–4898.

Nestler, Eric J, and George K Aghajanian. 1997. “Molecular and Cellular Basis of Addiction.” *Science (New York, N.Y.)* 278 (5335) (October 3): 58–63.

Ontl, T, Y Xing, L Bai, E Kennedy, S Nelson, M Wakeman, and K Magnusson. 2004. “Development and Aging of N-Methyl-D-Aspartate Receptor Expression in the Prefrontal/frontal Cortex of Mice.” *Neuroscience* 123 (2) (January): 467–479.

Pan, L, J Xu, R Yu, M-M Xu, Y-X Pan, and G W Pasternak. 2005. “Identification and Characterization of Six New Alternatively Spliced Variants of the Human Mu Opioid Receptor Gene, Oprm.” *Neuroscience* 133 (1) (January): 209–220.

Pasternak, Gavril W. 2011. “Molecular Insights into Mu Opioid Pharmacology: From the Clinic

to the Bench." *Clin J Pain* 26 (Suppl 10): 1–15.

Quock, R M, T H Burkey, E Varga, Y Hosohata, K Hosohata, S M Cowell, C a Slate, F J Ehlert, W R Roeske, and H I Yamamura. 1999. "The Delta-Opioid Receptor: Molecular Pharmacology, Signal Transduction, and the Determination of Drug Efficacy." *Pharmacological Reviews* 51 (3) (September): 503–532.

Rahman, W, M R Dashwood, M Fitzgerald, A Aynsley-Green, and a H Dickenson. 1998. "Postnatal Development of Multiple Opioid Receptors in the Spinal Cord and Development of Spinal Morphine Analgesia." *Brain Research. Developmental Brain Research* 108 (1-2) (June 15): 239–54.

Ranger, Manon, Cecil M Y Chau, Amanmeet Garg, Todd S Woodward, Mirza Faisal Beg, Bruce Bjornson, Kenneth Poskitt, et al. 2013. "Neonatal Pain-Related Stress Predicts Cortical Thickness at Age 7 Years in Children Born Very Preterm." *PloS One* 8 (10) (January): e76702.

Rodríguez-Muñoz, María, Elena de la Torre-Madrid, Gema Gaitán, Pilar Sánchez-Blázquez, and Javier Garzón. 2007. "RGS14 Prevents Morphine from Internalizing Mu-Opioid Receptors in Periaqueductal Gray Neurons." *Cellular Signalling* 19 (12) (December): 2558–2571.

Rodríguez-Muñoz, María, Elena de la Torre-Madrid, Pilar Sánchez-Blázquez, Jia Bei Wang, and Javier Garzón. 2008. "NMDAR-nNOS Generated Zinc Recruits PKCgamma to the HINT1-RGS17 Complex Bound to the C Terminus of Mu-Opioid Receptors." *Cellular Signalling* 20 (10) (October): 1855–1864.

Ross, E M, and T M Wilkie. 2000. "GTPase-Activating Proteins for Heterotrimeric G Proteins: Regulators of G Protein Signaling (RGS) and RGS-like Proteins." *Annual Review of Biochemistry* 69 (January): 795–827.

Sadraie, Seyed Homayoon, Gholam Reza Kaka, Hedayat Sahraei, Hosein Dashtnavard, Hosein Bahadoran, Mahmood Mofid, Hossein Mahdavi Nasab, and Fatemeh Jafari. 2008. "Effects of Maternal Oral Administration of Morphine Sulfate on Developing Rat Fetal Cerebrum: A Morphometrical Evaluation." *Brain Research* 1245 (December 15): 36–40.

Schulz, Stefan, Dana Mayer, Manuela Pfeiffer, and Ralf Stumm. 2004. "Morphine Induces Terminal L -Opioid Receptor Desensitization by Sustained Phosphorylation of Serine-375." *The EMBO Journal* 23 (16): 3282–3289.

Sharma, A, D Tallchief, J Blood, T Kim, A London, and E D Kharasch. 2013. "Perioperative Pharmacokinetics of Methadone in Adolescents." *Anesthesiology* 115 (6): 1153–1161.

Singhal, P C, a a Kapasi, N Franki, and K Reddy. 2000. "Morphine-Induced Macrophage Apoptosis: The Role of Transforming Growth Factor-Beta." *Immunology* 100 (1) (May):

57–62.

Singhal, P C, P Sharma, a a Kapasi, K Reddy, N Franki, and N Gibbons. 1998. “Morphine Enhances Macrophage Apoptosis.” *Journal of Immunology (Baltimore, Md. : 1950)* 160 (4) (February 15): 1886–1893.

Slater, Rebeccah, Anne Cantarella, Shiromi Gallella, Alan Worley, Stewart Boyd, Judith Meek, and Maria Fitzgerald. 2006. “Cortical Pain Responses in Human Infants.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 26 (14) (April 5): 3662–3666.

Slikker, William, Xiaoju Zou, Charlotte E Hotchkiss, Rebecca L Divine, Natalya Sadovova, Nathan C Twaddle, Daniel R Doerge, et al. 2007. “Ketamine-Induced Neuronal Cell Death in the Perinatal Rhesus Monkey.” *Toxicological Sciences : An Official Journal of the Society of Toxicology* 98 (1) (July): 145–158.

Spain, J W, B L Roth, and Carmine J. Coscia. 1985. “Differential Ontogeny of Multiple Opioid Receptors (p, 6, and K)’.” *The Journal of Neurosciance* 5 (3): 584–588.

Stemland, Christopher J, Jurgen Witte, Douglas a Colquhoun, Marcel E Durieux, Loralie J Langman, Ravi Balireddy, Swapna Thammishetti, Mark F Abel, and Brian J Anderson. 2013. “The Pharmacokinetics of Methadone in Adolescents Undergoing Posterior Spinal Fusion.” *Paediatric Anaesthesia* 23 (1) (January): 51–57.

Sternini, C, M Spann, B Anton, D E Keith, N W Bunnett, M von Zastrow, C Evans, and N C Brecha. 1996. “Agonist-Selective Endocytosis of Mu Opioid Receptor by Neurons in Vivo.” *Proceedings of the National Academy of Sciences of the United States of America* 93 (17) (August 20): 9241–9246.

Tang, W J, and J H Hurley. 1998. “Catalytic Mechanism and Regulation of Mammalian Adenylyl Cyclases.” *Molecular Pharmacology* 54 (2) (August): 231–240.

Taylor, D a, and W W Fleming. 2001. “Unifying Perspectives of the Mechanisms Underlying the Development of Tolerance and Physical Dependence to Opioids.” *The Journal of Pharmacology and Experimental Therapeutics* 297 (1) (April): 11–18.

Thomas, Elizabeth A, Patria E Danielson, and J Gregor Sutcliffe. 1998. “Rapid Communication RGS9 : A Regulator of G-Protein Signalling With Specific Expression in Rat and Mouse Striatum.” *Journal of Neuroscience Research* 52 (February): 118–124.

Thornton, S R, a F Wang, and F L Smith. 1997. “Characterization of Neonatal Rat Morphine Tolerance and Dependence.” *European Journal of Pharmacology* 340 (2-3) (December 11): 161–167.

Trujillo, K a, and H Akil. 1994. “Inhibition of Opiate Tolerance by Non-Competitive N-Methyl-D-Aspartate Receptor Antagonists.” *Brain Research* 633 (1-2) (January 7): 178–188.

Tseng, L F, K a Collins, and Q Wang. 1995. “Differential Ontogenesis of Thermal and Mechanical Antinociception Induced by Morphine and Beta-Endorphin.” *European Journal of Pharmacology* 277 (1) (April 13): 71–76.

Van Crugten, J T, a a Somogyi, R L Nation, and G Reynolds. 1997. “The Effect of Old Age on the Disposition and Antinociceptive Response of Morphine and Morphine-6 Beta-Glucuronide in the Rat.” *Pain* 71 (2) (June): 199–205.

Vanderah, T, A E Takemori, M Sultana, P S Portoghese, H I Mosberg, R C Haaseth, T O Matsunaga, and F Porreca. 1994. “Interaction [D-Pen2, D-Pen5]enkephalin and [D-Ala2,Glu4]deltorphinwith Δ-Opioid Receptor Subtypes in Vivo.” *European Journal of Pharmacology* 252: 133–137.

Walker, S M, and M Fitzgerald. 2007. “Characterization of Spinal Alpha-Adrenergic Modulation of Nociceptive Transmission and Hyperalgesia throughout Postnatal Development in Rats.” *British Journal of Pharmacology* 151 (8) (August): 1334–1342.

Walker, Suellen M, Richard F Howard, Kevin a Keay, and Maria Fitzgerald. 2005. “Developmental Age Influences the Effect of Epidural Dexmedetomidine on Inflammatory Hyperalgesia in Rat Pups.” *Anesthesiology* 102 (6) (June): 1226–1234.

Wang, D., W. Sadee, and J. M. Quillan. 1999. “Calmodulin Binding to G Protein-Coupling Domain of Opioid Receptors.” *Journal of Biological Chemistry* 274 (31) (July 30): 22081–22088.

Wang, Hong, K Noelle Gracy, and Virginia M Pickel. 1999. “Receptors Are Often Colocalized in Spiny Neurons Within Patches of the Caudate-Putamen Nucleus.” *The Journal of Comparative Neurology* 146 (April): 132–146.

Wang, Yan, James Mitchell, Kumi Moriyama, Ki-jun Kim, Manohar Sharma, Guo-xi Xie, and Pamela Pierce Palmer. 2005. “Age-Dependent Morphine Tolerance Development in the Rat.” *Anesthesia and Analgesia* 100 (6) (June): 1733–1739.

Ward, Robert M, David R Drover, Gregory B Hammer, Christopher J Stemland, Steve Kern, Martin Tristani-Firouzi, Ralph a Lugo, Kristin Satterfield, and Brian J Anderson. 2014. “The Pharmacokinetics of Methadone and Its Metabolites in Neonates, Infants, and Children.” *Paediatric Anaesthesia* 24 (6) (June): 591–601.

Westin, B David, Suellen M Walker, Ronald Deumens, Marjorie Grafe, and Tony L Yaksh. 2010. “Validation of a Preclinical Spinal Safety Model: Effects of Intrathecal Morphine in the Neonatal Rat.” *Anesthesiology* 113 (1) (July): 183–199.

Whistler, J L, H H Chuang, P Chu, L Y Jan, and M von Zastrow. 1999. “Functional Dissociation of Mu Opioid Receptor Signaling and Endocytosis: Implications for the Biology of Opiate Tolerance and Addiction.” *Neuron* 23 (4) (August): 737–746.

Williams, John T, M A C Donald J Christie, and Olivier Manzoni. 2001. “Cellular and Synaptic Adaptations Mediating Opioid Dependence.” *Physiological Reviews* 81 (1): 299–343.

Wilson, L D, S a Ross, D a Lepore, T Wada, J M Penninger, and P Q Thomas. 2005. “Developmentally Regulated Expression of the Regulator of G-Protein Signaling Gene 2 (Rgs2) in the Embryonic Mouse Pituitary.” *Gene Expression Patterns : GEP* 5 (3) (February): 305–311.

Wolozin, B L, and G W Pasternak. 1981. “Classification of Multiple Morphine and Enkephalin Binding Sites in the Central Nervous System.” *Proceedings of the National Academy of Sciences of the United States of America* 78 (10) (October): 6181–6185.

Workman, Alan D, Christine J Charvet, Barbara Clancy, Richard B Darlington, and Barbara L Finlay. 2013. “Modeling Transformations of Neurodevelopmental Sequences across Mammalian Species.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 33 (17) (May 24): 7368–7383.

Xie, Guo-xi, and Pamela Pierce Palmer. 2005. “RGS Proteins: New Players in the Field of Opioid Signaling and Tolerance Mechanisms.” *Anesthesia and Analgesia* 100 (4) (April): 1034–1042.

Xu, Jin, Mingming Xu, Yasmin L Hurd, Gavril W Pasternak, and Ying-Xian Pan. 2009. “Isolation and Characterization of New Exon 11-Associated N-Terminal Splice Variants of the Human Mu Opioid Receptor Gene.” *Journal of Neurochemistry* 108 (4) (February): 962–972.

Yon, J-H, J Daniel-Johnson, L B Carter, and V Jevtovic-Todorovic. 2005. “Anesthesia Induces Neuronal Cell Death in the Developing Rat Brain via the Intrinsic and Extrinsic Apoptotic Pathways.” *Neuroscience* 135 (3) (January): 815–827.

Young, Chainllie, Vesna Jevtovic-Todorovic, Yue-Qin Qin, Tatyana Tenkova, Haihui Wang, Joann Labruyere, and John W Olney. 2005. “Potential of Ketamine and Midazolam, Individually or in Combination, to Induce Apoptotic Neurodegeneration in the Infant Mouse Brain.” *British Journal of Pharmacology* 146 (2) (September): 189–197.

Zhao, Guo-min, Dunli Wu, Y I Soong, Megumi Shimoyama, Irena Berezowska, Peter W Schiller, and Hazel H Szeto. 2002. “Profound Spinal Tolerance after Repeated Exposure to a Highly Selective □ -Opioid Peptide Agonist : Role of □ -Opioid Receptors.” *The Journal of Pharmacology and Experimental Therapeutics* 302 (1): 188–196.

Zhao, X, R Rosenke, D Kronemann, B Brim, S R Das, a W Dunah, and K R Magnusson. 2009. "The Effects of Aging on N-Methyl-D-Aspartate Receptor Subunits in the Synaptic Membrane and Relationships to Long-Term Spatial Memory." *Neuroscience* 162 (4) (September 15): 933–945.

Zhu, Hongbo, and Gordon a Barr. 2003. "Ontogeny of NMDA Receptor-Mediated Morphine Tolerance in the Postnatal Rat." *Pain* 104 (3) (August): 437–447.

Zhu, Y, M a King, a G Schuller, J F Nitsche, M Reidl, R P Elde, E Unterwald, G W Pasternak, and J E Pintar. 1999. "Retention of Supraspinal Delta-like Analgesia and Loss of Morphine Tolerance in Delta Opioid Receptor Knockout Mice." *Neuron* 24 (1) (September): 243–252.

Zhu, Yanxin, Ming-sing Hsu, and John E Pintar. 1998. "Developmental Expression of the M, Δ, and K Opioid Receptor mRNAs in Mouse." *The Journal of Neurosciance* 18 (7): 2538–2549.

Zimprich, A, T Simon, and V Höllt. 1995. "Cloning and Expression of an Isoform of the Rat Mu Opioid Receptor (rMOR1B) Which Differs in Agonist Induced Desensitization from rMOR1." *FEBS Letters* 359 (2-3) (February 13): 142–146.

Zissen, M H, G Zhang, A McKelvy, J T Propst, J J Kendig, and S M Sweitzer. 2007. "Tolerance, Opioid-Induced Allodynia and Withdrawal Associated Allodynia in Infant and Young Rats." *Neuroscience* 144 (1) (January 5): 247–262.

Zubieta, J K, R F Dannals, and J J Frost. 1999. "Gender and Age Influences on Human Brain Mu-Opioid Receptor Binding Measured by PET." *The American Journal of Psychiatry* 156 (6) (June): 842–848.

Zukin, R Suzanne, Mahboubeh Eghbali, Diane Olive, Ellen M Unterwald, and A N N Tempel. 1988. "Characterization and Visualization of Rat and Guinea Pig Brain K Opioid Receptors : Evidence for κ1 and κ2 Opioid Receptors." *Proceedings of the National Academy of Sciences of the United States of America* 85 (June): 1–5.