Ph.D. thesis

The role of spinal $\mu$ and $\delta$ opioid receptors in the development of opioid tolerance

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INTRODUCTION AND AIMS OF THE STUDY

For thousands of years it has been known that in case of long term use tolerance can develop to most of the actions of the poppy extract. In the medicine certain alkaloids of the poppy (morphine, codeine) and similarly acting drugs are mostly used as analgesics. So far the most popular and most commonly used opioid is still the morphine (Sertürner, 1805). Morphine application is limited by its significant unwanted effects (respiratory depression, euphoria, sedation) and the development of tolerance to most of its effects and dependence. The aims of pharmacological research targeting the opioids are to develop medicines similarly effective as morphine but possessing fewer side effects and limited or no tolerance developing potential.

Endogenous opioid system, opioid receptors, receptor subtypes and ligands

Opioids, similarly to most of medically used drugs, act through receptors. So far three main types of opioid receptors called \( \mu \), \( \kappa \) and \( \delta \) have been identified both pharmacologically and by means of genetic approaches. The receptors were pharmacologically described together with the endogenous opioids that are called enkephalins, endorphins and dynorphins. The latest discovered endogenous opioids are the \( \mu \) receptor selective endomorphines. Every endogenous opioid is a peptide and synthesized from bigger precursor peptide molecules. The precursors are called proenkephalin for the enkephalins, prodynorphin for the dynorphins and proopiomelanocortin for the \( \beta \)-endorphin. The precursors of the endomorphines have not been identified yet.

The opioid ligands are different both structurally and by the binding and receptor activating capabilities. For example we know peptides like the \( \mu \) receptor selective agonist DAMGO and DALDA, the \( \mu \) antagonist peptide CTAP and the heterocyclic compound naloxonazine. We know several \( \delta \) selective peptides (DPDPE, deltorphin II, DALCE, TIPP\( \psi \)) and non-peptides (naltrindole, naltribene, benzilidene-naltrexone, naltrindol-izothiocyanate) as well as \( \kappa \) selective ligands such as the non-peptide agonists ethylketocyclazocine, U50488H and U69593 and the antagonist nor-binaltorphimine.

The molecular biological studies revealed the genetic sequences of the opioid receptors so now we know the amino acid sequences as well. Genetically the existence of one allele has been proven for all three receptors, respectively.

All three opioid receptors belong to the superfamily of the seven transmembrane domain containing G-protein coupled receptors. There is a high homology can be observed among the opioid receptors especially the amino acid sequences of the transmembrane regions are similar. The opioid receptors may activate the \( G_i/G_o \) and \( G_q \) proteins so they are coupled to the following second messenger systems: adenylyl cyclase (inhibition), \( Ca^{++} \)-channels (closing), \( K^+ \)-
channels (opening) and the phosphoinositol cascade (activation). The analgesic activity of the opioid drugs can be explained by all the above cellular activations and inhibitions.

The pharmacological studies have described receptor subtypes for all three opioid receptors. So far we know three µ receptor subtypes: µ1, µ2 and µ3; two δ receptor subtypes: δ1 and δ2; and four κ receptor subtypes: κ1, κ2, κ3. The δ receptor subtypes are distinguished by differences of the binding and activity of several δ selective compounds: the DPDPE, DALCE and benzilidene-naltrexone sensitive δ1 receptor and the deltorphin II, naltrindole-isothyocyanate and naltribene sensitive δ2 receptor. At the spinal level some research groups could not detect inhibition by DALCE against DPDPE and deltorphin II in tail-flick test, so they suggested the lack of δ1 receptors in the spinal cord.

The role of opioid receptors in the analgesia

The opioid studies have revealed that the type of opioid receptor mediating the analgesic effect depends on the applied painful stimulus, the tested opioid agonist and its way of application. The antinociceptive tests based on thermal pain are mostly sensitive for the µ receptor mediated analgesic effects while the chemical irritation tests are very useful to measure the antinociceptive effects of the κ receptor agonists.

The role of δ opioid receptors in the analgesia is not quite clear so far. The antinociceptive activities of the δ agonists depend on the region of the central nervous system where they are applied. For example, it has been proven that the δ selective DPDPE given intracerebroventricularly (icv.) acts through δ receptors while given intrathecally (it.) it behaves as a µ receptor agonist. The δ2 selective deltorphin II remains δ agonist when given it. These results raised the possibility that the δ1 receptor is only a functional subtype of the δ opioid receptor.

The studies done by the antisense-oligo-DNA against δ receptor suggest that the cloned δ receptor is identical with the pharmacologically described δ2 receptor.

The studies on mice lacking the µ receptor and on µ knock-out mice suggest that the existence of the µ receptor is essential for the δ receptor-mediated antinociception. They proved that in the µ receptor deficient CXBK mice the antinociceptive potencies of the icv. given δ agonists decreased. In the µ knock-out mice morphine lost its antinociceptive efficacy as well as the icv. given δ1 selective DPDPE.

Opioid receptor interactions

Opioid receptors may interact with one another either locally, within a central nervous system region, or non-locally in different brain or spinal cord regions. If
the local interactions are mediated by opioid receptors on the same neuron the physical association of the receptors cannot be ruled out.

Local interactions have been described both in the spinal cord and the brain. In the spinal cord the δ agonist Leu-enkephalin potentiates the antinociceptive action of morphine, DPDPE enhances that of DAMGO.

Recently some research groups proved the possibility of the physical association of the μ and δ opioid receptors. Both DAMGO and DPDPE bind with relatively high affinity for the μδ complex, in both cases their affinities are one tenth of their original affinities for their own receptors. Besides the μδ dimer there have been described δκ heterodimer and μμ, δδ homodimers. It is still uncertain whether the receptor complexes have physiological importance. Today the κ2 (δκ) and the δ2 (μδ) subtypes seem to be receptor complexes but it has not yet been a proven fact.

**Some possible mechanisms of the opioid tolerance**

The morphine tolerance has been known for a very long time. According to the classical pharmacological definition we speak about tolerance (in the case of any drug) when in case of continuous administration increasing dose of the medicine is necessary to reach the original effect.

In the development of opioid tolerance several mechanisms may play a role. Opioids cause receptor adaptation which includes desensitization, internalization and receptor down-regulation. Different opioid compounds have different capabilities to induce the above processes, chronic morphine treatment can even increase the number of the opioid binding sites so it actually causes up-regulation which comes from the increased δ receptor density. Chronic morphine application may start other adaptive processes: the levels of G proteins, adenylyl cyclase and protein kinases may also change. Furthermore, the neuronal networks and the interactions between the neurotransmitter systems may also play an important role in the adaptive changes.

Opioid receptor interactions may also be involved in the development of opioid tolerance. In the local changes the μ and δ receptor interactions seem important. The pretreatment with δ antagonist attenuated the development of tolerance to the antinociceptive effect of morphine and the spinal potentiating synergism between DAMGO and DPDPE disappeared.

**Polymorphism of the opioid receptors**

There can be huge individual differences in the sensitivity to drugs. This difference is most likely based on several mechanisms; one of these can be the single nucleotide polymorphism (SNP) of the receptor encoding gene. In humans several μ receptor polymorphisms have been detected, one of these SNP-s
increased the affinity of β-endorphin for the μ receptor. Other studies, however, have not found relationship between the opioid receptor polymorphisms and the probability of the development of opioid dependence. These negative results raised the possibility of the importance of the mutations in the non-coding regions. The non-coding regions may regulate the level of gene expression. In contrast to the lack of relationship between the opioid receptor polymorphism and the opioid dependence one SNP in the δ receptor has a higher prevalence in opioid addicts, though this SNP has not altered the amino acid sequence of the δ receptor.

The investigation of the opioid dependence is very difficult in humans, similarly to other behaviors, because of the multiple, genetic and non-genetic alterations. In animal studies the opioid tolerance, though it can only be a component of the dependence, is a well-defined model and it can be studied well. Therefore one of the first approaches to understand the opioid dependence can be the investigation the relationship between the chronic morphine treatment caused tolerance and the possible SNP-s in the opioid receptor sequences.

In our studies we tried to answer the following questions:

1. Is there any difference between the levels of opioid tolerance in different mouse strains? If there are differences, are they related to the possible polymorphisms of the genes encoding the opioid receptors?
2. Does a cross-tolerance develop to the analgesic effect between morphine and the highly μ receptor selective opioid peptide [Dmt¹]DALDA?
3. Do the interactions between the spinal μ and δ receptors play a role in the development of opioid tolerance in mice?
METHODS

Experimental animals

To study the relationship between morphine tolerance and the opioid receptor polymorphism we used six mouse strains, all derived from an original Swiss strain. This way these mouse strains may be very closely related genetically to one another. We used the following strains (the vendor in parentheses, then the abbreviations used in this work):

1. NIH Swiss (Harlan Sprague-Dawley Inc. Indianapolis, IN, USA) → H/NIH Swiss
2. ND4 (Harlan Sprague-Dawley Inc. Indianapolis, IN, USA) → H/ND4
3. ICR (Harlan Sprague-Dawley Inc. Indianapolis, IN, USA) → H/ICR
4. Simonsen Swiss Webster (Simonsen Laboratories, Gilroy, CA, USA) → S/SW
5. Hilltop Swiss Webster (Hilltop, Scottsdale, PA, USA) → Hilltop/SW
6. Charles River Swiss Webster (Charles River Laboratories, Wilmington, MA, USA) → CR/SW

We used the CR/SW and H/ND4 mice in the receptor interaction studies, the H/ICR mice for the experiments with [Dmt$^1$]DALDA.

Making the mice tolerant to morphine

We implanted a 75mg morphine base containing pellet under the skin of the back of the mice. On the fourth day after the implantation we did the experiments leaving the pellet intact.

Materials

Heterocyclic opiates
Morphine-sulphate; naloxone, naloxone-methiodid, nor-binaltorphimine

Opioid peptides

[Dmt$^1$]DALDA ((2’6’-dimetil-Tyr)-D-Arg-Phe-Lys-NH$_2$); DAMGO (H-Tyr-D-Ala$^2$-Gly-(N-Me)Phe-Gly-ol); DPDPE ((D-Pen$^{2-5}$)-enkephalin); TIPP$^\psi$ (H-TyrTicPsi[CH$_2$NH]Phe-Thr-OH), CTAP (D-Phe-Cys-Tyr-D-Arg-Thr-Pen-Thr-NH$_2$)
**Treatment of the animals during the experiments**

When given subcutaneously, morphine-sulphate, [Dmt\(^1\)]DALDA, DAMGO, naloxone and naloxone-methiodid were dissolved in saline, the doses were given in µmol/kg. The antinociceptive action was measured 30 minutes after the injection, except for [Dmt\(^1\)]DALDA since this compounds showed the highest activity two hours after the injection in mouse tail-flick test.

We applied the former compounds and the other opioid peptides i. and icv., in these cases the doses were given in nmol/mouse. When we tested antagonists the agonist and antagonist were co-administered. The antinociceptive action was measured 30 minutes after the injection in mouse tail-flick test.

**Evaluation of the data and the statistical analysis**

The AD\(_{50}\) values of the opioid compounds were determined from a dose-response curve established from at least three different doses. Each dose was applied to 8-10 animals. The 95% confidence limits of the AD\(_{50}\) values were calculated according to Litchfield and Wilcoxon.

**Determining the degree of opioid tolerance**

We determined the AD\(_{50}\) values of the sc. given morphine in the control and morphine pelleted animals then divided the two values. We calculated the ratio of these two AD\(_{50}\) values as the degree of tolerance to sc. given morphine. The significance between the differences in the tolerance degrees were calculated by ANOVA.

**Calculation of the pA\(_2\) values of the µ antagonist CTAP**

We determined the pA\(_2\) value of CTAP by Schild regression.

**Molecular biology methods applied in the polymorphism study and DNA sequencing**

Genomic DNA from spleen and total RNA from brain and spinal cord were isolated by the standard molecular biological methods. The receptor genes were amplified by PCR and RT-PCR. After purification the amplified DNA sequences were sent for sequencing, the results were analyzed by the Megalign program (DNASTar, Madison, WI, USA). The gene bank accession numbers for the sequences used for comparison were as follows: #L11064 for δ receptor, #U26915 for µ receptor, #L11065 for κ receptor. The gene bank sequences come from the inbred C57 mouse strain.
DISCUSSION

Morphine dependence in humans is a very complex phenomenon, besides the physiological, social and environmental factors also contribute to its development. Even the physiological factors are more than simply the development of tolerance and dependence; it is especially true for the craving. Still, the mouse model of opioid tolerance/dependence is an excellent tool toward the understanding the human addiction. There can be a lot of factors in the development of opioid tolerance. In our studies we investigated the role of the opioid receptors.

**Development of morphine tolerance in different mouse strains**

*Tolerance development after morphine pellet implantation*

In our studies the degree of tolerance was calculated as the ratios of $AD_{50}$ values of opioid compounds measured in morphine tolerant and naïve mice. We chose six outbred mouse strains descending from a small group of Swiss mice. This way we assumed that the genetic diversity is not too significant. Surprisingly enough, we found low degree of tolerance to sc. given morphine in three strains (H/NIH Swiss, H/ND4 and S/SW) (2-3-fold difference in the $AD_{50}$ values) and high degree of tolerance to sc. morphine (H/ICR, CR/SW and Hilltop/SW) (7-8-fold difference in the $AD_{50}$ values) in the other three strains. The difference between the tolerance degrees was significant (p<0.05). The difference can be resulted from several pharmacodynamic and pharmacokinetic or other reasons. For example, changes may occur in the opioid receptor sequences, opioid mediated signal transduction pathways such as the G proteins or the adenylyl cyclase. SNP-s or other changes may influence these molecules too. Furthermore, other neurotransmitter systems, e.g. glutamate through NMDA receptors may also influence the opioid system.

*Tolerance development to it. given morphine in the mouse strains*

Unlike the tolerance to sc. given morphine, the morphine pellet implantation resulted in significant degree of tolerance to the antinociceptive effect of it. given morphine in one (H/ICR) of the six mouse strains.

**Polymorphism of the opioid receptor genes in the chosen mouse strains**

We determined the nucleotide sequences of all three opioid receptors from genomic DNA in the six strains as well as the cDNA sequences in two strains (Hilltop/SW and S/SW).

We have not found SNP-s in the nucleotide sequences of $\mu$ and $\kappa$ opioid receptors. We have not found connection between the 5 SNP-s found in the
nucleotide sequence of the δ opioid receptor and the different degrees of morphine
tolerance in the mouse strains. Furthermore, these δ receptor SNP-s have not
altered the amino acid sequence of the receptor. When we compared the cDNA and
genomic sequences there were no differences so we could rule out RNA editing,
exon skipping or intron transcription. Since we isolated RNA from mouse brain
and spinal cord we could have seen differences in the sequences of the brain and
spinal cord receptors if there had been any. The analysis did not reveal any more
SNP-s, each sequence was identical with genomic DNA-s. These results suggest
that in the chosen mouse strains the different degrees of opioid tolerance to sc.
morphine cannot be explained with the different primary structures of their opioid
receptors.

Cross-tolerance between opioid antagonists after subcutaneous administration

Both DAMGO and [Dmt1]DALDA possess antinociceptive activity after
subcutaneous administration. The AD50 value of DAMGO is identical with that of
morphine (12.5µmol/kg and 10.0µmol/kg respectively), the equipotent dose of
[Dmt1]DALDA was 0.3µmol/kg. This result suggests that [Dmt1]DALDA can
penetrate through the blood brain barrier. After sc. administration [Dmt1]DALDA
showed incomplete cross-tolerance with morphine. The AD50 value of the peptide
hardly increased in the morphine tolerant animals while the tolerance degree was
4-5-fold for sc. DAMGO and 8-9-fold for sc. morphine. Apparently – though
[Dmt1]DALDA and morphine act on the same receptor (see later) – we observed
differences in morphine tolerant animals. Others also verified the lack of cross-
tolerance. The incomplete cross-tolerance between morphine and [Dmt1]DALDA
can be explained by the differently induced conformational change of the receptor
which may decrease the affinity of morphine for the receptor and does not affect
the affinity of [Dmt1]DALDA. Another explanation involves the interaction of
[Dmt1]DALDA with other receptor subtypes such as μ2 or μ3.

Spinal antinociceptive effects of different opioid agonists in morphine tolerant
mice

Antinociceptive effects of [Dmt2]DALDA in naïve animals

[Dmt1]DALDA, the analog of the natural opioid peptide dermorphine, is 20
times as potent as morphine on the electrically evoked contractions of the
longitudinal muscle preparation of the guinea pig ileum and 7 times as potent in the
mouse vas deferens bioassay. Its affinity for the μ receptor is seven times higher
than that of morphine, its μ receptor selectivity over the δ receptor is 10000-fold.
In our experiments the sc. given [Dmt1]DALDA is 40-fold as potent as morphine
or DAMGO in mouse tail-flick test. Intrathecally the potency of [Dmt1]DALDA is
6-14-fold higher than that of it. DAMGO and 500 times higher than that of it. morphine. The classic opioid antagonist naloxone inhibited the systemic antinociceptive effect of [Dmt₁]DALDA, the results with the it. given CTAP suggested that [Dmt₁]DALDA interacts with the same spinal receptor population like DAMGO. After it. administration neither the κ selective antagonist nor-binaltorphimine nor the δ selective TIPPψ did not inhibit the antinociceptive effect of [Dmt₁]DALDA. These results well correlate with other studies showing that [Dmt₁]DALDA binds to μ opioid receptors in vitro too.

After sc. administration the antinociceptive effect of [Dmt₁]DALDA could not be antagonized by the peripherally acting antagonist naloxone-methiodid while the peripherally and centrally acting naloxone inhibited its effect in μ selective dose. The two methyl group in the 2’-6’ positions probably make [Dmt₁]DALDA more lipophilic then its parent compound DALDA this way increasing the penetration capability through the blood brain barrier. It is also interesting that [Dmt₁]DALDA is a very long acting compound, it reached its maximum effect two hours after the sc. administration.

*The spinal antinociceptive effects of DAMGO, DPDPE and [Dmt₁]DALDA in morphine tolerant mice*

When morphine is given systemically, either subcutaneously or by pellet implantation, significant tolerance develops to sc. morphine within three days but there is no tolerance to it. given morphine. We verified this phenomenon in six different mouse strains. Tolerance also developed to the it. given μ selective DAMGO and the δ selective DPDPE. To it. given morphine we detected significant tolerance in only one chronically treated mouse strain. Tolerance developed to the it. given δ agonist DPDPE in all six strains, and to the it. given DAMGO in 4 strains (data not shown). The degree of tolerance was high but did not correlate with the tolerance to the sc. given morphine.

We found that the lack of spinal tolerance to morphine is a general phenomenon, it can be seen in several mouse strains. In our earlier studies we could see that in Hilltop/SW mice the it. given selective μ antagonist CTAP inhibited the antinociceptive effects of it. DAMGO and it. DPDPE in the same dose. The it. given selective δ antagonist naltrindole inhibited the antinociceptive effects of both agonists in the same dose. These results and other findings suggest that DPDPE acts through μ receptors in the mouse spinal cord. We found these results in the recent experiments. We chose two strains of the six, a low tolerant (H/ND4) and a high tolerant (CR/SW) strain. CTAP inhibited the antinociceptive potencies of both agonists with equal potency in both strains. The identical pA₂ values of CTAP against DAMGO and DPDPE suggest that DPDPE acts through μ receptors in the mouse spinal cord.
There was no cross-tolerance between the it. given [Dmt₁]DALDA and the sc. morphine to the antinociceptive effect (not published own data).

**Interactions between µ and δ opioid receptors in the spinal cord of morphine tolerant mice**

In the following experiments all compounds were administered intrathecally. Cross-tolerance developed between morphine and DAMGO, and morphine and DPDPE. In case of DAMGO to reach the similar antinociceptive effect to the naïve control 7-fold higher dose was needed in the low degree tolerance strain H/ND4 and 20-fold higher dose in the high degree tolerance strain CR/SW. When we tested DPDPE the it. degree of tolerance was 3-fold in both strains. It is worth to mention that in morphine tolerant CR/SW mice in most of the experiments it. DPDPE did not cause 50% of antinociception even in extremely high (120nmol/mouse) dose.

In the two selected strains we determined the antagonistic effect of the it. given selective µ antagonist CTAP against DAMGO and DPDPE. In both strains the antagonistic potency of CTAP was similar against both agonist compounds. This result suggests that DPDPE exerts its antinociceptive action through µ receptors in the mouse spinal cord.

We studied the role of the δ opioid receptor in the it. given µ and δ opioid agonists in naïve and morphine tolerant mice with the highly δ selective antagonist peptide TIPPψ. In naïve H/ND4 and CR/SW mice even 30nmol/mouse TIPPψ had no influence on the antinociceptive effects of DAMGO or DPDPE. In morphine tolerant mice, in both strains, 0.2 and 2nmol/kg TIPPψ potentiated the antinociceptive effect of DAMGO in a dose dependent manner. In H/ND4 mice showing low sc. morphine tolerance 2nmol/mouse TIPPψ completely restored the antinociceptive potency of it. DAMGO to the naïve control. In CR/SW mice which showed high degree of tolerance to sc. morphine the restoration was partial. The enhancing effect of the δ antagonist TIPPψ on the antinociceptive effect of DAMGO suggests the role of δ receptors in the development of spinal opioid tolerance.

It is known that in the spinal cord of naïve mice µ and δ receptors show potentiating interactions in tail-flick test. This potentiation disappears during the development of morphine tolerance. We showed similar potentiation in H/ND4 and CR/SW strains. 2nmol/mouse DPDPE decreased the AD₅₀ values of DAMGO 3.fold and 6-fold, respectively. This potentiation was completely blocked by 30nmol/mouse TIPPψ which indicates the role of δ receptors in the spinal synergistic antinociceptive effect of DAMGO and DPDPE in naïve mice.
Hypothetic model of the development of spinal opioid tolerance

Based on the receptor complex theory we can suggest a common explanation of our results. Chronic morphine treatment increases the number of δ opioid receptors this way the number of μδ complexes may also raise. This increase in the number of the complexes decreases the number of the sole μ receptors. If the affinity or the efficacy of DAMGO is significantly lower for the μδ complex than for the μ receptor then we can detect an increasing AD50 of DAMGO. This proposal is supported by the results showing decreased affinities of both DAMGO and DPDPE for the μδ complex. Similarly, even if DPDPE binds to the μδ complex it may not activate it so due to the decreased number of free μ receptors also decreases the antinociceptive potency of DPDPE. On the other hand, morphine and [Dmt1]DALDA may bind and activate the μδ complex. Since the structure of [Dmt1]DALDA is different from that of DAMGO the conformational change of the receptors induced by [Dmt1]DALDA may also be different from the DAMGO-induced receptor conformations.

Ligands induce conformational changes of the receptors, so it can be suggested that some ligands may even promote the formation of the μδ complexes or, on the contrary, others may disconnect the previously existing μδ complexes. If we assume that the selective δ antagonist TIPPψ - either directly, by binding to the μδ complex or indirectly, by decreasing the number of the empty δ receptors – disconnects the μδ complex this way it restores the original μ receptor density so the μ receptors become available for the μ agonists again. The low degree of tolerance to it. morphine in morphine pelleted mice can be explained by the weak partial δ agonistic effect of morphine which results a similar μδ disconnecting effect to TIPPψ. This way morphine sets free μ receptor for its own.

Summary of the new results

1. In the six studied Swiss mouse strains the different degrees of tolerance developing by 3-day morphine pellet implantation do not depend on the polymorphism of the opioid receptors.
2. In morphine pelleted mice we could not find connection between the tolerance degrees to sc. and it. given morphine.
3. Morphine pellet implantation resulted in a significant degree of tolerance to it. morphine in only one of the six strains.
4. In morphine tolerant animals significant tolerance developed to the selective μ agonist DAMGO and the selective δ agonist DPDPE.
5. The highly μ selective peptide [Dmt1]DALDA proved to be more potent than morphine or DAMGO both sc. and it.
6. The sc. given [Dmt1]DALDA acts significantly longer than morphine in mouse tail-flick test.
7. In morphine tolerant animals there was no cross-tolerance with [Dmt\(^1\)]DALDA either sc. or it.
8. The it. given δ selective TIPP\(\psi\) potentiated the antinociceptive effect of the it. given DAMGO in morphine tolerant mice. TIPP\(\psi\) almost completely restored the antinociceptive potency of DAMGO to the value measured in naïve mice.
9. In case of it. administration TIPP\(\psi\) completely antagonized the synergistic effect of the δ agonist DPDPE to the antinociceptive effect of the μ agonist DAMGO.

We hope that our work has contributed to the better understanding of the mechanisms involved in opioid tolerance and elucidating the role of opioid receptors which may facilitate the invention of new, potent opioid medicines with less side-effects.
ACKNOWLEDGEMENTS

I must express my gratitude to my parents who have always stood beside me with unconditional love, supported my aims and accepted my decision to go to research instead of the clinical practice.

Miklós Péter Kalapos showed me the life of a researcher, he guided me, a medical student, at the 1st Department of Chemistry and Biochemistry, Semmelweis University of Medicine and I could work in Joseph Mandl’s laboratory. Right after graduation I started working at the Department of Pharmacology, Semmelweis University of Medicine. For 13 years spent here I couldn’t have gone so far without the help and guidance of Tamás Friedmann who has taught me to the way of thinking of a pharmacologist, who gave me advices to my work and sometimes to my private life. I feel he is not just a tutor but also a friend of mine. I also say thank András Rónai for the methods he taught me and the knowledge he passed me.

Difficult to be grateful enough to professor Susanna Fürst, the head of the Department of Pharmacology and the leader of the Opioid Research Group. With her help I could work in Nancy M. Lee’s laboratory in San Francisco. With the guidance of Nancy M. Lee I could work on the mechanisms of opioid tolerance and publish the papers which have become the basis of this PhD thesis. I am very grateful to Nancy for it. Among my colleagues in San Francisco I thank He Li for teaching the intrathecal injection technique and Tracy M. Gant for the molecular biology experiments.

For helping in making this dissertation I express my gratitude to professors Susanna Fürst and Valéria Kecskeméti, to Tamás Friedmann and László Köles, my good friend and colleague, who gave valuable advices.

Last but not least I am very grateful to my wife, Melinda, who showed infinite patience and love during the preparation and provided the warmthness of our home. I am especially grateful to my children, Enikő and Miklós, who had to miss playing with their daddy.
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