33rd Meeting of the
International Narcotics Research Conference
Asilomar, Pacific Grove, CA

July 9-14, 2002

Program Committee

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General Information

❖ BADGES

Name badges must be worn at all times during the conference. Badges are required for entrance into all meals, events and scientific venues.

❖ REGISTRATION DESK

The registration desk will assist in all conference needs. The registration desk will be at Asilomar and will be open from Tuesday, July 9 until Sunday, July 14 at the following times:

Tuesday, July 9	14:00 – 19:00
Wednesday, July 10	07:30 – 15:00
Thursday, July 11	08:00 – 15:00
Friday, July 12	08:00 – 12:00
Saturday, July 13	08:00 – 15:00
Sunday, July 14	08:00 – 12:00

❖ MEALS

All meals are provided by Asilomar Conference Center for those registrants staying at Asilomar.

❖ SOCIAL PROGRAM

Tuesday, July 9	Opening Reception
Wednesday, July 10	Wine Reception at Poster Session I
Friday, July 12	Excursions and Beach Barbeque
Saturday, July 13	Banquet at the Monterey Bay Aquarium

❖ ACCOMODATIONS

Asilomar Conference Center
800 Asilomar Boulevard, P.O. Box 537
Pacific Grove, CA 93950
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ACKNOWLEDGMENTS

INRC thanks the generous support from:

Wheeler Center for the Neurobiology of Addiction

National Institute on Drug Abuse

Conference Program

Tuesday July 9

12:00 – 19:00	Registration, Asilomar Conference Office
18:00 – 19:00	Dinner, Asilomar Dining Room
19:00 – 21:00	Opening Reception

Wednesday July 10

8:15 – 8:30	Opening remarks: **C. Chavkin**
8:30 – 9:30	**Plenary Lecture (P1)** **G. Koob**: The Dark Side of Drug Addiction: Corticotropin Releasing Factor, Stress, and Allostasis
9:30 – 10:00	*Coffee Break*
10:00 – 12:30	**Symposium 1. Opiate dependence and addiction: the neuropeptide/ GABA connection**
10.00 – 10:05	Chair **T. Shippenberg** and **R. Maldonado**: Introduction
10.05 – 10:25	**Y. Hurd**: Heroin use on opioid neuropeptide genes in GABAergic neuronal populations in the human striatum (**S1**)
10:25 – 10:45	**C. Gadd**: Absence of self-administration and behavioral sensitization to morphine, but not cocaine, in mice lacking NK1 receptors (**S2**)
10:45 – 11:05	**J. Williams**: Opioids inhibit release of GABA from arcuate POMC neurons (**S3**)
11:05 – 11:25	**M. Christie**: opioid-withdrawal induced superactivation of the cAMP cascade in GABAergic neurons (**S4**)
11:25 – 11:40	**A. Stadlin**: Association studies of opioid and GABAA-gamma2 receptor genes between Chinese heroin-dependent and control subjects (**S5**)
11:40 – 11:55	**F. Noble**: Imbalance in endogenous CCK/opioid systems may be involved in vulnerability to drug dependence **(S6)**
11:55 – 12:10	**A. Svingos**: Delta- and kappa-opioid receptors in the VTA: cellular distributions and targeting to mesocortical neurons **(S7)**
12:10 – 12:25	**T. Nakagawa**: Role of the central nucleus of amygdala in morphine withdrawal-induced conditioned place aversion in rats **(S8)**
12:30 – 14:00	*Lunch*

14:00 – 16:30	**Symposium 2. Enzymes and new horizons**
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14:10 – 14:35	**L. Fricker**: "Neuropeptidomics" – identification and quantitation of opioid peptides **(S9)**
14:35 – 15:00	**J. Pintar**: Mutational analysis of mice defective in endogenous opioid peptide production **(S10)**
15:00 - 15:25	**R. Tyndale**: Pharmacogenetic and environmental variation in drug metabolizing enzymes alters drug dependence **(S11)**
15:25 – 15:40	**L. Toll**: Computational methods for novel neuropeptide identification **(S12)**
15:40 – 15:55	**K. Lutfy**: Nocioception, tolerance and dependence in PC2 knockout mice **(S13)**
15:55 – 16:10	**B. Reed**: Metabolic products of dynorphin a (1-17): microdialysis and mass spectrometry **(S14)**
16:10 – 16:25	**G. Bakalkin**: Biogenesis and trafficking of prodynorphin are regulated by the ubiquitin proteasome pathway (UPP) **(S15)**
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17:00 – 18:00	**Founders Lecture** **Lars Terenius**: Opioid peptides, the extended family
19:30 – 21:00	**Poster Session 1 and Wine Reception**

Thursday July 11

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10:30 – 11:00	**G. Schoenbaum**: Linking affect to action: the role of neural representations in mesocorticolimbic structures in goal-oriented behavior **(S18)**
11:00 – 11:30	**H. Breiter:** Functional circuitry of reward and aversion in the human: potential implications for analgesia and addiction **(S19)**
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15:35 – 15:50	**L. Sim-Selley**: Time course for normalization of CB1 receptor levels and G-Protein activation following cessation of chronic cannabinoid treatment **(S29)**
15:50 – 16:05	**B. Szabo**: Depression of GABAergic neurotransmission by cannabinoids in the ventral tegmental area **(S30)**
16:05 – 16:20	**T. Rubino**: MAP kinase pathway plays a role in cannabinoid tolerance **(S31)**

16:30 – 18:00 **Poster Session 2 and Coffee**

19:30 – 20:30 **TK Lecture Sponsored by ICRS**
R. Tsien: Understanding how CNS synapses remodel themselves

Friday July 12

8:30 – 12:30 **Symposium 5. Genetic Approaches to Complex Disorders**
8:30 – 8:45 Chair **S. Watson** and **G. Pei**: Introduction

8:45 – 9:30 **U. Heberlein**: Molecular genetics of drug-induced behaviors in *DROSOPHILA* **(S32)**

9:30 – 10:00 *Coffee Break*

10:00 – 10:30 **H. Akil:** Microarray analysis in the study of neural circuits of stress and emotion **(S33)**

10:30 – 11:00 **P. Levitt**: Gene profiling of neuropsychiatric diseases: schizophrenia as a disease of the synapse **(S34)**

11:00 – 11:15 **L. Moulédous**: Proteomic analysis of laser-capture microdissected brain samples: what works and what doesn't **(S35)**

11:15 – 11:30 **K. Xu**: Haplotype-based of DRD2 gene association with heroin abuse in a Chinese Han population **(S36)**

11:30 – 11:45 **S. Izenwasser**: Depletion Of Serotonin Blocks The Regulation Of Dynorphin By Kappa-Opioid Agonists **(S37)**

11:45 – 12:00 **D. Proudnikov**: Massive parallel analysis of gene expression by Taqman in application to neurobiological studies **(S38)**

12:00 – 12:15 **G. Barr**: Changes in gene expression induced by precipitated morphine withdrawal in preweanling rats **(S39)**

12:30 – 13:30 *Lunch*

13:30 – 14:00 *Business Meeting*

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19:00 *Beach Barbeque*

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10:00 – 10:05	Chair **H. Ueda** and **P. Panula**: Introduction
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10:25 – 10:45	**S. Fukusumi**: Identification of novel peptides with C-terminal RF-amide and their receptors **(S41)**
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11:35 – 11:50	**M. Inoue**: Prepro-Nociceptin/Orphanin FQ 160-187 plays role in modality-specific pain pathways **(S45)**
11:50 – 12:05	**F. Fernandez**: Nociceptin/Orphanin FQ (N/OFQ) is anxiogenic in tests of rat neophobia **(S46)**
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14:30 – 14:55	**B. Martin**: Interrelationships of cannabinoid and opioid dependence mechanisms **(S49)**

14.55 – 15:20	**W. Fratta**: Functional interactions between cannabinoids and opioids in animal models of drug self-administration **(S50)**
15:20 – 15:45	**G. Kunos**: Endocannabinoids and appetite: possible interactions with other neurotransmitter system **(S51)**
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16:00 – 16:15	**H. Ozsoy**: CB1 antagonist SR141716a-induced blockade of mu-receptor desensitization in the SH-SY5Y cell line **(S53)**
16:15 – 16:30	**D. Cichewicz**: The effects of oral administration of D9-THC on morphine tolerance and physical dependence **(S54)**
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9:00 – 11:00	**Symposium 8** Chair **C. Evans** and **L. Toll**
9:00 – 9:15	**A. Persson**: Opioid-induced changes in adult hippocampal progenitors **(S55)**
9:15 – 9:30	**J. McLaughlin**: Kappa opioid systems mediate stress responses **(S56)**
9:30 – 9:45	**J. Whistler**: Regulation of opioid receptor trafficking and morphine tolerance by mu opioid receptor oligomerization **(S57)**
9:45 – 10:00	**N.-J. Xu**: Opiate abuse induces translocation of glutamate transporters at hippocampal synapses **(S58)**
10:00 – 10:15	**P. Skoubis**: Persistence of naloxone-induced conditioned place aversion in beta-endorphin but not enkephalin knock-out mice **(S59)**
10:15 – 10:30	**S. Schulz**: DAMGO-stimulated mu-opioid receptor activates phospholipase D2 in an ARF-dependent manner **(S60)**
10:30 – 10:45	**R. Rodriguez**: Characterization of ZFOR3, a new putative opioid receptor from the teleost zebrafish **(S61)**
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11:00	*End of meeting. Pass the bell ceremony.*

POSTERS

The number after each title refers to the poster board number.

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Differential Sensitivities Of Mouse Strains To Morphine And [Dmt^1]Dalda Analgesia **(1)**
C L Neilan, M A King, M Ansonoff, J Pintar, P W Schiller, GW Pasternak

Alkaloids From Brugmansia Arborea (L.) Lagerhein Reduce Morphine Withdrawal In Vitro **(4)**
Anna Capasso, Gianni Saladino and Vincenzo De Feo

NMDA Receptor Antagonists Prevent And Slowly Reverse Opiate-Induced Behavioral And Neural Plasticity **(7)**
D.J. Peterson, I. A. Mendez, R.M. Lewellen, and K.A. Trujillo

Sensitization To The Locomotor Stimulant Effect Of Morphine **(10)**
K.A. Trujillo, K.P. Warmoth, K. Kubota, D.J. Peterson and E. Ruzek

Buprenorphine Substitution In The Treatment Of Morphine-Dependent Rat Pups **(13)**
Dawn C Stoller and Forrest L Smith

Tan-67 Improves Rather Than Interupts Vagal Bradycardia **(16)**
M. Farias, K. Jackson, D. Yoshishige, and J.L. Caffrey

Mu Opioid Receptor Knockout Mice Have Altered Behavioral Responses To Cocaine **(19)**
M. Hummel, M. Ansonoff*, J. Pintar*, E.M. Unterwald

Up-Regulation Of PGE_2-Receptor, But Not PGI_2- And Opioid-Receptors, In The Mouse Spinal Cord Following Peripheral Inflammation **(22)**
M. Shimamura, M. Narita, Y. Yajima, J. Khotib, C. Kubota and T. Suzuki

Electroacupuncture-Induced Activation Of Endogenous Anti-Analgesic System In Rats **(25)**
Y. Fukazawa, S. Kishioka, T. Maeda, N. Shimizu, X. Fan, C. Yamamoto, H. Yamamoto

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Several Delta Ligands Display No Subtype Selectivity In Cells Stably Transfected With The Human Delta Opioid Receptor **(28)**
Amy L. Parkhill and Jean M. Bidlack

Pharmacological Consequences Of Mutagenesis Of A Conserved Aspartic Acid Residue In The Mu Opioid Receptor **(31)**
G. D. Dalton, T. G. Metzger, D. E. Selley

III. **RECEPTOR TRAFFICKING**

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Molecular Evidence For The Involvement Of The Phosphatidylinositol Metabolism Cascade In The Morphine-Induced Rewarding Effect **(2)**
Keisuke Mizuo, Minoru Narita and Tsutomu Suzuki.

The Role Of Neuropeptides In The Diabetic Rat **(5)**
M.M. Backer, Z. Nevo, A. Shahar and C.G. Pick

Inconsistent Effects Of NMDA Receptor Antagonists On Opiate Analgesia **(8)**
K. Watorski and K.A. Trujillo

Profound Spinal Tolerance After Repeated Exposure To A Highly Selective Mu-Opioid Agonist: Role Of Delta Opioid Receptors **(11)**
Dunli Wu, Yi Soong, Guo-Min Zhao and Hazel H. Szeto

Topical Capsaicin-Induced Thermal Allodynia In Primates: Effects Of Kappa-Opioid Agonists **(14)**
E.R. Butelman, J.W. Ball, M.J. Kreek

Effect Of Acamprosate On Morphine Conditioned Place Preference **(17)**
M.F. Olive

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P. Kosson, D. Kosson, I. Maszczynska-Bonney, B. Kwiatkowska-Patzer, A. Misicka, D. B. Carr, A. W. Lipkowski

Effects Of Supraspinal Endomorphin-1 And Endomorphin-2 On Allodynia And Rewarding In Rats **(23)**
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O. Valverde, F. Berrendero, C. Ledent, M. Parmentier, R. Maldonado

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II. **SIGNAL TRANSDUCTION**

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V. **NOVEL LIGANDS**

VI. **OPIOID RELATED SYSTEMS**

PLENARY LECTURE

P1

THE DARK SIDE OF DRUG ADDICTION: CORTICOTROPIN RELEASING FACTOR, STRESS, AND ALLOSTASIS

Dr. George F. Koob

The Scripps Research Institute, San Diego, CA

Drug addiction is a chronic relapsing disorder characterized by compulsive drug intake, loss of control over intake and impairment in social and occupational function. Animal models have been developed for various stages of the addiction cycle with a focus on the motivational effects of withdrawal, craving and protracted abstinence. A conceptual framework focused on allostatic changes in reward function that lead to excessive drug intake provides a heuristic framework by which to identify the neurobiologic mechanisms involved in the development of drug addiction. Neuropharmacologic studies in animal models have provided evidence for the dysregulation of specific neurochemical mechanisms in specific brain reward and stress circuits that provide the negative motivational state that drives addiction. These changes in the reward and stress systems are hypothesized to maintain hedonic stability in an allostatic state as opposed to a homeostatic state and as such convey the vulnerability for relapse in recovering drug addicts. The brain reward system implicated in the development of addiction is comprised of key elements of a basal forebrain macrostructure termed the extended amygdala and includes the central nucleus of the amygdala, the bed nucleus of the stria terminalis and a transition zone in the medial (shell) part of the nucleus accumbens. The brain stress systems such as corticotropin releasing factor that are recruited during the development of dependence and contribute significantly to the allostatic state are also localized to the extended amygdala. The allostatic model not only provides a model by which to integrate molecular, cellular and circuitry neuroadaptations in brain motivational systems produced by chronic drug ingestion with genetic vulnerability, but also provides the key by which to translate advances in animal studies to the human condition.

SYMPOSIUM 1. Opiate dependence and addiction: the neuropeptide/ GABA connection

S1

HEROIN USE ON OPIOID NEUROPEPTIDE GENES IN GABAergic NEURONAL POPULATIONS IN THE HUMAN STRIATUM.

Yasmin L. Hurd.

Karolinska Institute, Dept. Clinical Neuroscience, Psychiatry Section, Stockholm, Sweden.

There is a tight interaction between GABA and opioid neuropeptides within the striatum, a brain region that integrates reward, motivation, and motor functions and thus has been central to drug addiction studies. All striatal output neurons contain GABA, but they differentially express opioid neuropeptides; striatonigral neurons predominantly express dynorphin, whereas striatopallidal neurons primarily express enkephalin. Post-mortem brain sections from humans and animal models were studied to characterize the neuropeptide gene expression within discrete striatal GABAergic output neurons following the use of addictive substances. In contrast to stimulant drugs which differentially alter the distinct output neuronal populations (increased prodynorphin, decreased proenkephalin), pilot studies of human opiate users show a down-regulation of the prodynorphin and proenkephalin mRNA expression in both sensorimotor- and limbic-related striatal subregions. Evidence of reduced GAD65/67 mRNA expression was also evident in heroin users. Overall, the findings indicate that both striatonigral and striatopallidal GABAergic systems are impaired in association with heroin use.

S2

ABSENCE OF SELF-ADMINISTRATION AND BEHAVIORAL SENSITIZATION TO MORPHINE, BUT NOT COCAINE, IN MICE LACKING NK1 RECEPTORS

C. Gadd, T. Ripley, P. Murtra, C. De Felipe, D. Stephens, S. Hunt

Department of Anatomy and Developmental Biology, UCL, London, England; Laboratory of Experimental Psychology, University of Sussex, Brighton, England; Instituto de Neurociencias, Universitas Miguel Hernández, Alicante, Spain

Mice lacking the NK1 receptor, the preferred receptor for substance P, do not show conditioned place preference (CPP) or locomotor stimulation to morphine. We have extended these findings by examining self-administration and locomotor sensitization behavior in these mice. After learning an operant lever-press response to obtain food, WT mice increased lever presses to receive infusions of morphine (0.2 mg/kg/infusion i.v.), whereas NK1-/- mice continued to operate rewarded and non-rewarded levers at low rates. NK1-/- mice also failed to sensitize to the locomotor stimulant effects of chronic morphine (15 mg/kg i.p.). These deficits were specific to opiates, since NK1-/- mice showed normal responses to cocaine. We have also examined the behavioral effects of ablation of NK1-expressing neurons in forebrain nuclei using the neurotoxin substance P-saporin. Ablation of these neurons in the amygdala, but not the nucleus accumbens, reduced both CPP and hyperlocomotion to morphine.

S3

OPIOIDS INHIBIT RELEASE OF GABA FROM ARCUATE POMC NEURONS

J.T. Williams, S. Hentges, S. Arttamangkul, D. Grandy, M. Nishiyama, M.P. Stenzel-Poore, M. Low.
Vollum Institute, Dept of Physiology and Pharmacology and Dept of Molecular Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR, USA

Neurons from the arcuate nucleus were grown in dissociated cell culture and whole cell recordings made in order to study the pre- and postsynaptic actions of opioids. Living POMC neurons were isolated and positively identified from transgenic mice where the expression of Green Fluorescent Protein (GFP) was driven by the POMC promotor. When grown in dispersed cell culture, these neurons made recurrent synapses (autapses), such that when an action potential was evoked in the axon, a short latency synaptic current was recorded in the same cell. Under these conditions the POMC cells released GABA that resulted in the activation of GABA-A receptors. Double label *in situ* hybridization studies using brain slices confirmed the colocalization of POMC and GAD65/67 mRNA, suggesting that the release of GABA from cultured POMC neurons was not the result of the *in vitro* conditions. Opioid agonists decreased the amplitude of the autaptic IPSCs, activated potassium currents and inhibited voltage dependent calcium currents through activation of mu opioid receptors. In addition, experiments with fluorescently labeled dermorphin (DERM-BTR) showed both binding and time-dependent internalization of the opioid agonist. These cultured neurons will allow the examination of both acute and more prolonged actions of opioids on several different effector systems and will be a powerful tool to test the link between receptor desensitization and trafficking in brain cells. This system also offers a unique possibility to study the synaptic release of beta-endorphin.

S4

OPIOID-WITHDRAWAL INDUCED SUPER-ACTIVATION OF THE cAMP CASCADE IN GABAERGIC NEURONS

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A common set of adaptations in GABAergic neurons in several brain regions may contribute to different aspects of opioid withdrawal. We have identified two adaptations in GABAergic PAG neurons undergoing opioid withdrawal using patch-clamp recording. Opioid-sensitive PAG neurons from dependent rats and mice were electrically hyperexcited when withdrawn in slices. Hyperexcitation was due to superactivation of the AC/PKA cascade that opens an opioid modulated cation conductance that is sensitive to the GAT-1 inhibitor NO711. Combined c-fos immunohistochemistry and *in situ* hybridisation for GAD65/67 indicated that many of the hyperexcited PAG neurons were GABAergic. We also found hyperexcitation of GABAergic synaptic neurotransmission in the PAG during opioid withdrawal that was mediated by superactivation of the AC/PKA cascade. In mice (but not rats) presynaptic hyperexcitation was damped by extracellular adenosine generated from superactivation of AC. The stimulation of protein kinase A dependent neurotransmission in GABAergic terminals, together with hyperexcitation of GABAergic cell bodies are likely to contribute to the role of PAG and other regions in opioid withdrawal.

S5

ASSOCIATION STUDIES OF OPIOID AND GABAA-GAMMA2 RECEPTOR GENES BETWEEN CHINESE HEROIN-DEPENDENT AND CONTROL SUBJECTS

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A previous study showed that there is a significant association between heroin dependence and the polymorphisms A118G of exon 1 and C1031G of intron 2 of the human mu opioid receptor (Szeto et al. 2001, Neuroreport 12:1103) in 200 Chinese heroin-dependent subjects when compared to the controls. The present study further showed that in the same cohort, a significant association was also shown for the polymorphism T921C of exon 3 of the human delta opioid receptor. Six single nucleotide polymorphisms (SNP) across the four GABAA subunit genes clustered on 5q33 and the K289 mutation were furthered examined by using RFLP assays. Pair-wise haplotype LD analysis demonstrated that the frequencies of every haplotype consist of an intronic SNP at the GABAA-gamma2 subunit gene (intron 7, nucleotide-123) that was significantly associated with heroin dependence. Chi-square test also demonstrated a significant ($p<0.001$) association between the intronic SNP and heroin dependence. The rest of the haplotypes and SNPs were not associated with heroin dependence.

S6

IMBALANCE IN ENDOGENOUS CCK/OPIOID SYSTEMS MAY BE INVOLVED IN VULNERABILITY TO DRUG DEPENDENCE

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One of our hypotheses to explain individual differences in vulnerability to drug dependence is a deficit, either pre-existing or occurring after administration of the product in the endogenous opioid system and/or in counteracting systems such as that involving brain cholecystokinin (CCK). Disruption of the emotional homeostasis maintained by CCK/opioid balance may be one of various factors making it difficult for addicts to adapt to environmental variables. Enkephalins may activate pathways reducing stress, and activating rewarding effects. In contrast, to adapt the organism to different kind of aggression, CCK system could increase vigilance, and could participate in the memory processes related to the addicted states. We investigated this possibility in two inbred rat strains, Lewis and Fischer rats differing in propensity to drug consumption (high in Lewis rats and very low in Fischer rats). This was done by measuring the binding parameters of CCK and opioid receptors, the behavioral responses induced by CCK2 antagonists and by the dual inhibitor of enkephalin-degrading enzymes RB 101 and by evaluating the extracellular levels of CCK and enkephalins by microdialysis in the two inbred rats.

S7

DELTA- AND KAPPA-OPIOID RECEPTORS IN THE VTA: CELLULAR DISTRIBUTIONS AND TARGETING TO MESOCORTICAL NEURONS

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Stimulation of delta- and kappa-opioid receptors (DOR, KOR) modulates neurotransmission in the VTA, yet their activation sites in local and medial prefrontal cortex (mPFC) projection neurons have not been investigated.we used electron microscopic immunocytochemistry and retrograde tract-tracing to determine if dor and kor have comparable distributions, and are located in mesocortical neurons. DOR and KOR immunoreactivity was primarily axonal (55%, 59%) and was similarly localized among presynaptic structures. Both receptors were comparably observed in glia (DOR, 14%, KOR, 15%). However, dendritic DOR and KOR labeling, which was proportionally similar (21%, 17%), differed in the size of dendrites targeted and in subcellular distribution. DOR was distributed intracellularly in variously sized dendrites, and kor was localized to plasma and cytosolic membranes of small dendrites. Retrogradely labeled cell bodies and dendrites contained DOR and KOR. KOR also was detected in apposing terminals and glia. These results suggest that DOR and KOR have differences in VTA dendritic targeting, and that both directly modulate mesocortical activity. Supported by NIDA DA11768.

S8

ROLE OF THE CENTRAL NUCLEUS OF AMYGDALA IN MORPHINE WITHDRAWAL-INDUCED CONDITIONED PLACE AVERSION IN RATS

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Chronic use of morphine is well known to lead physical and psycological dependence, and cessation of drug administration produces withdrawal symptom. We investigated the role of the amygdala in the negative affective component of morphine abstinence by evaluating naloxone-precipitated withdrawal-induced conditioned place aversion (CPA) in morphine-dependent rats. Excitotoxic lesion of the central nucleus of the amygdala (CeA), but not basolateral nucleus, significantly attenuated the CPA. *In vivo* microdialysis studies revealed that both extracellular glutamate and noradrenaline levels in the CeA were elevated during naloxone-precipitated withdrawal. Microinjection of glutamate receptor antagonists such as CNQX, MK-801 and D-CPPene into the bilateral CeA significantly attenuated the CPA. Similarly, microinjection of beta-adrenoceptor antagonists such as propranolol, timolol, atenolol and butoxamine into the CeA significantly attenuated the CPA. These results suggest that both glutamatergic and noradrenergic systems within the CeA are involved in the naloxone-precipitated morphine withdrawal-induced CPA.

SYMPOSIUM 2. Enzymes and new horizons

S9

"NEUROPEPTIDOMICS" – IDENTIFICATION AND QUANTITATION OF OPIOID PEPTIDES

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We have developed a technique to isolate a large number of neuropeptides, both known and novel, using a single affinity column1. The basis for this procedure is the defect of carboxypeptidase E in mice homozygous for the *fat* mutation. These mice accumulate peptide-processing intermediates with C-terminal basic residues that can be isolated on an anhydrotrypsin agarose column. Liquid chromatography coupled to mass spectrometry provides partial sequence. Using this approach, a large number of known peptides were identified as well as many peptides that resulted from novel cleavage sites or post-translational modifications of proenkephalin and proopiomelanocortin.

To obtain information on the relative levels of opioid and other peptides present in brain, we have developed a quantitative peptidomics method using stable isotopic tags. Unlike other techniques (such as radioimmunoassay), the quantitative peptidomics method can provide information on the exact form of the peptide that is being measured, and can also detect unknown peptides (or novel modifications of known neuropeptides). This approach is currently being used to study the regulation of opioid and other peptides in a variety of paradigms.

1) Fricker, US Patent 6043023; Fricker et al, J. Neurosci. 2000; Che et al, PNAS, 2001.

S10

MUTATIONAL ANALYSIS OF MICE DEFECTIVE IN ENDOGENOUS OPIOID PEPTIDE PRODUCTION

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Disruption of endogenous opioid peptide production leads to significant alterations in behavior and responses to exogenous opiates. As one example, morphine tolerance fails to develop in enkephalin KO mice following daily morphine injection. Thus regulation of peptide production can be expected to impact multiple processes including analgesia. Biosynthesis of all peptides including opioid peptides can be regulated at several enzymatic steps as well as by chaperones that regulate specific processing enzyme activities. The phenotypes of mutations that affect specific enzymatic steps on prenatal development, prohormone processing, and HPA activity will be presented. Specifically, we will demonstrate required roles for PC2 and 7B2 in opioid peptide production, show that transient, severe disruption of HPA axis function occurs in neonatal 7B2 KO mice, document developmental changes in the processing of the presumptive PC1 regulator proSAAS, and present initial characterization of the embryonic lethal phenotype that accompanies disruption of the major mammalian amidating enzyme PAM.

S11

PHARMACOGENETIC AND ENVIRONMENTAL VARIATION IN DRUG METABOLIZING ENZYMES ALTERS DRUG DEPENDENCE

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Interindividual variation in drug metabolism by genetically polymorphic hepatic enzymes can alter the relative risk for specific drug dependencies, the amount of a drug consumed, and related toxicities. This can be shown for genetically variable drug activation (e.g. codeine to morphine by *CYP2D6*) and inactivation (e.g. nicotine to cotinine by *CYP2A6*) using a variety of approaches (e.g. drug abuse liability, kinetic, genetic studies). Therapeutic intervention by inhibition or induction (phenocopying) can also be performed. In addition these CYP enzymes are found in the CNS where they show cell-specific expression and regulation. For example chronic low-dose nicotine increases the nicotine, PCP and amphetamine metabolizing CYP2B1 and ethanol metabolizing CYP2E1 in rat brain; they are also found at higher levels in brains from human smokers and alcoholics. Many drugs (e.g. codeine, SSRIs, neuroleptics) are metabolized by CYP2D6; this enzyme is also found at higher levels in specific brain regions and cells of alcoholics. These data suggest that genetic and environmental variation in drug metabolizing enzymes (hepatic and CNS) may alter drug use, efficacy, tolerance and neurotoxicity. Funded by NIDA and CIHR and a CR Chair.

S12

COMPUTATIONAL METHODS FOR NOVEL NEUROPEPTIDE IDENTIFICATION

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Peptide hormones or neuropeptides are a string of amino acids ranging from 3 to approximately 50 residues. They are found within a larger protein (a preprohormone), and the production of the actual hormone usually follows specific rules. Preprohormones are secreted proteins. Each has a signal sequence that is necessary for the transport of the protein out of the Golgi and into a secretory vesicle for processing and secretion. Within the secretory vesicle processing takes place. In general, the active neuropeptides are surrounded by a pair of double basic residues, i.e. Arg-Arg, Arg-Lys, Lys-Arg, or Lys-Lys is found directly adjacent to the putative hormone. These double basic residues act as recognition sites for processing enzymes that cleave the prohormone to liberate the active peptide. One other crucial property suggests the likely presence of a hormone or neuropeptide. The active neuropeptides are usually well conserved among species, while the intervening sequences, because they presumably are simply discarded, are not well conserved. Utilizing these principles, we have developed a Hidden Markov Model based method for the identification of potential neuropeptides. This method utilizes a comparison of human and other (usually mouse) protein sequences and can screen the entire complement of human and mouse genes. As a proof of principle, the screening of over 100,000 sequences in SwissProt identified 90% of the peptide hormones found in that database, with a limited number of false positives. Screening of the Celera predicted human and mouse proteins has identified possible neuropeptides that may be the endogenous ligands for orphan G protein coupled receptors.

S13

NOCIOCEPTION, TOLERANCE AND DEPENDENCE IN PC2 KNOCKOUT MICE

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The prohormone convertase PC2 is a key enzyme in the biosynthesis of the endogenous opiates, [beta]-endorphin, dynorphin (Dyn) and met-enkephalin (Met-Enk). Mice with a knockout of PC2 have reduced levels of mature Met-Enk-Arg-Phe, Leu-Enk and Met-Enk and elevated levels of Enk precursors. They have a complete absence of Dyn A-8 and a substantial reduction of Dyn B-13. In their neurointermediate lobe, there is reduced (but some) [beta]-endorphin. In contrast, in the hypothalamus and amygdala of these mice, conversion of [beta]-LPH to [beta]-endorphin was impaired, but due to decreased PC2-mediated carboxy-shortening of [beta]-endorphin, its levels were increased in these brain regions of the PC2 knockout (KO) mice. We hypothesized that the altered biosynthesis of the three lines of endogenous opiates in these mice would lead to alterations in the acute and chronic effects of morphine. Basal nociceptive response was similar in PC2 KO, heterozygous (HT) and wild-type (WT) mice in the tail-flick (measures spinal cord-mediated nociception) and hot-plate assays (measures centrally-mediated nociception). In the PC2 KO mice, morphine injection (2.5 mg/kg, sc) produced a mildly greater anti-nociceptive effect than in WT and HT mice, possibly due to opioid receptor up-regulation due to lack of endogenous opiate peptides. Tolerance, as assessed by the response to an acute sc injection of 2.5 mg/kg of morphine following 6 days of daily injection of 10 mg/kg of morphine, was diminished (but still observed) in the PC2 KO mice, compared to HT and WT mice. The diminished tolerance was more pronounced in the hot-plate assay compared to the tail-flick assay and more diminished in male mice compared to female mice. Dependence was assessed by naloxone-precipitated withdrawal. Most interestingly, the frequency of jumping was most diminished in the PC2 HT mice and mildly diminished in PC2 KO mice. The onset of diarrhea was delayed in PC2 HT mice and mildly delayed in PC2 KO mice. Other parameters of withdrawal were not affected in the PC2 KO mice. We conclude that tolerance is the parameter most affected by depletion of the endogenous opiates, dynorphin and Met-Enk. The lack of effect on nociception and only mild effect on dependence may be related to i) the continued biosynthesis of [beta]-endorphin in the PC2 KO mice, ii) compensation by other opiate-generating enzymes or iii) the impaired biosynthesis of agents that promote nociception, such as substance P or orphanin FQ/nocieceptin.

S14

METABOLIC PRODUCTS OF DYNORPHIN A (1-17) – MICRODIALYSIS AND MASS SPECTROMETRY

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Dynorphin A(1-17) [DynA] is a biotransformation product of preprodynorphin, the mRNA for which has been found to be expressed in the caudate putamen. Furthermore, DynA injection into mesolimbic brain regions has been shown to regulate dopamine levels, presumably via interaction with kappa opioid receptors. To investigate the possibility that bioactive fragments result from the degradation of this opioid peptide, DynA was injected into the caudate-putamen of rats, and microdialysis was used to recover the metabolic products. Following concentration and desalting, matrix-assisted laser desorption/ionization mass spectrometry was used to identify the resulting fragments. The production of both C-terminal and N-terminal fragments has been shown, with results suggesting a cleavage at the R7-I8 position, as well as carboxypeptidase and aminopeptidase activity. The relative contributions that different enzymes make to the degradation of DynA are dependent on the concentration of DynA. Thus, for example, following the infusion of 20 nmol of DynA, series of both N-terminal and C-terminal degradation products are found; conversely, following infusion of 2 nmol of DynA, the predominant products involve internal cleavage.

S15

BIOGENESIS AND TRAFFICKING OF PRODYNORPHIN ARE REGULATED BY THE UBIQUITIN PROTEASOME PATHWAY (UPP)

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Prodynorphin possesses potential PEST regions, which target proteins for destruction by the UPP. To test whether prodynorphin is degraded by the UPP, we treated cells expressing prodynorphin with proteasome inhibitors. Following treatment, a 5-10-fold increase in the prodynorphin levels and the formation of large round prodynorphin-positive inclusions in the cytoplasm, similar to protein aggregates, aggresomes, were observed. Prodynorphin appears to dislocate from the ER back to the cytosol where it is polyubiquitinated and degraded by the UPP. Sequences that target prodynorphin for degradation partially overlap with PEST regions. Degradation by the UPP may be the way to eliminate misfolded prodynorphin molecules or to regulate the transit of the protein through the secretory pathway and, consequently, generation of dynorphins. Perturbations with prodynorphin trafficking appear to affect downstream events; the regulated secretion of dynorphin A and prodynorphin is impaired in cells exposed to proteasome inhibitors.

SYMPOSIUM 3. WHEELER SYMPOSIUM
Limbic mechanisms of reinforcement and motivation

S16

NEUROPLASTICITY IN PREFRONTAL CORTEX REGULATION OF DIRECTED BEHAVIOR.

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Cognitive processing is known to be impaired and alterations in prefrontal cortical metabolism have been demonstrated in neuroimaging studies of addicts. Using the reinstatement of drug-seeking behavior in rats trained to self-administer cocaine, it was found that cocaine-primed reinstatement was prevented by inhibiting neuronal activity in the dorsal prefrontal cortex with a microinjection of the GABA agonists muscimol and baclofen. Moreover, it was shown that drug-primed reinstatement was associated with an increase in the release of glutamate from prefrontal cortical projections to the nucleus accumbens. To investigate how the physiology of the prefrontal cortex may have been altered by drug self-administration, two experiments were conducted. 1) Using intracellular recording from pyramidal cells in the prefrontal cortex *in vivo* it was shown that after repeated cocaine bistability in membrane potential was eliminated. 2) Levels of various proteins were examined in the prefrontal cortex after repeated drug administration and it was found that the Gi inhibitor AGS-3 was markedly elevated. These findings will be presented and integrated into a model of drug-induced cortical dysfunction that alters motivated behavioral responding.

S17

BEHAVIORAL SENSITIZATION AND SYNAPTIC PLASTICITY IN THE MESOLIMBIC DOPAMINE SYSTEM

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The long-lasting sensitization to the locomotor stimulatory effects of drugs of abuse is often used as an animal model for certain core features of addiction. Modifications in the ventral tegmental area (VTA) appear to be involved in the induction of behavioral sensitization whereas modifications in the nucleus accumbens (NAc) are involved in its long-term maintenance. As a first step in elucidating the changes in neural circuits that mediate behavioral sensitization, we have examined changes in excitatory synaptic transmission in slices of the VTA and NAc in mice previously exposed to cocaine *in vivo*. Evidence will be presented that a single *in vivo* exposure to cocaine causes an LTP-like effect at excitatory synapses in the VTA while more prolonged *in vivo* cocaine exposure causes LTD in the NAc. Thus mechanisms thought to be important for adaptive forms of experience-dependent plasticity may also play an important role in the etiology of pathological behaviors such as addiction.

S18

LINKING AFFECT TO ACTION: THE ROLE OF NEURAL REPRESENTATIONS IN MESOCORTICOLIMBIC STRUCTURES IN GOAL-ORIENTED BEHAVIOR

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Orbitofrontal cortex (OFC) and basolateral amygdala (ABL) are crucial to behavior based on the acquired incentive value of otherwise neutral cues. This presentation will consider the behavioral data that support this position and then turn to recent neurophysiological findings to explore the roles of these areas in mediating such goal-directed behavior. In one experiment, recordings of neurons were made in OFC and ABL of rats during learning and reversal of odor discrimination problems. Although neural activity in both structures reflected acquired incentive value during cue sampling, there were crucial differences between these representations in the two structures. Cells in OFC encoded incentive value in conjunction with performance, whereas cells in ABL encoded incentive value before performance changes. These differences suggest that ABL encodes acquired incentive value while networks in OFC utilize this information to guide responses to specific cues. To test this hypothesis, we again recorded from OFC in rats with bilateral neurotoxic lesions of ABL. In ABL-lesioned rats, fewer OFC cells altered firing selectivity as a result of learning or reversal training, and more cells were selective simply on the basis of odor identity. These results confirm the importance of connections between ABL and OFC in linking affective or incentive-based information to appropriate behavior.

S19

FUNCTIONAL CIRCUITRY OF REWARD AND AVERSION IN THE HUMAN: POTENTIAL IMPLICATIONS FOR ANALGESIA AND ADDICTION

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Since the time of Spinoza, Bentham, and Acquinas, there has been a question of how the experience of reward was similar across category of reinforcer, and how these reinforcers were experienced relative to aversive or painful events. Studies using functional magnetic resonance imaging (fMRI) across multiple categories of rewarding and aversive stimuli now suggest that at the scale of their measurements, there is a generalized circuitry for processing pleasure and pain, comprised of subcortical gray matter [including the nucleus accumbens (NAc), amygdala, sublenticular extended amygdala, ventral tegmentum, and thalamus], and paralimbic cortices [orbitofrontal cortex, insula, anterior cingulate, parahippocampus, and temporal pole]. In our lab, there is strong evidence that the motivationally salient features of monetary gains and losses, infusions of drugs of abuse, visual processing of beautiful faces, and somatosensory experience of pain, are evaluated by these brain regions, and produce putative signatures for rewarding vs. aversive events. Some of these studies have further begun to dissect the functions of reward and aversion into their subcomponent processes, including segregating expectancy functions and valuation functions around reward and aversion. These experiments aimed at dissecting reward and aversion functions point to a general model for the processing of rewarding and aversive information that is convergent with ideas developed from animal physiology studies, and have a number of implications for understanding clinical phenomena such as analgesia and addiction. In particular, the differing activation produced by addictive compounds with analgesic effects, such as morphine, vs. acute pain, in regions such as the NAc and thalamus, suggest a means for using neuroimaging to segregate the potential for addiction to a compound from the potential for analgesia from it.

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S20

SITES FOR MU-OPIOID AND DOPAMINE D2 RECEPTOR ACTIVATION ARE HIGHLY CO-EXPRESSED MEDIAL PREFRONTAL CORTEX DENDRITES

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Opiates and psychostimulants regulate mu-opioid (MOR) and dopamine D2 receptor systems in reward-linked brain structures, including the medial prefrontal cortex (mPFC). We investigated the electron microscopic immunocytochemical localization of MOR and D2 receptors to determine the 1) cellular distributions, and 2) functional sites for potential interactions in this region of rat brain. Mor immunoreactivity was primarily postsynaptic (84%), and was localized mainly to dendrites (74%), but also cell bodies (14%), while only 5% was seen in axonal profiles. In comparison, D2 receptor labeling was more evenly distributed among presynaptic (41%) and postsynaptic (46%) structures. From all dually labeled profiles, 72% were dendrites, and 25% were cell bodies, however, less than 1% were presynaptic. Our results indicate that in the mPFC, MOR ligands mainly effect the postsynaptic responsivity of neurons, while D2 receptor activation can modulate both pre- and postsynaptic actions. In addition, these results suggest that dendrites in the mPFC are the primary cellular targets for opiate and psychostimulant regulation of MOR and D2 receptors. Supported by NIDA grant DA11768.

S21

ENDOMORPHIN-1 AND -2 IMMUNOREACTIVE CELLS IN THE HYPOTHALAMUS ARE LABELED BY FLUORO-GOLD INJECTIONS TO THE VENTRAL TEGMENTAL AREA

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Endomorphin–1 and –2 are endogenous opioids with high affinity and selectivity for the mu opioid receptor. Cells containing endomorphin-like immunoreactivity (EM-LI) are present in the hypothalamus, and fibers containing EM-LI are present in many brain areas, including the ventral tegmental area (VTA). The VTA is one of the most sensitive brain regions for the rewarding effects of opioids, which are thought to disinhibit dopamine transmission to the nucleus accumbens. We tested whether EM-LI cells project to the VTA. The retrograde tracer Fluoro-Gold (FG) was injected into the VTA in rats. Nine days later, colchicine was injected, and 24 h later the tissue was processed for immunofluorescence. Specific subpopulations of cells were double-labeled with EM1-LI or EM2-LI and FG. An anterior/posterior topographic correlation was observed between the VTA and hypothalamus. The results support the idea that EM-LI neurons in the hypothalamus project to the VTA, where they may modulate reward and locomotor circuitry.

S22

MODULATION OF MAPK ACTIVATION *IN VIVO* FOLLOWING ACUTE mOPIOID TREATMENT

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There is increasing evidence that opioid receptor activation leads to activation of MAPK (ERK1 and ERK2) in cell culture. However, little is known about MAPK activation *in vivo* following acute opioid treatment. In this study, using antibodies against the phosphorylated form of ERK1 and ERK2, we examined acute opioid signaling in the mouse brain *in vivo*. We found activation of MAPK in the cingulate cortex, claustrum, sensory cortex, locus coeruleus and lateral amygdala. Decreased activation was found in the angular cortex, nucleus accumbens, and striatum. These results indicate that MAPK modulation occurs in brain areas known to be involved in opioid related behaviors including analgesia and reward. Confocal analysis of double-labeled sections revealed that phospho-MAPK staining occurred primarily in cells that do not express the mu opioid receptor, indicating that MAPK modulation is mainly secondary to the activation of neurons containing mu opioid receptors. The data suggests that activation of the MAPK pathway is indirectly involved in opioid-related behaviors *in vivo*.

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S23

GENE EXPRESSION PATTERNS IN THE AMYGDALA AND BASAL GANGLIA FOLLOWING ACUTE AND CHRONIC MORPHINE TREATMENT

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We analyzed gene expression in the amygdala and basal ganglia following acute and prolonged morphine exposure in mice. Animals in the acute group were injected with morphine (10 mg/kg i.p.) and brain tissues removed at 1 and 4 hrs. In the chronic group, animals were implanted with a morphine pellet (75mg) and tissues isolated at 3 days or following administration of naltrexone (4hr) (1 mg/kg i.p.). RNA samples were isolated from the amygdala and basal ganglia and subsequently analyzed by TOGA®. 16,949 RNAs were detected by TOGA® and more than 200 showed at least a two-fold change with morphine treatment. A subset of these 37 genes was further studied in morphine-treated mice using real-time PCR and *in situ* hybridization techniques. Morphine-regulated genes were identified as components of signal tranduction pathways and involved in transcriptional regulation, mitochondrial respiration and cytoskeletal organization. These changes in gene expression may play roles in neuronal mechanisms of opioid abuse.

This was supported by DGT and KBN grant 6PO5A 107 20.

SYMPOSIUM 4. Joint with ICRS
THC/Opioid Receptor Function/Regulation

S24

MEMBRANE TRAFFICKING OF OPIOID RECEPTORS IN NEURAL CELLS

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Membrane trafficking processes are thought to play a fundamental role in regulating CNS opioid receptors in response to physiological and pharmacological stimuli. However cellular and molecular mechanisms that mediate opioid receptor trafficking have been studied primarily using non-neural model systems. We have utilized several approaches to investigate regulated membrane trafficking of opioid receptors in neurosecretory cells and neurons. While certain aspects of opioid receptor membrane trafficking are similar in neural and non-neural cell types, our studies suggest the existence of two additional features of opioid receptor trafficking which are observed specifically in neurons. First, in contrast to their constitutive targeting to the plasma membrane in non-neural cells, recently synthesized delta opioid receptors (DOR) are sorted from the trans-Golgi network to a regulated export pathway in neurosecretory cells and neurons. Studies of the underlying molecular mechanism define a novel membrane sorting signal in the cytoplasmic tail of DOR and identify an unanticipated role of receptor tyrosine kinase signaling in regulating anterograde membrane trafficking of opioid receptors. Second, we have observed an unanticipated process of regulated membrane trafficking of MOR occurring selectively in dendrites of CNS neurons. This process regulates both recombinant and endogenously expressed MOR in nucleus accumbens neurons *in vivo* and may play an important role in mediating physiological adaptation of CNS neurons to opiate drugs such as morphine.

S25

CHRONIC DRUG EFFECTS ON OPIOID AND CANNABINOID RECEPTOR ACTIVATION OF G-PROTEINS IN BRAIN

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Mu opioid receptor and CB1 cannabinoid receptors both belong to the same family of G-protein-coupled receptors that activate Gi/o proteins. In most cases, chronic agonist exposure produces a varying degree of receptor/G-protein uncoupling and receptor downregulation, but the relationship between these actions and development of opioid and cannabinoid tolerance remains unclear. Chronic treatment of rats with THC produces profound decrease in CB1 activation of G-proteins, with the degree of uncoupling varying between regions. This uncoupling is accompanied by significant decreases in CB1 receptor number, occurring with a time course later than that of the G-protein uncoupling. Chronic treatment of rats with mu agonists (morphine, heroin, or methadone) also produces a region-selective decrease in receptor/G-protein uncoupling, but no accompanying decrease in mu receptor number. The time course of mu receptor uncoupling varies across different brain regions, with brainstem areas uncoupling first, followed by midbrain and finally forebrain. This uncoupling is reversible, with mu receptor-G-protein uncoupling returning to normal levels before the disappearance of withdrawal symptoms, and in regions in the reverse order of the onset of uncoupling. These studies reveal numerous similarities and differences in the ways in which CB1 and mu receptors respond to chronic agonist exposure in brain. Supported by grants DA02904, DA06634 and DA06784 from NIDA.

S26

CELLULAR BASIS FOR STRIATAL CANNABINOID-OPIOID INTERACTIONS

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Activation of cannabinoid subtype 1 (CB1) receptors as well as deletions of the CB1 gene in CB1 (-/-) mice produce hypolocomotion, and catalepsy. Opiates active at mu-opioid receptors (MOR) can also produce similar behavioral responses through mechanisms involving the caudate-putamen nucleus (CPN) or nucleus accumbens shell (NAcSH), respectively. We used electron microscopic immunolabeling of CB1 and MOR to show that in normal rats and mice, spiny neurons in each region show dendritic plasma membrane labeling of both receptors. More often, however, the CB1 receptor was located on excitatory-type terminals presynaptic to spines containing MOR. In the NAcSH of CB1 (-/-) mice, the plasma membrane density of MOR immunogold was significantly less than in wild-type controls. Together, the results suggest that cannabinoids and opiates dually modulate the output of single striatal neurons, and that chronic absence of CB1 receptors can decrease the availability of MOR in the targeted neurons. (Supported by grants DA04600; CB1 (-/-) mice generously provided by Dr. A. Zimmer).

S27

OPIOID-CANNABINOID CROSS TALK: A ROLE FOR RECEPTOR-RECEPTOR INTERACTIONS.

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A number of previous studies have reported functional interactions between opioid and cannabinoid receptors. However, molecular mechanisms for this phenomenon have not been well explored. We addressed this by examining if opioid and cannabinoid receptors physically interact with each other and if these interactions modulate their function. We find that, when co-expressed in heterologous cells, mu, delta and kappa opioid receptors physically interact with CB1 cannabinoid receptors. We examined the functional implications of these interactions in rat striatum- a tissue that has been reported to express all of these receptors. The examination of the effect of individual or a combination of ligands on [^{35}S]GTPgammaS binding showed that the dose-dependent increase in [^{35}S]GTPgammaS binding by the cannabinoid receptor agonist WIN-2 is augmented in the presence of the kappa selective agonist or antagonist. Interestingly, WIN-2 mediated [^{35}S]GTPgammaS binding is significantly decreased by the mu receptor agonist, DAMGO. These results suggest that interactions between opioid and cannabinoid receptors differentially affect their function. In order to further characterize the interactions between mu opioid and CB1 cannabinoid receptors we have initiated studies in F-11 cells; a cell line that endogenously expresses these receptors. Our results examining the level of mitogen activated protein kinase phosphorylation in response to opiates in the presence of CB1 receptor ligands are consistent with the proposal that interactions between these receptors differentially regulate their activity.

S28

DETECTION OF ORL1 DIMERIZATION IN LIVING CELLS USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

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Recently, several GPCRs, including opiate receptors, which were supposed to be monomeric proteins, have been shown to co-immunoprecipitate and appeared as multimers on SDS-PAGE analysis. The multimer state seemed regulated in the presence of ligands. In order to address the question of ORL1 oligomerization in living cells, we expressed in HEK 293 cells ORL1 carrying at the C terminus one molecule of EBFP (as fluorescence donor) or one EGFP (as acceptor). Both ORL1-EBFP and ORL1-EGFP were expressed in a 1:1 ratio and clones expressing increasing levels of receptors were selected. The receptor oligomerization was monitored by FRET by recording the fluorescence emission of the cell surface after laser excitation at 360 nm using a microspectrofluorimeter. For the cells expressing ORL1 up to 5 picomoles/mg of protein, the addition of 10-7 M of nociceptin (Noc) induced, in 56% of the cells, a shift of 30% of the fluorescence from 460 nm (blue) to 520 nm (green). The other cells showed no shift (27%), or a reverse shift (17%), while untransfected cells and cells expressing only ORL1-EBFP or only ORL1-EGFP showed no transfer. No FRET was observed at 4°C whereas at 22°C, the kinetics showed an increasing FRET with a maximal at 2 min. The non peptide agonist Lofentanil (10-6 M) induced ORL1 oligomerization as well. For the clones expressing between 6 and 16 picomoles of ORL1/mg protein, a spontaneous, expression-dependent emission at 520 nm was detected in the absence of agonist, indicating vicinity of the receptors. Application of Noc induced for few cells (20%) some additional transfer, suggesting that ORL1 was probably already dimerized at these receptor densities. Our results suggest that agonist binding induces ORL1 dimerization, the agonist effect being less efficient when receptor density increases.

S29

Time course for Normalization of CB1 RECEPTOR Levels and G-Protein ACTIVATION Following Cessation of Chronic Cannabinoid Treatment

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Previous studies showed that chronic administration of Δ9-THC or WIN 55,212-2 produced CB1 receptor downregulation and desensitization and *in vivo* tolerance. Furthermore, the magnitude and rate of development of cellular effects varied among brain regions. Region-specific effects may be reflected *in vivo* because studies have shown that the rate of recovery from tolerance to hypoactivity was faster than tolerance to antinociception. Therefore, these studies were conducted to determine the time course for normalization of CB1 receptor binding and G-protein activation in different regions following cessation of cannabinoid treatment. Mice were injected twice daily (s.c.) with Δ9-THC or WIN 55,212-2 at initial doses of 10 mg/kg and 3 mg/kg, respectively. These doses were doubled every three days to final doses of 160 mg/kg for Δ9-THC and 48 mg/kg WIN 55,212-2 at day 15. Brains were collected at 1, 3, 7 and 14 days after the final injection and dissected into cerebellum, striatum and hippocampus. Decreased cannabinoid-stimulated [^{35}S]GTPγS binding was found in membrane homogenates from all regions at the 1-day point, but the reduction in cerebellum was small and this region was not analyzed further. The Emax for cannabinoid-stimulated [^{35}S]GTPγS binding in vehicle-treated mice was 98% in striatum and 150% in hippocampus. Cannabinoid-stimulated [^{35}S]GTPγS binding in the striatum at day 1 was approximately 70% of vehicle control Δ9-THC and WIN 55,212-20-treate mice. By day 3, percent control binding was 95% in WIN55,212-2 and 88% in Δ9-THC-treated mice, which did not significantly differ from vehicle. In the hippocampus, percent control binding in the WIN 55,212-2-treated mice was 61% on day 1 and did not return to vehicle levels until day 14 where it was 89% of control. Similarly, percent control binding in Δ9-THC-treated mice was 53% of control on day 1 and returned to 83% of vehicle control at day 14, which did not significantly differ from vehicle. No significant changes in the levels of Gαi or Gαo were found in these regions, suggesting that decreases in cannabinoid-stimulated [^{35}S]GTPγS binding resulted from receptor uncoupling or loss of binding sites. These data demonstrate that cannabinoid receptor desensitization persists for 3-14 days following cessation of treatment and that the persistence of desensitization varies among regions. These findings suggest that different factors may contribute to CB1 receptor desensitization in different brain regions. Furthermore, regional differences in the persistence of desensitization may produce differences in the time course for recovery from tolerance to cannabinoid-mediated effects *in vivo*.

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S30

DEPRESSION OF GABAERGIC NEURO-TRANSMISSION BY CANNABINOIDS IN THE VENTRAL TEGMENTAL AREA

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It was shown recently that Δ9-tetrahydrocannabinol, like many other drugs eliciting euphoria, increases dopamine release in the nucleus accumbens of conscious rats (Tanda et al., Science 276:2048-2050, 1997). Since cannabinoids have no direct action on terminals of dopaminergic neurons in the nucleus accumbens (Szabo et al., J Neurochem 73:1084-1089, 1999), they probably increase their firing rate. The hypothesis of the present work was that cannabinoids depress GABAergic inhibition of dopaminergic neurons in the VTA which project to the nucleus accumbens.

Electrophysi-ological properties of VTA neurons in coronal rat midbrain slices were studied with the patch-clamp technique. The GABAA receptor antagonist bicuculline (20 µM) did not change the firing rate and the holding current of the neurons, indicating absence of a GABAergic inhibitory tone in our slice preparation. GABAA receptor-mediated inhibitory postsynaptic currents (IPSCs) were evoked by electrical stimulation in the vicinity of the recorded neurons. The amplitude of IPSCs was depressed by the synthetic mixed CB1/CB2 cannabinoid receptor agonist (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)-methanone mesylate (WIN55212-2; 1 and 10 µM) (Fig. 1). The CB1-selective antagonist N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A; 1 µM) abolished the inhibition produced by WIN55212-2 (10 µM) (Fig. 1). Two kinds of experiments were carried out to determine whether IPSCs were depressed with a pre- or postsynaptic mechanism. Currents evoked by ejection of muscimol from a pipette in the vicinity of the recorded neurons were only slightly changed by WIN55212-2 (10 µM). The frequency and amplitude of miniature IPSCs recorded in the presence of tetrodotoxin were also not changed by WIN55212-2 (10 µM).

The results indicate that activation of CB1 cannabinoid receptors inhibits GABAergic neurotransmission in the VTA with a presynaptic mechanism. Depression of the GABAergic inhibitory input of dopaminergic neurons would increase their firing rate *in vivo*. Accordingly, dopamine release in the projection region of VTA neurons, the nucleus accumbens, would also increase.

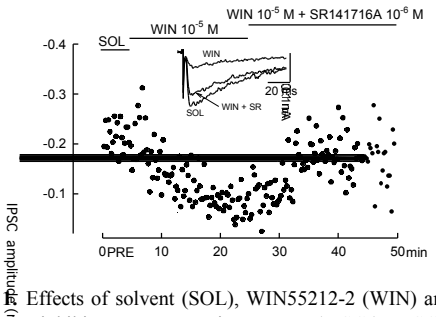

Fig. 1. Effects of solvent (SOL), WIN55212-2 (WIN) and SR141716A (SR) on inhibitory postsynaptic currents (IPSCs). IPSCs were evoked every 15 s by single electrical pulses. The inset shows averaged IPSCs obtained during superfusion with SOL, WIN 10-5 M and SR 10-6 M + WIN 10-5 M.

S31

MAP KINASES PATHWAY PLAYS A ROLE IN CANNABINOID TOLERANCE

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Cannabinoids exert most of their psychoactive effects through the CB1 receptor. This G protein-coupled receptor has been shown to be functionally coupled to inhibition of adenylyl ciclase and modulation of calcium and potassium channels. Moreover in non-neuronal cells, stimulation of transfected CB1 receptor leads to activation of MAP kinases of the ERK subfamily, as well as JNK. Furthermore in hippocampal slices, stimulation of CB1 receptor activates p38 MAPK.However intraneuronal signaling events mediated by CB1 receptor stimulation in the brain *in vivo* remain to be established.

Starting from these considerations, the present work was organized to evaluate the cellular alteration in the MAP kinase cascade induced by *in vivo* THC in specific brain regions measuring the level of phosphorilated ERK1/ERK2, JNK and p38 MAP kinases in rats acutely or chronically treated with THC. For the acute studies animals were injected with THC at the dose of 15 mg/kg, ip, while for the chronic ones THC (15 mg/kg, ip) was administered in rats twice a day for 6.5 days. Thirty minutes after the last injection, brains were quickly removed and the cerebral areas for western blot analysis were obtained by gross dissection on ice.

THC acutely injected in rats did not significantly affect the phosphorilation level of MAP kinases, while chronic THC activated (phosphorilated) ERK1/ERK2 signalling in specific brain areas, with the higher increases in the areas showing the densiest CB1 receptor content such as the striatum and the cerebellum.

In order to confirm the involvement of MAP kinases signalling in the chronic effects of THC we evaluated the acute and chronic effects of THC in mice lacking the ras-GRF1 gene. The exchange factor ras-GRF1 activates ras proteins that represent the molecular switch to trigger the sequential activation of the members of MAP kinase cascade. Acute injection of THC (10 mg/kg s.c) did not alter in ko mice the occurrence of two classical cannabinoid effects such as analgesia and hypolocomotion. On the contrary in mice lacking ras-GRF1, chronic injection of THC (10mg/kg s.c. twice a day for 5 days) did not develop tolerance to the analgesic and hypomotility effects.

Our results support the role of this signalling pathways in some biochemical and behavioural responses related to the development of cannabinoid addiction.

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SYMPOSIUM 5. Genetic Approaches to Complex Disorders

S32

"MOLECULAR GENETICS OF DRUG-INDUCED BEHAVIORS IN *DROSOPHILA"*

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Drug abuse and addiction are among the most devastating social and medical problems in our society, yet little is known about the mechanisms by which these drugs regulates behavior. Our laboratory uses the fruitfly *Drosophila*, with its accessibility to behavioral, genetic, and molecular analysis, to help establish the missing links between genes and drug-induced behaviors.

Flies display many of the behaviors observed in mammals after both, acute and chronic exposure to ethanol and the psychostimulants cocaine and nicotine. We have developed quantitative behavioral assays to ascertain the effect of these drugs on fly behavior, including assays for postural control and locomotion. Using these assays, we are carrying out genetic screens for mutations with aberrant drug-induced behaviors. The behavioral, genetic, and molecular characterization of some of these mutants will be presented.

To define the neuroanatomical sites that regulate ethanol-induced behaviors we have used targeted expression of transgenes encoding tetanus toxin or protein kinase A inhibitors to specific brain regions. These studies have revealed that dopaminergic and serotonergic systems mediate the acute effects of ethanol, cocaine, and nicotine; octopaminergic systems are involved in the development of ethanol tolerance.

S33

MICROARRAY ANALYSIS IN THE STUDY OF NEURAL CIRCUITS OF STRESS AND EMOTION

H. Akil, S. Evans and S. J. Watson.

Genomic approaches are being used for the study of central nervous system function and for understanding complex brain disorders. The use of microarray technology is both powerful and fraught with difficulties when applied to the study of brain function. This talk will outline some of the general technical issues, and will then focus on some of our animal studies using microarray technology. These animal studies include analysis of the effect of adrenalectomy on hypothalamic gene expression as a model of a perturbation with both known and unknown consequences that can be used as a validation tool. They also include gene profiling to characterize the effect of chronic stress on animals with different emotional reactivities (high and low responders). The last component of the talk will discuss the extension of these strategies to human postmortem studies to understand the neural phenotype associated with severe mood disorders.

S34

GENE PROFILING OF NEUROPSYCHIATRIC DISEASES: SCHIZOPHRENIA AS A DISEASE OF THE SYNAPSE

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The level of cellular and molecular complexity of the nervous system, and the polygenic and epigenetic etiology of neuropsychiatric disorders, create unique challenges for scientists utilizing functional genomics to investigate brain diseases. Using cDNA microarrays, we demonstrate altered patterns of gene expression in dorsolateral prefrontal cortex (DLPFC) between subjects with schizophrenia and controls. First, transcripts encoding proteins regulating presynaptic function were decreased. Second, the most changed gene was regulator of G protein signaling 4, which influences the extent of postsynaptic responsiveness of G-protein coupled receptors. Third, there were changes select metabolic groups that could influence neuronal communication. These findings suggest fundamental problems in the efficacy of synaptic transmission, consistent with our neurodevelopmental hypothesis, which proposes that impaired mechanics of synaptic transmission during childhood and adolescence may cause altered synapse formation and/or pruning in specific neural circuits, manifested post-adolescence.

S35

PROTEOMIC ANALYSIS OF LASER-CAPTURE MICRODISSECTED BRAIN SAMPLES: WHAT WORKS AND WHAT DOESN'T

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Powerful and precise microdissection techniques are needed to analyze protein changes in small brain nuclei. Laser Capture Microdissection (LCM) is a technique that is precise enough to dissect single cells within a tissue section. The goal of this study was to determine the effects of tissue staining procedures associated with LCM on protein extraction and separation on 2D gels. LCM rat brain samples obtained after several histological or immunostaining steps and fresh-frozen unstained manually dissected samples underwent protein extraction and 2D gel electrophoresis. Gels were analyzed to determine the number of proteins detected from stained samples compared with unstained controls. Our results indicated that histological staining of the tissue greatly reduced protein recovery while immunofluorescent staining had only minimal effects. Moreover, fixation and LCM without staining did not significantly affect protein recovery. These results indicate that LCM of fixed, unstained or immunostained brain tissue could be used to dissect discrete brain regions for proteomic analysis.

S36

HAPLOTYPE-BASED OF DRD2 GENE ASSOCIATION WITH HEROIN ABUSE IN A CHINESE HAN POPULATION

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Evidence is growing that dopamine 2 receptors (DRD2) play a central role in the molecular mechanism of addictive behaviors including heroin abuse. However, there are a large number of contradictory genetic linkage and association findings of DRD2 gene with substance abuse. Haplotype-based approach is more powerful than individual SNP association. To further understand the role of DRD2 gene as a susceptibility gene of heroin abuse, this study examined the association of 10 SNPs with heroin abuse in a large case control study population of Han Chinese. We found that TaqIB 1 allele was significantly abundant in the case group ($X2 = 16.94$, $p=0.000038$). 10 SNP haplotype analysis has shown that two haplotypes were significantly higher in the control group than the case group ($X2 = 10.87$, $p=0.000098$ for haplotype A and $X2 =8.189$, $p=0.004$ for haplotype B. The 9 SNPs haplotype analysis, which excluded TaqIB, indicated there was no longer significant difference between the case and control groups ($X2 =3.012$ $p=0.0826$). These results strongly support the association of DRD2 with heroin abuse.

S37

DEPLETION OF SEROTONIN BLOCKS THE REGULATION OF DYNORPHIN BY KAPPA-OPIOID AGONISTS

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Chronic treatment with U-69593 increases dynorphin gene expression in the hypothalamus, and decreases it in striatum and hippocampus. To determine whether serotonin plays a role in the regulation of dynorphin mRNA, rats were treated with parachloroamphetamine (PCA) to deplete serotonin, followed by five daily injections of either U-69593 or vehicle. Three days later, dynorphin mRNA was decreased in the hypothalamus in rats treated with PCA+vehicle, suggesting that serotonin is necessary to sustain normal levels of dynorphin message in this brain region. This was not true in either hippocampus or striatum, where dynorphin mRNA was unchanged. PCA blocked the effects of U-69593 on dynorphin message in both the hippocampus and striatum, but not the hypothalamus. These data suggest that regulation of dynorphin mRNA by kappa-opioid agonists requires the presence of serotonin in some, but not all brain regions. Thus the mechanism by which kappa-opioid agonists regulate the endogenous ligand for the kappa-opioid receptor appears to be complex and differs across brain regions. Supported by DA 11960.

S38

MASSIVE PARALLEL ANALYSIS OF GENE EXPRESSION BY TAQMAN IN APPLICATION TO NEUROBIOLOGICAL STUDIES

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Real time fluorescent PCR (TaqMan), an ultrasensitive technique for mRNA quantification, makes possible the simultaneous analysis of many genes in a small tissue sample. We performed multiple gene expression analyses from brain regions of male Fisher rats after 1 day "binge" saline or cocaine administration (3 x 15 mg/kg cocaine, i. p., at hourly intervals). The levels of mRNAs for opioid and opioid-like receptors (mu, kappa, delta, orphanin FQ), peptides (preprodynorphin, preproenkephalin, orphanin FQ, substance P), dopamine receptors (D1, D2, D3), glutamate receptors (GluR1, GluR2), N-methyl D-aspartate receptor type 1 (NMDAR1), glucocorticoid receptors (type 1 and 2), immediate early gene *c-fos*, and housekeeping genes (actin, cyclophilin, glyceraldehyde-3-phosphate-dehydrogenase) in RNA extracts from rat caudate putamen and nucleus acumbens were measured in parallel by the TaqMan technique. 18S ribosomal RNA was used to normalize the level of expression of genes of interest. Significant changes in preproenkephalin, *c-fos*, NMDAR1 and substance P mRNA levels after cocaine were found ($p<0.05$, one-tail t-test). Some of these findings have been reported using independent methods.

S39

CHANGES IN GENE EXPRESSION INDUCED BY PRECIPITATED MORPHINE WITHDRAWAL IN PREWEANLING RATS

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Infant rats undergo opiate withdrawal as do adults of many species, but the signs and symptoms are distinct. Evidence suggests both similarities and differences in the fundamental neurobiology of withdrawal during development. To assess further the developmental changes in the basic mechanisms underlying withdrawal during development, we examined changes in gene expression at 7 days of age, when the infant form of withdrawal is seen, and at 21 days of age when the more adult-like form is expressed. Pups were treated for 7 days with morphine and tested at 7 or 21 days of age. Withdrawal was precipitated by naltrexone and pups sacrificed at 1 or 4 hours afterwards. Total mRNA was extracted from the lumbar enlargement of the spinal cord and changes in gene expression assessed by the Affymetrix U34A gene chip, which contains probes for about 8700 rat genes. Analysis of the data, including comparison of experimental groups to controls, and analysis of the relationship of gene expression levels at different ages showed both age- and time-dependent patterns of change. Supported by DA13794 to GAB.

PLENARY LECTURE

P2

NEUROTRANSMITTER TRANSPORTERS ON SECRETORY VESICLES

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Since most classical neurotransmitters are made in the cytoplasm, exocytotic release depends on their transport into secretory vesicles. Indeed, work over the last few decades has shown that synaptic vesicles exhibit multiple, distinct transport activities for different classical transmitters. A number of psychoactive drugs interfere with the transport of monoamines into vesicles, indicating the importance of this transport for behavior. However, we lack drugs specific for most of the other vesicular neurotransmitter transporters. In addition, the proteins responsible for the transport of any transmitter into secretory vesicles have been identified only over the last few years. They belong to three distinct families of proteins, each with a characteristic mechanism of ionic coupling, and with divergent pathways of membrane trafficking. Although generally considered to reside in different neurons, consistent with Dale's law that a neuron releases only one classical transmitter, certain cells express multiple vesicular neurotransmitter transporters. The expression of a vesicular glutamate transporter by monoamine neurons will in particular challenge our understanding of the role played by these cell populations in brain function.

SYMPOSIUM 6. New Opioids and Opioid Related Systems

S40
INTERACTION OF OPIOID RECEPTORS: A NEURAL CIRCUIT ANALYSIS.
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Dramatic progress has been made in understanding opioid receptor function at the molecular and cell biological level. Attempts to understanding how these actions relate to behavior and to clinically meaningful effects has been much more problematic. In this talk I will discuss how the synaptic actions of mu, kappa and ORL1 agonists can be placed in the context of two neural circuits: the midbrain-medulla-spinal cord pain modulatory network and the midbrain dopaminergic projection to the nucleus accumbens, which has been implicated in reinforcement and motivation. Some general principles emerge from these studies: mu agonists produce behavioral effects by inhibiting GABAergic interneurons and disinhibiting projection neurons while kappa agonists directly inhibit the projection neurons and have actions that generally oppose those of mu agonists.

S41
IDENTIFICATION OF NOVEL PEPTIDES WITH C-TERMINAL RF-AMIDE AND THEIR RECEPTORS
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We have recently identified RFamide-related peptide (RFRP) gene that would encode three peptides (i.e., RFRP-1, -2, and -3) in human, and demonstrated that synthetic RFRP-1 and -3 act as specific agonists for a G protein-coupled receptor OT7T022. RFRP and OT7T022 mRNAs were expressed in the hypothalamus, and the intracerebroventricular administration of RFRP-1 evidently increased prolactin secretion in rats. We prepared a monoclonal antibody for RFRP-1, and purified endogenous RFRP-1 with 35-amino acid length from bovine hypothalamus on the basis of the immunoreactivity. By immunohistochemical analysis, RFRP-1-positive nerve cells were detected in the hypothalamus in rats. RFRP-1-positive fibers were widely distributed in the brain: not only in the hypothalamus but also in the brain stem (periaqueductal gray in the midbrain and parabrachial nucleus in the pons, which are the regions critical for the control of nociception). RFRPs and neuropeptide FF (NPFF) have a common C-terminal structure and cross-react their receptors each other. As anti-opioid effects of NPFF are well known, those of RFRPs will be discussed.

S42
IDENTIFICATION OF THE FIRST DIMETHYL-4-(3-HYDROXYPHENYL)-PIPERIDINE AND 5-(2-HYDROXYPHENYL)MORPHAN DERIVATIVES TO POSSESS HIGHLY POTENT AND SELECTIVE OPIOID KAPPA RECEPTOR ANTAGONIST ACTIVITY
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Since the discovery of three distinct opioid receptors (mu, delta, and kappa), researchers studying the underlying mechanism of opiate addiction have sought highly potent and receptor subtype selective antagonists. While many agonist structures have been discovered for the opioid receptor system, very few structures displaying potent pure antagonist activity have been identified. Even fewer antagonists are available that show opioid subtype selectivity. In this presentation the development of potent and selective kappa opioid antagonists from the dimethyl-4-(3-hydroxyphenyl)piperidine and the 5-(3-hydroxyphenyl)morphan classes of opioids will be presented. Supported under NIDA grant DA09045.

S43
OVARIAN SEX STEROIDS ACTIVATE SPINAL DYNORPHIN ACTIVITY VIA THE LOSS OF NEGATIVE NOCICEPTIN AND OPIOID MODULATION.
D. Gupta, L. Toll, M. Szucs, A.R. Gintzler
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Ovarian sex steroids are potent modulators of spinal opioid activity via the selective disinhibition of spinal dynorphin neurons. In spinal cord obtained from untreated animals, nociceptin (orphanin FQ; N/OFQ) as well as kappa- or delta- opioid agonists inhibit the stimulated release of dynorphin. However, in spinal tissue obtained from ovariectomized animals treated with estrogen / progesterone, there is a reduction in functional N/OFQ receptors and evoked dynorphin release is no longer negatively modulated by N/OFQ. Ovarian steroid treatment also results in the loss of opioid inhibition of evoked dynorphin release, which in fact is now facilitated by delta-opioid receptor activation. As a result of these changes, the magnitude of spinal dynorphin signaling increases ♠ 2-fold. On the other hand, spinal methionine-(met-) enkephalin release is differentially regulated. Neither delta- nor kappa-opioids modulate its release. Moreover, although met-enkephalin release is inhibited by N/OFQ, it is not negated by ovarian steroid treatment, which also fails to alter the magnitude of met-enkephalin signaling. We conclude that N/OFQ functions as an endogenous negative modulator of spinal dynorphin and met-enkephalin release. The offset of the former but not the latter by ovarian steroid treatment indicates that regulation of the activity of multiple interactive spinal opioids is independent and asymmetrical.

S44

OPIOID AND "ANTIOPIOID" PEPTIDES IN THE NPFF-KO MOUSE CNS

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In situ hybridization revealed that expression of other RFamide peptides (PrRP, RFRP, and Kiss-1), particularly in posterior hypothalamus and in the medulla-spinal cord was not altered in NPFF KO mice. In contrast, the expression of opioid peptide mRNA:s appeared to be altered in some specific brain regions. In the behavioural analysis the NPFF-KO mice did not reveal a clear phenotype related to acute pain. Similarly, acute effects of morphine were normal. The NPFF KO model is valuable in evaluating the functions of NPFF, RFamides and well as opioid peptides. In particular, this model may be suitable for studies of pathological pain, which is in part regulated by NPFF.

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S45

PREPRO-NOCICEPTIN/ORPHANIN FQ 160-187 PLAYS ROLE IN MODALITY-SPECIFIC PAIN PATHWAYS

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Prepro (pp)-nociceptin/orphanin FQ 160-187, a C-peptide of its prohormone was recently found to exist in the brain and spinal cord. We have previously demonstrated that intraplantar (i.pl.) injection of C-peptide induced nociception through Gs-mechanisms in the newly developed algogenics-induced nociceptive flexor response (ANF) test in mice. The nociception was blocked by intrathecal (i.t.) injection of MK-801 (NMDA receptor antagonist), but not by CP-99994 (NK1 receptor antagonist). Unlikely N/OFQ-induced nociception, the C-peptide-induced nociception was not affected by neonatal capsaicin-pretreatment. Anti-C-peptide IgG (i.t.) increased the threshold in the thermal- and mechanical-nociception tests. The IgG also blocked the nociception induced by the i.pl. injection of capsaicin or ATP, but not by the substance P or prostagrandin I2 agonist injection. All these results suggest that the C-peptide plays a modality-specific role in pain-regulation.

S46

NOCICEPTIN/ORPHANIN FQ (N/OFQ) IS ANXIOGENIC IN TESTS OF RAT NEOPHOBIA.

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N/OFQ and its receptor are highly abundant in limbic structures that integrate anxiety- and stress-provoking stimuli. We previously reported that N/OFQ administration activates the HPA axis, and enhances hormonal responses to a mild stressor. We also reported that acute stress causes release of N/OFQ from forebrain neurons. In light of these findings, we investigated the effects of N/OFQ in rodent tests of anxiety. N/OFQ (0.001, 0.01, 0.10, and 1.00 nmol, i.c.v.) produced anxiety-related behaviours in the light/dark, open field, and elevated plus maze tests of anxiety in independent groups of rats. Specifically, rats that were injected with N/OFQ showed longer latencies to enter a brightly lit environment, and spent less time in that environment than did rats that were injected with vehicle. Rats that were injected with N/OFQ also exhibited longer latencies to enter an open field from a small start box, spent less time in the open field, and explored the center of the field less than did vehicle-treated rats. N/OFQ-treated rats showed longer latencies to enter and less time on the open arms of an elevated plus maze than did the vehicle-treated rats. Furthermore, plasma ACTH and corticosterone concentrations were higher in the N/OFQ-treated rats than they were in the vehicle-treated rats. Accordingly, N/OFQ administration appears to increase anxiety-related behaviour while it increases HPA axis activity.

S47

BIVALENT LIGANDS DESIGNED TO PROBE OPIOID RECEPTOR HETERODIMERS/HETERO-OLIGOMERS: PHARMACOLOGICAL EVIDENCE FOR INTERACTION BETWEEN OPIOID RECEPTOR TYPES

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Growing evidence has demonstrated that G protein-coupled receptors, specifically opioid receptors, can exist in dimeric/oligomeric complexes. The exact significance of dimerization/oligomerization in opioid receptor function is unclear at this time; however, recent reports suggest signal transduction pathways and receptor trafficking may be different for homomeric vs. heteromeric receptors. Bivalent ligands may be useful tools to probe the structural organization of opioid receptor dimers/oligomers and may help clarify differences in the functional roles between opioid receptor homomers and heteromers. We have designed and synthesized hetero-bivalent ligands containing a delta antagonist and a kappa agonist connected together by a spacer comprised of oligoglycyl units centered around a central variable length diacid core. When evaluated in electrically stimulated guinea pig ilea muscle preparations, these bivalent ligands showed substantial enhancement of kappa agonist potency compared to appropriate matched monovalent control ligands. However, only moderate enhancement was observed in mouse vas deferentia experiments. The possible significance of these results will be discussed.

SYMPOSIUM 7. Joint with ICRS
New possibilities for opioids and cannabinoids

S48

ATTENUATION OF CANNABINOID WITHDRAWAL IN DOUBLE MU AND DELTA OPIOID RECEPTOR KNOCKOUT MICE

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Several studies have shown functional relationships between the endogenous cannabinoid and opioid systems. However, acute delta9-tetrahydrocannabinol (THC) pharmacological responses and physical dependence were not modified in knock-out mice with single delection of mu, delta or kappa opioid receptors. To further investigate the neurobiological basis of cannabinoid dependence, we have evaluated THC responses in double mu and delta opioid receptor knock-out mice. Antinociception and hypolocomotion induced by acute THC administration remain unaffected whereas the acute hypothermic effects were slightly attenuated in these double mutants. During chronic THC treatment, knock-out mice developed slower tolerance to the hypothermic effects but the development of tolerance to antinociceptive and hypolocomotor effects was almost unaffected. The rewarding properties of THC were abolished in knock-out mice. Interestingly, the somatic manifestations of THC withdrawal were also significantly attenuated in mutant mice, suggesting that a cooperative action of mu and delta opioid receptors is required for the entire expression of THC dependence.

S49

INTERRELATIONSHIP OF CANNABINOID AND OPIOID DEPENDENCE MECHANISMS

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Opioids and cannabinoids are two distinct classes of compounds with unique pharmacological properties. However, they do share some common pharmacological traits, one of which is antinociception. Moreover, it is now well documented that opioids and cannabinoids interact, at least in part, to produce antinociception. Historically, there has been considerable interest in establishing commonalties in the dependence properties of both cannabinoids and opioids. In recent times, several laboratories have demonstrated at least some form of cross-dependence between opioids and cannabinoids. In our own laboratory, we demonstrated that precipitated THC withdrawal was ameliorated in mu opioid receptor knockout mice and failed to occur in mice devoid of CB1 cannabinoid receptors. Morphine decreased withdrawal signs in THC-dependent mice. Morphine dependence was attenuated in CB1 receptor knockout mice. Acute treatment of THC attenuated some morphine dependence signs. Acute administration of specific mu (morphine), delta (SNC 80), and kappa (U50,488 and enadoline) agonists ameliorated precipitated withdrawal in THC-dependent mice. Taken together these findings implicate a role for opioid receptor subtypes in the modulation of cannabinoid dependence. These findings taken implicate a reciprocal relationship between the cannabinoid and opioid systems in dependence.

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S50

FUNCTIONAL INTERACTIONS BETWEEN CANNABINOIDS AND OPIOIDS IN ANIMAL MODELS OF SELF-ADMINISTRATION.

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Despite the fact that cannabis is one of the oldest abused drugs, it has been difficult for a long time to demonstrate cannabinoid rewarding effects in laboratory animals. Recently, we have developed two models of cannabinoid intravenous self-administration: a model of acute intravenous self-administration in drug-naive mice and a model of chronic intravenous self-administration in Long-Evans rats. We have shown that cannabinoids are self-administered by mice and rats in a way similar to other drugs of abuse. Furthermore, we have hypothesized a functional interaction between opioid and cannabinoid self-administration. In fact, CB1 receptor antagonist SR 141716A antagonized morphine self-administration whereas cannabinoid self-administration was antagonized by the opioid antagonist naloxone. We have also shown that mice lacking the CB1 receptor do not self-administer morphine and morphine is unable to stimulate dopamine release in nucleus accumbens of these mice. These data strongly support the hypothesis of a mutual functional interaction between cannabinoid and opioid systems for the expression of rewarding effects of both opioids and cannabinoids.

S51

ENDOCANNABINOIDS AND APPETITE: POSSIBLE INTERACTIONS WITH OTHER NEUROTRANSMITTER SYSTEMS.

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Previous studies have indicated that endocannabinoids acting via CB1 receptors contribute to the hunger-induced increase in food intake in mice. Since CB1 receptors in the brain are presynaptically located, a possible mechanism of a CB1-mediated orexigenic effect could involve presynaptic inhibition of the release of a tonically active anorexigenic mediator, such as a-MSH, CRH or CART. SR141716 (3 mg/kg ip) did not affect food intake in food-restricted CART knockout mice, whereas it significantly reduced food intake in their wild-type littermates. Of brain regions implicated in appetite control, abundant CART immunostaining was found in various hypothalamic areas including the arcuate, paraventricular and dorsomedial nuclei, as well as in the amygdala and the nucleus accumbens, with very low levels in the cingulate cortex. In contrast, CB1 was minimally detectable in the hypothalamus and nucleus accumbens, but high levels were found in the cingulate cortex and amygdala. These findings suggest that CART may be a downstream mediator involved in the orexigenic effect of endocannabinoids, alhough the site and exact nature of their possible interaction remains to be identified.

S52

WIN 55212-2, A CANNABINOID AGONIST, AND U-50,488H, A KAPPA OPIOID AGONIST, PRODUCE SYNERGISTIC HYPOTHERMIA

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Opioids and cannabinoids share a number of physiological actions, including thermoregulation. Kappa and mu receptors mediate the hypothermic and hyperthermic effects, respectively, of opioids in rats. Cannabinoid agonists produce hypothermia through CB1 receptors. Given the functional similarities between opioids and cannabinoids, we investigated their interaction on body temperature in male SD rats. WIN 55212-2 (WIN; 1-10 mg/kg, i.m.), a selective cannabinoid, or U-50,488H (U50; 5-20 mg/kg, i.m.), a selective kappa opioid, evoked dose-dependent hypothermia. Pretreatment (24 hr) with nor-BNI (1 mg/kg, s.c.), a selective kappa antagonist, attenuated the response to WIN, suggesting that kappa receptors contribute to cannabinoid-induced hypothermia. Neither SR141716, a selective CB1, nor SR144528, a selective CB2, antagonist altered the effect of U-50 (10 mg/kg). Using the dose-response relations of WIN and U50, we studied a fixed-ratio combination (0.525 U50: 0.475 WIN). The observed effects exceeded the expected (additive) for each dose pair, thereby demonstrating a synergistic interaction for the two drugs. (DA06650, DA13429, DA 09793, DA07237)

S53

CB1 ANTAGONIST SR141716A-INDUCED BLOCKADE OF MU-RECEPTOR DESENSITIZATION IN THE SH-SY5Y CELL LINE

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Interactions between opioid and cannabinoid systems regarding tolerance and dependence have been demonstrated in *in vivo* studies. Cellular mechanisms underlying these functional interactions are unknown. Chronic morphine (MS) treatment (24 h) produces a significant desensitization of mu receptor-mediated inhibition of forskolin-stimulated cAMP accumulation in SH-SY5Y neuroblastoma cells that endogenously express mu and CB1 receptors. Protein kinase C (PKC), PKC-alpha and PKC-epsilon, were upregulated by chronic MS treatment. Also chronic MS treatment decreased G-protein (Gi and Go) solubility in sodium cholate. Concominant use of the CB1 antagonist SR141716A (SR) with MS blocked MS-induced mu receptor desensitization. In addition SR blocked the MS-induced PKC-alpha and PKC-epsilon upregulation, whereas it did not block the MS-induced Gi and Go solubility shift in sodium cholate. Our data suggests that the CB1 antagonist, SR141716A, interferes with MS-induced changes in PKC levels that may contribute to its ability to block mu receptor desensitization.

S54

THE EFFECTS OF ORAL ADMINISTRATION OF Δ9-THC ON MORPHINE TOLERANCE AND PHYSICAL DEPENDENCE

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Recent reports from our laboratory indicate that a low dose of delta-9 tetrahydrocannabinol (Δ9-THC) enhances the potency of oral opioids in a mouse acute pain model (Cichewicz et al., *J Pharmacol Exp Ther*, 289: 859-867, **1999**). The analgesic effect of a low dose combination of these drugs is similar to that of a high dose of morphine. Due to the common occurrence of tolerance in human patients treated with morphine for chronic pain, we were eager to determine whether a Δ9-THC/morphine combination would provide an alternate pain therapy without the development of tolerance or dependence. Thus, we hypothesized that 1) morphine tolerance would develop in mice after 7 days of oral administration; 2) co-administration of Δ9-THC during tolerance development would diminish or eliminate morphine tolerance and physical dependence; and 3) a combination of low doses of Δ9-THC and morphine would provide effective analgesia without tolerance or physical dependence. Morphine tolerance was produced in ICR mice by administration of high doses of morphine p.o. twice daily for 7 days. This paradigm resulted in a 3-fold shift to the right of the morphine dose-response curve. When Δ9-THC at a low dose of 20 mg/kg p.o. was administered along with each morphine dose for 7 days, there was no significant difference in the ED50 of morphine as compared to vehicle-treated mice. Thus, the co-administration of Δ9-THC was effective in preventing the development of morphine tolerance. Similarly, there was no difference in the dose-response curves for morphine in mice treated with a combination of low doses of both Δ9-THC and morphine (20 mg/kg p.o.) for 7 days as compared to vehicle-treated mice. These results suggest that chronic administration of a low dose-combination may provide analgesia similar to that required by patients on long-term morphine without the development of tolerance to the drug. We also examined the propensity of this combination to inhibit the development of physical dependence to morphine. After chronic treatment, mice were challenged with 1 mg/kg naloxone s.c. to precipitate opioid withdrawal and observed for a 10-minute period for jumping behavior and forepaw tremors. Morphine-tolerant mice showed platform jumping behavior with a mean jumping latency of 5-6 minutes, while mice treated with Δ9-THC and high doses of morphine showed a reduction in jumping and an increased jumping latency. The chronic administration of a low dose combination of Δ9-THC and morphine (20 mg/kg) completely attenuated the expression of withdrawal signs. These data indicate that combination treatment with Δ9-THC and morphine may be a successful analgesic therapy while prolonging or preventing the development of physical dependence to morphine.

Acknowledgements: This work was supported by NIDA Grants DA-07027, DA-05274 and K02-DA-00186.

SYMPOSIUM 8.

S55

OPIOID-INDUCED CHANGES IN ADULT HIPPOCAMPAL PROGENITORS

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New cells are born throughout life in hippocampus. Isolated adult hippocampal progenitors (AHPs) expressed DORs and MORs but no KORs and released beta-endorphin. Stimulation with beta-endorphin increased the level of $[Ca^{2+}]i$. Incubation with naloxone for ten days resulted in increased neurogenesis and decreased gliogenesis. MOR and DOR antagonists decreased proliferation probably caused by regulation of several cell cycle genes mediated through decreased phosphorylation of ERK1/2 and decreased activation of c-fos and c-jun. Both beta-endorphin and synthetic opiates such as morphine were shown to increase proliferation. Increased proliferation was mediated through changed calcium levels and the MEK-ERK-pathway. Both treatment with beta-endorphin and opioid antagonists resulted in regulation of gene expression on cDNA arrays. Naltrindole increased the number of BrdU-positive cells in dentate gyrus of hippocampus *in vivo*. It also lowered the levels of corticosterone which is known to decrease neurogenesis in hippocampus.

S56

KAPPA OPIOID SYSTEMS MEDIATE STRESS RESPONSES.

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To investigate the role of endogenous kappa opioids in stress, C57Bl/6 mice were administered vehicle or nor-BNI (10 mg/kg, ip) and subjected to the Forced Swim Test (FST) followed by the 55°C warm-water tail-immersion assay. On the 2nd day of testing, vehicle-treated mice were significantly more immobile than nor-BNI treated mice. Moreover, vehicle-treated mice demonstrated stress-induced analgesia in the tail-immersion test after FST, but nor-BNI treated mice did not. Dynorphin (DYN) WT mice demonstrated an increase in both FST immobility time and in tail-immersion latency, but DYN KO mice showed no increases in either assay. Brain protein isolated from swim-stressed mice was analyzed by Western blots using the phosphoselective KOR antibody, KOR-P. Vehicle-treated FST mice showed a 48±14% increase in KOR phosphorylation compared to controls, whereas the nor-BNI treated FST mice demonstrated a decrease of 20±11%. Immunolabeling with KOR-P of FST-stressed brains detected labeling differences between vehicle- and nor-BNI-treated mice, suggesting the involvement of specific brain regions in the opioid-mediated response. Thus, dynorphins released during stress induce immobility and analgesia by activating kappa opioid receptors.

S57

REGULATION OF OPIOID RECEPTOR TRAFFICKING AND MORPHINE TOLERANCE BY MU OPIOID RECEPTOR OLIGOMERIZATION.

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The utility of morphine for the treatment of chronic pain is hindered by the development of analgesic tolerance. Morphine is unusual in its failure to promote desensitization and endocytosis of the mu opioid receptor (MOR), processes that for many receptors contribute directly to tolerance. This apparent paradox has led us to revise the idea that receptor desensitization and endocytosis are solely responsible for tolerance and withdrawal to morphine, and instead test the hypothesis that these side effects occur due to abnormally prolonged MOR signaling. We report here that MOR mutations that facilitate endocytosis reduce the development of cellular tolerance and cAMP superactivation, a cellular hallmark of withdrawal. Moreover, mutant receptors with reduced endocytosis produce exacerbated superactivation. Furthermore, here we demonstrate that DAMGO can facilitate the ability of morphine to stimulate MOR endocytosis, an effect we attribute to the oligomeric nature of the MORs. As a consequence, rats treated chronically with both drugs show reduced tolerance compared to rats treated with morphine alone.

S58

OPIATE ABUSE INDUCES TRANSLOCATION OF GLUTAMATE TRANSPORTERS AT HIPPOCAMPAL SYNAPSES

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∗Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, †Laboratory of Sensory System, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, People's Republic of China.

Opiate abuse involves adaptive changes in several processes of synaptic transmission. Here, we show that chronic morphine treatment enhanced glutamate uptake by increasing the number of functional glutamate transporter GLT1 in hippocampal synaptosomes. The increase was due to redistribution, clustering of GLT1 at nerve terminals rather than an increase of its mRNA expression. Direct morphine treatment in cultured hippocampal neurons was sufficient to induce a surface expression and clustering of GLT1 in neurites and an increase in glutamate uptake. The induction of GLT1-mediated uptake is related to an increase in glutamate release probability during morphine withdrawal period. These results indicate that neuronal GLT1 mediated-glutamate uptake at synapses is inducible and significant for the glutamate clearance under opiate abuse, which may be a neuronal component of synaptic strength in the pathological state.

S59

PERSISTENCE OF NALOXONE-INDUCED CONDITIONED PLACE AVERSION IN BETA-ENDORPHIN BUT NOT ENKEPHALIN KNOCK-OUT MICE

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Tonic activity of the opioid system may be important for the maintenance of hedonic homeostasis. Naloxone is well-known to produce aversion in drug-naïve subjects, and our previous data from mu- knock-out mice demonstrate a vital role for the mu-opioid receptor in this regard. The simplest explanation for naloxone's aversive effect is that it is blocking the action of an endogenous ligand that normally upholds a certain level of hedonic state. Likely candidate peptides are beta-endorphin and enkephalins, given their affinity for the mu-receptor, rewarding properties when administered exogenously, and dense distribution in sites known to be important for this behavior. Here we present data from our study of transgenic mice selectively deficient in beta-endorphin or enkephalin. Whereas beta-endorphin knock-out mice retain naloxone conditioned place aversion (10 & 1 mg/kg), naloxone becomes ineffective in enkephalin knock-out mice (10 mg/kg). These data suggest a crucial role for the action of tonically released enkephalin peptides in sustaining hedonic homeostasis. Supported in part by NIDA grants DA05010 and DA09359; P.D.S was supported by NIDA Training Grant T32DA07272 and the Hatos Center for Neuropharmacology.

S60

DAMGO-STIMULATED MU-OPIOID RECEPTOR ACTIVATES PHOSPHOLIPASE D2 IN AN ARF-DEPENDENT MANNER

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The rat mu-opioid receptor (rMOR1) couples to a variety of downstream effectors including adenylate cyclase, phospholipase C, and mitogen activated protein kinase. Using a yeast two-hybrid method, the rMOR1 was found to interact with the NH2-terminus of the phospholipase D2 (PLD2), a phospholipid-specific phosphodiesterase located in the plasma membrane. The NH2-terminal interaction site represents the Phox homology (PX) domain of the PLD2. The association between rMOR1 and a full length PLD2 was confirmed by coimmunoprecipitation experiments with stably cotransfected HEK293 cells and was shown to be agonist-independent. However, in the rMOR1/PLD2 expressing HEK293 cells treatment with DAMGO led to an increase in the activity of PLD2, which was blockable by naloxone. Moreover, the DAMGO-mediated activation could be inhibited by brefeldin A, an inhibitor of ADP-ribosylation factor (ARF) but not by the PKC inhibitor calphostin, indicating that receptor-mediated activation of PLD2 is ARF-dependent but not PKC-dependent. Consistent with these findings, we observed an enhanced coimmunoprecipitation of rMOR1 with ARF after DAMGO treatment.

S61

CHARACTERIZATION OF ZFOR3, A NEW PUTATIVE OPIOID RECEPTOR FROM THE TELEOST ZEBRAFISH

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The teleost Zebrafish (*Danio rerio)* has recently been named as the canonical vertebrate due to its usefulness as a research tool for investigating development, genomics, genetics and human disease. The characterization, from this fish, of a new clone, ZFOR3 (EMBL accesion nº AF285173), with high similarity to the mammalian kappa opioid receptor is presented here. Stable HEK293 cell expression yielded naloxone blockable binding for the non-selective ligand diprenorphine (Bmax=72pmol/mg protein; Kd= 1.6nM), for the mu selective ligand DAMGO (Bmax=227pmol/mg protein; Kd=20.8nM) and for the kappa selective ligand U69593 (Bmax= 72 pmol/mg protein , Kd=9.9nM). There was no appreciable recognition of the specific delta ligand DPDPE. To determine whether the receptor encoded by ZFOR3 can transduce a physiological signal the agonist activity of several opioid compounds was evaluated in the 35SGTPgammaS assay. Greater stimulation was seen with D-Arg-Dyn A (79.5%), although other agonists also produced a positive effect. ISH studies showed that ZFOR3 mRNA is expressed at high levels in all subdivisions of the CNS of the zebrafish. These results will also be compared with data from other zebrafish opioid receptor-like recently cloned by our group.

S62

RECEPTOR BINDING AND FUNCTIONAL STUDIES ON NOVEL SYNTHETIC NOCICEPTIN PEPTIDE ANALOGS

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Several synthetic nociceptin derivatives were prepared and studied in receptor binding assays, functional tests and on mouse *vas deferens* bioassays. In binding experiments the compounds exhibited moderate to high affinity in competing the binding of the [3H]nociceptin ligands to rat brain membranes. A new putative antagonist structure, Ac-RYYRIK-ol was described in the mouse *vas deferens* tests, showing a Ke of 2 nM, and no agonist effect at 10 microM ligand concentration. The Schild plot indicated a clearly competitive interaction. G-protein activation by some of the peptides was further measured in [35S]GTPgammaS binding assays using native tissue preparations and membranes from cultured CHO cells expressing nociceptin receptors. Ac-RYYRIK-ol was only weak stimulator alone, but it inhibited the stimulation induced by nociceptin. Because Ac-RYYRIK-ol displayed high potency in the binding assays and in the pharmacological tests, this new compound can serve as pure, competitive antagonist at the nociceptin receptor. Supported by the Hungarian Scientific Research Fund OTKA T-035211, T-033078, T-030841, and the Ministry of Education, NKFP 1/027 Hungary.

POSTER SESSION 1
Wednesday July 10, 19:30 – 21:00

I. BEHAVIOR

1

DIFFERENTIAL SENSITIVITIES OF MOUSE STRAINS TO MORPHINE AND [DMT[1]]DALDA ANALGESIA

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The dermorphin-derived peptide [Dmt[1]]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2), is a highly potent and selective mu-opioid agonist. It displays a pharmacological profile distinct from morphine, as evidenced by lack of cross tolerance to morphine and an insensitivity to MOR-1 antisense probes that reduce morphine analgesia. Moreover, the binding of [^{3}H][Dmt[1]]DALDA is insensitive to divalent cations, Na^+, and GTP analogs. The following study was undertaken to determine the abilities of [Dmt[1]]DALDA and morphine to produce analgesia in genetically different strains of mice, including mice lacking exon1 of MOR-1. We show that in contrast to morphine, s.c [Dmt[1]]DALDA retains its sensitivity in mu$^{-/-}$ animals. In addition, s.c and i.c.v administered [Dmt[1]]DALDA produce analgesia in morphine-insensitive CXBK mice. In C57BL/6J mice, s.c [Dmt[1]]DALDA analgesia was 10-fold less potent than in CD-1 mice, whereas s.c morphine analgesia did not differ. In conclusion, [Dmt[1]]DALDA clearly produces analgesia via a unique mechanism of action and further illustrates the differing sensitivities of various mouse strains to opioid analgesia.

4

ALKALOIDS FROM *BRUGMANSIA ARBOREA* (L.) LAGERHEIN REDUCE MORPHINE WITHDRAWAL *IN VITRO*

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Traditional medicine is a primary source for the study of medicinal plants. In some countries, the knowledge about the therapeutical use of medicinal plants is very deep and very often "magical" plants also are used to diagnose and treat illnesses. The study of these plants can help in the research of metabolites active on central and peripheral nervous system.

Brugmansia arborea (L.) Lagerheim (Solanaceae) is used in the northern Peruvian Andes for magic-therapeutical purposes and the present study examined the effect of three pure tropane alkaloids from *Brugmansia arborea* (L.) Lagerhein (Solanaceae) on the morphine withdrawal in vitro. All the tropane alkaloids isolated from *Brugmansia arborea* (L.) (10^{-7}-$5x10^{-7}$-10^{-6} M) significantly and in a concentration dependent manner reduced the morphine withdrawal indicating that the above alkaloids of *B. Arborea* exert important control on morphine withdrawal phenomena.

The results of the present study suggest that these alkaloids may be a potential anti-addictive agent and further studies are necessary to better elucidate the mechanism underlying this interaction.

7

NMDA RECEPTOR ANTAGONISTS PREVENT AND SLOWLY REVERSE OPIATE-INDUCED BEHAVIORAL AND NEURAL PLASTICITY

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Evidence suggests that N-methyl-D-aspartate (NMDA) receptors have an important role in the development of opiate tolerance and sensitization. The present studies extended this work by examining the ability of the NMDA receptor antagonist MK-801 to prevent and reverse opiate tolerance and sensitization in rats. When MK-801 (0.1 mg/kg) was administered prior to morphine (10 mg/kg) each day, it inhibited the development of tolerance and sensitization to the locomotor effects, as well as tolerance to the analgesic effect. When MK-801 was administered prior to the locomotor stimulant phase of morphine (10 mg/kg) each day, it selectively inhibited the development of sensitization. MK-801 also inhibited the development of sensitization to the stimulant effect of a lower dose of morphine (3.0 mg/kg). When the NMDA receptor antagonist was administered with morphine (10 mg/kg) over a series of days to tolerant animals, it slowly reversed tolerance to morphine analgesia. The results suggest that NMDA receptors are involved in several different types of opiate-induced behavioral and neural plasticity. [Supported by NIGMS (GM59833)].

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SENSITIZATION TO THE LOCOMOTOR STIMULANT EFFECT OF MORPHINE

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Chronic administration of several drugs of abuse leads to sensitization to their stimulant effects. Sensitization is thought to be responsible for the escalating desire for drugs in addicts (craving), and therefore of key importance to the development of addiction. Although sensitization to psychomotor stimulants has been widely studied, sensitization to opiates has been less well-characterized. Based on studies with stimulants, it is believed that sensitization develops following repeated intermittent injections, but not chronic infusions. The present studies examined sensitization to the locomotor stimulant effect of morphine, using a variety of different experimental protocols. As expected, sensitization developed to both a low dose (3.0 mg/kg) and a high dose (10 mg/kg) of morphine, when a repeated dosing protocol was used (once daily injections). However, sensitization also developed when a chronic infusion protocol was used (75 mg morphine pellets). The results suggest that opiate sensitization may differ from psychomotor stimulant sensitization, in that it is induced by both repeated injections and chronic infusions. [Supported by NIGMS (GM59833)].

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BUPRENORPHINE SUBSTITUTION IN THE TREATMENT OF MORPHINE-DEPENDENT RAT PUPS

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Infants receiving ECMO or mechanical ventilation require continuous infusions of morphine and exhibit signs of withdrawal upon cessation of morphine. Currently, the morphine dose is tapered over 2- to 3- weeks to avoid withdrawal. Studies on dependent adults demonstrate that buprenorphine effectively reduces opiate withdrawal. Alzet 1003D osmotic minipumps were implanted into the subcutaneous space of post-natal day 14 rats to deliver morphine at 2 mg/kg/h. After 72-h, the effectiveness of single and repeated doses of buprenorphine was assessed in rats undergoing spontaneous withdrawal. Each group was observed for 30-min at specific time-points for 5 days. Signs of wet-dog shakes, abdominal stretches, and forepaw tremors were counted, and signs of splayed hind limbs, ptosis, and evoked vocalization were noted as either absent or present. Vehicle-injected rats exhibited a robust spontaneous withdrawal from morphine. A single dose of buprenorphine (1 mg/kg) administered 30-min before pump removal reduced the early signs of morphine withdrawal. Repeated doses of buprenorphine suppressed withdrawal throughout the entire 5-day period.

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TAN-67 IMPROVES RATHER THAN INTERUPTS VAGAL BRADYCARDIA

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To determine the opiate receptor subtype through which met-enkephalin-arginine-phenylalanine (MEAP) inhibits vagal bradycardia, anesthetized dogs were instrumented with a microdialysis probe in the sinoatrial (SA) node. Previous data indicated that the delta-2 agonist, deltorphin, blocked the bradycardia produced by vagal stimulation while the delta-1 agonist, DPDPE, did not. To confirm the absent delta-1 response, the more selective delta-1 agonist, TAN-67 was tested. Heart rate, frequency-responses were constructed by stimulating the right vagus nerve at 1, 2, and 3 Hz. Sequential doses of TAN-67 were infused into the nodal interstitium. The frequency response was re-tested after 5 min of exposure to each dose. The probe was flushed with vehicle and allowed to reequilibrate after each test. TAN-67 had no vagolytic effect at any dose. In contrast, TAN-67 consistently improved vagal bradycardia. The improved vagal function was reversed by the delta-1 antagonist, BNTX. These data support the hypothesis that MEAP interrupts vagal bradycardia through delta-2 opiate receptors on parasympathetic terminals within the cardiac pacemaker and that nearby delta-1 receptors are vagotonic.

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MU OPIOID RECEPTOR KNOCKOUT MICE HAVE ALTERED BEHAVIORAL RESPONSES TO COCAINE

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This study investigated the importance of mu opioid receptors (MOR) in cocaine's actions by examining locomotor activity and behavioral sensitization in MOR knockout mice and in wild-type mice treated chronically with naltrexone. Mice received single daily injections of saline or 15 mg/kg cocaine for 10 days. All mice received a cocaine challenge on day 17. On days 1 and 10, the locomotor-stimulating effect of cocaine seen in +/+ and +/- mice was absent in the MOR null -/- mutants. Daily cocaine exposure sensitized both +/+ and +/- mice to the locomotor-activating effects of cocaine on day 17. Sensitization was greatly attenuated in the -/- mice. Similar results were found in C57Bl/6J mice pretreated with naltrexone (10 mg/kg/day) delivered continuously by osmotic minipumps. Naltrexone pretreated mice showed attenuated responses to cocaine-induced activation and sensitization. These data demonstrate that MOR gene deletion or mu receptor blockade suppresses the locomotor-activating effects of cocaine and the expression of behavioral sensitization.

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UP-REGULATION OF PGE_2-RECEPTOR, BUT NOT PGI_2- AND OPIOID-RECEPTORS, IN THE MOUSE SPINAL CORD FOLLOWING PERIPHERAL INFLAMMATION

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We previously found that the expression of COX-2 mRNA was markedly increased in the spinal cord obtained from mice with intraplantar injection of complete Freund's adjuvant (CFA). In addition, the CFA-induced thermal hyperalgesia was significantly suppressed by chronic intrathecal treatment with a selective COX-2 inhibitor etodolac. The present study was then to examine the ability of PGE_2, PGI_2 and endogenous opioid peptides to activate G-proteins using [^{35}S] $GTP_{gamma}S$ binding assay. CFA-injected mice revealed the enhancement of the increased [^{35}S] $GTP_{gamma}S$ binding induced by PGE_2, but not PGI_2, in spinal cord membranes. In contrast, there were no differences in [^{35}S] $GTP_{gamma}S$ binding induced by endomorphins, [Met]^5-enkephalin, dynorphin A (1-17) and beta-endorphin in the spinal cord between CFA- and saline-injected mice. The present data provide evidence that the specific up-regulation of PGE_2 receptor function to activate G-proteins is implicated in thermal hyperalgesia caused by CFA injection in mice. Now, we are investigating whether chronic morphine treatment could affect the PGE_2- and PGI_2- stimulated $GTP_{gamma}S$ binding.

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ELECTROACUPUNCTURE-INDUCED ACTIVATION OF ENDOGENOUS ANTI-ANALGESIC SYSTEM IN RATS

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Our previous study indicated that subcutaneous morphine analgesia was significantly attenuated following electroacupuncture (EA), suggesting the existence of anti-analgesic system activated by EA. We examined the effects of various EA-morphine injection intervals and routes of morphine administration, and of N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 on EA-induced activation of anti-analgesic system. EA (3 Hz, 0.1-msec duration for 45 min) was applied to acupoints, ST-36 or LI-4, and pain thresholds were estimated by the hind-paw pressure test in male SD-rats. The attenuation of subcutaneous morphine analgesia following EA was inversely proportional to the time-interval between EA and morphine injection. Intrathecal, but not intracerebroventricular injection of morphine following EA showed significant decrease in analgesic effect. The attenuation of morphine analgesia following EA was not affected by the pretreatment with MK-801 though EA-induced analgesia was suppressed. These results suggest that the anti-analgesic system activated by EA exists in the spinal cord and NMDA receptor may not be involved in the activation of this system.

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PHARMACOLOGICAL CONSEQUENCES OF MUTAGENESIS OF A CONSERVED ASPARTIC ACID RESIDUE IN THE MU OPIOID RECEPTOR

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An aspartic acid residue in the second transmembrane domain is highly conserved among G-protein coupled receptors. To investigate the role of aspartate 114 in the rat mu opioid receptor (MOR), this residue was mutated to asparagine (D114N), and the wild-type (WT) and D114N-MORs were stably expressed in HEK-293 cells. The D114N mutation reduced the affinity and abolished Na+ sensitivity of opioid agonist binding, and greatly decreased the efficacy of most opioid agonists for G-protein activation. However, 4-anilidopiperidine derivatives retained high efficacy at the D114N-MOR. The D114N-MOR was insensitive to up- or down-regulation of receptor levels following chronic treatment with various opioid ligands, although chronic treatment with full agonists produced D114N-MOR desensitization. These data demonstrate that the D114N mutation produces a hypoactive MOR, with dramatic consequences to the acute and chronic actions of most opioid drugs. However, these data also suggest that the D114 residue is not critical to MOR activation by 4-anilidopiperidines.

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II SIGNAL TRANSDUCTION

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SEVERAL DELTA LIGANDS DISPLAY NO SUBTYPE SELECTIVITY IN CELLS STABLY TRANSFECTED WITH THE HUMAN DELTA OPIOID RECEPTOR.

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Pharmacological studies performed *in vivo* suggested that the delta opioid receptor could exist as two distinct subtypes, delta1 and delta2, while *in vitro* studies are inconclusive. Therefore, we measured the binding and functional selectivity of several putative delta1-and delta2-selective compounds in membranes from Chinese hamster ovary cells stably expressing the human delta opioid receptor. The compounds characterized were the agonists DPDPE (delta1) and deltorphin II (delta2), and the antagonists 7-benzylidenenaltrexone (delta1), naltriben (delta2), 5'-naltrindole-isothiocyanate (delta2), and naltrindole (delta1 and delta2). In competition binding assays, all compounds tested showed no preference for the [^{3}H]DPDPE, [^{3}H]deltorphin II, or [^{3}H]naltrindole sites. BNTX also showed no delta-mu selectivity. In functional assays, the stimulation of [^{35}S]GTP-gamma-S binding induced by either DPDPE and deltorphin II was potently inhibited by both delta1– and delta2–selective antagonists. Together, these results indicate that these compounds are not selective for the delta1 or delta2 sites in binding or functional assays.

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INVOLVEMENT OF RAF-1 KINASE IN CHRONIC DELTA OPIOID AGONIST MEDIATED ADENYLYL CYCLASE SUPERACTIVATION.

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Chronic delta opioid agonist (SNC 80, 1 μM, >4h) treatment of CHO cells stably expressing the human delta opioid receptor (hDOR/CHO) leads to increased cAMP formation after the removal of the inhibitory agonist (AC superactivation). Previously we demonstrated that chronic SNC 80 treatment (1 μM, >4 h) of the hDOR/CHO cells leads to a 2.5-fold increase in the phosphorylation of the adenylyl cyclase VI isoenzyme. Phosphorylation of AC VI was SNC 80 dose- and pretreatment time-dependent and was antagonized by naltrindole (1 μM). Phosphorylation and activation of AC VI by Raf-1 protein kinase was recently demonstrated. Consequently we tested the effect of the Raf-1 kinase inhibitor, GW5074, on delta opioid agonist-mediated signaling in hDOR/CHO cells. We found that GW5074 pretreatment (10 μM, 30 min) attenuated both delta opioid agonist-mediated MAPK phosphorylation and AC superactivation. Preliminary data indicate that at least two parallel pathways may be simultaneously involved in delta opioid agonist mediated Raf-1 activation in hDOR/CHO cells. (Supported by grants from ADCRC and NIDA)

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ACTIVATION OF THE MU OPIOID RECEPTOR INCREASED PHOSPHORYLATION OF THE p38 MAP KINASE IN CHO CELLS.

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Stimulation of the rat μ opioid receptor stably expressed in CHO cells enhanced phosphorylation of p38 mitogen activated protein kinase (MAPK) in a naloxone-sensitive and dose-dependent manner. DAMGO-induced enhancement was biphasic with the first peak at 5 min and the second at ~1 h. Morphine and DAMGO exhibited a shorter duration of p38 phosphorylation than did methadone and buprenorphine. Pertussis toxin partially inhibited p38 phosphorylation, although it completely inhibited p42/44 phosphorylation. Neither the p42/44 nor p38 MAPK phosphorylation was sensitive to either beta/gamma scavenger proteins or the PI3K inhibitor wortmannin. Wortmannin, even at a dose as low as 10 nM, increased p38 MAPK phosphorylation. The PKC inhibitor R031824, which failed to inhibit p42/44 phosphorylation, inhibited morphine-, but not DAMGO-induced p38 phosphorylation. The p38 phosphorylation was partially sensitive to MEKK1 inhibitor U0126 at doses which inhibited p42/44 MAPK phosphorylation. Hence mu opioids regulate p38 MAPK pathways, which are important in cellular growth, differentiation and adaptation. (supported by NIDA grants)

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MORS AND MORPHINE TOLERANCE

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MORs plays an important part in morphine tolerance and dependence. However, the mechanisms are not well understood. In the present study, autoradiographic analysis of MOR binding and receptor-activated G-proteins were used to assess the MOR density and G-protein activity in different brain regions of rats tolerant to and dependent on morphine. Rats were chronically administrated morphine i.c.v. and analgesia was measured by tail-flick for 7 days. On day 7 rats were given naloxone to precipitate withdrawal. All rats developed tolerance to morphine and had significant withdrawal signs. [^{3}H]-DAMGO binding, agonist-stimulated [^{35}S]-GTPgammaS binding and immunohistochemistry was used to assess receptor density, function and distribution. Several brain regions, such as striatum, NAc, hippocampus, amygdala, VTA and PAG were evaluated. No significant differences of [^{3}H]-DAMGO between morphine-treated rats and controls were observed; however, in most of those regions, morphine-stimulated [^{35}S]-GTPgammaS binding was decreased relative to controls. Therefore, our results indicated that decreases in receptor G-protein coupling, not the receptor density, contribute to the development of morphine tolerance and dependence.

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CHRONIC MORPHINE-INDUCED CHANGES IN MU OPIOID RECEPTOR- COUPLED SIGNAL TRANSDUCTION *IN VITRO*

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We assessed forskolin stimulated adenylyl cyclase (AC) activity in CHO cell membranes stably transfected with rat mu opioid receptors (MOR-CHO). While 10^{-6} M DAMGO inhibited AC activity by 40% in opioid naive cells, its effect decreased by about 50% after 48 hrs morphine treatment indicating the development of tolerance. The mu opioid antagonist CTAP abolished DAMGO inhibition in opioid naive, but produced a significant cAMP 'overshoot' in tolerant membranes. Pertussis toxin treatment revealed AC stimulation by DAMGO in both opioid naive and tolerant membranes. However, QEHA (AC 2 956-982), a Gbetagamma. blocker reversed this DAMGO stimulation of AC activity to a significant inhibition only in preparations rendered tolerant. These data suggest that chronic morphine exposure unmasks and/or induces stimulatory mu opioid signaling via Gbetagamma derived from non Gi/Go-coupled mu opioid receptors. This might involve AC isoforms different from those modulated by acute morphine. Supported by NIH-DA12251 (A.R.G.) and OTKA T-33062 (M.S.) research funds.

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MAP KINASE MODULATES MU OPIOID DESENSITIZATION IN SENSORY NEURONS.

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The mu agonist DAMGO inhibits voltage-gated Ca^{2+} channels in sensory neurons. This effect diminishes during prolonged agonist exposure. We reported previously that blocking PI3 kinase attenuated DAMGO desensitization in mouse dorsal root ganglion (DRG) neurons. This study further investigated the role of MAP kinase, which can be activated by PI3 kinase, in DAMGO desensitization. Whole-cell Ca^{2+} currents were recordings from small to medium sized DRG neurons in cultures. Acute application of DAMGO (1 microM) induced rapid reduction of Ca^{2+} currents in untreated DRG neurons (47+/-3%, n=57), but produced much less current inhibition in neurons pretreated with DAMGO for 1, 4 and 24 hr (17+/- 4%, 17+/- 2 % and 7+/-2%, N = 10-13). Co-pretreatment with a MAP Kinase inhibitor PD98059 (10 microM) partially reversed DAMGO desensitization. In neurons pretreated with both drugs for 1, 4 and 24 hr, acute DAMGO reduced Ca^{2+} currents by 35+/-7 %, 30 +/-3% and 19+/-3%, respectively (N=7-10, $P<0.05$ for all time points as compared to DAMGO alone). Thus, MAP kinase may be one of the key components in the signaling pathways underlying mu opioid desensitization in DRG neurons.

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NALOXONE INDUCES MAPK ACTIVATION IN AMYGDALA AND BNST IN WILD-TYPE BUT NOT ENKEPHALIN-KO MICE

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In drug naive mice naloxone is aversive, and recent data demonstrate that mice lacking proenkephalin (enk-ko) show no naloxone-aversion (Skoubis et al, INRC 2002 abstract). Naltrexone induces Fos-like immunoreactivity in the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST) and the nucleus accumbens shell (NAcSh). Recent evidence suggests that the extended amygdala including the NAcSh, BNST, and the CeA, plays a role in opiate dependence and in reinstatement of drug seeking behavior. In this study we report that acute administration of naloxone induces MAPK activation in the CeA and the BNST in drug naive mice. Moderate activation was also observed in the lateral and basolateral amygdala. We then examined the effect of naloxone on MAPK activity in enk-ko mice. An elevated basal level of MAPK activity was observed in the CeA in the enk-ko mice, but naloxone failed to further activate MAPK. Additionally, naloxone did not activate MAPK in the BNST of enk-ko animals. The correlation between naloxone-induced behavior and MAPK activation suggests a role for MAPK signaling in enkephalin-regulated hedonic tone.

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ISOLATION AND CHARACTERIZATION OF A NEW SPLICE VARIANT, mMOR-1R, OF THE MOUSE MU-OPIOID RECEPTOR GENE

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Our previous studies has identified and characterized fourteen splice variants of the mouse mu-opioid receptor (MOR-1) gene. Here we report isolation of an additional mouse MOR-1 splice variant, mMOR-1R, using an RT-PCR strategy. Sequence analysis indicates that mMOR-1R contains another new exon, exon 15, with an exon composition of 1, 2, 3 and 15. Exon 15 has been mapped to downstream of the exon 3 with similar location as the fourth exon of hMOR-1R. Exon 15 is predicted to encode 70 amino acids in the C-terminal of the mMOR-1R, which contains putative phosphrylation sites for both cAMP- and cGMP-dependent protein kinase and protein kinase C. Northern blot analysis of mouse brain total RNA using an exon 15 probe showed a distinct band, approximately 1.8 kb. Expression of the mMOR-1R in mouse brain was also determined by Western blot analysis with a specific mMOR-1R antibody. Pharmacological studies in CHO cells stably transfected with the mMOR-1R cDNA suggested that mMOR-1R encodes a mu-opioid receptor. This work is supported by DA00296 (Y.-X. P.) and DA02615 and DA02615 (G.W.P.).

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STUDIES ON TWO G PROTEIN COUPLED RECEPTOR MOTIFS PRESENT IN TM 6 OF THE MU OPIOID RECEPTOR

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Molecular modeling and sequence homology analyses of G protein coupled receptors (GPCRs) and channel membrane proteins by Weinstein and colleagues have previously identified "TP" and "aromatic cluster" (FX3WX3F) structural motifs; both motifs are present with subtle or no variation in TM 6 of the mu opioid receptor (mOR). A threonine preceding a conserved TM proline residue ("TP") has been shown to enhance the proline-induced kink in channel protein α-helices, with functional consequences upon its alteration. Aromatic residues aligned on the same α-helical, ligand accessible face of TM 6 of the 5HT2A receptor apparently promote agonist-mediated signaling via a concerted conformational shift. We have addressed the role of each motif in the mOR via site-directed mutagenesis. The TM 6 "TP" motif was unexpectedly tolerant to threonine modification, as replacement of this sequence with "AP" only mildly affected the binding, potency and efficacy of the several ligands tested. The C-terminal phenylalanine (6.52) of the 5HT2A receptor TM 6 aromatic cluster is replaced with histidine in the mOR (FX3WX3H), the position proposed in the Weinstein model as a ligand-triggered "toggle switch" for receptor activation. Mutation of several positions within this mOR motif altered the intrinsic efficacies of opiate alkaloids, and the nature of the H6.52 substitution influenced ligand-mediated activation of the mOR. Our results are consistent with predictions regarding the aromatic cluster motif, but the TP motif does not appear to play the critical role identified in other GPCRs. Thus, the roles of some structural motifs in the mOR may be more complex than previously thought and may depend on local structural differences that require further elucidation.

III. RECEPTOR TRAFFICKING

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HETERODIMERIZATION OF MU-OPIOID RECEPTOR AND SUBSTANCE P RECEPTOR. A ROLE IN RECEPTOR TRAFFICKING

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Several recent studies suggest that heterodimerization can modulate the ligand binding, signaling and trafficking properties of G protein-coupled receptors. We have previously shown that heterodimerization of the mu-opioid receptor (MOR1, MOP) and the sst2A somatostatin receptor selectively cross-modulates phosphorylation, internalization and desensitization of these closely related receptors. Given the fact that the μ-opioid receptor (MOR1, class A receptor) and the Substance P receptor (NK1, class B receptor) coexist in pain processing pathways, we examined dimerization of these distantly related receptors in stably transfected HEK 293 cells. In co-immunoprecipitation studies, we provide direct evidence for heterodimerization of MOR1 and NK1. Although heterodimerization did not substantially change ligand binding and signalling, it dramatically altered the internalization properties of these receptors. Exposure of the MOR1-NK1 heterodimer to either DAMGO or Substance P promoted co-internalization of both MOR1 and NK1 suggesting that physical interaction may cross-modulate surface expression of these receptors.

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AGONIST EFFICACY AND THE ROLE OF BARRESTIN-2 IN MU OPIOID RECEPTOR REGULATION

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G protein-coupled receptor regulation by GRKs and beta-arrestins leads to receptor desensitization. While it appears that beta-arrestins do not play a major role in regulating MOR responsiveness in cell culture, removal of even one allele of the beta-arrestin2 (Barr2) gene in mice leads to enhanced and prolonged MOR-mediated antinociception. These mice do not develop morphine hot-plate antinociceptive tolerance suggesting that the Barr2 protein plays an essential role in MOR regulation *in vivo*. These observations suggest a paradoxical role of Barr2. In this study, the contribution of Barr2 to the regulation of the MOR was examined in both HEK293 cells and in Barr2-KO mice following treatment with several opiate agonists. A green fluorescent protein (GFP) tagged receptor was used to track MOR internalization in living cells and the interaction of Barr2 with the receptor was visualized by Barr2-GFP translocation. Opiate agonists that induced rapid internalization and Barr2-GFP translocation, produced similar analgesia profiles in WT and Barr2-KO mice. It appears that the agonist can determine the contribution of Barr2 to the regulation of the receptor. *DA-02749 LAD, DA-14600 LMB, & DA-13511 MGC.*

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DIFFERENT REGULATION OF HUMAN DELTA OPIOID RECEPTORS (hDOR) BY ENKEPHALINS AND SNC80

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We previously reported a differential regulation of hDOR endogenously expressed in the human neuroblastoma SK-N-BE cells by peptide and alkaloid agonists. We extend this study to other opioid agonists. Binding experiments using Leu and Met-enkephalin and a synthetic agonist SNC80 revealed the presence of two binding sites with high Ki values. They promoted inhibition of cAMP accumulation with similar efficacy and potency. Differential time of exposure with SNC80 (30minutes) and enkephalins (2h) were needed to promote a similar level of desensitization. Resensitization experiments showed that after Leu and Met-enkephalin exposure, hDOR displayed a stronger ability to re-inhibit adenylyl cyclase than SNC80 suggesting a differential trafficking. Relationships between desensitization and internalisation were studied using sucrose 0.5 M and we showed that sequestration was partially responsible for desensitization. Taken together our data suggest that the differential hDOR regulation would rely on the selectivity of the agonists rather than their chemical nature and the molecular mechanism of hDOR desensitization by these agonists are underline study.

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ETORPHINE AND LEVORPHANOL BLOCK DYNORPHIN A- AND U50,488H-INDUCED INTERNALIZATION OF THE HUMAN KAPPA OPIOID RECEPTOR

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Following agonist exposure, most GPCRs undergo internalization. We previously observed that U50,488H promoted internalization of the human kappa opioid receptor (hkor) stably expressed in CHO cells using radioligand binding method. In this study, using fluorescence flow cytometry and immunofluorescence staining, we found that dynorphin A, like U50,488H, promoted internalization of the Flag-tagged hkor in a time- and dose-dependent manner. The antagonists naloxone and nor-BNI had no effect on Flag-hkor internalization and effectively blocked dynorphin A- and U50,488H-induced internalization. Interestingly, the full agonists etorphine or levorphanol did not cause internalization of the Flag-hkor, but significantly reduced dynorphin A- or U50,488H -induced internalization in a dose-dependent manner. Treatment with 100 nM etorphine or 10 uM levorphanol shifted the internalization dose response curve of dynorphin A and U50,488H to the right. It is likely that conformations of the hkor required for activation of G proteins are different from those for initiation of internalization. (supported by NIDA grants)

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RELATIONSHIP BETWEEN MORPHINE TOLERANCE AND MU RECEPTOR DESENSITIZATION

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Chronic morphine treatment produces tolerance and brain region-specific desensitization of mu receptors. These studies characterized the relationship between the degree of tolerance and the regional distribution and magnitude of desensitization. Three groups of mice were used: 1. placebo pellet, 2. morphine pellet, 3. morphine pellet + supplemental morphine injection every 12 hours. After 3 days, mice were tested for antinociception using the tail flick assay or sacrificed for [^{35}S]GTPgammaS autoradiography. Morphine pelleted mice showed an 8-fold increase in the ED50 value of morphine to produce antinociception, whereas morphine pellet + supplemental morphine-treated mice showed a 50-fold increase. DAMGO-stimulated [^{35}S]GTPgammaS binding was reduced in most regions of mice that exhibited 50-fold tolerance compared to vehicle, and this decrease was significant in caudate-putamen, parabrachial nucleus and nucleus of the solitary tract. The level of DAMGO-stimulated [^{35}S]GTPgammaS binding in 8-fold tolerant mice was intermediate between vehicle-control and 50-fold tolerant mice, and did not differ significantly from either group in any region. These results suggest that greater tolerance to morphine may be associated with greater desensitization of mu receptor-mediated G-protein activity.

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REGIONAL DIFFERENCES IN RECEPTOR MULTIMERIZATION

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Several labs have recently reported that G-protein coupled receptors can form either monomers, dimers, or multimers. However, there appears to be considerable variability in the relative amount of the various forms found in the different laboratories. To investigate this, and to rule out variations in the sample, a cell line expressing mu and delta opioid receptors was frozen in several aliquots, and these aliquots were taken to different laboratories where the amount of each form was determined. In Ann Arbor, Michigan, the major form was found to be a stable heterodimer. Although dimers were also found to predominate in San Francisco, the dimers in this location were largely homodimers. When assayed in Los Angeles, both homo and heterodimers were found along with higher oligomerization states (trimers, tetramers, and more). Interestingly, these higher oligomerization states predominated when the temperature was increased to 42° in the presence of high concentrations of alcohol (termed the "hot-tub" conditions). Finally, when tested in New York City, no results could be obtained due to the rapid degradation of the protein in this environment. Taken together, these results indicate that the variation observed by the different research groups is due to an environmental factor that affects the oligomerization state of the receptor, and not to differences in the preparation. It is further hypothesized that the environmental factor(s) that influences the state of the receptor also influences human behavior. Further studies are in progress.

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DESENSITIZATION OF THE HUMAN DELTA OPIOID RECEPTOR: INVOLVEMENT OF DIFFERENT KINASES.

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In a previous study, we observed a differential regulation of human delta opioid receptor (hDOR) endogenously expressed in the neuroblastoma cell line SK-N-BE. Indeed, upon peptide agonists treatment (DPDPE or deltorphin I), we noticed a strong desensitization, observed on adenylate cyclase inhibition, compared to a alkaloid agonist, etorphine.

So, we speculated that this difference could be related to a differential recruitment in kinase involved in desensitization. This hypothesis was assayed either by using chemical inhibitors of PKA, PKC, CaMKinase or tyrosine kinases, or by transfecting the cDNA of the mutant negative dominant of GRK2. The effects of this different inhibitors was measured by functional studies on desensitization involve either by etorphine or peptide agonists. We observed that PKC, GRK2 and to a lesser extent tyrosine kinases contribute to etorphine-induced desensitization of hDOR. For peptide agonists, DPDPE and deltorphin I, only tyrosine kinase seems to be involved in desensitization. We can conclude that one or more kinase could be implicated in hDOR desensitization and that its depends on the agonist used.

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HSP 40 FAMILY MEMBER HLJ 1 BINDS TO THE CARBOXYL TERMINAL OF THE HUMAN MU OPIOID RECEPTOR (HMOP)

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A yeast two-hybrid screen, using the carboxyl tail (aa 333-400) of the human mu opioid receptor (hMOP) as bait and a human brain library as target, indicated that the carboxyl tail (aa 227-337) of the human Hsp 40 family member HLJ 1, interacts with the receptor C-tail. This finding was confirmed by obtaining positive colonies, using the cDNA of the C-tail of hMOP as bait and that of HLJ 1 C terminal as target in a two-hybrid screen. To determine if direct *in vitro* binding occurs between these two proteins, overlay experiments were performed, using the GST fusion protein of the receptor C tail (GST-C) and a His fusion protein of the C terminal of HLJ 1. Using an antihistidine antibody, positive results were obtained in the lanes with GST-C but not in the lanes containing, as controls, a GST-fusion protein of hMOP third cytoplasmic loop or GST alone. We are currently assessing co-localization of HLJ 1 and hMOP by fluorescence confocal microscopy. It is well known that HSP 40 family members, complexed with HSP 70 and other heat shock proteins, are chaperones involved in protein folding and play a role in protein trafficking. The function of this protein in opioid receptor function and/or regulation is under investigation. (supported by Grants DA00017 and KO5-DA00364 to EJS and a NIDA Postdoctoral Fellowship to NAT from Training Grant DA-07254).

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TRAFFICKING OF OPIOID RECEPTORS IN MESOLIMBIC NEURONS

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Multiple cellular mechanisms of opioid tolerance have been proposed, many of them focusing on endocytosis and recycling of opioid receptors. Less attention has been paid to the initial trafficking of receptor protein from trans-Golgi network to plasma membrane. We have developed a primary neuronal culture system that allows imaging of the trafficking of receptor protein from the perinuclear soma to the plasma membrane. Given the salience of the mesolimbic system in opioid reward, cultures are taken from ventral tegmental area (VTA) and nucleus accumbens. VTA tissue from embryonic (E17) rats and accumbens tissue from neonatal (P1) rats are dissected from coronal slices, dissociated enzymatically and mechanically, and plated on coated coverslips. These cultures have been characterized by morphological and immunohistochemical evaluation. At 7 to 10 days in culture, cells are transfected with fluorescently-tagged mu (MOR1) or delta (DOR1) opioid receptor DNA. The progress of receptor protein can be visualized by either static or live fluorescence microscopy. Determining the kinetics of trafficking through analysis of live imaging will allow comparison of control and drug-exposed conditions.

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OPIOID RECEPTOR SELECTIVE MONOCLONAL ANTIBODIES.

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The isolation of opioid receptor cDNAs has enabled a number of studies characterizing their biochemical properties. The majority of these studies have used antisera against the epitope-tagged receptor expressed in heterologous cells. This is mainly due to the lack of sensitive and selective antisera that are able to recognize the endogenous receptors in the native form. In order to facilitate studies that enable detection of receptors from endogenous sources as well as in live cells we generated monoclonal antisera using peptides derived from the N-terminal region of mouse mu, delta or kappa receptors as immunogens. These antisera are highly selective towards the receptor type and do not exhibit significant cross reactivity with other receptors. Furthermore, these antisera recognize the receptor both in live cells and fixed cells and are able to selectively immunoprecipitate the receptor from overexpressing cells as well as from endogenous tissue. Using these antisera we have been able to immunoprecipitate mu-delta complexes from mouse spinal cord membranes. We have recently examined if the antisera can distinguish between activated and non-activated receptors. We find that agonist treatment leads to a loss of recognition by the antisera and this is blocked by the antagonist. Taken together these results suggest that the opioid receptor selective antisera exhibit unique properties that should prove to be useful for a number of studies characterizing biochemical properties of opioid receptors.

This work was supported in part by grants DA 08863 and DA 00458 (to L.A.D).

IV. GENE REGULATION/GENETICS

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INVOLVEMENT OF AP-1 IN TRANSCRIPTIONAL REGULATION OF THE HUMAN MU-OPIOID RECEPTOR GENE

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Expression of mu-opioid receptors is regulated by cytokines (e. g. IL-1), hormones (e. g. estrogens), various drugs like cocaine and the phorbolester TPA. This study characterizes molecular mechanisms for regulation of human mu-opioid receptor (h-MOR) gene transcription in the neuroblastoma cell line SH SY5Y. Transfection experiments with reporter gene constructs showed that the h-MOR promoter is responsive to both TPA and TNF-alpha. Measuring activation of transcription factors in SH SY5Y cells, we found that TPA activates both AP-1 and NFkappaB, whereas TNF-alpha only activated NFkappaB. To distinguish between AP-1 and NFkappaB effects, the specific NFkappaB inhibitor sulfasalazine was used. Using gelshift and immunoshift assays in combination with transfection experiments, two functional AP-1 elements were identified at positions -2388 and -1434 on the h-MOR promoter out of six putative elements which differ in one nucleotide each from the classical AP-1 binding site. Transcriptional regulation of mu-opioid receptors by AP-1 may be of importance, because many agents which are known to regulate the human mu-opioid receptor can activate members of the AP-1 transcription factor family.

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NF-KAPPA-B REGULATES HUMAN MU-OPIOID RECEPTOR GENE EXPRESSION IN IMMUNE CELLS

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Gene expression of mu-opioid receptors (MORs) in neuronal and immune cells is regulated by various cytokines. This study demonstrates upregulation of human MOR gene transcription by the proinflammatory cytokine TNF-alpha in immune cells and characterizes underlying molecular mechanisms. Selective "Knocking-out" of various transcription factors by specific "decoy" oligonucleotides combined with quantitative real time PCR revealed that NF-kappa-B is the key factor for TNF-alpha-mediatied upregulation of MOR transcription in Raji and U937 immune cells. Out of six putative motifs, three NF-kappa-B binding sites were delineated to positions –2174, -557 and –207 on the human MOR promoter in transfection experiments. Independent of their orientation, these elements were active in front of the heterologous thymidine kinase promoter. NF-kappa-B binding to these elements was confirmed using electrophoretic mobility shift and immunoshift assays. A functional polymorphism within the –557 element reduces its trans-activating potency and impaires binding of NF-kappa-B. In conclusion, regulation of MOR gene expression by the cytokine TNF-alpha presents another facette of the multiple opio-immune-interactions.

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TRANSLATION OF GUINEA PIG PREPROENKEPHALIN mRNA

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In previous studies we have shown that preproenkephalin mRNA in the guinea pig brain is cleaved at a specific site in the 3' untranslated region (UTR) to yield a 1130 base truncated transcript and a 165 base 3' terminal fragment that also contains the poly (A) tail. Evidence from polysomal association suggests that the full-length form of the mRNA may be more transcriptionally active. To test the hypothesis that the 3' truncation of the guinea pig preproenkephalin mRNA alters translational effeciency, we first constructed a cDNA library from guinea pig brain mRNA and screened it with probe from the guinea pig preproenkephalin gene, which we isolated previously. Using a full-length cDNA clone as template, oligonucleotide-directed PCR mutagenesis was used to construct plasmid clones of full length and 3' truncated preproenkephalin cDNA with or without an 80 base poly (A) tail. RNA was synthesized *in vitro* from these template clones. Transcribed full-length or 3' truncated RNA, either with or without the poly (A) tail was tested in reticulocyte lysate translated systems. Results of these studies will be presented.

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QUANTIFICATION OF OPIOID RECEPTOR-mRNA UPREGULATION IN DORSAL ROOT GANGLIA DURING INFLAMMATION

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Using real time Light Cycler PCR, we have established a method for mRNA quantification of DOR, KOR and MOR. First we investigated the amount of opioid receptor mRNA in rat dorsal root ganglia (DRG) in untreated control animals. The MOR-mRNA-content was ten times higher than for DOR and three times higher than for KOR. Earlier investigations showed an increase in axonal transport of opioid receptors towards the periphery in response to inflammation induced by intraplantar injection of Freund's Complete Adjuvant (FCA). To examine whether this increase is related to transcription, we quantified opioid receptor-mRNA during the inflammatory process. The mRNA-content for DOR remained unchanged, while MOR showed a significant upregulation at 1-2 hours after FCA-induced inflammation. KOR was also upregulated with a significant increase 12 hours after onset of inflammation. Taken together, these data indicate that the increased axonal transport of opioid receptors in response to peripheral inflammation is accompanied by an increase in mRNA content in the DRG. Therefore, the increase in opioid receptors may be transcriptionally regulated under inflammatory conditions.

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SIBLING SIMILARITY FOR PERSONALITY TRAITS: TEMPERAMENT AND CHARACTER INVENTORY AND THE OPIOID RECEPTOR GENES AND CATECHOL O-METHYLTRANSFERASE GENE

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We investigated the genetic influence on personality trait in humans including the role of single nucleotide polymorphisms (SNPs) in opioid receptor (OPR) genes and catechol O-methyltransferase (COMT) gene. Paper-and-pencil form of the Temperament and Character Inventory (TCI) was given to normal subjects (306 females and 194 males). Scores for four temperament dimensions – Novelty Seeking (NS), Harm Avoidance (HA), Reward Dependence (RD), Persistence (P) - and three character dimensions, i.e. Self Directedness (SD), Cooperativeness (C), and Self Transcendence (ST) were supplied. We evaluated the correlations of TCI traits from 103 sibling pairs. SNPs in the OPRD1 T80G, T921C, OPRM1 C17T, A118G, and COMT G1947A were evaluated. The sibling-sibling correlations ranged from 0 to 0.29. TCI traits showed gender and ethnic difference. Among the TCI traits, RD and C showed association with OPR SNPs, but not with COMT G1947A associated with Val 158 Met amino acid substitution. These findings suggest that human personality traits are as moderately heritable and that OPR may play more of a role in this behavior than COMT.

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GENE EXPRESSION CHANGES FOLLOWING DEVELOPMENT OF TOLERANCE TO MORPHINE AND THEIR REVERSAL BY MK-801.

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Chronic opioid treatment leads to a multitude of changes at both the behavioral and biochemical levels. Prolonged morphine treatment also leads to changes in gene expression that could be either a cause or a consequence of the addiction process. Among affected genes could be those involved in variety of morphine's actions, including potential changes in opiate metabolizing enzymes, changes in transcripts related to pain processing, and changes related to the tolerance and dependence processes *per se*. It has been well established that morphine tolerance development can be diminished by the co-administration of a variety of agents, including NMDA antagonists. In order to identify changes in gene expression directly pertaining to tolerance development following chronic morphine treatment, mice were treated with PBS, morphine, morphine plus MK-801 (0.3 mg/kg), and MK-801 alone. After demonstrating the development of tolerance and its reversal by co-administration of MK-801, striata from the four groups of mice were obtained. Gene expression was determined using a custom prepared DrugAbuse cDNA array containing about 2000 mouse genes in duplicate. From these four conditions, by using regression-clustering analysis we have identified a number of genes up- or down-regulated following morphine administration, but not altered after co-injecting morphine with MK-801. Functional interpretation of these changes will be discussed.

V. NOVEL LIGANDS

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POSSIBLE INTRAPROTEIN FRAGMENTS AS OPIOID RECEPTOR LIGANDS

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We predict that steric requirements for bioactive opioid pharmacophores may be achieved by side chains of amino acid residues within proteins. The phenol group of tyrosine, near to the basic residue of arginine or lysine may mimic the topographic features of the tyramine moiety required for opioid peptide bioactivity. To explore for structural requirements for opioid bioactivity of nearby amino acids side chains, we synthetized and screen small peptide peptidomimetic library for bioactivity. We found that peptides with a sequence related to one fragment of HIV capsid protein (...-AA-Tyr-Arg-Arg-Phe-AA-....) expressed affinity for the mu opioid receptor. This data indicates the possibility that the viruses with functional opioid receptor ligand activity may employ interaction as a possible route of cell entry.

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BIOCHEMICAL, FUNCTIONAL AND PHARMACOLOGICAL CHARACTERIZATION OF A SYNTHETIC MEAP ANALOGUE

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The endogenous opioid heptapeptide (Tyr-Gly-Gly-Phe-Met-Arg-Phe; MEAP) and its radiolabelled form [^{3}H]MEAP were shown to interact with opioid (kappa>delta) and non-opioid sites in various tissues. To achieve more stable analogues of MEAP, new derivatives with D-amino acid substitutions were prepared and studied in binding assays and in pharmacological tests. One compound with the structure of Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe (DADN) had only moderate affinities in competing with [^{3}H]MEAP, whereas this peptide displayed the highest potency in producing antinociception following intrathecal administration. DADN was also prepared in tritiated form having 41 Ci/mmol specific radioactivity. Specific binding of [^{3}H]DADN was reversible, saturable, stereo-selective and of high affinity. The results from the [^{35}S]GTPγS binding assay confirmed the peptide's opioid agonist effect. Chemical stability, increased mu-receptor selectivity and hydrophobicity of the compound all contribute to the high potency observed in the pharmacological assays.

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BIVALENT LIGANDS: NOVEL BRIDGES IN OPIOID RESEARCH

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Bivalent ligands are composed of two pharmacophores joined by various connecting bridges. These ligands are useful pharmacological tools for studying the structure of opioid receptors, which may form dimers and oligomers. We evaluated the binding and functional selectivity of the morphinan (-)cyclorphan, its N-cyclobutylmethyl derivative (MCL-101), and nine bivalent derivatives. The novel bivalent ligands consisted of two morphinans connected by various spacer groups at the 3-hydroxy position. Similar to the parent morphinans, most of the bivalent compounds had Ki values less than 0.5 nM for the kappa receptor, which was 2- and 20-fold greater for the mu and delta receptors, respectively. Preliminary data indicated that most of the bivalent ligands were potent kappa agonists and mu antagonists as measured in [35S]GTPgammaS binding assays. In conclusion, the bivalent derivatives of (-)cyclorphan or MCL-101 are a novel series of opioids useful for studying receptor structure.

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METABOLISM OF L-α-ACETYLMETHADOL (LAAM) BY HUMAN PLACENTA.

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Methadone is considered the standard of care for treatment of the opiate addict. However, several complications associated with treatment of the pregnant addict have been reported. Buprenorphine (BUP) and LAAM are being considered as alternatives to methadone for treatment of these women in the United States though reports on the use of BUP in other countries has been made. Drugs used for treatment of pregnant woman may have direct and indirect effects on the fetus, thus data on the kinetics for their transplacental transfer (TPT), effects on and metabolism by the placenta are needed. Data on the TPT of LAAM indicated that it was retained by the placenta with the ratio of tissue/maternal being 5.6 and tissue /fetal of 36.6. We report here on the identification and localization of the enzyme catalyzing the metabolism of LAAM to norLAAM in human placental tissue. The highest activity for the enzymatic reaction was present in the microsomal fraction. The product of the reaction was identified by HPLC and Mass Spectroscopy as norLAAM. The reaction exhibits saturation kinetics with an apparent Km and Vmax values of 75 μM and 50-75 pmoles/mg protein.min. Inhibitors selective for CYP450 isoforms are utilized to identify the enzyme catalyzing the reaction in placental tissue. Monoclonal antibodies raised against isoforms of CYP450 are used to confirm the identity of placental enzyme. Supported by grant DA-13431 to M.S.A.

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PHARMACOLOGICAL CHARACTERIZATION OF AR-M1000390 AT HUMAN DELTA OPIOID RECEPTORS (HDOR)

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We investigated the pharmacological properties of a newly synthesised delta agonist, AR-M1000390, at the hDOR, endogenously expressed in the neuroblastoma cell line SK-N-BE. Binding and functional experiments showed a weak affinity (Ki = 106 ± 34 nM) correlated with a weak potency (EC50 = 111 ± 31 nM) to inhibit forskolin-stimulated cAMP accumulation. Sustained activation of opioid receptors in the presence of maximal inhibitory concentration of AR-M1000390 produced a rapid and strong desensitization. In order to examine the contribution of internalization and down-regulation in desensitization processes, binding and functional experiments were conducted in the presence or in the absence of hypertonic sucrose solution to block clathrin-dependent endocytosis. We observed both the inability of AR-M1000390 to down-regulate opioid receptors and the absence of any effect of sucrose on desensitization. The lack of hDOR internalization by AR-M1000390 was further corroborated by confocal microscopy using antibody directed against the delta-opioid receptor. These data suggest that uncoupling rather than internalization is responsible for hDOR desensitization by AR-M1000390.

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DESIGN, SYNTHESIS AND OPIOID ACTIVITY OF ANALOGS OF ARODYN, A NOVEL KAPPA-SELECTIVE ANTAGONIST

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Arodyn (*aro*matic *dyn*orphin), a novel analog of the opioid peptide dynorphin (dyn) A, was designed based on a lead peptide identified from a combinatorial library prepared in our laboratory using solid phase peptide chemistry. Arodyn displays nanomolar binding affinity and significant selectivity for kappa opioid receptors. It was anticipated that arodyn may display different structure-activity relationships (SAR) from Dyn A. An alanine scan was performed to determine the residues that are critical for binding to kappa opioid receptors in comparison to Dyn A. An *N*-methyl amino acid scan in the *N*-terminal 'message' sequence was performed to determine how changes in backbone conformation would alter the binding affinity for kappa receptors. The pharmacological evaluation of these arodyn analogs using Chinese hamster ovary cells stably expressing opioid receptors suggests that arodyn displays somewhat different SAR from Dyn A.

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VI. OPIOID RELATED MOLECULES

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***IN VITRO* RECONSTRUCTION OF RAT MESOLIMBIC SYSTEM USING ORGANOTYPIC SLICE CULTURE**

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The nucleus accumbens (NAc) is involved in drug dependence. NAc activity is controlled by dopaminergic (DA) afferents of the ventral tegmental area (VTA) and glutamatergic (Glu) afferents of the prefrontal cortex (PFC). We reconstructed the mesolimbic system using organotypic slice co-culture of VTA, NAc and PFC (triple culture), and examined effects of DA agents on field excitatory postsynaptic potential (fEPSP) in PFC–NAc synapses. A triple culture, prepared from newborn rats (P2), was maintained on a multi-electrode dish. Neurite outgrowth of PFC to NAc and of VTA to both NAc and PFC was identified by DiI labeling and an immunohistochemical (anti-tyrosine hydroxylase antibody) study, respectively. Electrical stimulation of PFC evoked Glu antagonist-sensitive fEPSP in NAc. The amplitude of fEPSP was decreased by D1-like receptor (D1R) agonist SKF38393, but not D2-like receptor (D2R) agonist quinpirole. Cocaine reduced the amplitude of fEPSP, which was reversed by D1R antagonist SCH23390, but not D2R antagonist sulpiride. These results indicate that Glu synaptic transmission is subject to exogenous and endogenous DA modulation in the triple culture, as shown in *in vivo*.

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HIGH AFFINITY HEXAPEPTIDES FOR ORL1 RECEPTORS: *IN VITRO* ACTIVITY AND *IN VIVO* NOCICEPTIVE EFFECTS

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We have designed and tested a series of hexapeptides for ORL1 (OP4) receptors with a broad range of *in vitro* efficacies. These compounds have a high affinity for ORL1 receptors as determined by *in vitro* measurements of [35S]GTPγS binding and their binding affinities range from 0.1-30 nM. A high affinity antagonist and agonist were tested *in vivo* for nociceptive activity. Animals received i.c.v. injections of the drug and were tested for tail-flick latencies at 5-, 10-, and 20-min post-injection. Similar to the prototypic ORL1 agonist N/OFQ, the hexapeptide agonist dose-dependently attenuated the antinociceptive effects of morphine. High doses of the antagonist alone increased tail-flick latencies at 10- and 20-min post-injection. In combination with morphine, the antagonist attenuated morphine-induced analgesia, at 5-min post-injection. Surprisingly, the antagonist administered with N/OFQ, attenuated morphine-induced analgesia to a similar extent as N/OFQ suggesting that the antagonist did not block the effects of N/OFQ. The results indicate that compounds with very low-efficacy at ORL1 receptors *in vitro* can display N/OFQ-like actions *in vivo*. (Supported by: DK55457 (to A.K.J) and DA 06682 (to L.T.))

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NOVEL, POTENT, ORL-1 RECEPTOR AGONIST PEPTIDES CONTAINING ALPHA-HELIX PROMOTING CONFORMATIONAL CONSTRAINTS

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The Orl-1 receptor has recently been cloned and is implicated in a wide variety of physiological and pathophysiological processes. Toward the goal of elucidating important features of the receptor-bound conformation of the endogenous ligand, nociceptin (NC), several conformationally constrained analogs were prepared. Alpha-aminoisobutyric acid (AIB) or N-methyl alanine (NMA) were inserted alanine replacements in the native NC sequence (FGGFTGARKSARKLANQ). *In vitro* assays measuring human ORL-1 receptor affinity, functional potency, and efficacy were performed for each new peptide. The receptor affinities of the AIB-containing peptides (i.e. NC peptidomimetics) generally matched or exceeded NC, showing Ki's in the range of 0.1 – 0.5 nM. By comparison, the receptor affinities of the NMA-containing peptides were significantly diminished.Peptide VIIa (FGGFTG[AIB]RKS[AIB]RKLANQ-NH2), which contains two constrained alanine residues was found to be a very potent agonist with Ki = 0.05 nM and EC50 = 0.08 nM in the human ORL-1 assays. The data support a hypothesis that the receptor-bound form of NC might adopt an amphipathic helix in the "address" segment of the sequence.

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ANTITUSSIVE EFFECT OF WIN 55212-2, A CANNABINOID RECEPTOR AGONIST

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Several lines of evidence indicate an interaction between opioid and cannabinoid systems. In the present study, the effect of WIN 55212-2, a high affinity cannnabinoid receptor agonist, on capsaicin-induced cough and the possible involvement of opioid receptors in the antitussive effect of WIN 55212-2 in mice. WIN55212-2, at doses of 0.3-3 mg/kg, i.p., produced dose-dependent antitussive effect. Antitussive effect of WIN 55212-2 was antagonized by pretreatment with SR141716A (3 mg/kg, i.p.), a CB1 receptor antagonist. Blockade of mu-opioid receptors by pretreatment with beta-funaltrexamine (40 mg/kg, s.c.) significantly reduced the antitussive effect of WIN 55212-2. However, pretreatment with nor-binaltorphimine (20 mg/kg, s.c.), a kappa-opioid receptor antagonist, did not affect the antitussive effect of WIN 55212-2. Furthermore, pretreatment with naloxonazine (35 mg/kg, s.c.), a mu1-opioid receptor antagonist, did not affect the antitussive effect of WIN55212-2. These results indicate that the antitussive effect of WIN 55212-2 is mediated by the activation of CB1 receptors and mu2 (naloxonazine-insensitive)-opioid receptors, but not mu1- and kappa-opioid receptors.

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INTERACTIONS BETWEEN NEUROPEPTIDE FF AND HUMAN DELTA OPIOID RECEPTOR

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There is evidence of the interaction between neuropeptide FF (NPFF) and delta opioid receptor (DOR) but the molecular mechanisms behind this interaction are not known. We have created a stable Chinese hamster ovary cell line expressing MYC-tagged human DOR (MYChDOR) that was used to study the effect of the stable NPFF-analogue [(1DMe)NPYF] on the localization of hDOR and on the hDOR signaling pathways.

Stimulation of cells with DPDPE caused phosphorylation of ERK1/2. (1DMe)NPYF did not activate ERK1/2 but it significantly diminished the DPDPE-induced phosphorylation.

DPDPE caused approximately 50% inhibition of forskolin-stimulated cAMP accumulation, whereas (1DMe)NPYF was uneffective. (1DMe)NPYF significantly decreased the inhibition of cAMP accumulation caused by DPDPE. Immunofluorescent staining showed that DPDPE induced rapid internalization of hDOR. (1DMe)NPYF delayed the internalization induced by DPDPE but did not affect the receptor localization alone. Taken together, (1DMe)NPYF does not directly activate hDOR. However, it seems to have an indirect effect on the cellular localization and signaling of the receptor. The mechanism remains to be identified.

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COMPARISON OF NOCICEPTIN AND NOCISTATIN LEVELS IN CSF BETWEEN CHRONIC PAIN PATIENTS AND NORMAL VOLUNTEERS

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We have developed a unique identification method using HPLC combined with RIA specific for nociceptin (NCP/OFQ) and nocistatin (NST), which enabled us to detect less than 1 picomple of the both peptides in cerebrospinal fluid (CSF). This method allowed us to detect NCP/OFQ, NST-17 and NST-30 together with their precursors. Our aim is to find possible relationships between pain perception and CSF levels of NCP/OFQ and NST. CSF samples were mainly collected from chronic low back pain and osteoarthritis patients. Further 6 CSF samples of male healthy volunteers were also analyzed. The crude CSF extracts were subjected to HPLC with a ODS or Phenyl column. The eluates were collected at every 1 ml/min, and NCP/OFQ , NST-17and -30 were determined by RIA respectively. The amounts of NCP/OFQ and NST in normal CSF appeared to be lower than those of the both chronic pain patients. Interestingly the levels of NCP/OFQ and NST in CSF seemed to be different between chronic back pain and ostheoarthrites patients groups.

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BASAL AND MORPHINE-EVOKED DOPAMINERGIC NEUROTRANSMISSION IN THE NUCLEUS ACCUMBENS OF MOR AND DOR KNOCKOUT MICE.

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Conventional and no net flux microdialysis were used to quantify basal and morphine-evoked extracellular dopamine (DAext) levels and the rate of DA uptake in the nucleus accumbens (NAc) of wild type (WT) mice and those with a constitutive deletion of MOR (MOR KO) or DOR (DOR KO). Locomotor activity was assessed in these same animals. No difference between genotypes in dialysate DA levels was seen. No net flux studies revealed significant decreases in the extraction fraction of DA (a measure of the rate of DA uptake) in MOR- and DOR-KO mice. DAext, however, was unchanged suggesting that basal DA release is decreased in KO mice. MOR KO exhibited only a small increase in morphine-evoked DA levels and failed to exhibit a behavioral response to morphine. In contrast, morphine increased dialysate DA levels and locomotor activity in DOR KO and WT. These data indicate that constitutive deletion of either MOR or DOR results in decreased basal DA release and a compensatory decrease in DA uptake. The contrasting effects of MOR and DOR ablation upon responsiveness to morphine are consistent with the predominant role of MOR receptors in the regulation of DA neurotransmission and DA-dependent behaviors.

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DIFFERENT EXPRESSION OF μ-OPIATE RECEPTOR IN CHRONIC AND ACUTE WOUNDS AND THE EFFECT OF β –ENDORPHIN ON TGF–β TYPE II RECEPTOR AND CYTOKERATIN 16 EXPRESSION

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There is evidence that neuropeptides, especially the opiate receptor agonists are involved in wound healing. We have previously observed that β-endorphin, the endogenous ligand for the μ-opiate receptor, stimulates the expression of cytokeratin 16 (CK16) in a dose dependant manner in human skin organ cultures. Cytokeratin 16 is expressed in hyperproliferative epidermis such as psoriasis and wound healing. Therefore we were interested to study if epidermal μ-opiate receptor expression is changed at the wound margins in acute and chronic wounds. Using classical and confocal microscopy, we were able to compare the expression level of μ-opiate receptors and the influence of β-endorphin on TGF−β type II receptor in organ culture. Our results show indeed a significantly decreased expression of μ-opiate receptors on keratinocytes close to the wound margin of chronic wounds compared to acute wounds. Additionally β-endorphin upregulates the expression of TGF−β type II receptor in human skin organ cultures. These results suggest a crucial role of opioid peptides not only in pain control but also in wound healing. Opioid peptides have already been used in animal models in treatment of wounds, they induce fibroblast proliferation, growth of capillaries, accelerate the maturation of granulation tissue and the epithelialization of the defect. Furthermore opioid peptides may fine-tune pain and the inflammatory response while healing takes place. This new knowledge could be potentially used to design new locally applied drugs to improve the healing of painful chronic wounds.

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THE ROLE OF NEUROPEPTIDES IN THE DIABETIC RAT

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Sensory diabetic polyneuropathy is initially accompanied by hypersensitivity and followed by hyposensitivity. In order to study this bi-phasic pattern of neuropathic nociception, streptozotocin treated rats were used. The hyperalgesic and hypoalgesic responses were observed by tail-flick and hot-plate tests. The hyperalgesia was noticed at the first 4-6 weeks after the STZ injection. Later on, this response gradually and inversely turned into hypoalgesia. The neuropeptides, CGRP and met-Enkephalin were proposed to be involved in this bi-phasic pattern of diabetic nociception. CGRP and met-Enkephalin antibodies staining density was measured with computing image analysis. The CGRP content was reduced in DRG of the hyperalgesic and the hypoalgesic diabetic rats and increased in PAG of both experimental groups without change in the spinal cord. Met-Enkephalin content was significantly reduced in DRG and spinal cord of hypoalgesic rats and increased in PAG of hyperalgesic rats. Therefore, the hyperalgesic response may be induced due to down regulation of met-Enkephalin, while hypoalgesic response is due to decreasing of CGRP.

POSTER SESSION 2
Thursday July 11, 16:30 – 18:00

I. BEHAVIOR

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MOLECULAR EVIDENCE FOR THE INVOLVEMENT OF THE PHOSPHATIDYLINOSITOL METABOLISM CASCADE IN THE MORPHINE-INDUCED REWARDING EFFECT

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Recently, various studies have indicated that the G-protein coupled receptor-mediated several pharmacological actions can be regulated by phosphatydilinositol metabolism cascade. The present study was then to investigate the role of phosphatidylinositol metabolism cascade via inositol-1, 4, 5-triphosphate (IP3) in the morphine-induced rewarding effect. Pretreatment i.c.v. with either antisense oligodeoxynucleotide to Ggamma or specific antibody to Gbeta/gamma produced a significant and concentration-dependent suppression of the morphine-induced place preference. Under these conditions, the morphine-induced place preference was almost reversed by i.c.v. injection of a IP3 inhibitor xestospongin C. Furthermore, heterozygous IP3 type 1 receptor knockout mice were virtually devoid of the roboust morphine-induced place preference. It has been shown that IP3 production can be mediated by Gbeta/gamma including Ggamma_. Taken together, the present data suggest the possibility that the activation of phosphatidylinositol metabolism cascade may contribute to the morphine-induced rewarding effect.

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INCONSISTENT EFFECTS OF NMDA RECEPTOR ANTAGONISTS ON OPIATE ANALGESIA

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There are conflicting results on the ability of N-methyl-D-aspartate (NMDA) receptor antagonists to modify opiate analgesia, with some studies showing enhancement, others showing inhibition, and yet others finding no effects. To further explore this controversy we examined the ability of several NMDA antagonists to modify the acute analgesic effects of morphine (3.0 mg/kg) and fentanyl (0.05 mg/kg), as assessed by the tail-flick test in rats. The NMDA antagonists targeted each of the major sites on the receptor complex (doses in mg/kg): MK-801 (0.1 & 0.3), dextromethorphan (10 & 30), memantine (3 & 10), LY235959 (1 & 3), (+)-HA-966 (10 & 30), and ifenprodil (1 & 3). Neither morphine nor fentanyl analgesia was affected by the low dose of any antagonist (doses that are known to block NMDA receptors). The higher dose of LY235959 produced a mild analgesic effect on its own and enhanced both morphine and fentanyl analgesia. Fentanyl, but not morphine analgesia was enhanced by the higher dose of HA-966 and dextromethorphan. The results demonstrate no consistent effects of NMDA receptor antagonists on opiate analgesia, but suggest that selected antagonists may enhance the effects of certain opiates. [Supported by NIGMS (GM59833)].

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PROFOUND SPINAL TOLERANCE AFTER REPEATED EXPOSURE TO A HIGHLY SELECTIVE MU-OPIOID AGONIST: ROLE OF DELTA OPIOID RECEPTORS.

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Recent findings suggesting that the delta opioid receptor plays an important role in the development of morphine tolerance led us to hypothesize that tolerance may be minimized with a highly selective mu agonist. [Dmt1]DALDA (Dmt-D-Arg-Phe-Lys-NH2) is a dermorphin analog with very high selectivity for mu opioid receptor (mu:delta>14,000) and extraordinary intrathecal potency. However, our results showed profound spinal tolerance (40-50x shift in i.t. ED50) after 5 doses of s.c. [Dmt1]DALDA in mice. Morphine efficacy was severely compromised in these tolerant mice. Co-administration of naltriben with [Dmt1]DALDA reduced level of tolerance, while delta antagonists had no effect on [Dmt1]DALDA potency in naïve mice. Most surprisingly, [Dmt1]DALDA sensitivity could be partially restored with concurrent administration of naltriben or TIPP[ψ] in tolerant mice. These findings suggest that agonist activation of delta receptors is not required for development of tolerance; however, delta receptors play an important modulatory role in the maintenance of the tolerant state.

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TOPICAL CAPSAICIN-INDUCED THERMAL ALLODYNIA IN PRIMATES: EFFECTS OF KAPPA-OPIOID AGONISTS

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The aims of this study were 1) To develop a model of topical capsaicin-induced thermal allodynia in rhesus monkeys. 2) To determine effectiveness of kappa–opioid agonists in blocking topical capsaicin-induced allodynia in this model. Capsaicin was applied topically to the tail of rhesus monkeys (0.3 ml of 0.004 M capsaicin over a 1 cm2 area; 15 min contact; n=4), according to a technique previously used in human and non-human primates. Monkeys were tested in the warm water tail withdrawal assay, using thermal stimuli which are normally non-noxious (38, 42 degrees C; cutoff latency=20 sec). Topical capsaicin (0.004 M) produced robust thermal allodynia (i.e., latencies decreased to approximately 2 sec), with peak effects observed 15-30 min after capsaicin removal. A lower capsaicin concentration (0.0012 M) was ineffective. The proposed kappa-1 agonist U69,593 (0.01-0.1 mg/kg, s.c.) partially prevented topical capsaicin-induced allodynia, whereas the proposed kappa-2 agonist, GR89,696 (0.00032-0.001 mg/kg, s.c.) fully prevented this allodynia. This model may thus be used to test topical capsaicin-induced allodynia and its modulation by kappa-opioid ligands. Supported by NIDA grants DA11113, DA00049, DA05130.

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EFFECT OF ACAMPROSATE ON MORPHINE CONDITIONED PLACE PREFERENCE

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Acamprosate (calcium acetylhomotaurinate) is a taurine derivative that reduces alcohol consumption and relapse in both animals and humans. However, little attention has been given to the study of interactions between acamprosate and other drugs of abuse, including opiates. The present study was conducted to determine if acamprosate modulates the conditioned place preference (CPP) produced by morphine. Male C57BL/6J mice were allowed access to one of two conditioning chambers for 15 min immediately following alternating injections of saline or morphine sulfate (5 mg/kg ip) for 8 days. On the day following conditioning, mice were given access to both chambers and displayed a robust preference for the morphine-paired chamber (average 450 sec/30 min). Subsequent to the conditioning phase, mice were injected with saline or acamprosate (300 mg/kg ip) daily for 10 days. Upon re-testing 30 min following the last injection, both groups of mice retained the preference for the morphine-paired chamber, indicating that acamprosate does not prevent the sustained expression of morphine CPP. Further studies will determine if co-administration of acamprosate during the conditioning phase inhibits the acquisition of morphine CPP.

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OPIOID PEPTIDE ANALOGUES AS A POTENTIAL ANALGESICS IN PATHOPHYSIOLOGICAL CONDITIONS. THE INCREASE OF ANALGESIC ACTIVITY OF INTRAVENOUS (IV) BIPHALIN IN RATS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE)

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Biphalin is a bivalent opioid peptide analogue. The analgesic intrathecal (IT) activity of biphalin is thousand times higher than morphine but intravenous (IV) activity in healthy rats is similar to morphine. This discrepancy between IT and IV activity reflects lower blood-brain barrier (BBB) permeability of peptide in comparison to morphine. Clinical pain often arises in the context of pathophysiological conditions affecting a number of homeostatic regulatory elements, including BBB permeability. To check the effect of neuroinflammation on biphalin analgesic activity we tested responses to IV drug in Lewis rats in a model of EAE. The analgesic potency of IV biphalin correlate well with the progression of EAE. This observation suggests that peptide analgesics may be much more effective in gaining access to the central nervous system in disease states.

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EFFECTS OF SUPRASPINAL ENDOMORPHIN-1 AND ENDOMORPHIN-2 ON ALLODYNIA AND REWARDING IN RATS

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Two potent endogenous opioid peptides, endomorphin-1 (EM-1) and –2 (EM-2), which are selective μ-opioid agonists, have been recently isolated from bovine and human brain. These endomorphins were shown to produce a potent anti-allodynic effect at spinal level. In the present study, we further investigated their supraspinal anti-allodynic effects and rewarding effects. In a neuropathic pain model (sciatic nerve crush in rats), EM-1 and EM-2 (15 μg, i.c.v.) both showed significant effects in the cold-water allodynia test but EM-1 is more potent than EM-2. Naltrexone (15 μg) was able to completely block the effects of EM-1 and EM-2. In conditioned place preference tests, only EM-2 showed significant positive rewarding effect. We also found that only acute EM-2 significantly increased dopamine turnover in the shell of NAc in the microdialysis experiments. From these results, it could be suggested that EM-1 may have better supraspinal anti-allodynic effect and less rewarding effect than EM-2.

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EVALUATION OF THE FUNCTIONAL SIGNIFICANCE OF OPIOID RECEPTOR DIMERS *IN VIVO*: EFFECTS OF DELTA/KAPPA AND DELTA/MU AGONIST MIXTURES IN RHESUS MONKEYS

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In vitro binding and functional studies suggest that opioid receptor heterodimerization may have functional consequences. To assess the possible functional significance of delta/kappa and delta/mu receptor heterodimers *in vivo*, this study tested the hypothesis that combinations of delta/kappa or delta/mu agonists would produce synergistic behavioral effects. Mixtures of the delta agonist SNC80 with either the kappa agonist U69,593 or the mu agonist fentanyl were prepared according to the relative potency of the component drugs, and the effects of these mixtures were evaluated in 5 adult male rhesus monkeys (*Macaca mulatta*) responding under a fixed ratio 30 schedule of food reinforcement. Data were analyzed with isobolographic analysis. Mixtures of SNC80 and U69,593 produced only additive effects. Mixtures of SNC80 and fentanyl produced subadditive effects, suggesting that SNC80 and fentanyl mutually attenuated each other's effects. These results suggest that delta/kappa and delta/mu agonist mixtures may not produce synergistic effects in rhesus monkeys. Supported by RO1-DA11460 from NIDA.

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INVOLVEMENT OF CB1 AND A2A RECEPTORS IN MORPHINE WITHDRAWAL SYNDROME

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The involvement of A2a adenosine and CB1 cannabinoid receptors in morphine dependence has been investigated by using single A2a knockout mice and the recently new generated double mutants deficient in A2a and CB1 receptors. First, we have evaluated the spontaneous behavioural responses of these double mutants. Spontaneous locomotion was reduced in double knockout as compared to wild-type mice. These double mutants showed an increased anxiety-like response in the elevated plus-maze and the light-dark box. Double mutants were less sensitive to thermal nociceptive stimulation (tail-immersion test) compared to wild-type mice. The specific involvement of A2a receptors in morphine dependence was evaluated in knockout mice deficient in A2a receptors. The severity of morphine withdrawal was significantly increased in the absence of adenosine A2a receptors. We also investigated the somatic expression of morphine abstinence in double mutants lacking A2a and CB1 receptors. No modification of morphine withdrawal was observed in these double mutants. These results suggest that both A2a and CB1 receptors participate in an opposite way in the expression of opiate physical dependence.

II. SIGNAL TRANSDUCTION

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HOMODIMERIZATION OF HUMAN MU-OPIOID RECEPTOR OVEREXPRESSED IN SF9 INSECT CELLS

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In this study, we demonstrate that human mu-opioid receptors do form SDS-resistant homodimers and examine the ability of human mu-opioid receptors to dimerize and the role of agonists in the dimerization. Increasing concentrations and longer exposure of agonists reduce the levels of dimer with a corresponding increase in the levels of monomer. This effect is achieved with both peptide and alkaloid opioid agonists and it is antagonist reversible. These results suggest that human mu-opioid receptors are present as receptor oligomers and interconversion between dimeric and monomeric forms may be important for biological activity.

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KAPPA OPIOID RECEPTORS ARE DIFFERENTIALLY LABELED BY ARYLACETAMIDES AND BENZOMORPHANS

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Using CHO cell membranes that stably expressed the human kappa opioid receptor, we investigated the hypothesis that kappa1 and kappa2 opioid receptors, historically defined as being selectively labeled by either arylacetamides (i.e., U69,593) or benzomorphans (i.e., bremazocine) are, in fact, different affinity states of the same kappa opioid receptor. Binding studies with GTPgammaS showed that it potently inhibited [^{3}H]U69,593 binding, while virtually no inhibition was seen for [^{3}H]bremazocine binding. Scatchard experiments showed a 3-fold decrease in [^{3}H]U69,593 affinity in the presence of GTPgammaS and no GTPgammaS effect on [^{3}H]bremazocine affinity. The kappa antagonist, nor-BNI, had a 4-fold higher affinity for [^{3}H]U69,593-labeled receptors than for [^{3}H]bremazocine-labeled receptors. U69,593 stimulated [^{35}S]GTPgammaS binding to a greater extent than bremazocine did. These results suggest that GTP and nor-BNI have different effects on arylacetamide and benzomorphan binding and activity.

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6-BETA-NALTREXOL, AN OPIOID NEUTRAL ANTAGONIST, PRECIPITATES LESS SEVERE WITHDRAWAL COMPARED TO THE INVERSE AGONIST NATREXONE: DOSE-RESPONSE AND TIME COURSE STUDIES

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Elucidation of basal signaling in GPCR systems has led to the reclassification of many classic antagonists as inverse agonists or neutral antagonists. Naloxone and naltrexone display inverse agonist properties in opioid dependent states, while 6-beta-naltrexol is a "neutral" antagonist. The compounds antagonize CNS effects of morphine in ICR mice, with naltrexone being 3-9 times more potent. In contrast, 6-beta-naltrexol is ~80-times less potent in precipitating withdrawal jumping. More comprehensive withdrawal measures indicate that 6-beta-naltrexol precipitates a less severe withdrawal than equieffective antagonist doses of naltrexone. Acute and chronic morphine exposure produces long lasting changes in basal signaling in mouse brain tissue and corresponding signs of precipitated withdrawal with naltrexone. In contrast, 6-beta-naltrexol produces minimal withdrawal. These studies indicate that opioid neutral antagonists may have broad applicability to the treatment of a variety of opioid mediated side-effects.

Funding for this project was provided by NIDA grants DA04166 and DA 06284

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ELUCIDATION OF ACTIVATION AND BINDING PROFILES OF OPIOID LIGANDS IN TRANSFECTED CELLS EXPRESSING MU, DELTA OR KAPPA OPIOID RECEPTORS.

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Cloning of opioid receptor genes has permitted biochemical and functional characterization of these receptors. Transfected cells can be used to characterize each receptor subtype in the absence of other receptors. The goal of our studies was to characterize the activation and binding profiles of fifteen opioid ligands in transfected HEK cells expressing opioid receptors. All ligands bound opioid receptors with binding affinities of 0.2-300 nM. Activation profiles of these ligands were assessed by measuring inhibition of forskolin stimulated adenylyl cyclase activity. Agonists demonstrated efficacies and potencies between 0.3 nM to 2 μM, with maximum inhibitory effect of 29% to 70%. These results provide a detailed characterization of several opioid ligands, including "mixed agonist/antagonists", at each opioid receptor subtype. In combination with *in vivo* data on the activity of each ligand, the present results will be used to describe the features of the ideal opioid drug, ultimately leading to the design of novel opioid analgesics with minimal side effects.

This work was supported by a grant from NIH, DA12539.

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DUAL ACTIONS OF MU OPIOID RECEPTORS: ACUTE HETEROLOGOUS ACTIVATION AND CHRONIC DOWN REGULATION OF EGF RECEPTORS IN RAT ASTROCYTES

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In previous studies, we found that acute and chronic mu opioids display proliferative and anti-proliferative actions, respectively, via the endogenous mu opioid receptor (MOR) and ERK in rat C6 cells, an astrocytic model system. Here, we correlate acute and chronic DAMGO regulation of EGF receptor (EGFR) activation with ERK phosphorylation in rat astrocytes. Acute DAMGO acting via endogenous MOR initiates transactivation of EGFR subsequently resulting in ERK activation. Inhibitors of Ca^{2+} mobilization, PKC and metalloproteases attenuate MOR-induced ERK phosphorylation. Chronic DAMGO inhibits ERK phosphorylation by exogenous EGF and reduces EGFR levels. In contrast, kappa opioid, U69,593, can transactivate but does not down regulate EGFR; it induces a longer lasting ERK phosphorylation. Collectively, the findings suggest that MOR plays a dual role in the heterologous regulation of EGFR activity in rat astrocytes. The data also demonstrate that mu and kappa OR signaling to ERK differ in this cell line and they lend insight into the molecular mechanisms underlying offspring pathogenesis in maternal opiate abuse.

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MORPHINE TOLERANCE IN TRIGEMINAL NOCICEPTORS FROM MORPHINE TOLERANT MICE

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Morphine acts on sensory neurons to relieve pain, but the effects of prolonged morphine treatment on the opioid responses of sensory neurons is unknown. In acutely isolated C57 mouse trigeminal ganglion neurons MOR agonists inhibited *I*Ca in a population of small neurons that also expressed transduction proteins characteristic of nociceptors: VR1 channels, TTX-resistant *I*Na and Gs-coupled prostaglandin receptors. Neurons isolated from mice chronically treated with morphine (3 x 300 mg kg^{-1} morphine base s.c. for 6d) showed significantly reduced maximal responses to DAMGO (30 % decrease compared to vehicle controls) and morphine (45 % decrease) but no obvious change in their responses to nociceptin. *I*Ca density was slightly but significantly reduced (15%, $P < 0.01$) in the putative nociceptors. These data show that chronic morphine treatment leads to MOR tolerance in nociceptive sensory neurons, similar to what has been reported in LC and PAG neurons. Chronic morphine treatment may also lead to changes in *I*Ca expression in sensory neurons, which is different to what has been reported in LC and PAG, and suggests that nociceptors may adapt to the continued presence of morphine beyond simply becoming tolerant to morphine.

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ROLE OF PKC AND PKA IN THE EXPRESSION OF MORPHINE TOLERANCE IN MICE

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Evidence shows that the phosphatidylinositiol cascade plays an important role in the expression of opioid antinociceptive tolerance. Previous studies from our laboratory demonstrated that PKC inhibitors acutely reversed morphine tolerance when injected intracerebroventricularly 30-min before the radiant heat tail-flick test. Experiments in this study attempted to determine the duration of tolerance reversal by two structurally dissimilar PKC inhibitors, Go 7874 and sangivamycin. We found that these inhibitors persistently reversed morphine tolerance for up to 24-h. Other studies have shown that the PKA inhibitor, KT-5720, completely blocked 8-fold morphine tolerance. We have shown that the PKC inhibitors also completely blocked this level of tolerance. This led us to conclude that both PKC and PKA mediate morphine antinociceptive tolerance. We speculated that the individual contribution of both protein kinases to tolerance would be revealed in mice with profound morphine tolerance. We found that combined administration of both PKC and PKA inhibitors completely blocked a 50-fold morphine tolerance.

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PHARMACODYNAMICS OF CODONES AND MORPHONES AT MU AND DELTA OPIOID RECEPTORS

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This study compared the pharmacodynamics of codeine, oxycodone and hydrocodone with their O-demethylated metabolites. Relative efficacy values were determined in concentration-effect curves for G-protein activation in rat thalamic (mu) and NG108-15 cell (delta) membranes, by comparison to the full agonists DAMGO and SNC-80, respectively. Oxymorphone and hydromorphone were ~10-fold more potent than morphine at the mu receptor, with the order of efficacy: oxymorphone ≥ morphine > hydromorphone (50-65% of DAMGO). Codeine was ~10-fold less potent than morphine and the codones were 30-40-fold less potent than their respective morphones. The relative efficacy of hydrocodone was similar to hydromorphone, but codeine and oxycodone were less efficacious. At the delta receptor, morphine and the morphones were of similar potency and efficacy (~30% of SNC-80). Codeine was 30-fold less potent than morphine, and codones were 4-5 fold less potent than the morphones. Oxycodone, but not codeine and hydrocodone, was significantly less efficacious than morphones. These data predict that O-demethylation would enhance codone activity *in vivo*.

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ROLE OF GBETA-GAMMA AND GALPHA-I IN ADENYLYL CYCLASE SUPERACTIVATION

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Transfecting adenylyl cyclase (AC) isozymes into COS cells, we found that acute/chronic opioid treatments inhibit/superactivate AC-I, V, VI and VIII. Two approaches were taken to investigate the role of AC molecular domains and of Galpha-i and Gbeta-gamma in superactivation: (i) Using AC-VIII splice variants, we found that acute mu-receptor activation inhibits AC-VIII-A and B but not C (which lacks most of the C1b domain). Agonist withdrawal after chronic treatment induced superactivation of all three splice variants, demonstrating that C1b is not critical for AC superactivation. (ii) Producing point mutations in AC-V and AC-I, we found that several C1a mutations (AC-V DNV469-471AAA, AC-V F481Y, and AC-I F314Y) led to reduced superactivation, showing that these amino acids play a role in this process. Moreover, contrary to wt AC-V and AC-I, neither Gbeta-gamma nor constitutively active Galpha-i inhibited the activity of the mutated molecules. To discriminate between the roles of Gbeta-gamma and Galpha-i, we prepared AC-V C1a mutants less sensitive to Gαi. These showed normal inhibition by Gbeta-gamma and normal superactivation. Thus, Gbeta-gamma has a crucial role in superactivation. *Supported by NIDA and BSF.*

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MECHANISMS OF DELTA OPIOID RECEPTOR ACTIVATION

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We have previously investigated structure-activity relationships of DOR by site-directed mutagenesis and identified aminoacid residues involved in ligand recognition or receptor activation. To further investigate molecular determinants for receptor activation without preconceived structural hypothesis, we now have developed a random mutagenesis approach to identify a large number of constitutively active mutant (CAM) receptors. We have optimized PCR conditions to randomly mutate the entire hDOR cDNA under conditions that statistically introduce one point-mutation per receptor molecule. We have transiently expressed the receptor library into HEK 293 cells and used a high-throughput reporter gene assay in conjunction with the inverse agonist ICI174864 to identify cells expressing CAM receptors. We have obtained several mutant receptors and identified the nature and localization of mutations by DNA sequencing. A set of activating mutations will be presented on a 3D-model of hDOR, together with hypotheses on possible mechanisms for hDOR activation. This strategy offers a unique way to generate a general picture of receptor activation, and both the approach and some of the conclusions may be applicable to other GPCRs. It also provides mutant receptors that will be useful to screen for compounds with inverse agonist properties.

NOVEL OPIOID CANNABINOID SIGNAL TRANSDUCTION PATHWAY

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Certain signal transduction pathways of G protein coupled receptors have been well characterized, and it is known that phosphorylation plays a large role in receptor actions and trafficking. It is know that receptor phosphorylation by proteins such as β-Adrenergic Receptor Kinase (BARK) can lead to internalization, and is involved in desensitzation and down regulation. In addition, receptor mediated phosphorylation by MAPKs can lead to changes in gene expression. We have recently discovered another signal transduction system specific for receptors mediating the action of abused drugs. The cannabinoid receptor can be activated by PhosphO-Transferase (POT). The activated receptor leads to paranoia and an increase in the ingestion of Twinkies in lab rats. The δ-opioid receptor can also be phosphorylated by a related enzyme called Delta Opioid Phosphorylating Enzyme (DOPE). The protein β-arrestin is not effective in diminishing receptors activated by these enzymes, possibly due to a suppression of the β-arrestin releasing factor (BARF)-response. However, if one uses either POT or DOPE, these actions can be arrested by Cannabinoid Opioid Phosphatase (COP). Found to be even more effective than COP in arresting the utilization of POT and DOPE was an undercover tetrapeptide Asn-Ala-Arg-Cys (NARC). The actions of NARC are sly, as it is derived from the C-terminal tail of DOR, and thus looks like it would use either POT or DOPE, but really activates an isoform of COP. Studies are continuing to determine the relationship between POT- and DOPE- activated receptors and intracellular signaling using the Multiple Analytical Response In Joined User And Nonuser Array (MARIJUANA).

III. TRAFFICKING

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MORPHINE-INDUCED SPINAL-MEDIATED ANTI-NOCICEPTION AND TOLERANCE IN BARRESTIN-2 KNOCKOUT MICE.

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Morphine induces analgesia by activating MORs in spinal and supraspinal regions of the CNS. Beta-arrestin2 (Barr2) regulates the MOR *in vivo*. We have previously shown, in the hot plate test, that mice lacking Barr2 experience enhanced morphine-induced analgesia and do not become tolerant to morphine. To determine the general applicability of the Barr2-MOR interaction in other neuronal systems, we have tested the Barr2-KO mice using the warm water tail immersion paradigm which primarily assesses spinal reflexes to painful thermal stimuli. In this test, the Barr2-KO mice have greater basal nociceptive thresholds and markedly enhanced sensitivity to morphine. However, after a delayed onset, they do ultimately, though to a lesser degree, develop morphine tolerance. In the Barr2-KOs, but not in the WTs, morphine tolerance can be completely reversed with a low dose of PKC inhibitor. These findings provide *in vivo* evidence that the MOR is differentially regulated in diverse regions of the CNS and that the Barr2-KO mice represent an animal model wherein the contributions of other mechanisms can be evaluated in the absence of the predominant Barr2-mediated desensitization. *DA-14600 LMB, DA-13511 MGC.*

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TIPP[ψ] RESTORES [DMT1]DALDA POTENCY IN DESENSITIZED SH-SY5Y CELLS

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Pharmacological and molecular studies have suggested that the delta opioid receptor plays an important role in the development of morphine tolerance. We were therefore surprised that even short-term treatment with a highly selective mu-opioid agonist, [Dmt1]DALDA (Dmt-D-Arg-Phe-Lys-NH2; mu:delta > 14000) resulted in profound spinal tolerance (40-50 fold shift in ED50) in mice. Furthermore, concurrent administration of a delta antagonist (naltriben or TIPP[ψ]) significantly restored the potency of [Dmt1]DALDA in tolerant animals. We have used the SH-SY5Y cell line in an attempt to study the mechanisms behind the interaction between [Dmt1]DALDA and TIPP[ψ]. We found that pretreatment with [Dmt1]DALDA produced much greater desensitization in undifferentiated (45x shift in IC50) than in differentiated (6.6x shift) cells. Furthermore, TIPP[ψ] completely restored [Dmt1]DALDA sensitivity in undifferentiated cells but not in differentiated cells. Finally, we found that TIPP[ψ] behaved as a partial agonist in undifferentiated but not in differentiated cells. These data suggest that undifferentiated SH-SY5Y cells may be a more suitable cell model than differentiated cells.

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MU OPIOID RECEPTORS DESENSITIZE LESS RAPIDLY THAN DELTA OPIOID RECEPTORS.

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Receptor desensitization in Xenopus oocytes revealed distinct differences in the kinetics of GRK and arrestin regulation of the closely related opioid receptors. We demonstrated that desensitization of MOR proceeds dramatically slower than DOR. The GRK3 phosphorylation sites required for DOR and KOR desensitization reside in the carboxy-terminal tail, while MOR depends on T180 in the second intracellular loop, which is not important for internalization in mammalian cell lines. Increasing the amount of arrestin expressed accelerated the rate of MOR desensitization bringing it closer to that of DOR. Similarly, coexpression of a constitutively active arrestin2(R169E) desensitized both MOR and DOR at rates that were indistinquishable. Together, these data suggest that the activation of arrestin, rather than its binding is rate-limiting for MOR desensitization. In addition, mutation of T161 in DOR significantly inhibited the faster desensitization of DOR. These results suggest that DOR desensitization involves phosphorylation of both the carboxy-terminal tail and the second intracellular loop that together lead to a more efficient activation of arrestin and thus faster desensitization. Supported by DA11672, DA07278.

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SWIM STRESS PROMOTES TRANSLOCATION OF THE DELTA OPIOID RECEPTOR (DOR) IN PERIAQUEDUCTAL GRAY AXON TERMINALS

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Immunolabeling for DOR is primarily localized to axon terminals in the ventrolateral periaqueductal gray (vlPAG). However, rather then on the plasma membrane DOR immunoreactivity is usually in the cytoplasmic compartment, often in association with dense-core vesicles. In this study the hypothesis that a cold water swim stress (CWSS) could initiate the translocation of DOR to the plasma membrane of axon terminals was tested. The subcellular distribution of DOR was followed ultrastructurally using a pre-embedding immunogold method. In comparison to control rats, rats perfused 8-15 minutes following a CWSS (3 min at 4 degrees) had a significantly greater fraction of DOR immunoreactivity on the plasma membrane. In addition after a CWSS DOR immunoreactivity was more often associated with vesicles that had a clear core. Moreover, vesicles containing DOR immunoreactivity could occasionally be visualized as fusion intermediates after a CWSS. These data suggest the involvement of DOR in the vlPAG in the behavioral response to CWSS. Furthermore, the results reveal that by virtue of the association with dense-core vesicles, the presynaptic plasma membrane distribution of DOR can be regulated in a state-dependent manner.

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LIGAND BINDING AFFECTS THE LATERAL DIFFUSION OF THE MU OPIOID RECEPTOR ON THE CELL SURFACE AS REVEALED BY SINGLE PARTICLE TRACKING

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The dynamic changes of the human μ-opioid receptor (MOR) expressed at the cell surface after binding to the mu agonist DAMGO have been investigated by Single Particle Tracking. In this purpose, NRK fibroblast cells were stably transfected with the cDNA encoding the MOR tagged with a T7 sequence at its N terminus, which allows its specific labelling by colloidal gold particles (40 nm) coupled to a monoclonal anti-T7 antibody without affecting ligands interaction. The lateral movements of gold particles were followed in Nomarski contrast videomicroscopy at 40 ms time resolution during 2 min with a precision in particle position of 15 nm. Basically, the receptor exhibited two kinds of behaviours : a directed diffusion mode (diffusion coefficient $D<10\text{-}11 cm^2/s$) and a " walking confined diffusion " mode, more rapid, consisting in a combination of confined and directed diffusion. In the absence of ligand, 10% of the receptors were in directed diffusion mode and 90% exhibited a " walking confined diffusion " mode. The addition of 10^{-6} M DAMGO shifted each mode to 50%, thus significantly reducing receptor mobility. Our results point out that the signalling process involves changes in the dynamic membrane organisation of the G protein coupled receptors.

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NEITHER DOR1-IR NOR MOR1-C-IR ARE EXPRESSED IN AXONS OF PAG NEURONS PROJECTING TO THE RVM.

M Wessendorf and N JensenDept Neuroscience, Univ Minnesota, Minneapolis, MN 55455 Previous studies have shown that the cloned delta-opioid receptor (DOR1) and the cloned mu-opioid receptor (MOR1) are expressed by neurons in the periaqueductal gray matter (PAG) that project to the rostral ventromedial medulla (RVM: PAG-RVM neurons). Since both DOR1-immunoreactivity (-ir) and that for the splice variant MOR1-C are expressed in axons, we examined whether either DOR1-ir or MOR1-C-ir existed in RVM axons that had been anterogradely labeled from the PAG. Rats were anesthetized and biotinylated dextran-amine (BDA) was injected into the PAG. Five to seven days later, rats were deeply anesthetized and killed by vascular perfusion with fixative. Frozen sections (50 um) were cut and immunofluorescently stained for either DOR1 or MOR1-C using a Cy3-labeled secondary antibody; BDA was visualized using Cy2-streptavidin. Sections of RVM were examined for double-labeled axons or terminals. Although many single-labeled structures (both Cy2 and Cy3) were seen, no convincing examples of double-labeling were observed. These findings suggest that delta- and mu-opioid receptors modulate the actions of PAG-RVM neurons at their somata and/or dendrites, but not at their terminals. Supported by DA 09642.

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TRAFFICKING AND SIGNALING OF THE MU AND DELTA OPIOID RECEPTORS FOLLOWING ALTERATION TO THE CYTOSKELETON

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The cytoskeleton has been reported to regulate the function of G-protein coupled receptors by affecting receptor clustering, endocytosis and/or post-endocytic trafficking. The receptor trafficking properties of mu opioid receptors (MOR) and delta opioid receptors (DOR) were examined in stably transfected HEK 293 cells following disruption to the actin and tubulin cytoskeleton using either latrunculin B, an inhibitor of F-actin polymerization or nocodazole which inhibits tubulin polymerization. Internalization of neither MOR nor DOR was significantly altered by treatment with latrunculin B. In contrast nocodazole affected the trafficking of both MOR and DOR following treatment with either DAMGO or DPDPE respectively. In double stably transfected HEK293 cells containing MOR and a CRE-luciferase reporter gene, nocodazole but not latrunculin B significantly inhibited the ability of MS to inhibit FSK-stimulated CRE-luciferase activity. Mutation of phosphorylation sites in the C-terminal tail of MOR to alanine enhanced the ability of MS to inhibit FSK-stimulated CRE-luciferase activity in the presence of nocodazole.

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EXPRESSION AND TRAFFICKING OF PRODYNORPHIN IN THE BRAIN

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Here we characterize the trafficking and biochemical properties of prodynorphin in the brain. The distribution of prodynorphin generally correlates with that of prodynorphin mRNA and dynorphins. However, prodynorphin and dynorphin peptides are present in high amounts in the CA3 region of the hippocampus, where the levels of prodynorphin mRNA are negligible; the precursor may be transported from the soma of neurons in the dentate gyrus, where it is synthesized, to the synaptic terminals projecting into the CA3 region where it is processed. Prodynorphin is present in these structures as insoluble oligomers with monomers linked via disulfide bridges. Oligomer formation appears to occur in the ER, it is promoted by Brefeldin A, which blocks protein transport from the ER to the Golgi apparatus, and may serve as the mechanism for prodynorphin sorting into secretory granules. Prodynorphin oligomers in the synaptic terminals may also represent protein deposits from which opioid peptides may be processed.

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INTERACTIONS OF MU AND DELTA RECEPTORS ENHANCE SIGNALING BY OPIATES.

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While morphine exerts its analgesic effects primarily via mu opioid receptors, a number of studies have shown that delta receptor selective drugs enhance the potency of morphine. Despite considerable effort by a number of investigators the molecular basis for these findings has been elusive. We had previously reported that when co-expressed in heterologous cells mu and delta receptors associate with each other and exhibit unique ligand binding and signaling properties. In the present study we examined mu-delta interactions in living cells by the proximity-based bioluminescence resonance energy transfer (BRET) assay. We find that co-expression of mu and delta receptors leads to an increase in the BRET signal suggesting an association between these two receptors. We also find that these interactions affect the affinity and efficacy of clinically relevant drugs. In cells co-expressing mu-delta receptors low doses of morphine, fentanyl or methadone enhance the binding of the delta agonist tritiated-deltorphin II, and potentiate delta receptor signaling. Furthermore, signaling of mu receptor by morphine is enhanced by the delta receptor antagonist TIPP[psi]. Finally, this potentiation of signaling is seen in wild type mice but not in mice that lack delta receptors. These findings have important clinical ramifications and provide new foundations for more effective therapies.

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IV. GENE REGULATION/GENETICS

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OPIATES AND GENE EXPRESSION PATTERN: MICRO ARRAY STUDIES

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Micro arrays can be used to investigate the mechanisms of drug action. We are currently using micro arrays consisting of approximately 7000 thousands of individual gene sequences printed in a high-density array on a glass slide.

Pheochromocytoma (PC12) cell line was transfected stably with the human mu-opioid receptor and was used as a model to investigate cellular response to opiates. This model has been characterized functionally including ligand binding, immunocitochemistry and cAMP assay. The transfected cells were treated with a mu-opioid receptor agonist and an antagonist to study changes in gene expression using micro array. Numbers of genes responded to these treatments. The gene expression profile indicated that genes responding to opiates could be placed in a variety of functional categories. The results and statistical analysis of opiate treatment will be presented.

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INDUCTION OF CHRONIC FRAS FOLLOWING ESCALATING DOSE MORPHINE OR HEROIN

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Induction of the transcription factor delta fosB was measured in order to examine long-lasting molecular changes following repeated opiate administration. The 35-37 kDa isoforms of delta fosB, the chronic Fras, were measured in the caudate putamen and nucleus accumbens of male Sprague-Dawley rats following three escalating dose schedules of morphine for 6 or 10 days. Heroin was also tested to determine if the findings extend to other opiates. Results from Western blots using a fosB antibody demonstrate that intermittent escalating dose morphine produced a significant increase in chronic Fra immunoreactivity in both the caudate putamen and nucleus accumbens. The effects of morphine were both time and dose dependent. Likewise, heroin administered twice daily for 10 days by an escalating dose schedule also induced chronic Fras in the caudate putamen and nucleus accumbens. Studies are currently underway to investigate the mechanism underlying the induction of chronic Fras by opiates.

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EFFECTS OF ACUTE "BINGE" COCAINE ON DYNORPHIN, ENKEPHALIN, POMC AND CRH RECEPTOR mRNA LEVELS IN THE STRIATUM AND HYPOTHALAMIC-PITUITARY-ADRENAL AXIS OF MU-OPIOID RECEPTOR KNOCKOUT MICE

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Cocaine administration increases activity at dopamine receptors, increases dynorphin (Dyn) gene expression in the caudate-putamen (CPu), and activates the stress responsive hypothalamic-pituitary-adrenal (HPA) axis. To examine the hypothesis that mu-opioid receptors (MOR) may play roles in these cocaine effects, we tested the effects of acute "binge" pattern cocaine administration in mice with targeted disruption of the MOR gene. Wild type (+/+) and homozygous MOR deficient (-/-) mice received three injections of 15 mg/kg cocaine at 1-hour intervals. Mice were sacrificed 30 min after the last injection, and mRNAs for Dyn and enkephalin (Enk) in the CPu and nucleus accumbens (NAc), and for type I corticotropin-releasing hormone receptor (CRH1 receptor) and pro-opiomelanocortin (POMC) in the hypothalamus and pituitary, were measured by solution hybridization RNase protection assays. Cocaine elevated Dyn mRNA in the CPu, but not NAc, of both the MOR -/- and +/+ mice. Enk mRNA in the CPu, but not NAc, was lower in MOR -/- mice than in MOR +/+ mice following cocaine administration. Hypothalamic CRH1 receptor and POMC mRNAs were expressed at similar levels in untreated and in cocaine treated mice of each genotype. However, there were lower basal levels of CRH1 receptor mRNA in the anterior pituitary of the MOR -/- mice than in MOR +/+ mice, and the MOR -/- mice failed to show the cocaine-induced decreases in CRH1 receptor mRNA found in the wild type mice. Cocaine activated the HPA axis similarly in MOR -/- and +/+ mice, as reflected in similar increases in plasma corticosterone levels in both genotypes. These results support a specific role for MORs in acute cocaine's effects on striatal Enk gene expression, and fail to support critical roles for these receptors in acute cocaine's effects on either Dyn gene expression or HPA activation. MOR -/- mice are useful models for studying cocaine effects on Enk gene expression that could aid interpretation of the similar post-mortem phenomena found in human cocaine addicts.

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CHARACTERIZATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE CODING REGION OF THE HUMAN CANNABINOID RECEPTOR GENE

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The endogenous cannabinoid system is an important part of the brain reward system. Recent studies indicate a functional cross-interaction between the cannabinoid and the opioid system, and polymorphic variants of the cannabinoid receptor gene (CNR1) are suggested to be associated with a higher vulnerability to alcohol and opiate dependence. To further elucidate the role of the CNR1 gene polymorphisms in drug dependence, we are currently studying in our ongoing research on the genetics of vulnerability to addiction single-nucleotide polymorphisms (SNPs) in a cohort of 171 human unrelated individuals. This cohort includes well-characterized opiate addicts with and without co-dependencies, as well as, control individuals that have been earlier studied with regard to association between opiate addiction and SNPs in the opioid receptor gene (Bond et. al., 1998). Using PCR, we amplified the entire coding region of the human CNR1 gene. Sequence specific primer for amplification and sequencing were based on the reported genomic human CNR1 sequence. Allele frequencies of identified SNPs in the CNR1 gene and results of association analyses between drug dependent and control subjects will be presented.

V. NOVEL LIGANDS

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[NPHE1,ARG14,LYS15]N/OFQ-NH2 (UFP-101) A NOVEL LIGAND FOR THE NOCICEPTIN / ORPHANIN FQ RECEPTOR

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Nociceptin/orphanin FQ (N/OFQ) modulates several biological functions by activating a GPCR named NOP. Few molecules are available that selectively activate or block this receptor. Here we describe the *in vitro* and *in vivo* pharmacological profile of a novel NOP receptor ligand, [Nphe1,Arg14,Lys15]N/OFQ-NH_2 (UFP-101). UFP-101 binds to the human recombinant NOP receptor expressed in Chinese hamster ovary (CHO) cells with high affinity (pKi 10.2) and shows more than 3000 fold selectivity over classical opioid receptors. UFP-101 competitively antagonizes the effects of N/OFQ on GTPγ^{35}S binding in CHOhNOP cell membranes (pA2 9.1) and on cAMP accumulation in CHOhNOP cells (pA2 7.1), being *per se* inactive at concentrations up to 10 μM. In isolated peripheral tissues of mice, rats and guinea pigs, and in rat cerebral cortex synaptosomes preloaded with [^{3}H]-5-HT, UFP-101 competitively antagonized the effects of N/OFQ with pA2 values in the range of 7.3 – 7.7. In the same preparations, the peptide was inactive alone and did not modify the effects of classical opioid receptor agonists. UFP-101 is also active *in vivo* where it prevented the depressant action on locomotor activity and the pronociceptive effect induced by 1 nmol N/OFQ i.c.v. in the mouse. In the tail withdrawal assay, UFP-101 at 10 nmol produces *per se* a robust and long lasting antinociceptive effect. UFP-101 is a novel, potent and selective NOP receptor antagonist which appears to be a useful tool for future investigations of the N/OFQ-NOP receptor system.

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REPLACEMENT OF TYR1 WITH DMT1 IN DALDA ANALOGS: BINDING AND BIOLOGICAL ACTIVITY STUDIES.

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DALDA (Tyr-D-Arg-Phe-Lys-NH_2) is a dermorphin analog with very high affinity and selectivity for mu opioid receptor. Its ability to inhibit NE uptake in the spinal cord contributes to its intrathecal potency. Replacement of Tyr1 with 2',6'-dimethyltyrosine (Dmt) ([Dmt1]DALDA) resulted in increased affinity and potency. When given it, [Dmt1]DALDA was 225 times more potent and longer-acting than DALDA. We have now examined the effect of Dmt1 substitution in a series of DALDA analogs. Dmt1 substitution increased mu ([^{3}H][Dmt1]DALDA) binding affinity by 20-90-fold and GTPγS binding by 14-215 fold. The effect on efficacy (GTPγS Emax) was inconsistent. Inhibition of NE uptake was increased by 2->100 fold. Although [Dmt1]TAPP (Dmt-D-Ala-Phe-Phe-NH2) and [Dmt1,Orn4]DALDA (Dmt-D-Arg-Phe-Orn-NH2) showed similar properties *in vitro*, [Dmt1,Orn4]DALDA was 88-fold more potent and much longer-acting than [Dmt1]TAPP when given i.t. Despite their 3+ net charge, [Dmt1]DALDA and [Dmt1,Orn4]DALDA were potent and long-acting sc analgesics.

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TRANSLOCATION OF A 3+ NET CHARGE DERMORPHIN TETRAPEPTIDE ACROSS PLASMA MEMBRANE OF MAMMALIAN CELLS.

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Polypeptides are thought to have poor permeability across biological membranes. However, SD (Dmt-D-Arg-Phe-Lys-NH_2), a highly selective mu opioid agonist with 3+ net charge, was found to be very potent after sc injection in mice. We found that SD can readily penetrate the cell membrane in several mammalian cell lines (Caco-2, SH-SY5Y, HEK293, CRFK). [^{3}H]SD uptake occurred at 37^{o}C and 4^{o}C, was concentration-dependent but independent of pH. Cells preloaded with [^{3}H]SD exported >90% of internalized SD by 60 min at 37^{o}C. Transport studies using Caco-2 cells grown on transwells showed progressive accumulation of SD on the receiver side. Comparison with Gly-Sar uptake in Caco-2 cells revealed that SD uptake did not involve peptide transporter PEPT1. Confocal fluorescence microscopy using the dansylated analog Dmt-D-Arg-Phe-dnsDap-NH_2 revealed diffuse cytoplasmic localization excluding nuclei. These findings suggest that this highly polar tetrapeptide can cross plasma membranes by energy-independent mechanisms. Similar findings have been reported for several cationic peptides.

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METABOLISM OF METHADONE BY HUMAN PLACENTA.

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Methadone is considered the standard for treatment of the pregnant opiate addict and is the only drug approved for this patient population in the United States. However, several unexplained clinical observations are associated with its use e.g. the absence of a correlation between maternal dose and intensity of neonatal abstinence syndrome. The latter prompted us to investigate its metabolism by placental tissue.We report here on the identification of the placental enzyme responsible for the metabolism of methadone and the product(s) formed. Microsomal fractions of placental tissue catalyzed the demethylation of methadone to 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), identified by HPLC. This reaction is catalyzed by CYP450 3A4 in human liver. Analysis of the saturation isotherm for the demethylation reaction catalyzed by placental microsomes revealed an apparent Km and Vmax values of 570 ± 192 μM and 483 ± 128 pmoles/mgP.min respectively. Inhibitors selective for CYP isoforms were utilized to identify the placental enzyme. Troleandomycin (CYP 3A) caused 60-70 % inhibition, α-naphthoflavone (CYP 1A1) and 4-methylpyrosole (CYP 2E1) resulted in 40 % inhibition while other inhibitors caused less than 10%. These data suggest that CYP 3A4 is major enzyme responsible for the N-demethylation of methadone in placentas obtained from term healthy pregnancies. Supported by NIDA grant DA-13431 to MSA.

ASTROMORPHIN: AN OPIOID PEPTIDE ISOLATED FROM ASTEROIDS

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While it is well established that amino acids and other "biological" molecules are found in space rocks and asteroids, previous studies have not reported whether peptides are present in these samples. In the present study, asteroids were analyzed for the presence of peptides, with a focus on the opioid peptides. Using a radioreceptor assay based on the mu opioid receptor, binding activity was detected in a variety of asteroid samples. This activity was named "astromorphin." Purification and sequence analysis of astromorphin showed that it was identical to the mammalian brain peptide known as endomorphin, except that the amino acids were all of the D conformation. Injection of astromorphin into rat brain produced analgesia in addition to a generally spaced-out behavior, consistent with the astral-origin of the compound. The levels of astromorphin ranged from 1 to 10 ug per kg of asteroid. Based on this level, the asteroid that slammed into the earth 65 million years ago would have contained from 5,400 to 54,000 kg of astromorphin, and if 10% of this survived the impact and was lofted into the atmosphere, the levels of astromorphin in the air would have been sufficient to intoxicate in nearly all life forms on the planet. Thus, it is possible that the extinction of the dinosaurs was caused not by the dust from the impact, as commonly believed, but rather from the aerosolized astromorphin which created a planet of drug addicts.

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NATIONAL INSTITUTE ON DRUG ABUSE DRUG SUPPLY & ANALYTICAL SERVICES PROGRAM

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The NIDA Drug Supply Program has grown from a source of marijuana, LSD, and a small number of research compounds in the early 1970's to the major source of research compounds for the drug abuse research community. Initially, the Drug Supply Program focused on THC and other naturally occurring cannabinoids, and then gradually began to fulfill the demand for metabolites of these compounds, various isotope-labeled derivatives, and other drugs of abuse. The Drug Supply Program has been responsive to the needs of the research community over the past years when a variety of new compounds were required: opiate antagonists, phencyclidine derivatives, cocaine metabolites and analogs, and a variety of receptor ligands for studies of opiate receptors. When "designer drugs" became of interest to researchers, meperidine and fentanyl derivatives, and more recently, new substituted amphetamines were synthesized in response to the interest generated by the increased abuse of MDMA on college campuses.

Since starting in 1967 with only LSD, hundreds of different compounds were made available to the research community. Today, the Drug Supply Program provides basic research investigators with authentic stimulants, hallucinogens, narcotics, cannabinoids, opioid peptides, and marijuana. Labeled compounds are also available which incorporate deuterium, tritium, 14C or 125I isotopes. New compounds are periodically added to the Drug Supply inventory as needed by the research community. Resources updates which list newly added or deleted chemicals and/or services are mailed to investigators upon request.

In addition to the Drug Supply Program, research resources are provided for the quantitative analysis of drugs of abuse by RIA or GC/MS, and determination of 3-dimensional structure parameters by x-ray crystallography. The Drug Supply and Analytical Services Program also provides analytical support for the analysis of cannabinoids by providing quantitative stock solutions to investigators.

Prsonnel at the NIDA Drug Supply and Analytical Services Program at the Division of Neuroscience and Behavioral Research are available to answer your questions and to assist you in using these services. In order to utilize the services or obtain materials offered by NIDA, interested research investigators are required to submit their requests along with research protocols detailing the work to be carried out. The protocols are reviewed by NIDA staff and/or external experts for scientific merit and compatibility with NIDA's goals. If the research protocols meet basic requirements, the requests are processed subject to resource availability.

VI. OPIOID RELATED MOLECULES

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COMPARISON OF INJURY-INDUCED RESPONSES IN WILD-TYPE AND NOCICEPTIN/ORPHANIN FQ KNOCK-OUT MICE

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We have reported earlier that nociceptin/orphanin FQ (N/OFQ) mRNA levels and immunoreactivity are strongly induced in the vicinity of penetrating brain injury, while receptor (NOP) levels for N/OFQ did not change. We used a N/OFQ knock-out mouse strain to investigate the function of N/OFQ in injury-induced responses in the CNS. Apoptotic cell death after stab wound injury was evaluated by TUNEL staining and a similar number of cells were found positive in wild-type (WT) and N/OFQ knock-out (KO) animals. Stab wound injury triggers expansion and activation of glial cells. Astroglial proliferation occurred at the same rate in WT and N/OFQ-KO mice as revealed by GFAP immunostaining. Cell-proliferation following injury was assayed by BrdU staining and was found essentially the same in the WT and KO animals. Traumatic brain injury elicits infiltration and activation of polymorphonuclear leukocytes (PMNs) and NOP receptors were found on PMNs. However we found no difference in leukocyte infiltration evaluated by CD45-immunostaining in WT and N/OFQ KO mice. Supported by a grant from NIDA.

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SEQUENCE AND HOMOLOGY OF A MU OPIOID-LIKE RECEPTOR IN AN AMPHIBIAN

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It is not known what specific sequences or domains of opioid receptors determine the type selectivity of opioid agents. The present studies took a comparative approach to understanding the mechanisms of opioid receptor selectivity and opioid analgesia. To date, the type of opioid receptor mediating analgesia in non-mammalian species is not known. Much data exists on the analgesic effects of mu, kappa, and delta opioids and on the radioligand binding of selective opioids in an amphibian model. To complement these previous data, we cloned and sequenced *Rana pipiens* opioid-like receptors using a PCR-based strategy and degenerate primers based on mammalian opioid receptors. A mu opioid-like receptor, termed RPMOR, shows 75-85% homology to existing mu opioid receptors. Phylogenetic analysis using CLUSTAL-W and MEGalign programs produced a nearest-neighbor dendrogram consistent with RPMOR arising from common ancestors of mu opioid receptors in mammalian species. Comparison of extracellular (ECL) receptor domains among 10 vertebrate mu opioid receptors shows that ECL1 diverged least suggesting that mu opioid selectivity is conferred by that domain. Support from NIH, NIDA grant 12448.

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A ROLE FOR THE ORL-1 RECEPTOR IN THE ACTIONS OF BUPRENORPHINE

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Buprenorphine (BUP), an opioid with mixed agonist/antagonist activity at the classical opioid receptors, has recently been shown to act as an agonist at the opioid receptor-like (ORL-1, also known as NOP) receptor. Orphanin FQ/nociceptin, the endogenous agonist ligand of the ORL-1 receptor, has been demonstrated to block opioid-induced antinociception, raising the possibility the antinociceptive action of BUP, mediated by the mu opioid receptor, could be altered by its ability to concomitantly activate the ORL-1 receptor. In the present study, we determined the antinociceptive effect of BUP in mice lacking the ORL-1 receptor or in the presence of J-113397, an ORL-1 receptor antagonist. BUP produced a submaximal antinociception in wild type mice. The antinociceptive effect of BUP was enhanced in the ORL-1 receptor knockout mice as compared to the wild type littermates. The action of BUP was also enhanced by J-113397 in wild type, but not ORL-1 receptor knockout, mice. Repeated BUP treatment in mice produced tolerance, which was significantly greater in mice lacking the ORL-1 receptor. Taken together, the present data suggest that antinociception and tolerance induced by BUP were modulated by its ability to concomitantly activate the ORL-1 receptor. (Supported by NIDA DA 05010; KL was supported by a KO1 award DA00411 from NIDA)

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NOCICEPTIN INDUCED EFFECTS ON DRUG REWARD IN MICE

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Several studies have reported that nociceptin blocks acquisition of place preference to rewarding drugs in rats. On the other hand, few reports have studied this effect in mice. The aim of the current study was to establish a method and protocol to examine nociceptin effects on drug reward in mice. C57BL6 mice were implanted with chronic indwelling intracerebroventricular (i.c.v.) cannulae and then tested in a conditioned place preference paradigm. The effects of three doses of nociceptin (0.1, 1.0, 10nmol i.c.v.) on morphine (7.6mg/kg) induced place preference were examined. 1nmol and 10nmol of nociceptin, but not 0.1nmol, administered during morphine conditioning sessions inhibited the development of place preference to morphine. 0.1nmol of nociceptin partially facilitated morphine-induced sensitization expressed as locomotion during the conditioning sessions. Conditioning with 10nmol nociceptin alone induced neither preference nor aversion. Thus, similar to the rat, nociceptin blocks acquisition of morphine-induced place preference in mice. We plan to expand these studies of nociceptin-induced effects to other rewarding drugs using this model.

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THE EFFECTS OF NOISE-STRESS ON THE NEURO-TRANSMITTERS OF MONOAMINES IN RAT'S BRAIN

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Noise is considered as one of the sources of stressor. It has been shown that noise can also influence the cochlear system, the cardiovascular system, gastrointestinal system, immune system, hormonal system and central nervous system. In addition, noise was reported to raise the pain threshold in experimental animal. In our previous study, it has shown that such effects may be related to monoaminergic neurons and opioid sytem in mice. In the present study, we try to determine the effects of noise on the neurotransmitters in rat's brain by *in vivo* microdialysis and glyoxylic acid fluorecence histochemistry. The result shows that noise (110 dB) increases the concentration of epinephrine in striatum and dorsal raphe nucleus (41.55% and 38.89%, respectively), with subsequent reduction to basal levels after the noise exposure is terminated. Noise also decreases the concentraion of DOPAC in striatum and cortex (maximal to 98.65% and 53.21%, respectively); however, the concentration of DOPAC remains low even after noise exposure is terminated. Besides, noise can decrease significantly the concentration of HVA in striatum (28.24%) and the concentration of 5-HIAA in striatum and dorsal raphe nucleus. Another study using fluorecence histochemistry fails to show any significant changes of neurotransmitters affected by noise. From the above results, it is suggested that there must be a close relationship between noise-stress and some neurotransmitters in brain. The mechanisms of noise-stress to affect neurotransmitters and to raise the pain threshold remain to be elucidated.

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EFFECT OF PAEONIFLORIN AND ANTISENSE ODNs OF NMDA RECEPTOR ON HIGH-DOSE MORPHINE-INDUCED BEHAVIOR AND ON NMDA RECEPTOR EXPRESSION

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Paeoniflorin, a major component from *Paeoniae lactifloria*, has several pharmacological actions including analgesic, anti-inflammatory, antispasmodic and anti-allergic effects. Based on our previously reported paeoniflorin attenuated the seizure-like excitation induced by high dose morphine and NMDA-induced biting and scratching behavior, we tried to explore the effects of paeoniflorin by co-administration with antisense ODNs of NMDA receptor. The combination administration significantly enhanced the inhibitory effect of antisense ODNs of NMDA receptor on high-dose morphine induced excitatory behavior and on NMDA-induced biting and scratching behavior. Moreover, paeoniflorin potentiated the inhibitory effect of antisense ODNs of NMDA receptor subunit on the NMDA receptor protein expression by immunohistochemistry and western blotting. From above results, it is revealed that the inhibitory effect of paeoniflorin on high-dose morphine or NMDA-induced excitatory behavior might be via the inactivation of NMDA receptor.

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EFFECT OF CHEMOKINES ON THE ACTIONS OF A RECEPTOR-SELECTIVE OPIOID ON ANALGESIA AND THERMOREGULATORY RESPONSES

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We investigated the possible involvement of chemokines in the hyperthermia and analgesia induced by the selective mu opioid receptor agonist DAMGO. Male S-D rats weighing 250-300 g were used. A sterilized stainless-steel 21-gauge cannula guide was implanted above the preoptic anterior hypothalamus (POAH) or periaqueductal gray (PAG). Body temperature (Tb) was monitored using a biotelemetry system, and the cold water tail-flick test was used to assess the analgesic responses. Microinjected into the POAH, DAMGO (0.4 μg) induced hyperthermia; microinjected into the PAG, it produced analgesia. SDF (1 –100 ng) or RANTES (1-100 ng) alone had no effect on Tb, and neither affected DAMGO-induced hyperthermia when given 30 min before the opioid. However, pretreatment with SDF or RANTES caused a dose-dependent reduction in analgesia induced by DAMGO. RANTES or SDF given into the PAG did not produce analgesia or hyperalgesia. These data indicate that chemokines are involved in the analgesic effect induced by DAMGO but not in DAMGO-induced hyperthermia. (Supported by NIDA grants DA00376, DA06650 and DA13429)

POSTER SESSION 3
Saturday July 13, 16:30 – 18:00

I. BEHAVIOR

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ENKEPHALIN AND PREJUNCTIONAL SYMPATHETIC CONTROL OF HEART RATE

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Leucine-enkephalin (LE) and the sympathetic regulation of the cardiac pacemaker was examined. LE was delivered into the interstitium of the canine sinoatrial node by microdialysis during sympathetic stimulation. LE added to the dialysis inflow reduced the tachycardia. The inhibition was identical at 5 and 20 min, providing no evidence for a progressively evolving response or for desensitization. LE reduced vagally mediated bradycardia by 50% at the same intervals. Naltrindole only restored 30% of the sympathetic tachycardia but fully restored the coincident vagal bradycardia suggesting the sympatholytic effect was non-opioid or non-delta. In study two, norepinephrine added to the dialysis inflow increased heart rate 35.2 ± 1.8 bpm. When LE was combined with norepinephrine, the tachycardia was unaltered by LE or the subsequent addition of naltrindole. LE again reduced vagal bradycardia by 50% which was fully restored by naltrindole. These studies support the conclusion that the local, nodal, sympatholytic effect of LE resulted from a reduction in the effective interstitial concentration of norepinephrine and not from post-junctional interactions between LE and norepinephrine.

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REGULATION OF BASAL SIGNALING ACTIVITY OF MU OPIOID RECEPTOR IN MOUSE BRAIN AND ITS ROLE IN NARCOTIC DEPENDENCE

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Mu opioid receptor (MOR) is one of a number of G protein coupled receptors shown to display spontaneous (basal) G protein coupling in the absence of agonist. Using a ^{35}S-GTP-γ-S binding assay, we have demonstrated that morphine treatment of mice enhances basal MOR activity in brain tissue homogenates and causes a persistent change in the effects of naloxone and naltrexone. These antagonists did not affect basal MOR signaling (acting as 'neutral antagonists') in untreated brain tissue but suppressed basal signaling (acting as 'inverse agonists') after morphine pretreatment. These results extend and confirm our earlier findings in tissue cultures expressing MOR. Naltrexone's ability to suppress basal MOR signaling in brain homogenates paralleled the time course of naltrexone-induced withdrawal in morphine-dependent mice. In contrast, 6β-naltrexol acted as a neutral antagonist regardless of morphine pretreatment and caused significantly fewer withdrawal symptoms. These results indicate that basal MOR signaling plays a key role in narcotic dependence. Moreover, neutral MOR antagonists have promise in the treatment of narcotic addiction. Supported by a grant from NIDA, Rockville MD, DA04166.

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NMDA RECEPTOR ANTAGONISTS INHIBIT TOLERANCE AND SENSITIZATION TO THE LOCOMOTOR EFFECTS OF OPIATES

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There is growing evidence that N-methyl-D-aspartate (NMDA) receptors may be involved in opiate tolerance, sensitization and physical dependence. However, the majority of studies in this area have relied exclusively on morphine to explore these phenomena. The present studies examined the ability of the non-competitive NMDA antagonist, MK-801 (0.1 mg/kg), and the competitive antagonist, LY235959 (3.0 mg/kg), to affect tolerance and sensitization to the locomotor effects of different opiates. Morphine, fentanyl, methadone, or buprenorphine was administered once daily for 10 days to male rats in the presence or absence of the NMDA antagonist. Tolerance and sensitization were assessed by determining the locomotor effects of a challenge dose of the opiate on day 11. Changes characteristic of tolerance and/or sensitization developed to each of the opiates. Animals treated chronically with the opiate in the presence of the antagonists showed little evidence of tolerance or sensitization. These results support the idea that NMDA receptors are involved in several different types of opiate-induced behavioral and neural plasticity. [Supported by NIGMS (GM59833)].

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ANTI-ILEUS EFFECTS OF ADL 10-0102, A NOVEL KAPPA AGONIST, IN RATS

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Post-operative ileus, or bowel paralysis, occurs after abdominal surgery, and narcotic analgesics commonly administered after surgery, exacerbate it. The goal of this study was to characterize the effect of ADL 10-0102, a novel kappa opioid receptor (KOR) agonist, in a model of surgically induced ileus. Rats were anesthetized with isoflurane and a 3.5 cm laparotomy was followed by externalization and manipulation of the small intestine and cecum for 5 min. Sham rats received a skin incision only. ADL 10-0102 or vehicle was administered 30 min after surgery, followed by charcoal slurry p.o. 30 min later. After an additional 30 min, rats were euthanized and gastrointestinal transit (GIT) was measured. The ED50 to reverse surgically induced ileus for ADL 10-0102 s.c. was 0.072 mg/kg, while the oral ED50 was 3.4 mg/kg. The KOR-specific antagonist, n-BNI, completely antagonized the anti-ileus effects of ADL 10-0102. These findings indicate that ADL 10-0102 potently reversed the slowing of GIT produced by surgical manipulation, and this effect is KOR-mediated. Further studies are warranted to characterize the potential anti-ileus effects of KOR agonists.

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THE EFFECTS OF U-50488, A KAPPA OPIOID AGONIST, ON MORPHINE REWARDING AND DEPENDENCE

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In our previous study, it was found that the kappa-opioid receptor agonist, U-50488 could partially prevent the development of tolerance to morphine analgesia. Therefore, we further investigate the possible effect of U-50488 on morphine rewarding and dependence in the present study. In our behavioral experiments in rats, conditioned place preference (CPP) and naloxone-precipitated withdrawal were used to examine psychological and physical dependence respectively. The results revealed that U-50488 could almost completely abolish the morphine-induced CPP and attenuate certain naloxone-precipitated withdrawal signs. To investigate the neurochemistry of morphine rewarding, we used the technique of microdialysis to determine the change of extracellular dopamine turnover in the shell of nucleus accumbens (NAc). In consistent with the other reports, the acute administration of morphine resulted in a drastic increase of dopamine turnover in NAc. More importantly, this increase could be attenuated by U-50488. According to the present results, U-50488 may possess an ability to prevent morphine-induced physical and psychological dependence, which could be of great therapeutic potential.

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ROLE OF MORPHINE IN ALTERING FREE RADICAL SCAVENGER SYSTEM PROFILE AND Na^+ K^+ - ATPase ACTIVITY OF THE IMMUNE SYSTEM IN CHRONIC ADDICTS.

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The pesent study has been aimed to investigate the effects of opioids on free radical scavenger system profile of immune system of chronic addicts to highlight the role of morphine on oxidative stress and on immune system. Total glutathione content , and activities of some related enzymes of glutathione oxidant system and Na^+, K^+ - ATPase were studied in the lymphocytes 30 minutes after the intravenous administration of abused drug. The present study revealed that morphine suppressed the total Glutathione content and total enzyme activities of Glutathione -S- Transferase, Glutathione reductase, catalase and Na^+, K^+ - ATPase significantly as compared to control values. Nonsignificant suppression of total lymphocyte count and body weight have also been found as compared to controls. As the generation of reactive oxygen species and scavengers of oxygen radicals are site specific, these results provide an explanation in part for the greater susceptibility of immune system to free radical damage under opioidergic modulation.

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BIMODAL OPIOID EFFECTS ON VAGAL BRADYCARDIA

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Introducing met-enkephalin-arginine-phenylalanine (MEAP) into the interstitium of the canine sinoatrial (SA) node by microdialysis interrupts vagal bradycardia. In contrast, raising endogenous MEAP by occluding the SA node artery improves vagal bradycardia. This study was conducted to test the hypothesis that vagal responses to enkephalin are bimodal and that ultra-low-dose enkephalin is vagotonic. Doses of MEAP well below those previously determined as vagolytically ineffective were introduced into the nodal interstitium and carefully evaluated. Heart rate, frequency-responses were constructed by stimulating the right vagus nerve at 1, 2, and 3 Hz. Ultra-low MEAP infusion rates produced a 50-100% increase in bradycardia during vagal stimulation. The maximal improvement was at 500 fmol/min. The vagal improvement returned to control when naltrindole was added at a dose (5 pmol/min) previously determined as completely ineffective versus the vagolytic effect of MEAP. When MEAP was later re-introduced in the same animals at 10,000 X the dose (5 nmol/min), a clear vagolytic effect was observed. These data support the hypothesis that opioid effects within the SA node are bimodal in character.

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IN VIVO STUDIES OF P-GLYCOPROTEIN AND OPIOIDS; DOES CALCIUM CHANNEL BLOCKERS ACT AS P-GLYCOPROTEIN INHIBITOR?

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It is well known that pretreatment with verapamil (VER) and diltiazem (DTZ) produced a dose-dependent potentiation of the morphine (MOR) analgesia, and these drugs are inhibitors of P-glycoprotein (P-gp; drug efflux pump in CNS) in *in vitro* studies. In this experiment, we examined the effects of VER and DTZ on brain content and serum concentration of MOR in male P-gp knock out (KO) and ICR mice, using HPLC-ECD method. Brain content, but not serum concentration, of MOR in KO mice (45 min after MOR; 4 mg/kg, s.c.) was higher than that in wild-type mice, indicating that MOR is a substrate of P-gp. Pretreatment with VER and DTZ increased both brain content and serum concentration of MOR in ICR mice. However, the brain/serum ratios were decreased in a dose-dependent manner, although the ratios are constant after morphine 1–132 mg/kg in naive ICR mice. Moreover, both VER and DTZ potentiated MOR analgesia in KO mice. VER decreased the brain/serum ratios of MOR, though it increased the brain content and serum concentration. These results suggest that VER and DTZ can not act as P-gp inhibitor in *in vivo*, as shown in *in vitro*.

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MU OPIOID RECEPTOR BINDING; EFFICACY AND POTENCY IN DORSAL ROOT GANGLIA AND INFLAMMATION

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Mu-, delta- und kappa-opioid receptors are expressed in primary sensory neurons and local administration of low doses of agonists elicits potent analgesia in inflamed, but not in non-inflamed tissue. In the present study we have addressed the role of inflammation on opioid binding, efficacy and potency in dorsal root ganglia (DRG). 96 hours after induction of hindpaw inflammation we found a small increase in MOR expression in DRG. The affinity of several mu-ligands and the maximal level of [^{35}S] GTPγS binding stimulated by the different agonists did not change significantly during inflammation. Compared with hypothalamus (HT) the intrinsic efficacy in inflamed and non-inflamed DRG was significantly lower. However, the potencies to activate G-proteins were comparable between HT and DRG from noninflamed hindlimbs. Interestingly, potencies for G-protein coupling shifted 14fold between inflamed versus non-inflamed tissue. This suggests that agonist occupancy of MOR in inflamed tissue may activate more or different types of G protein. These results are consistent with observations regarding the increased analgesic effects of peripheral opioids during inflammation.

II. SIGNAL TRANSDUCTION

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HETEROLOGOUS MU-RECEPTOR MODIFICATION BY REPEATED STIMULATION OF KAPPA-RECEPTOR IN THE MOUSE BRAIN

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The present study was designed to investigate the effect of repeated administration of a selective kappa-agonist (-)U-50,488H on the antinociception and G-protein activation induced by mu-agonists in mice. Repeated s.c. administration of (-)U-50,488H resulted in the development of tolerance to (-)U-50,488H-induced antinociception. Using the [^{35}S]GTPgammaS binding assay, we found that (-)U-50,488H was able to produce an enhancement of G-protein activation in the mouse thalamus. Repeated administration of (-)U-50,488H caused a significant reduction in the (-)U-50,488H-stimulated G-protein activation in this region. Under these conditions, we demonstrated that repeated injection of (-)U-50,488H significantly enhanced the antinociceptive effect of selective mu-agonists, endomorphin-1, endomorphin-2 and DAMGO. Furthermore, chronic treatment with (-)U-50,488H exhibited an increase in the mu-agonist-activated G-protein in the mouse thalamus. These results suggest that repeated stimulation of kappa-receptors leads to the up-regulation of mu-receptor functions in the mouse thalamus, which may be associated with the supersensitivity of mu-receptor-mediated antinociception.

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EFFICACY DIFFERENCES FOR AGONIST SIGNALING TO MULTIPLE PATHWAYS THROUGH MU OPIOID RECEPTORS

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Most clinically-relevant opioid drugs act mainly through mu opioid receptors (MOR). This study compared relative efficacies of several opioid ligands at multiple levels of signal transduction in MOR-expressing CHO cells. The full agonist, [D-Ala2, MePhe4, Gly-OH5]enkephalin (DAMGO), activated G-proteins, inhibited adenylyl cyclase, activated extracellular signal regulated kinases (ERK) and protein kinase B (PKB), and stimulated cellular proliferation in serum-deficient media. All of these effects were blocked by naloxone or pretreatment with pertussis toxin. Morphine, sufentanil, and methadone also were full agonists for G-protein activation and adenylyl cyclase inhibition, whereas meperidine, buprenorphine, butorphanol and nalbuphine were partial agonists. Similar relative efficacies of these ligands were observed for activation of ERK and PKB. However, only DAMGO and morphine were full agonists in stimulating proliferation, whereas sufentanil and methadone were partial agonists. These results suggest pathway-dependent relative efficacies of opioid agonists in MOR-CHO cells.

Supported by DA-10770 from NIDA.

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MU OPIOID RECEPTOR MOR-1 AND ITS SPLICE VARIANTS IN THYMUS.

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Opioid receptors are widely expressed in the central nervous system where they mediate the analgesic actions of opioids and modulate numerous endogenous functions. However, evidence has been accumulating for peripheral actions as well as in immune cells. Since the initial description of the cloned mu opioid receptor (MOR-1, also described as MOP1), a number of splice variants have been reported. In the current studies, we now report the presence of MOR-1 and several additional splice variants in thymus. Using RT-PCR, we obtained evidence for the expression of MOR-1, as well as MOR-1G, MOR-1K and MOR-1I. These variants have been cloned and sequenced and correspond to the same splice variants previously reported in mouse brain (*Proc. Natl. Acad. Sci. USA* **98**,14084-14089). Additional work exploring the significance of these variants will hopefully extend our understanding the effects of opioids on the immune system.

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CHRONIC AGONIST TREATMENT CONVERTS ANTAGONISTS INTO INVERSE AGONISTS AT DELTA OPIOID RECEPTORS

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In cellular models, chronic exposure to mu-opioid agonists converts antagonists into inverse agonists at mu-receptors. Such adaptations could contribute to the development of tolerance and/or dependence. To determine if delta-receptors respond similarly, or if this adaptation is unique for mu-receptors, this study examined the effects of prolonged agonist exposure on the intrinsic activity of several delta-opioid ligands in GH3 cells expressing delta- receptors. In opioid naïve cells, delta-receptors were constitutively active and a series of delta-ligands displayed a range of intrinsic activities for G protein activation. Chronic treatment with the full delta-agonist [D-Pen2,5]enkephalin reduced the acute ability of [D-Pen2,5]enkephalin to stimulate, and the full inverse agonist ICI-174864 to inhibit G protein activation. In contrast, while naloxone and naltriben exhibited weak partial agonism in opioid naïve cells, both ligands acted as full inverse agonists to produce concentration-dependent inhibition of [^{35}S]GTPgamaS binding following prologned exposure to [D-Pen2,5]enkephalin or to the partial agonist morphine. This effect was reversed by a neutral delta-antagonist ICI-154129. Finally, as is also characteristic of inverse agonists, naloxone and naltriben demonstrated higher affinities for uncoupled delta-receptors in cells chronically treated with [D-Pen2,5]enkephalin, relative to opioid naïve cells. Therefore, this relatively novel adaptation is shared by both mu- and delta-opioid receptors and therefore may serve as an important common mechanism involved the development of tolerance and/or dependence.

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THE FGF RECEPTOR IS AT THE SITE OF CONVERGENCE BETWEEN MU OPIOID RECEPTOR AND GROWTH FACTOR SIGNALING PATHWAYS IN C6 CELLS

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Although the mu opioid receptor (MOR) mediates stimulation of ERK via EGFR transactivation in HEK293 cells, the mechanism of acute MOR signaling to ERK has not been characterized in rat C6 glioma cells that appear to contain little EGFR. Here we implicate FGFR1 transactivation in the convergence of MOR and growth factor pathways leading to ERK activation in C6 cells. MOR agonists, endomorphin-1 and morphine, induced a rapid increase of ERK phosphorylation that was abolished by CTAP. By using selective inhibitors and overexpression of dominant-negative mutants, data were obtained to suggest that MOR signaling to ERK is transduced by G-beta-gamma and entails Ca^{2+}- and PKC-mediated steps while the FGFR1 branch of the pathway is Ras dependent. An intermediary role of FGFR1 transactivation was suggested by MOR- but not kappa opioid receptor- (KOR-) induced FGFR1 tyrosine phosphorylation. A dominant negative mutant of FGFR1 attenuated MOR- but not KOR- induced ERK phosphorylation. Thus, a novel transactivation mechanism entailing secreted endogenous FGF may link the MOR and growth factor pathways.

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PATTERNS OF ERK ACTIVATION DURING OPIOID EXPOSURE AND WITHDRAWAL IN NEURAL AND GLIAL CELL LINES

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Acute mu-opioid application has been shown to activate extra-cellular signal-related kinases (ERKs) in various non-neural cell lines. ERK activation has never been shown *in vivo* following acute morphine treatment, but has been observed *in vivo* during opioid withdrawal. The goal of this study was to determine if mu agonist treatment induced ERK activation acutely or after removal of opioid in various cell lines. Our results showed that acute application of fentanyl was able to activate ERK in a glial but not neuronal cell lines. This observation suggests that ERK could play a role in acute opioid effects in glial cells. In another set of experiments, cells were chronically treated with escalating doses of fentanyl. After 8 days, fentanyl was removed from the media and naloxone applied. ERK activation was not seen in any tested cell line acutely after fentanyl removal. This finding suggests that the ERK activation observed during opioid withdrawal *in vivo* may be an indirect phenomenon due to effects on neural homeostasis that cannot be reproduced in an *in vitro* model.

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CHARACTERIZATION OF OPIOID RECEPTOR TYPES IN AMPHIBIAN SPINAL CORD

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Recent cloning in our lab has revealed the presence of three distinct opioid receptor types in the amphibian, *Rana pipiens*. The present study utilizes tritiated opioid ligands to characterize the abundance of each opioid receptor type and the affinities of each receptor in spinal cord tissue homogenates using the Millipore Multiscreen cell separation system. Receptor density (Bmax) and affinity (Kd) values were ascertained first with the nonspecific opioid antagonist [^{3}H]-naloxone. Following this, spinal cord tissue homogenates were analyzed using the opioid type-specific agonists [^{3}H]-DAMGO (mu), [^{3}H]-U69593 (kappa), and [^{3}H]-DPDPE (delta). Cold competition studies were performed using unlabeled mu, kappa, and delta selective agonists and antagonists. Receptor density and affinity values are reported with comparisons to previously published amphibian brain and mammalian spinal cord studies. This is the first study evaluating the affinity, selectivity, and relative abundance of opioid binding sites in *Rana pipiens* spinal cord. Research supported by NIH grant DA12448.

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ACTIVATION OF THE MU OPIOID RECEPTOR INVOLVES CONFORMATIONAL REARRANGEMENTS OF MULTIPLE TRANSMEMBRANE DOMAINS.

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We identified the cysteine(s) within the TMs of the two constitutively active mutants (CAMs) D3.49(164)Y and T6.34(279)K that became accessible in the binding pocket. We have shown that the C7.38S mutant is insensitive to MTSEA, a charged, hydrophilic, sulfhydryl-specific reagent. Each Cys in TMs was mutated to Ser on the D3.49Y/C7.38S or T6.34K/C7.38S background. Either C6.47S triple mutants had no or very low binding. D3.49Y/C7.38S and T6.34K/C7.38S and the other triple mutants had similar affinities for [^{3}H]diprenorphine and DAMGO, indicating that they retained CAM phenotypes. The second-order rate constants for MTSEA reactions showed that C3.44S, C4.48S, C5.41S and C7.47S on the D3.49Y/C7.38S background became less sensitive to MTSEA. C4.48S, C5.41S and C7.47S were less sensitive than the T6.34K/C7.38S background. These results indicate that C3.44, C4.48, C5.41, C7.47 of the D3.49Y and C4.48, C5.41 and C7.47 of the T6.34K were rotated and/or tilted to become accessible in the binding pocket, suggesting that movements of TMs3, 4, 5 and 7 are associated with activation and there are different activated states. (supported by NIH grants)

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CHRONIC INVERSE AGONISM AT THE δ OPIOID RECEPTOR: A NOVEL MECHANISM FOR MIMICKING ASPECTS OF ACUTE AGONIST SIGNALING

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Chronic activation of opioid receptors, even by weak partial agonists, effectively induces cyclase supersensitivity. This adaptation occurs in part by increased coupling to stimulatory G-proteins and probably contributes to neuronal hyperactivity during opiate withdrawal. Since δ opioid receptors display constitutive activity, we reasoned that cells expressing δ receptors would have heightened basal sensitivity to cyclase stimulation. In this study we examined the effect of chronic treatment of ICI-174,864, a δ receptor inverse agonist, on cyclase stimulation in NG-108-15 cells. Chronic ICI-174,864 treatment produced inhibition of both forskolin and prostaglandin E1 stimulation of cAMP accumulation that was comparable to acute agonist inhibition with DPDPE. This novel phenomenon, termed cyclase subsensitivity, was also observed following inverse agonist treatment of HEK-293 and GH3 cells transfected with the δ receptor. This finding reveals that the delta opioid receptor can be excitatory in the absence of agonist stimulation and is a novel mechanism for mimicking aspects of acute agonist signaling. Inverse agonism at the δ receptor might be a new target for drug discovery and the treatment of pain.

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III. TRAFFICKING

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OPIOID RESPONSES ARE REGULATED BY TYR-PHOSPHORYLATION

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Tyrosine phosphorylation of opioid-activated potassium channels inhibits basal conductance and affects G-beta-gamma activation. To define the underlying mechanisms, we reconstituted MOR, GIRK, and trkB in *Xenopus laevis* oocytes and found that BDNF activation of trkB accelerated GIRK deactivation kinetics during the opioid response. Overexpression of the GTPase activating protein (GAP) RGS4 similarly accelerated the kinetics. GTP-gamma-S blockade of GTPase activity abrogated the trkB-dependent acceleration of GIRK deactivation. Similarly, substitution of phenylalanine for N terminal tyrosines in the channel blocked the trkB effect. Kinetic profiles were consistent with the hypothesis that Tyr-phosphorylation of GIRK increased the channel's intrinsic GTPase activity rather than decreasing affinity for G-beta-gamma. The results suggest that the GIRK tyrosines form part of a GAP motif. We conclude that tyrosine phosphorylation of GIRK unmasks a GTPase-activating sequence in the N-terminus, accelerating GIRK inactivation and regulating the opioid response. Supported by DA11672 and GM07270.

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A ROLE FOR FILAMIN A IN MU OPIOID RECEPTOR DOWN-REGULATION

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Using the yeast two-hybrid system, we detected proteins capable of interacting with the human mu opioid receptor (hMOP). As previously reported, the hMOP carboxyl tail (hMOP-C) binds to the carboxyl terminal region of human filamin A. Direct binding between these proteins was demonstrated. To investigate the role of filamin A in opioid receptor function we used a melanoma cell line M2, which does not express filamin A and its subclone A7, stably transfected with human filamin A. Both cell lines were stably transfected with cDNA encoding myc-tagged hMOP. Binding experiments, using [^{3}H]diprenorphine and [^{3}H]DAMGO, showed no significant difference in binding affinity (Kd~0.25nM and ~2.5nM, respectively) between M2 and A7 cells. Down-regulation studies revealed that after 24 hr treatment with 5μM DAMGO, filamin A-containing cells showed a decrease in total receptor binding sites of 55-60%, whereas in cells lacking filamin A, down-regulation of hMOP was virtually abolished. A concentration-dependence curve indicated that DAMGO treatment of cells lacking filamin A had an EC50 for down-regulation 3-orders of magnitude higher than cells containing filamin A. Filamin A or the actin cytoskeleton, via filamin A, plays a role in MOP down-regulation, perhaps, through effects on receptor trafficking to the lysosomes. (supported by grants R01-DA00017 and KO5-DA00364 to EJS).

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SUPPRESSION OF μ OPIOID RECEPTOR DESENSITIZATION AND PHOSPHORYLATION BY A PROTEIN KINASE C-INTERACTING PROTEIN IN CHO CELLS

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This study was designed to identify and functionally characterize novel intracellular protein(s) that participate in the cellular regulation of mu opioid receptor (mOR) signaling. A protein kinase C-interacting protein (PKCI) specially interacted with C-terminus of mOR was identified in yeast two-hybrid screening and the interaction was recapitulated in CHO cells stably expressing full-length mOR with transiently transfected PKCI. The affinity of mOR to opioid ligand and its ability to mediate the activation of G-protein and inhibition on adenylyl cyclase were not changed by the interaction. However, the association of PKCI with mOR induced partial suppression of mOR desensitization at G protein level and further amplified to complete inhibition at adenylyl cyclase level. The effect was specific to mOR since dOR desensitization was unaffected by PKCI. Furthermore, the PMA-induced mOR phosphorylation was partially inhibited by the co-expression of PKCI, suggesting the differential regulation effects of PKCI on mOR when exposed to different stimulation. These results indicated that PKCI is functioning as a negative regulator of mOR desensitization and phosphorylation, implying its role in tolerance and/or dependence development.

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MU-OPIOID RECEPTOR-MEDIATED ACTIVATION OF PHOSPHOLIPASE D2 ENHANCES RECEPTOR ENDOCYTOSIS IN HEK293 CELLS

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The rat mu-opioid receptor (rMOR1) was shown to stimulate phospholipase D2 (PLD2) in an ARF-dependent manner in HEK293 cells coexpressing rMOR1 and PLD2. Because PLD2 has been implicated in the formation of endocytotic vesicles, we tested the involvement of PLD2 in the agonist-mediated mu-opioid receptor endocytosis. Confocal microscopy analysis revealed a colocalization of rMOR1 with PLD2 in the plasma membrane, but no cointernalization of rMOR1 and PLD2 after treatment with the mu-agonist DAMGO. Interestingly, rMOR1-mediated activation of PLD2 was detected after DAMGO treatment, but not in response to morphine, which does not induce rMOR1 receptor endocytosis. Furthermore, we observed a DAMGO-stimulated ARF binding to rMOR1, which could not be detected under morphine treatment. However, stimulation of PLD2 activity by phorbol ester (PMA) led to an accelerated opioid receptor endocytosis after both DAMGO and morphine exposure, whereas inhibition of PLD2 activity by 1-butanol reduced agonist-mediated receptor endocytosis. These data suggest that PLD2 plays a key role in the regulation of agonist-induced receptor endocytosis.

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MU OPIOID RECEPTOR ACTIVATION IN VENTRAL TEGMENTAL NEURONS FOLLOWING SEXUAL BEHAVIOR

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Common mesolimbic reward circuits regulate drug abuse and natural motivated behaviors such as sexual behavior. Like drugs of abuse, sexual behavior causes dopamine (DA) release in the nucleus accumbens. This DA originates in the ventral tegmental area (VTA). Indeed, we previously showed sex induced activation of VTA DA neurons. Prior evidence suggests that mu opioid receptors (MOR) modulate these VTA DA neurons via inhibitory GABA interneurons. The goal of the present study was to determine if sexual behavior results in the release of endogenous opioid peptides in the VTA by measuring ligand induced internalization of MOR. Adult male rats were divided into 3 groups: Experienced animals (four prior exposures) that mated in home (EH) or test cage (ET) and naïve animals (NH). Males were sacrificed 1 hour after ejaculation, VTA sections were immunostained for MOR, and internalization was quantified using confocal microscopy. The results show significant sex-induced internalization of MOR, thus indicating that endogenous opioids are released in the VTA during natural motivation and are involved in activation of mesolimbic reward pathways.

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FUNCTIONAL CHANGES IN RAB4-ASSOCIATED MU-RECEPTOR RECYCLING, SYNAPTIC VESICLE MODIFICATION AND AXON GUIDANCE BY CHRONIC BLOCKADE OF MU-RECEPTORS IN MICE

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In the present study, we demonstrated that chronic treatment with an opioid receptor antagonist naloxone caused the supersensitivity to activate G-protein and the increase of mu-receptors on the membrane surface without changing in the levels of mRNA that encodes mu-receptors. Immunohistochemical study revealed that some cytosol-located mu-receptors were translocated to the plasma membrane by chronic naloxone treatment. Under these conditions, the level of Rab4, which is the small GTPase that has been shown to control recycling and endosomal fusion, was significantly increased in membranes of the spinal cord, whereas the levels of phosphorylated-synapsin I that can regulate neurotransmitter release in nerve terminals and a proteoglycan brevican were significantly decreased by chronic naloxone treatment. These findings suggest that chronic protection of mu-receptors leads to the functional changes in Rab4-associated mu-receptor recycling and induces a decline in the neuronal network with the decreased neurotransmitter release in the mouse spinal cord.

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EFFICACY OF MORPHINE DIFFERS FROM OTHER OPIOIDS FOR INTERNALISATION VERSUS G-PROTEIN ACTIVATION AND DESENSITIZATION IN A SINGLE MOR-EXPRESSING CELL-LINE

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Differences in agonist-induced MOR internalization may arise from differences in agonist efficacy or distinct agonist-induced receptor states. We used AtT-20 cells stably expressing FLAG-tagged MOR to compare the efficacies of agonists to (1) activate G-proteins using inhibition of calcium channel currents (ICa) as a reporter before and after inactivation of a fraction of receptors by β-CNA, (2) produce fast desensitization of ICa inhibition and (3) internalize receptors. Using receptor inactivation, relative efficacies for G-protein coupling were DAMGO > methadone ≥ morphine > pentazocine. For fast desensitization, the same rank order of efficacies was observed but much greater concentrations of agonist were required than for G-protein activation. By contrast, relative efficacies for promoting internalization were DAMGO > methadone >> morphine ≥ pentazocine. These results substantiate previous studies suggesting morphine uniquely induces a receptor state that is a poor substrate for endocytosis, but is relatively more efficacious at activating G proteins and producing fast desensitization.

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EXPRESSION OF MU-OPIATE RECEPTOR AND BETA-ENDORPHIN IN PERIPHERAL NERVE ENDINGS IN HUMAN SKIN

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The presence of the mu opiate receptor system in human epidermis adds a new dimension to the recent developed research in neuro-immuno-dermatology and in the neurogenic inflammation in skin diseases. We have previously shown that human epidermal keratinocytes express a functional active mu-opiate receptor and other authors show that the keratinocytes specifically bind and also produce the mu-opiate receptor ligand and POMC-derivative beta-endorphin. Using confocal imaging microscopy we could now demonstrate that mu-opiate receptors are not only expressed in keratinocytes but also on non-myelinated peripheral nerve fibers in dermis and epidermis. Some of the peripheral nerve fibers also express the ligand beta-endorphin. The beta-endorphin producing keratinocytes are clustered around the ends of the non-myelinated nerve fibers. These results show that there really exists a direct communication between nerve and epidermis through opiate receptor system. The keratinocytes can influence the non-myelinated nerve fibers in epidermis directly via secreting beta-endorphin. Through the K+-channels of the G-protein coupled mu-opiate receptor in the peripheral nerves and difference in membrane potentials sensations such as pain or itch could be modulated. On the other hand nerve fibers can also secrete beta-endorphin and influence the migration, differentiation and probably also the cytokine production pattern of keratinocytes. Our results reveals the new understanding of neurogenic inflammation and the influence of stress in various diseases.

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DESENSITIZATION OF μ-OPIOID RECEPTOR EVOKED POTASSIUM CURRENTS AND RECEPTOR REDISTRIBUTION

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Dependence to opioids is mediated by non-associative plasticity of μ-opioid receptor (MOR) transduction. Here we examine a very early step of opioid-associated neuroadaptation: the acute desensitization of MOR-evoked G-protein coupled inwardly rectifying potassium (GIRK) currents and investigate the role of receptor redistribution for this phenomenon. In neurons of acute slices of the locus coeruleus, GIRK currents substantially desensitized within 15 minutes. To specifically block MOR internalization through endocytosis of clathrin-coated vesicles, cells were loaded with a peptide of 15 amino acids coding for the interaction site of dynamin with amphiphysine. We found that under these conditions, the maximum currents elicited as well as the degree of desensitization for DAMGO and met-enkephalin did not differ significantly from control experiments without the peptide. Furthermore the application of various protein kinase inhibitors also left desensitization unchanged. Conversely, preliminary results from experiments to block exocytosis with NEM and the light chains of Botulinum Toxine apparently increased desensitization by reducing the steady state current after 15 minutes. These results suggest that desensitization occurs prior to MOR internalization, but depends on rapid receptor reinsertion.

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NEUROTROPHIN-INDUCED SORTING OF OPIOID RECEPTORS INTO A REGULATED SECRETORY PATHWAY

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Delta opioid receptors are targeted to the plasma membrane of non-neural cells but are present primarily in intracellular membranes of neurons. Here we describe a previously unanticipated role of neurotrophin signaling in specifically redirecting newly synthesized delta opioid receptors from a constitutive to a regulated export pathway following exit from the Golgi apparatus and in maintaining this intracellular pool for later release by depolarization-dependent exocytosis. These studies identify a mechanism by which neurosecretory cells form an intracellular reserve pool of specific signaling receptors which can be rapidly mobilized to the plasma membrane. They also establish a novel role of receptor tyrosine kinase-mediated signaling in regulating the export and sorting of integral membrane proteins from the trans-Golgi network in neural cells.

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DIFFERENTIAL GENE EXPRESSION IN THE RAT CAUDATE PUTAMEN AFTER "BINGE" COCAINE ADMINISTRATION: A MICROARRAY REPLICATE STUDY

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Rat Genome U34A (Affymetrix®) microarrays were used to analyze changes in gene expression in the caudate putamen (CPu) of Fischer rats induced by 1 day and 3 days of "binge" cocaine administration (3 x 15 mg/kg, ip, 1 h intervals). A triplicate chip assay on pooled RNA for each treatment group was used to evaluate the technical variability and sensitivity of the microarrays. In the triplicate chip analysis (Data Mining Tool, Affymetrix®) genes/ESTs with consistencies in change calls ≥66% were chosen and then analyzed using a t-test of signal intensities to examine group differences. Data analysis performed in triplicate produced more reproducible results than either single or duplicate chip comparisons and detected 89 up-regulated and 8 down-regulated genes after 1 day of "binge" cocaine. In contrast, 3-day cocaine treatment reduced the number of up-regulated genes to 21 and increased the number of down-regulated genes to 17. RNase protection assays for selected genes to confirm microarray results indicated that two novel genes, somatostatin receptor SSTR2 and the clock gene Per2 were differentially expressed after either 1 or 3-day "binge" cocaine. Support: NIDA P50-DA 05130 and K05-00049.

IV. GENE REGULATION/GENETICS

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REGULATION OF DYNORPHIN GENE EXPRESSION BY KAPPA-OPIOID AGONISTS AND COCAINE

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Kappa-opioid agonists decrease the behavioral effects of cocaine and chronic cocaine upregulates kappa-opioid receptors and alters dynorphin gene expression. In this study, rats were treated for 5 days with U-69593 or vehicle, and challenged 3 days later with either cocaine or saline. Two weeks later, prodynorphin mRNA was examined. Dynorphin gene expression was increased in hypothalamus, striatum, and frontal cortex of rats treated with U-69593 and challenged with saline. A single challenge injection of cocaine on day 8 did not alter the increased prodynorphin mRNA in hypothalamus and striatum. There were no changes in any brain region after vehicle + cocaine treatment. In contrast, a single injection of cocaine subsequent to the U-69593 treatment blocked the U-69593-induced increase in prodynorphin mRNA in the frontal cortex. These findings show that kappa-opioid receptor agonist treatment has long-term effects on dynorphin gene expression. In addition, the regulation of dynorphin gene expression by cocaine is altered subsequent to kappa-opioid agonist treatment. Supported by DA 11960.

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ONTOGENIC EXPRESSION PATTERNS OF MU OPIOID RECEPTOR SPLICE VARIANTS

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Differences in opioid pharmacology have implied the existence of multiple mu opioid receptors (MOR), yet only a single gene (Oprm) encoding such a receptor has been cloned. Posttranscriptional processing of Oprm through alternative splicing results in MOR diversity. MOR1 mRNA comprises exons 1 through 4. Two sets of alternatively spliced variants processed at either the 3' or 5' end of the gene have been identified. Regional differences in the brain distribution of the splice variants have been shown. The functional importance of MOR diversity is yet to be understood. Previous studies indicate a change in morphine analgesia and formalin-induced pain in rats during the first 25 days after birth, implying qualitative changes in pain processing.The present study aims to determine whether these pharmacological changes correlate with ontogenic expression patterns of the MOR variants. Immunohistochemistry, RT-PCR, and western blotting of rat and mouse brains at different postnatal days was performed using splice variant specific probes. Differential transcript expression and protein distribution of the variants during postnatal development was observed.

V. NOVEL LIGANDS

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GYKI 47261: ANOTHER POWERFUL AMPA ANTAGONIST WITH ANTINOCICEPTIVE ACTIVITY

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2,3-benzodiazepines are non-competitive and selective antagonists of the AMPA sensitive subtype of glutamate receptors. The prominent member of this class of drugs, talampanel, is now under phase II clinical trials as an antiepileptic agent. However, as reported earlier, GYKI 52466, the parent compound, also shows considerable antinociceptive potency in several conventionally used analgesia assays. GYKI 47261, a recently synthesized analog, chemically 6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo[1,2-c][2,3] benzodiazepine, has shown a similar pharmacological profile. *In vitro* it dose-dependently inhibits the AMPA induced spreading depression and inward cationic currents in isolated chicken retina and patch-clamped neonatal rat Purkinje neurons, respectively. In mice it potently inhibits the convulsions induced by electroshock and various chemoconvulsive agents. As for its antinociceptive activity, in mice it potently inhibits the phenylquinone-induced writhing and the pain reactions induced by intrapaw formalin. However, GYKI 47261 is somewhat weaker in mouse hot plate and rat tail-flick assays. Its antinociceptive action cannot be reversed by naloxone.

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NOVEL, ORALLY ACTIVE, DELTA/MU OPIOID ANALGESIC, RWJ-394674

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Although the mu opioid receptor is the primary target of marketed opioid analgesics, several studies suggest the advantageous effect of combinations of mu and delta opioids. RWJ-394674 bound with high affinity to the delta opioid receptor (0.2 nM), and somewhat weaker affinity to the mu receptor (72 nM). GTPgammaS assays demonstrated delta agonist and mu antagonist functionality. Mouse hot plate (48°C) evaluation of RWJ-394674 revealed potent oral antinociception (ED50=10.5 micromol/kg or 5 mg/kg), accompanied by a moderate Straub tail. Probe antagonist studies demonstrated the opioid nature of the antinociception (naloxone sensitive) as well as attenuation by subtype selective antagonists, delta (naltrindole) and mu (beta-funaltrexamine).
RWJ-394674 was metabolized to its N-des-ethyl analog, RWJ-413216, with a markedly different opioid receptor profile: Ki=47 and 0.26 nM at the delta and mu opioid receptors respectively, both agonist functionality. RWJ-413216 demonstrated potent oral antinociceptive effect [ED50=4.7 micromol/kg (2 mg/kg), 48°C MoHP]. Thus RWJ-394674 is a delta opioid that appears to augment its antinociceptive effect through biotransformation to a novel mu selective analog.

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A NEW TRITIATED TIPP ANALOGUE

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The delta-opioid receptor antagonist TIPP (Tyr-Tic-Phe-Phe-OH) has been reported as a highly potent and very selective peptide (1).
Some tritiated TIPP analogues ([^{3}H]-TIPP, [^{3}H]-TIPPψ, [^{3}H]-TICP) were developed in our laboratory. A systematic investigation of TIPP containing beta-methyl amino acids has resulted in the Tyr-Tic-(2S,3R)-beta-MePhe-Phe-OH which is a more potent and a more selective ligand for delta opioid receptors compared to the parent peptide (2). Here we present the synthesis and specific binding properties of tritium labelled new TIPP analogues (SA: 1.99 TBq/mmol) in rat brain homogenates.
According to the direct binding assays, the new tritiated peptide ligand is a very active and very selective to delta opioid receptors and a good tool due to the low non-specific binding.
1. P. Schiller et al: *Proc. Natl. Acad. Sci.* U.S.A. 89, 11871 (1992)
2. D. Tourwé et al: *J. Med. Chem.* 41, 5167 (1998)
Supported by grants OTKA T 30086 and NKFP /OM0217/2001

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BUPRENORPHINE AND ETORPHINE FAILED TO PRODUCE ANTINOCICEPTION, BUT BLOCKED THE ANTINOCICEPTIVE EFFECT OF U50,488H, IN MU RECEPTOR KNOCKOUT MICE

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Buprenorphine (BUP) and Etorphine (ETOR) have been shown to interact not only with mu but also with opioid receptor-like (ORL-1, also known as NOP) receptors as well as delta and kappa opioid receptors. However, whether ORL-1, delta or kappa opioid receptors could contribute to the antinociceptive action of these drugs remain to be elucidated. In the present study, we determined if BUP or ETOR would produce antinociception in mice lacking the mu opioid receptor using the tail flick test. BUP and ETOR each produced a dose-dependent antinociception in the wild type mice. As expected, BUP produced a sub-maximal antinociception, whereas etorphine produced a maximal antinociceptive response. However, both drugs failed to produce any antinociception in mice lacking the mu opioid receptor at 10-100 fold higher doses than used in the wild type mice. Interestingly, at these doses, however, both drugs blocked the antinociceptive action of U50,488H, a kappa opioid receptor agonist. Overall, our results indicate that the antinociceptive effect of BUP and ETOR was primarily mediated by the mu opioid receptor. Furthermore, the inhibitory action of BUP and ETOR on U50,488H-induced antinociception could be mediated via their actions at the ORL-1 receptor. (Supported in part by NIDA DA 05010; KL was supported by a KO1 award DA00411 from NIDA)

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KAPPA OPIOID ANTAGONISTS DERIVED FROM DYNORPHIN A, DYNORPHIN B AND ALPHA-NEOENDORPHIN

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We recently showed that replacement of Tyr1 by (2*S*)-2-methyl-3-(4-hydroxy-2,6-dimethylphenyl)propanoic acid [(2*S*)-Mdp] represents a generally applicable structural modification to convert opioid peptide agonists to antagonists. As we reported, [(2*S*)-Mdp1]dynorphin A (Dynantin) is a highly selective kappa antagonist with subnanomolar kappa receptor binding affinity. We now prepared (2*S*)-Mdp1-analogues of two dynorphin A octapeptide analogues, and of dynorphin B and alpha-neoendorphin. The (2*S*)-Mdp1-analogues of [MeTyr1,MeArg7,D-Leu8]dynorphin A(1-8)-NH2 and of its systemically active analogue with a C-terminal ethylamide group (E-2078) were found to be quite potent and selective kappa antagonists (Ke = 8 – 11 nM in the guinea pig ileum (GPI) assay). [(2-*S*)-Mdp1]dynorphin B showed quite high kappa receptor binding affinity (Ki = 9.12 nM) and antagonized the effects of dynorphins A and B, and of U50,488 (Ke = 46 – 179 nM) in the GPI assay. [(2*S*)-Mdp1]alpha-neoendorphin displayed similar antagonist properties. These compounds represent the first dynorphin B- and alpha-neoendorphin antagonists and may be useful as tools for studying kappa receptor subtypes.

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STUDIES OF WILD TYPE AND MUTANT mu OPIOID RECEPTORS WITH FLUOROGENIC REPORTER AFFINITY LABELS USING FLOW CYTOMETRY: EVIDENCE FOR CROSS-LINKING K233 and C235

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As a new approach to labeling opioid receptors, "reporter" affinity label involves the use of an aromatic ortho-dialdehyde moiety to cross-link neighboring lysine and cysteine residues at the receptor recognition site with the generation of a highly fluorescent isoindole. To overcome the shortcoming of compound 1 in following the kinetics of cross-linking by flow cytometry, compound 2 was synthesized. We studied the interaction of 2 with cloned wild type mu opioid receptor in the presence and absence of naltrexone (NTX) in order to establish the kinetics of specific fluorescence generation associate with the cross-linking. The finding that mutant mu opioid receptors, mu-K233R and mu-C235S, afforded no fluorescence intensity enhancement suggests that K233 and C235 are the cross-linking residues at the mu receptor recognition site, and molecular simulation data are consistent with these results.

VI. OPIOID RELATED MOLECULES

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THE NOP RECEPTOR MEDIATES THE URODYNAMIC EFFECTS OF INTRAVESICAL NOCICEPTIN/-ORPHANIN FQ IN PATIENTS WITH OVERACTIVE BLADDER

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Nociceptin/orphanin FQ (N/OFQ) modulates several biological functions by activating a GPCR named NOP. In a previous pilot study we have shown that N/OFQ inhibited the micturition reflex in patients with overactive bladder but not in normal subjects (Lazzeri et al., J Urol, 166, 2237, 2001). In the present study we evaluated the urodynamic effects of intravescical instillation N/OFQ and [desPhe1]N/OFQ (a N/OFQ metabolite that lacks affinity for the NOP receptor) in 14 patients with hyperreflexic bladder. Bladder capacity (BC, ml), threshold volume for appearance of detrusor hyperreflexia (VT-DH, ml) and maximal bladder pressure (MPB, cm H2O) were recorded by cistometrograms performed with saline and, after 30 min, with 1 μM N/OFQ (7 patients) or [desPhe1]N/OFQ (7 patients) according to a randomized double-blind design. Intravescical instillation of N/OFQ produced a statistically significant increase in BC (from 139 ± 18 to 240 ± 23) and VT-DH (from 84 ± 12 to 201 ± 26), while MPB was not changed. Intravescical instillation of [desPhe1]N/OFQ did not significantly modify any parameter. These results suggest that the inhibitory effect of N/OFQ on voiding reflex in patients with hyperreflexic bladder is due to NOP receptor activation. Thus, peripherally active NOP receptor agonists may represent an innovative approach to the management of neurogenic urinary incontinence due to detrusor hyperreflexia.

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PRENATAL HEROIN EXPOSURE ALTERS CHOLINERGIC RECEPTOR STIMULATED TRANS-LOCATION OF PKC ISOFORMS

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Mice were exposed prenatally to heroin by injecting dams with 10 mg/kg daily on gestational days 9-18. At adulthood, they showed behavioral deficits related to septohippocampal cholinergic synaptic function, concomitant with both pre- and postsynaptic cholinergic hyperactivity, including an increase in basal membrane-bound PKC activity, and a consequent desensitization of PKC to cholinergic input, both of which were highly correlated with behavioral performance. Cholinergic grafting reversed the biochemical and behavioral deficits. These results suggested that the primary defect might reside in disruption of signaling cascades. Further studies were carried out to pinpoint the changes in the behaviorally relevant PKC isoforms gamma and beta II (the latter was assessed with phospho-specific antibodies). There was a desensitization of both isoforms to cholinergic input, as attested to by the marked reduction in their carbachol induced translocation from cytosol to the cell membrane. (Supported by USPHS HD 40820, NC-Israel Partnership and Israeli Anti-Drug Authority)

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ANTINOCICEPTIVE EFFECT OF WIN55,212-2 IN DIABETIC MICE

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The antinociceptive effects of WIN55,212-2, a high affinity cannabinoid (CB) receptor agonist, were examined using the tail-flick test in non-diabetic and diabetic mice. Systemic administered WIN55,212-2, at doses of 0.3 – 3.0 mg/kg, i.p., produced dose-dependent antinociception in both non-diabetic and diabetic mice. The antinociceptive effect of i.p. WIN55,212-2 in diabetic mice was significantly greater than that in non-diabetic mice, as evidenced by a 3.8-fold leftward shift in the dose-response curve. The antinociceptive effect of WIN55,212-2 was significantly antagonized by either i.c.v. or i.t. pretreatment with SR141716A (10 micro-g), a selective CB1 receptor antagonist, in non-diabetic mice. In diabetic mice, the antinociceptive effect of WIN55,212-2 was also significantly antagonized by either i.c.v. (10 micro-g) or i.t. (30 micro-g) pretreatment with SR141716A. These results indicated that CB1 receptor-mediated antinociceptive systems may be enhanced in diabetic mice.

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MU OPIOID RECEPTOR REGULATION OF SEROTONIN2A (5-HT2A) RECEPTOR MEDIATED BEHAVIOR

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Based on *in vitro* electrophysiological evidence suggesting that mu opioid receptors modulate 5-HT_{2A} receptor activity, we have developed an *in vivo* model to test this hypothesis. Adult male New Zealand rabbits were used in experiments approved by our university's IACUC. Drugs were administered sc and behavioral observations were performed in the animal's home cage. Activation of 5-HT_{2A} receptors by the 5-$HT_{2A/2C}$ receptor agonist, DOI dose dependently elicits head bobs. Pretreatment with a low dose of the mu opioid receptor agonist, morphine, significantly attenuated DOI elicited head bobs. Morphine's effect was eliminated by pretreatment with the mu opioid receptor antagonist, naltrexone, establishing that morphine's effect is opioid receptor mediated. However, pretreatment with naltrexone did not increase spontaneous head bobs nor augment DOI elicited head bobs, suggesting that in the home cage (producing little or no stress) there is no endogenous opioid tone at the critical synapses mediating 5-HT_{2A} receptor function. These results demonstrate that mu opioid receptor/5-HT_{2A} receptor interaction occurs *in vivo* attenuating 5-HT_{2A} receptor mediated behavior. (Grant NIMH16841: J. Harvey)

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EFFECT OF A NON-PEPTIDIC κ-AGONIST, R-84760 ON EXTRACELLULAR DOPAMINE LEVELS IN THE CAUDATE PUTAMEN OF C57BL/6J MICE.

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The effect of the novel non-peptidic κ-agonist, R-84760, on dopamine levels in the caudate putamen was studied with *in vivo* microdialysis. Methods: Male C57/BL6J mice (6 weeks old) were allowed to acclimate for 1 week before surgery. A CMA guide cannula was implanted into the caudate putamen. After 4 days recovery, dialysis probes were lowered into the caudate putamen and mice were placed in individual microdialysis chambers. The next morning experiments were carried out on freely moving animals. R-84760 was administered i.p. (0.01, 0.05 and 0.1 mg/kg). Dialysates were collected in 20-min samples, up to 3 hrs. Results: ANOVA showed that R-84760 decreased dopamine levels in a dose dependent manner. The highest R-84760 dose (0.1 mg/kg) significantly decreased dopamine levels compared to vehicle, and this effect was completely blocked by pre-injection with 10 mg/kg of the κ-antagonist nor-BNI (Newman-Keuls *post hoc* tests). Conclusion: These findings suggest that the non-peptidic κ-agonist R-84760 reduced dopamine levels through the κ opioid receptors. Support: NIH-NIDA P50 DA05130 and KO5 DA00049 to MJK.

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