

Aus der Klinik für Anästhesiologie mit Schwerpunkt operative
Intensivmedizin der Medizinischen Fakultät Charité – Universitätsmedizin
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DISSERTATION

Leukocyte opioid receptors mediate analgesia via Ca²⁺-regulated
release of opioid peptides

zur Erlangung des akademischen Grades
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Zusammenfassung

Opioide sind der Goldstandard zur Behandlung von mittelstarken bis starken Schmerzen. Gemäß der klassischen Sichtweise bewirken sie eine Analgesie über die Regulation prä- und postsynaptischer Neuronen durch die Blockade des Ca^{2+} -Einstroms, und somit die Freisetzung exzitatorischer Neurotransmitter (z.B. Glutamat), sowie durch Hyperpolarisierung der synaptischen Membran über die Herabsetzung der intrazellulären K^+ -Konzentration. Die Anzahl der Verschreibungen von Opioiden ist innerhalb des letzten Jahrzehnts stark angestiegen, obwohl die Anwendung von Opioiden schwerwiegende, über das zentrale Nervensystem vermittelte Nebenwirkungen wie Atemdepression und Abhängigkeit zeigt. Diese Nebenwirkungen können möglicherweise durch die periphere Verabreichung von Opioiden umgangen werden. Immunzellen, welche sich an Verletzungen und Entzündungsherden anreichern, exprimieren Opioidrezeptoren und Opioidpeptide. In dieser Studie zeigen wir, dass die Aktivierung von Opioidrezeptoren auf Immunzellen diese zur Sekretion der endogenen Opioidpeptide Met-Enkephalin, β -Endorphin und Dynorphin A veranlasst, welche wiederum lokal Opioidrezeptoren auf peripheren sensorischen Neuronen binden und darüber eine Analgesie bewirken. Als Modell für neuropathischen Schmerz wurde eine chronische Kontstriktionsläsion des Ischiasnerves an Mäusen durchgeführt, welche zu mechanischer Hypersensitivität in der Pfote führte. Die lokale Verabreichung exogener Agonisten der δ -, μ - und κ -Opioidrezeptoren nahe der Verletzung und in der Gegenwart Opioidpeptid-exprimierender Immunzellen führte zu einer Schmerzminderung. Eine systemische Entfernung von Immunzellen sowie eine pharmakologische Inaktivierung von Opioidpeptiden führten zur Aufhebung dieses analgetischen Effekts. In Opioidpeptid-Knockout Mäusen war zudem die Schmerzlinderung durch lokal applizierte Opioidrezeptor-Agonisten verringert. In Immunzell-depletierten Mäusen führte ein Transfer von Immunzellen aus Wildtyp, nicht aber aus Opioidrezeptor-Knockout Mäusen zur Wiederherstellung der Opioid-induzierten Analgesie *in vivo*. *Ex vivo* bewirkten Agonisten der Opioidrezeptoren eine verstärkte Sekretion von Opioidpeptiden durch zuvor aus geschädigten Nerven isolierte Immunzellen. Die Peptidsekretion war abhängig von intrazellulärem Ca^{2+} . Eine Blockade der Gai/o und Gbg Untereinheiten heterotrimerer G-Proteine, der PLC und des IP_3 -Rezeptors führten zu einer auf basale Werte reduzierten Peptidsekretion, während eine Inhibition der PKC einen nur teilweise mindernden Effekt auf die Sekretion zeigte. Analog dazu konnte der analgetische Effekt lokal applizierter Opioide in Immunzell-depletierten Mäusen durch den Transfer von Immunzellen wiederhergestellt werden, wenn die Immunzellen *ex vivo* mit einem Chelator für

extrazelluläres Ca^{2+} behandelt wurden, nicht aber nach Behandlung der Immunzellen mit Inhibitoren der $\text{G}\alpha/\text{o}$ - und $\text{G}\beta\gamma$ Proteine oder einem Chelator für intrazelluläres Ca^{2+} . Diese Ergebnisse zeigen, dass Opioidrezeptoren auf Immunzellen über den Signalweg $\text{G}\alpha/\text{o}$ - $\text{G}\beta\gamma$ Protein-PLC- IP_3 Rezeptor, abhängig von intrazellulärem Ca^{2+} , *ex vivo* die Sekretion von Opioidpeptiden und *in vivo* Analgesie vermitteln. Dieser Signalweg stellt einen unkonventionellen Mechanismus zur Kontrolle pathologischer Schmerzen dar.

Abstract

Opioids are the gold standard for treatment of moderate to severe pain. Traditionally, opioids produce analgesia by regulating both pre- and post-synaptic neurons via blocking Ca^{2+} influx, consequently inhibiting the release of excitatory neurotransmitters (e.g., glutamate) and by hyperpolarizing the synaptic membrane via decreasing intercellular K^+ concentrations. Even though the number of prescriptions for opioids has greatly increased in the last decade, there are major problems with opioids due to their central side effects, such as respiratory depression and addiction, which might be avoided by peripheral administration of the opioids. Circulating leukocytes also produce opioid peptides and opioid receptors during injury and they accumulate at the inflammation site. Here we show that the activation of opioid receptors on leukocytes leads to the secretion of leukocyte derived endogenous opioid peptides Met-enkephalin, β -endorphin and dynorphin A, which bind to the local opioid receptors on peripheral sensory neurons to cause analgesia. As a model of neuropathy, chronic constriction injury of the sciatic nerve was performed on mice, which caused mechanical hypersensitivity. Pain was attenuated by the application of exogenous agonists of the δ -, μ -, and κ -opioid receptors at the injury site, which was infiltrated by opioid peptide containing leukocytes. The analgesic effect was abolished by systemic leukocyte depletion and pharmacological inactivation of opioid peptides. In addition, agonist induced pain relief was attenuated on opioid peptide knock-out mice. Adoptive transfer of leukocytes from wild-type into leukocyte depleted mice reconstituted agonist induced analgesia *in vivo*. Analgesia could not be reconstituted when leukocytes were transferred from opioid receptor knock-out mice. *Ex vivo*, opioid receptor agonists significantly elevated the secretion of opioid peptides from leukocytes isolated from damaged nerves. This secretion was dependent on intracellular Ca^{2+} and blocking $\text{G}\alpha\text{i/o}$ and $\text{G}\beta\gamma$ subunits, PLC and IP_3 receptor decreased the opioid peptide secretion back to the basal levels while PKC inhibition only had a partial effect. Similarly, leukocyte depletion resulted in the decrease of exogenous opioid analgesia *in vivo*. The analgesic effects could be re-established by transfer of leukocytes *ex vivo* pretreated with extracellular Ca^{2+} chelator, but was unaltered when leukocytes were pretreated with blockers of $\text{G}\alpha\text{i/o}$, $\text{G}\beta\gamma$ proteins or intracellular Ca^{2+} chelator. These findings demonstrate that both *in vivo* analgesia and *ex vivo* opioid peptide release were mediated by opioid receptors on leukocytes coupled to the $\text{G}\alpha\text{i/o}$ – $\text{G}\beta\gamma$ protein–PLC– IP_3 receptors pathway and dependent on intracellular Ca^{2+} which can be identified as an unconventional mechanism of pathological pain control.

Affidavit

I, Melih Özgür Celik, certify under penalty of perjury by my own signature that I have submitted the dissertation on the topic „Leukocyte opioid receptors mediate analgesia via Ca²⁺-regulated release of opioid peptides“. I prepared this dissertation independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Detailed Declaration of Contribution

Melih Özgür Celik had the following share in the following publication:

Publication: Melih Ö. Celik, Dominika Labuz, Karen Henning, Melanie Busch-Dienstfertig, Claire Gaveriaux-Ruff, Brigitte L. Kieffer, Andreas Zimmer, Halina Machelska, Leukocyte opioid receptors mediate analgesia via Ca²⁺-regulated release of opioid peptides, *Brain Behavior and Immunity*, 2016 doi: 10.1016/j.bbi.2016.04.018

Contribution in detail:

(i) Generation of single opioid receptor knockout (KO) mice by crossing the triple delta/mu/kappa opioid receptor (DOR/MOR/KOR) KO mice with the corresponding wildtype (WT) mice. (ii) Refreshing the PENK and PDYN KO lines by crossing with the corresponding WT mice. (iii) Generation of END KO mice by crossing END heterozygotes with the corresponding WT and heterozygote mice. (iv) Performing DNA extractions from each mouse and genotyping using PCR. (v) Performing chronic constriction injury in mice. (vi) Isolation of immune cells from injured nerves and blood. (vii) Counting isolated immune cells and verifying viability. (viii) Performing immunofluorescence staining for hematopoietic cell marker CD45. (ix) Performing opioid peptide release experiments from immune cells, including cellular mechanisms of opioid peptide secretion. (x) Preparation of isolated immune cells for intracellular opioid peptide measurements. (xi) Measurement of opioid peptide (ENK, END and DYN) levels by RIA and EIA. (xii) RNA extractions and cDNA preparations. (xiii) Performing quantitative RT-PCR for MOR, DOR and KOR. (xiv) Data analysis. (xv) Writing of the paper.

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Mark	Rank	Abbreviated Journal Title (linked to journal information)	ISSN	JCR Data ⁱ						Eigenfactor® Metrics ⁱ	
				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	Eigenfactor® Score	Article Influence® Score
<input type="checkbox"/>	1	NAT REV NEUROSCI	1471-003X	33792	29.298	35.142	5.603	58	8.0	0.07267	16.210
<input type="checkbox"/>	2	BEHAV BRAIN SCI	0140-525X	7873	20.415	23.842	1.700	10	>10.0	0.01290	11.205
<input type="checkbox"/>	3	TRENDS COGN SCI	1364-6613	21382	17.850	23.872	2.444	72	8.6	0.04734	10.525
<input type="checkbox"/>	4	NAT NEUROSCI	1097-6256	51112	16.724	16.874	4.100	221	7.8	0.15525	9.161
<input type="checkbox"/>	5	ANNU REV NEUROSCI	0147-006X	13125	14.265	22.563	3.000	21	>10.0	0.02076	11.517
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<input type="checkbox"/>	8	PROG NEUROBIOL	0301-0082	11877	13.177	11.181	1.318	44	9.7	0.02039	3.739
<input type="checkbox"/>	9	TRENDS NEUROSCI	0166-2236	18656	12.504	13.470	2.464	69	>10.0	0.03023	5.941
<input type="checkbox"/>	10	ACTA NEUROPATHOL	0001-6322	14701	11.360	10.616	2.743	109	6.7	0.03705	4.070
<input type="checkbox"/>	11	BIOL PSYCHIAT	0006-3223	42289	11.212	10.799	3.653	190	8.1	0.07406	3.719
<input type="checkbox"/>	12	BRAIN	0006-8950	46207	10.103	10.545	2.320	259	8.9	0.08391	4.110
<input type="checkbox"/>	13	ANN NEUROL	0364-5134	32995	9.638	10.880	2.277	177	>10.0	0.05956	4.360
<input type="checkbox"/>	14	J PINEAL RES	0742-3098	6914	9.314	7.140	2.391	87	6.1	0.00780	1.142
<input type="checkbox"/>	15	FRONT NEUROENDOCRIN	0091-3022	3407	8.852	9.045	1.297	37	6.5	0.00728	3.062
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<input type="checkbox"/>	17	CEREB CORTEX	1047-3211	26911	8.285	7.881	1.423	409	7.1	0.06829	3.383
<input type="checkbox"/>	18	SLEEP MED REV	1087-0792	4392	7.341	9.636	2.375	48	6.6	0.01038	3.463
<input type="checkbox"/>	19	NEUROSCIENTIST	1073-8584	4096	7.295	7.609	1.977	44	6.9	0.01011	2.903
<input type="checkbox"/>	20	MOL NEURODEGENER	1750-1326	2312	6.510	5.599	0.523	65	4.1	0.00936	1.968

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Mark	Rank	Abbreviated Journal Title (linked to journal information)	ISSN	JCR Data ⓘ						Eigenfactor® Metrics ⓘ	
				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	Eigenfactor® Score	Article Influence® Score
<input type="checkbox"/>	21	NEUROPSYCHOPHARMACOL	0893-133X	22869	6.399	7.825	1.794	291	6.7	0.05002	2.647
<input type="checkbox"/>	22	CURR OPIN NEUROBIOL	0959-4388	13090	6.373	6.958	1.671	152	8.5	0.03561	3.510
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<input checked="" type="checkbox"/>	27	BRAIN BEHAV IMMUN	0889-1591	9464	5.874	6.020	1.462	238	4.9	0.02405	1.795
<input type="checkbox"/>	28	BRAIN STRUCT FUNCT	1863-2653	3534	5.811	6.803	1.307	244	3.0	0.01508	2.514
<input type="checkbox"/>	29	J PSYCHIATR NEUROSCI	1180-4882	2690	5.570	6.207	1.136	44	6.5	0.00551	2.034
<input type="checkbox"/>	30	PAIN	0304-3959	32930	5.557	6.244	1.119	269	>10.0	0.04578	2.019
<input type="checkbox"/>	31	NEUROIMAGE	1053-8119	79475	5.463	6.797	1.160	770	6.7	0.18031	2.325
<input type="checkbox"/>	32	MOL NEUROBIOL	0893-7648	5142	5.397	5.392	1.155	283	3.4	0.01336	1.483
<input type="checkbox"/>	33	BRAIN PATHOL	1015-6305	4403	5.256	4.485	1.026	77	7.5	0.00880	1.550
<input type="checkbox"/>	34	ALZHEIMERS RES THER	1758-9193	1164	5.197	5.126	0.921	76	2.5	0.00538	1.726
<input type="checkbox"/>	35	FRONT MOL NEUROSCI	1662-5099	1592	5.154		0.329	79	3.2	0.00785	
<input type="checkbox"/>	36	NEUROBIOL AGING	0197-4580	19205	5.153	5.193	1.261	429	5.7	0.04583	1.663
<input type="checkbox"/>	37	SOC COGN AFFECT NEUR	1749-5016	4695	5.101	5.532	0.848	210	3.4	0.01840	2.073
<input type="checkbox"/>	38	HUM BRAIN MAPP	1065-9471	17184	4.962	5.638	0.931	378	6.5	0.04047	2.088
<input type="checkbox"/>	39	MOL AUTISM	2040-2392	847	4.961	5.184	0.921	63	2.5	0.00452	1.962
<input type="checkbox"/>	40	NEUROPHARMACOLOGY	0028-3908	17341	4.936	4.709	1.288	365	5.8	0.03944	1.460

Celik M. Ö., Labuz D., Henning K., Busch-Dienstfertig M., Gaveriaux Ruff C., Kieffer B.L., Zimmer A., Machelska H. Leukocyte opioid receptors mediate analgesia via Ca²⁺-regulated release of opioid peptides. *Brain Behav Immun.* 2016 57: 227-42.

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Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete list of publications

Celik M. Ö., Labuz D., Henning K., Busch-Dienstfertig M., Gaveriaux Ruff C., Kieffer B.L., Zimmer A., Machelska H. (2016) Leukocyte opioid receptors mediate analgesia via Ca²⁺-regulated release of opioid peptides. *Brain Behav Immun.* 57: 227-42. (Impact factor: 5.81)

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- Çelik M. Ö.**, Dominika L., M., Machelska H., 2013, Leukocyte opioid receptors and neuroimmune interactions in the control of neuropathic pain. [Poster, 2013 Berlin Brain Days (Berlin/Germany)]
- Çelik M. Ö.**, Dominika L., M., Machelska H., 2013, Neuroimmune interactions after nerve injury and opioid-mediated pain control. [Oral presentation and Poster, 2013 GEBIN meeting (Regensburg/Germany)]
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