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Die neuroprotektive Wirkung von Opioiden wird durch die Proteinkinase B / AKT vermittelt

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Inhaltsverzeichnis

Abkürzungsverzeichnis

AG879	3,5-di-t-Butyl-4-hydroxybenzylidene-cyano-thioacetamide
AG1024	3-Bromo-5-t-butyl-4-hydroxy-benzylidene-malonnitrile
AKT	Protein kinase B
cAMP	cyclic Adenosine-monophosphate
BAD	Bcl-2/Bcl-X_L-associated death promoter
Bax	Bcl-2/BCL2-associated X protein
CREB	cAMP response element-binding protein
DOR	δ-Opioid receptor
EBP50	Ezrin-radixin-moesin-binding phosphoprotein 50
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular-signal regulated kinase
GDP	Guanosine-diphosphate
GLUT4	Glucose transporter 4
GPCR	G Protein-coupled receptor
GRK2	G protein-coupled receptor kinase 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-triphosphate
JNK	N-terminal kinase (c-Jun)
MAP Kinase	Mitogen-activated protein kinase
LY294002	2-(4-Morphoinyl)-8-phenyl-4H-1-benzopyran-4-one
MAP3K	MAPKKK (MAP kinase kinase kinase)
MAPKK	MAPKK (MAP kinase kinase)
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
ORL1	Opioid receptor like 1
PDK1	3-Phosphoinositide-dependent protein kinase 1
PH-Domäne	Pleckstrin homologous domain
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PLC	Phospholipase C
PTX	Pertussistoxin
RTK	Receptor tyrosine kinase
ZNS	Zentrales Nervensystem

I Einleitung

1.1 Opioide

1.1.1 Definition

Als Opioide werden Substanzen bezeichnet, die ihre Wirkung über spezifische Zelloberflächenrezeptoren entfalten und die durch Naloxon antagonisierbar ist. In der Medizin werden sie hauptsächlich als stark wirksame Analgetika, Antitussiva und Antidiarrhoika eingesetzt. Aufgrund der ubiquitären Verteilung von Opioidrezeptoren in der ganzen Neuraxis gehen die klinisch erwünschten Wirkungen häufig mit unerwünschten Nebenwirkungen einher. Diese umfassen Übelkeit, Erbrechen, Konstipation, Atemdepression, Müdigkeit, Histaminfreisetzung, Vasodilatation, Hypotonie und, über die Hemmung dopaminerger Bahnen, auch Katalepsie und Katatonie. Des Weiteren können bei wiederholter Anwendung chronische Arzneimittelwirkungen wie Toleranz und Abhängigkeit entstehen [1].

1.1.2 Einteilung der Opioide

Die Begriffe „Opiat“ und „Opioid“ werden häufig synonym gebraucht, bezeichnen jedoch Liganden unterschiedlicher Herkunft. Während die im Rohopium, dem getrockneten Milchsaft des Schlafmohns (Papaver somniferum) vorkommenden Alkaloide als „Opiate“ bezeichnet werden, umfasst der Begriff „Opioide“ alle Verbindungen, die an Opioidrezeptoren binden [2].

Opioide können nach ihrem Ursprung (endogen, exogen, natürlich, halbsynthetisch, synthetisch), ihrer chemischen Struktur (Alkaloide, zu denen die Opiate Morphin, Codein und Thebain gehören, deren (halb-)synthetische Derivate sowie die endogenen Peptide Endorphin, Enkephalin und Dynorphin) und ihrer Spezifität gegenüber bestimmten Opioidrezeptoren eingeteilt werden. Auf molekularer Ebene sind drei Rezeptortypen (δ-, k-, μ-Rezeptoren) bekannt [3], die sich hinsichtlich ihrer pharmakologischen Eigenschaften weiter in die Subtypen (μ_1, μ_2, δ_1, δ_2, κ_1, κ_2, κ_3) unterscheiden lassen [4]. Die Ursachen der pharmakologischen Vielfalt sind nach wie vor unbekannt, als mögliche Grundlagen werden alternatives Splicing [5], Oligomerisierung [6] sowie unterschiedliche Rezeptorzustände [7] diskutiert.

1.1.3 Rezeptoren und Signaltransduktion

Opioidrezeptoren gehören zur Gruppe der G-Protein-gekoppelten Rezeptoren (GPCR), die aus sieben hydrophoben transmembranen Domänen sowie den sie verbindenden extra- und intrazellulären Schleifen bestehen. Sie bestehen aus ca. 380 Aminosäuren, der N-Terminus befindet sich auf der extrazellulären Seite, das C-terminale Ende kommt intrazellulär zu liegen [8]. An der intrazellulären C-terminalen Schleife binden die heterotrimeren G-Proteine, die aus je einer GDP-bindenden α-Untereinheit sowie einem funktionellen βγ-Dimer bestehen [9]. Die Einteilung der G-Proteine erfolgt nach den Eigenschaften der α-Untereinheit, wobei Opioidrezeptoren neben den Pertussistoxin (PTX)-sensitiven inhibitorischen G-Proteinen G_i/G_o auch die PTX-insensitiven G-Proteine der $G_{q/11}$, $G_{12/13}$ und G_{16} aktivieren können [10,11]. Daraus ergeben sich vielfältige Möglichkeiten der intrazellulären Signaltransduktion, die in Abbildung 1 dargestellt werden:

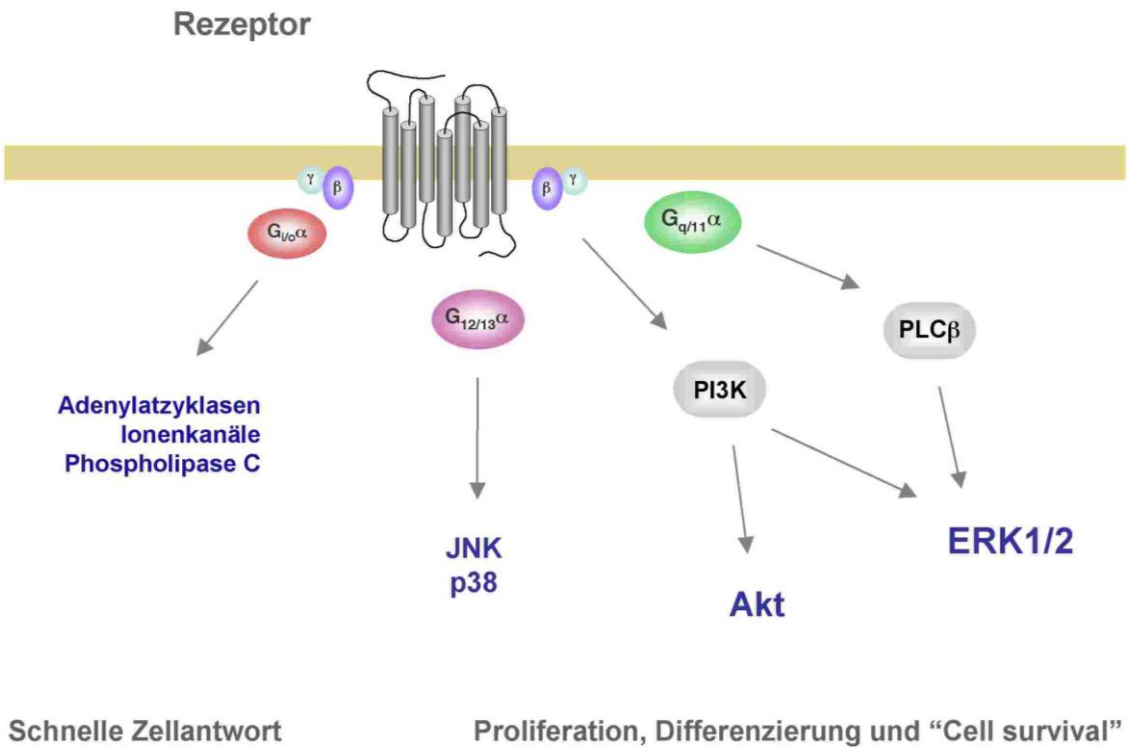

Abbildung 1: Regulation intrazellulärer Signalkaskaden durch Opioide.
Durch Interaktion mit verschiedenen G-Proteinen sind δ-Opioidrezeptoren in der Lage, unterschiedliche intrazelluläre Signalkaskaden zu induzieren. Die von G_i/G_o regulierten „klassischen" Effektorsysteme sind dabei für die schnelle Zellantwort verantwortlich. Neuere Untersuchungen weisen darauf hin, dass δ-Opioidrezeptoren über die Aktivierung von G-Proteinen der $G_{q/11}$ und $G_{12/13}$ Familie auch in die Zellproliferation, Differenzierung und Überlebens-mechanismen der Zelle eingreifen können.

Die Aktivierung des Rezeptors durch einen Agonisten bedingt eine Konformationsänderung an der α-Untereinheit, wodurch GDP durch GTP ersetzt wird und die nunmehr aktivierte, GTP-gebundene α-Untereinheit abdissoziiert. Sowohl die aktivierte α-Untereinheit als auch der freigesetzte βγ-Komplex können nun ihre entsprechenden Effektoren regulieren. Aufgrund der intrinsischen GTPase-Aktivität wird γ-Phosphat von GTP abgespalten, wodurch die GDP-gebundene α-Untereinheit wieder mit dem βγ-Komplex reassoziiert und für einen weiteren Aktivierungszyklus zur Verfügung steht [9].

Die von Opioidrezeptoren aktivierten inhibitorischen G-Proteine vermitteln eine schnelle Zellantwort über die Regulation verschiedener Effektormoleküle, wie zum Beispiel Adenylatzyklasen, spannungsabhängige Ca^{2+}-Kanäle, einwärts gerichtete K^{+}-Kanäle, oder die Phospholipase C (PLC) [12]. Neben diesen direkt regulierten Effektoren können Opioidrezeptoren aber auch indirekt Einfluss auf die Aktivität verschiedener intrazellulärer Effektoren, wie zum Beispiel den Mitogen-aktivierten Proteinkinasen (MAP-Kinasen) oder der AKT nehmen [13,14].

1.2 Opioid-regulierte Serin/Threoninkinasen

1.2.1 Mitogen-aktivierte Proteinkinasen

Die MAP Kinasen gehören zu den Serin/Threoninkinasen, die über eine konservierte Signalkaskade (MAP3K→ MAPKK→ MAP Kinase→ Substrat mit Serin/Threonin Phosphorylierungsstelle) aktiviert werden [15]. Beim Säuger werden mehrere MAP Kinasen unterschieden. Die am besten untersuchten MAP Kinasen stellen die Extrazellulär Signal-regulierten Proteinkinasen 1 und 2 dar (ERK1/2). Sie werden insbesondere durch Wachstumsfaktoren und Phorbolester aktiviert und sind für die Regulation der Zellproliferation von Bedeutung [16]. Von der p38 MAP Kinase existieren vier Isoformen (α, β, γ, δ), die sich in ihrem Verteilungsmuster und ihrer Substratspezifität unterscheiden. Ihre Aktivierung erfolgt Stress-assoziiert, zum Beispiel durch UV-Bestrahlung, oxidativen Stress und Ischämie, sowie durch inflammatorische Cytokine. Sie sind an der Regulation der Immunantwort sowie an Entzündungsreaktionen beteiligt [17]. Von den c-Jun N-terminalen Kinasen (JNK1, 2, 3) existieren verschiedene Splicingvarianten, die ebenfalls

überwiegend durch Stresssignale aktiviert werden. Sie sind wesentlich an der Regulation der Apoptose während der Organogenese sowie an der Zelltransformation beteiligt [18]. Darüber hinaus sind noch weitere MAP Kinasen, wie die ERK3/4, ERK5 (auch als Big MAP Kinase, BMK bezeichnet) und ERK7/8 beschrieben, deren Funktion allerdings noch weniger gut untersucht ist [19].

Die Aktivierung der MAP Kinasen erfolgt vorwiegend über Tyrosinkinasen (zum Beispiel Rezeptor-Tyrosinkinasen, Src Kinasen), sie können aber auch indirekt über GPCR reguliert werden. Von Opioidrezeptoren ist bisher bekannt, dass sie über bislang nur unvollständig charakterisierte Signalwege sowohl die ERK1/2 als auch die JNK und die p38 regulieren können [20,21,22].

1.2.2 AKT (Proteinkinase B)

Eine weitere, nicht mit den MAP Kinasen verwandte Serin/Threonin Kinase stellt die Proteinkinase B, auch als AKT bezeichnet, dar. Sie wurde 1991 von drei unabhängigen Forschergruppen entdeckt. Während die eine Gruppe herausfand, dass es sich um das Produkt des retroviralen Onkogens *v-AKT* (AKT8) handelt, bei dem das Onkogen die Fusion des zellulären AKT mit dem viralen Strukturprotein Gag codiert [23], wurden die beiden anderen Gruppen auf die AKT über ihre ausgeprägte Sequenzhomologie zu den Proteinkinasen A und C aufmerksam [24,25].

1.2.2.1 Aufbau

Von der AKT sind drei Subtypen beschrieben, die mit AKT 1, 2 und 3 (bzw. PKBα, β, γ) bezeichnet werden und eine Molekülgröße von ca. 57 kDa besitzen. Das Protein besteht aus einer N-terminalen Pleckstrin-homologen (PH) Domäne, die mit den Membranlipidprodukten PIP2 und PIP3 interagieren kann, einer zentralen, für die Kinaseaktivität verantwortlichen Domäne sowie einer C-teminalen, regulatorischen Domäne. Auf der zentralen Domäne befindet sich ein Threoninrest, dessen Phosphorylierung zusammen mit der Phosphorylierung eines Serinrestes in der C-terminalen Domäne eine maximale Enzymaktivität bewirkt [26]. Der Aufbau der

verschiedenen AKT Subtypen sowie die entsprechenden Phosophorylierungsstellen sind in Abb. 2 dargestellt:

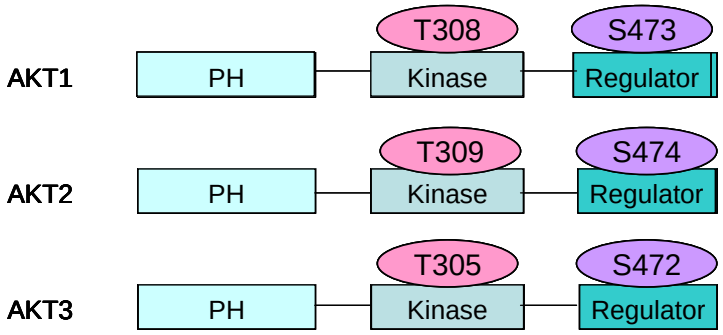

Abbildung 2: Aufbau und Phosophorylierungsstellen von AKT.
Die drei Subtypen der AKT weisen einen vergleichbaren Aufbau auf. Sie bestehen aus einer PH-Domäne, die für die Verankerung in der Zellmembran verantwortlich ist, einer Kinasedomäne und einer regulatorischen Einheit.

1.2.2.2 Mechanismen der AKT Aktivierung

Die Phosphorylierung und die damit einhergehende Aktivierung der AKT wird durch eine Reihe vorgeschalteter Aktivierungsschritte vermittelt. Zunächst wird die Phosphoinositid-3-Kinase (PI3K) entweder über G-Protein-gekoppelte Rezeptoren (GPCR) oder über Rezeptor Tyrosinkinasen (RTK) aktiviert und an die Zellmembran transloziert. Die PI3K vermittelt daraufhin die Umsetzung von PIP2 in PIP3. Letzteres ist dann wiederum in der Lage, die AKT an der Zellmembran zu verankern und, durch nachfolgende Konformationsänderung, für die 3-Phosphoinositid-abhängige Proteinkinase 1 (PDK1) zugänglich zu machen. Diese phosphoryliert beispielsweise an der AKT1 die OH-Gruppe des Threonin 308, wodurch eine Autophosphorylierung von Serin 473 induziert wird. Neben diesem grundsätzlichen Mechanismus ist aber auch eine PI3K-unabhängige Aktivierung der AKT beschreiben. Diese kann zum Beispiel über eine cAMP-abhängige Aktivierung der PKA oder über die Aktivierung der Ca^{2+}/Calmodulin-abhängigen Kinase erfolgen [27,28].

1.2.2.3 Substrate der AKT

Die physiologischen Wirkungen der AKT sind vielfältig und werden über eine Reihe

unterschiedlicher Substrate vermittelt. Die einzelnen Substrate werden dabei an entsprechenden Serin/Threoninresten phosphoryliert, wodurch sie in ihrer Wirkung entweder aktiviert oder inaktiviert werden. Wichtige Substrate der AKT sind an der Regulation der Apoptose, des Zellstoffwechsels, der Proliferation und des Zellzyklus beteiligt. So führt die Aktivierung der AKT über Hemmung des Caspase-9 und BAD Weges zu einer Hemmung der Apoptose [29]. Außerdem kontrolliert die AKT zahlreiche Transkriptionsfaktoren (zum Beispiel NFκB, JNK, p53, Bax, CREB), die ebenfalls an der Apoptose und am „Cell survival“ beteiligt sind [30]. AKT kann aber auch metabolische Funktionen beeinflussen. So führt die Insulin-induzierte Aktivierung der AKT zu einer vermehrten Einlagerung von Glucosetransportern (GLUT4) in die Zellmembran sowie zu einer Hemmung der Glucose Synthase Kinase 3 (GSK3). Neben der metabolischen Wirkung kann die GSK3 wiederum über die Regulation zahlreicher eigener Substrate in die Apoptose eingreifen. Schließlich stellt die eNOS ein weiteres Substrat der AKT dar, die ihrerseits eine NO-Freisetzung und, daraus resultierend, eine Vasodilatation induziert und an der Angiogenese beteiligt ist [31].

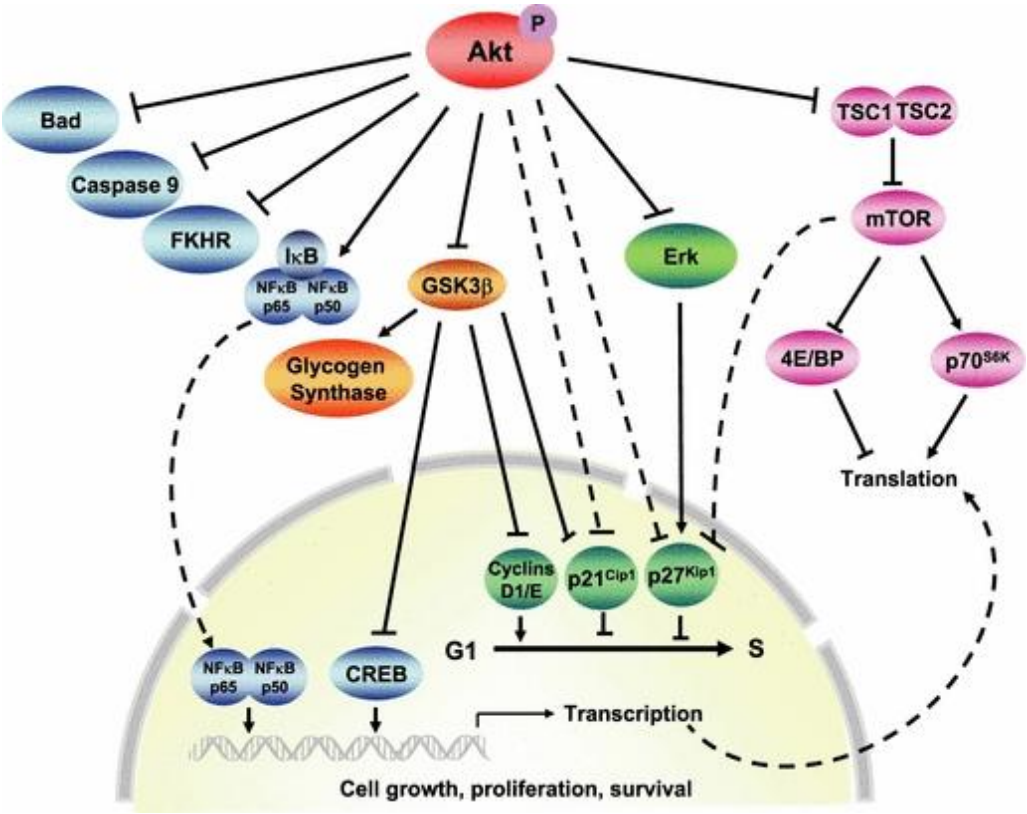

Abbildung 3: Von AKT regulierte Substrate.
Aktivierte AKT Isoformen können durch Phosphorylierung vielfältige zelluläre Substrate regulieren, die für das Überleben der Zelle (blau), den Glucosestoffwechsel (orange), den Zellzyklus (grün) und die Proteinsynthese (pink) von Bedeutung sind. Die gestrichelten Linien deuten eine Translokation der Proteine an [entnommen aus Ref. 28].

Aufgrund der zentralen Bedeutung der AKT können im Falle einer Fehlregulation zahlreiche Erkrankungen resultieren. So geht die Reduktion der Aktivität von AKT 2 mit einer Insulinresistenz einher [32], während eine überschießende AKT Aktivität mit cancerogenen Mechanismen in Verbindung gebracht wird [33]. Die AKT bietet daher über die pharmakologische Regulation ihrer Aktivität die Möglichkeit, in zentrale Mechanismen der Zellproliferation, Apoptose und des Glucosestoffwechsels einzugreifen [34].

Neben ihrer inhibitorischen Wirkung auf die neuronale Aktivität greifen Opioide auch auf vielfältige Weise in das Zellwachstum und die Zelldifferenzierung ein [35]. Diese Wirkungen können entweder über eine Stimulation der Zellproliferation oder eine Hemmung der Apoptose erfolgen. Hierdurch lassen sich nicht nur die tumorpromovierenden [36] und immunmodulierenden [37,38] Eigenschaften einiger Opioide erklären, sondern auch mögliche zytoprotektive Wirkungen ableiten. Diese werden derzeit intensiv auf ihre Fähigkeit untersucht, die Überlebensfähigkeit von Organen nach ischämischer Schädigung (zum Beispiel bei Herzinfarkt, Schlaganfall, Organtransplantation) zu verbessern [39,40,41].

1.3 Fragestellung

In den letzten Jahren wurden erhebliche Anstrengungen unternommen, die Bedeutung der Opioid-regulierten Serin/Threoninkinasen zu erforschen. Dabei stellte sich heraus, dass Opioide nicht nur proliferative Eigenschaften besitzen, sondern auch die Apoptose hemmen und das Überleben der Zellen verlängern können. Da die AKT eine wichtige Rolle bei der Vermittlung anti-apoptotischer Signale besitzt stellte sich die Frage, ob Opioide ihre zytoprotektive Wirkung über die Aktivierung dieser Serin/Threoninkinase vermitteln. Hierfür wurden Neuroblastoma x Glioma NG108-15 Hybridzellen gewählt, die große Mengen endogener δ-Opioidrezeptoren tragen und ein klassisches Modellsystem für die Untersuchung akuter und chronischer Opioidwirkungen darstellen. In der vorliegenden Studie konnte erstmals gezeigt werden, dass δ-Opioidrezeptoren in neuronalen Zellen in der Tat die AKT stimulieren können. Der zugrunde liegende Signalmechanismus ist komplex und schließt eine über IGF-1 Rezeptoren vermittelte Aktivierung der PI3K / AKT Kaskade ein. Die aktivierte AKT ist wiederum in der Lage, die Apoptose zu hemmen und das Überleben der Zellen nach Einwirkung exogener Stressfaktoren zu verlängern. Die

Untersuchungen zeigen damit, dass Opioide in NG108-15 Hybridzellen neuroprotektive Wirkungen besitzen und dass diese durch AKT vermittelt werden.

II Veröffentlichung

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δ-Opioid receptor-stimulated Akt signaling in neuroblastoma x glioma (NG108-15) hybrid cells involves receptor tyrosine kinase-mediated PI3K activation

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Abstract

δ-Opioid receptor (DOR) agonists possess cytoprotective properties, an effect associated with activation of the "pro-survival" kinase Akt. Here we delineate the signal transduction pathway by which opioids induce Akt activation in neuroblastoma x glioma (NG108-15) hybrid cells. Exposure of the cells to both [D-Pen^{2,5}]enkephalin and etorphine resulted in a time- and dose-dependent increase in Akt activity, as measured by means of an activation-specific antibody recognizing phosphoserine-473. DOR-mediated Akt signaling is blocked by the opioid antagonist naloxone and involves inhibitory $G_{i/o}$ proteins, because pre-treatment with pertussis toxin, but not over-expression of the $G_{q/11}$ scavengers EBP50 and GRK2-K220R, prevented this effect. Further studies with Wortmannin and LY294002 revealed that phophoinositol-3-kinase (PI3K) plays a central role in opioid-induced Akt activation. Opioids stimulate Akt activity through transactivation of receptor tyrosine kinases (RTK), because pre-treatment of the cells with inhibitors for neurotrophin receptor tyrosine kinases (AG879) and the insulin-like growth factor receptor IGF-1 (AG1024), but not over-expression of the Gβγ scavenger phosducin, abolished this effect. Activated Akt translocates to the nuclear membrane, where it promotes GSK3 phosphorylation and prevents caspase-3 cleavage, two key events mediating inhibition of cell apoptosis and enhancement of cell survival. Taken together, these results demonstrate that in NG108/15 hybrid cells DOR agonists possess cytoprotective properties mediated by activation of the RTK / PI3K / Akt signaling pathway.

Keywords

Akt, Apoptosis, Caspase-3, δ-Opioid Receptor, PI3K, Receptor Tyrosine Kinases

Abbreviations

DOR, δ-opioid receptor; DPDPE, [D-Pen^{2,5}]enkephalin; GRK2, G protein-coupled receptor kinase 2; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor; PI3K, phosphoinositol-3-kinase; RTK, receptor tyrosine kinase

Introduction

G protein-coupled opioid receptors (δ, κ, μ) regulate a number of cellular functions, including cell proliferation, differentiation and survival [1]. In this respect, activation of the δ-opioid receptor (DOR) is known to interfere with the immune system [2] and to protect neuronal cells and cardiomyocytes from apoptosis after ischemic injury [3,4]. Although the "pro-survival" serine/threonine kinase Akt has been suggested to play a critical role in the generation of anti-apoptotic opioid effects [4], the intracellular signal transduction pathways as well as the functional consequences of opioid-induced Akt activation remain largely unknown.

The activity of Akt, also known as protein kinase B (PKB), is regulated by class I phosphoinositol-3-kinases (PI3K) that catalyze the formation of phosphatidylinositol-3,4,5-tri- and 4,5-diphosphates (PtdIns-3,4,5-P_3, PtdIns-4,5-P_2). Upon formation, these membrane phospholipids facilitate the translocation of Akt to the plasma membrane [5], where it becomes activated by phosphorylation at threonine-308 through phosphoinositide-dependent kinase PDK-1 [6] and subsequent autophosphorylation at serine-473 [7]. Phosphorylated Akt in turn redistributes into the cytosol, where it regulates a number of different cellular and nuclear target proteins important for cell growth, metabolism and apoptosis [8].

Based on mechanistic properties, class I PI3Ks are further divided into class IA and IB subfamilies. Whereas Gβγ-subunits released from heterotrimeric G proteins activate class 1B PI3Ks [9], class IA PI3Ks are target for activation by receptor tyrosine kinases (RTKs). The latter can be achieved either directly by the RTK stimulated by extracellular growth factors [10] or indirectly through RTK-activated adaptor proteins [11,12]. PI3K-associated signal transduction pathways are also employed by G protein-coupled receptors (GPCRs), which stimulate Akt signaling through Gβγ-dependent mechanisms [13] or by transactivation of RTKs, *e.g.* the nerve growth factor binding Trk receptor [14]. Because PI3K might also become activated by the small GTPase p21Ras and the calcium sensing protein calmodulin [15,16], several additional possibilities exist by which GPCRs might activate the PI3K / Akt signaling pathway.

Neuroblastoma x glioma (NG108-15) hybrid cells carry high levels of endogenous DORs and provide an ideal model system for studying physiological aspects of acute and chronic opioid-induced signal transduction mechanisms at a single cell level [17,18]. In this respect, Akt has been recently shown to play a critical role in DOR-mediated phosphorylation of tuberin in this cell line [19]. Therefore, the present study was performed on NG108-15 cells in order to characterize the signal transduction pathway associated with DOR-mediated Akt signaling and to evaluate the physiological consequences of Akt stimulation on cell survival.

Material and Methods

Cell culture

NG108-15 hybrid cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), 100 µM hypoxanthine, 1 µM aminopterin, 16 µM thymidine, 2 mM glutamine, and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air as described [17]. Cells were transfected with plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) containing cDNAs coding for the HA-tagged Gβγ scavenger phosducin [20], the HA-tagged $G\alpha_q$ inhibitor EBP50 [21], and the inhibitor of $G_{q/11}$ signaling GRK2-K220T [22] using Metafectene® transfection reagent (Biontex Laboratories, Martinsried, Germany). Transfected cells were allowed to grow overnight before experimentation.

Akt kinase activation

Activation of Akt was determined in cells grown onto 12-well plates. Two hours before experimentation, cells were washed extensively and kept under serum-reduced (0.1 % FCS) conditions before reactions were started by the addition of [D-Pen^{2,5}]enkephalin (Bachem, Bubendorf, Switzerland), etorphine (National Institute on Drug Abuse, Bethesda, MD) and insulin-like growth factor (IGF-1) at the concentrations given in the text. In some experiments, cells were pre-treated with naloxone (10 µM, 10 min), pertussis toxin (PTX; 100 ng/ml, 18 h), the PI3K inhibitors Wortmannin (1 nM-10 µM, 30 min) and LY294002 (1 nM-10 µM, 30 min), the PI3K insensitive analog LY303511 (0.1-10 µM; 30 min) as well as the receptor tyrosine kinase inhibitors AG879 (100 µM, 30 min) and AG1024 (10 µM, 30 min). Reactions were terminated by aspiration of the medium and the addition of

sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v phenol red, and 200 µM sodium vanadate, pH 6.8). Cell lysates were boiled for 5 min at 95 °C and stored at -20 °C until they were assayed by immunoblotting.

Western blot

Solubilised proteins were resolved by electrophoresis over 12% SDS-polyacrylamide gels and subsequently transferred onto PVDF membranes (Millipore, Schwalbach, Germany). The blots were blocked with 5% non-fat milk in Tris-buffered saline, containing 0.1 % Tween (TBS/T), and incubated with antibodies for Akt (total Akt), phosphoserine-473 Akt (activated Akt), phosphoserine-21/9 GSK3α/β (Akt-phosphorylated GSK3), and cleaved caspase-3 (Cell Signaling Technologies, Frankfurt am Main, Germany; dilution 1:1,000 each). Over-expression of phosducin and EBP50 was monitored using an anti-HA antibody (Gramsch Laboratories, Schwabhausen, Germany), expression of GRK2-K220R by an anti-GRK2 antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Staining the blots with a β-tubulin antibody (Cell Signaling Technologies) was used to verify equal protein loading. The blots were developed using a horseradish peroxidase-conjugated secondary antibody and immuncomplexes were visualized by enhanced chemiluminescence (Amersham ECL Plus^{TM}, GE Healthcare, Munich, Germany). Immunoreactive bands were quantified by video densitometry using the Herolab EASY-5 system.

Immunocytochemistry

NG108-15 hybrid cells were plated onto 22-mm coverslips and stimulated with opioids and IGF-1 as indicated. After incubation, cells were fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature (RT), permeabilized with ice-cold methanol (10 min) and labeled with an overall reactive Akt antibody (1:500) over-night at 4°C. Cells were then washed three times with PBS and incubated for 60 min at RT with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:50; DAKO Deutschland, Hamburg, Germany). Cells incubated with the secondary antibody alone served as a negative control. Coverslips were mounted on glass slides and cells were analyzed by confocal microscopy (Carl Zeiss, Jena, Germany). The images shown were acquired using a 63 x 1.4 oil immersion objective.

Determination of cell death and survival

Caspase-3 Assay - Anti-apoptotic opioid effects were determined in NG108-15 hybrid

cells cultured for 24 h under serum-free conditions either in the absence or presence of 1 µM DPDPE, 1 µM etorphine, and the Akt kinase inhibitor 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazol[4,5-G]quinixalin-7-yl) phenyl) methyl)-4-piperidinyl-2H-benzimidazol-2-one (Akt inhibitor VIII; Merck Biosciences, Darmstadt, Germany). At the end of the incubation period, medium was removed and cells were lysed by the addition of Laemmli sample buffer. Induction of intrinsic apoptotic signals was evaluated by Western blot experiments using an antibody specifically recognizing cleaved caspase-3.

Annexin V/ propidium iodide staining - NG108-15 cells grown on poly-L-lysine coated cover slips were kept under serum-free conditions for 18 h either in the absence or presence of 1 µM DPDPE, 1 µM Wortmannin, and 2 µM Akt inhibitor VIII. Negative controls were grown in the presence of 10 % FCS. After incubation, cells were washed and stained for determination of early stages of apoptosis with Annexin V-fluorescein isothiocyanate (FITC) and for membrane permeability with propidium iodide (PI) using an apoptosis detection kit (Enzo Life Sciences GmbH, Lörrach, Germany). Coverslips were mounted on glass slides and analyzed by confocal microscopy as above using a dual filter set for FITC (green) and rhodamine (red).

Cell viability assay - Mitochondrial activity was evaluated by the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into the dark-blue dye formazan. For this, cells were plated on 96-well dishes and were allowed to attach overnight. Apoptosis was induced by serum deprivation and the effect of opioids on cell survival was determined by the addition of 1 µM DPDPE, 1 µM etorphine, 1 µM Wortmannin, and 2 µM Akt inhibitor VIII to the medium for 24 h. Negative controls were incubated in the presence of 10 % FCS. After incubation, color reactions were started by the addition of 20 µl of CellTiter 96 AQ_{ueous} One Solution reagent (Promega, Mannheim, Germany). Reactions were stopped after 2 h at 37°C and the amount formazan generated was determined spectrophotometrically at λ = 490 nM using a Microplate Reader (TECAN Spectra, Crailsheim, Germany). Cell survival is expressed as the percentage of viable cells as compared to serum-treated control cells.

Statistical analysis

All data are expressed as the mean values ± S.D. of the number of experiments indicated. IC_{50} values were calculated by non-linear curve fitting using GraphPad Prism™ software

(GraphPad Inc., San Diego, CA). Statistical differences were determined according to the two-tailed Student´s *t* test using InStat® software (Scottsdale, AZ). Differences were considered to be significant at $p < 0.05$.

Results and Discussion

Opioid activation of Akt in NG108-15 hybrid cells

To investigate whether DOR stimulation is associated with Akt signaling in NG108-15 hybrid cells, these were exposed for 5 min to increasing concentrations of the δ-selective peptide agonist DPDPE and the opioid alkaloid etorphine. Cells were lysed and examined for the active state of Akt by means of an antibody specifically recognizing phosphoserine-473 [23]. Compared to untreated controls, incubation of the cells with DPDPE and etorphine resulted in dose-dependent activation of Akt, starting for both agonists at a concentration of 10 nM and reaching maximum effects at 1 µM (Fig. 1A). The calculated EC_{50} values for DPDPE and etorphine were 64.2 and 17.1 nM (mean values of n = 3 independent experiments), respectively. These results demonstrate that in NG108-15 hybrid cells both the membrane permeable alkaloid etorphine as well as the membrane impermeable peptide agonist DPDPE induce Akt signaling at potencies comparable to those observed for activation of G proteins [24] and potassium channels [25] by these ligands.

The kinetics of Akt activation was determined for 5 to 60 min in the presence of 1 µM DPDPE and etorphine. Both opioids produced maximum Akt phosphorylation within 5 min, which then rapidly reversed to control levels after 30 min of incubation (Fig. 1B). The bell-shaped activation curve of Akt might be due to degradation of the enzyme or dephosphorylation of serine-473 by protein phosphatase 2A (PP2A) [26]. Because the overall amount of Akt remained unchanged (Fig. 1B, upper panel), the rapid decline in Akt activity appears to be mediated by protein dephosphorylation rather than degradation. A similar mechanism has been demonstrated previously for transient stimulation of phospholipase C in NG108-15 hybrid cells, which also involves PP2A [27]. Thus, rapid termination of opioid-induced Akt signaling is likely to be mediated by PP2A-induced dephosphorylation.

Opioid-induced Akt signaling is mediated by inhibitory $G_{i/o}$ proteins

Opioids mediate their cellular effects primarily by binding to their specific receptors (δ, κ, and μ), but there are also reports for receptor-independent processes insensitive to naloxone [28]. In NG108-15 cells, pre-treatment with the opioid receptor antagonist naloxone (10 μM, 10 min) completely prevented DPDPE- and etorphine-induced Akt phosphorylation at serine-473. Thus, stimulation of Akt signaling represents a specifically DOR-mediated effect that develops independent of agonist structure (Fig. 2A).

The DOR mediates its function by coupling G proteins of the $G_{i/o}$ and $G_{q/11}$ family [18,29]. Because both G protein families are also implicated in Akt signaling [13], we next determined the G proteins mediating this opioid effect. To discriminate between $G_{i/o}$ and $G_{q/11}$ proteins, NG108-15 cells were first pre-treated with PTX (100 ng/ml; 18 h). As shown in Fig. 2A, PTX pre-treatment completely abolished stimulation of Akt signaling by both DPDPE and etorphine without affecting basal levels of serine-473 phosphorylation and overall Akt abundance. From these results, one could conclude that opioid-induced Akt signaling is mediated through PTX-sensitive $G_{i/o}$ proteins. However, PTX has also been shown to interfere with Akt signaling of G_s-coupled β_2-adrenoceptors [30] and $G_{q/11}$-coupled histamine H1 receptors [31]. To rule out any additional contribution of $G_{q/11}$ family proteins, NG108-15 cells were transiently transfected with the $G\alpha_q$ inhibitor EBP50 [21]. As shown in Fig. 2B, over-expression of EBP50 affected neither Akt abundance nor opioid-induced Akt phosphorylation. Similar results were obtained when the cells were transfected with GRK2-K220R, another frequently used $G_{q/11}$ scavenger [22]. Again, disruption of DOR / $G_{q/11}$ interaction failed to interfere with DPDPE- and etorphine-induced serine-473 phosphorylation (Fig. 2B). These findings demonstrate that DOR-mediated Akt signaling is primarily mediated via inhibitory $G_{i/o}$ proteins rather than members of the $G_{q/11}$ family.

DOR-mediated Akt signaling involves PI3K activity

GPCRs may stimulate Akt signaling via PI3K-dependent and -independent pathways [32]. To discriminate between both possibilities, the effect of the PI3K inhibitors Wortmannin [33] and LY294002 [34] on DOR-mediated stimulation of Akt was determined. As shown in Fig. 3, both inhibitors concentration-dependently reduced DPDPE- and etorphine-stimulated Akt phosphorylation. Half-maximal effects were seen at about 10 nM Wortmannin and 1 μM LY 294002, values that correlate well with the IC_{50} values of these

compounds for PI3K inhibition [33,34]. Ten-fold higher concentrations of Wortmannin (0.1 μM) and LY 294002 (10 μM) completely blocked Akt signaling. In contrast, the PI3K-inactive LY-analog LY 303511 (0.1 – 10 μM) failed to affect opioid-induced Akt phosphorylation. These results demonstrate that DOR activation stimulates Akt activity via the PI3K pathway.

Gβγ is not involved in DOR-mediated stimulation of PI3K

GPCRs may stimulate the PI3K / Akt pathway by Gβγ-subunits released from activated $G_{i/o}$ proteins [13]. In addition, Pello et al. [35] have demonstrated that in monocytes DOR agonists activate Gβγ-stimulated class IB PI3Ks. To test whether in NG108-15 hybrid cells DOR-stimulated Akt signaling is also mediated by Gβγ-subunits, the cells were transiently transfected with phosducin, an inhibitor of Gβγ-signaling [20]. To our surprise, over-expression of phosducin not only failed to impair, but also even increased DPDPE- and etorphine-stimulated Akt phosphorylation (Fig. 4). Thus, Gβγ-dependent pathways appear to suppresses rather than stimulate opioid-induced Akt signaling in NG108-15 hybrid cells. Such a mechanism could be explained by the finding that in these cells DOR activation is associated with stimulation of Gβγ-regulated phospholipase C [27], which catalyzes the hydrolysis of PtdIns-3, 4-P_2 used for Akt activation [36]. Thus, inhibition of PLC activity by phosducin would result in an enhanced accumulation of the lipid messenger associated with an increased Akt phosphorylation [37]. Regardless of the underlying mechanism, our results indicate that opioid-induced activation of Akt signaling is unlikely to involve class IB PI3Ks regulated by Gβγ-subunits. This finding appears somewhat surprising, because it contrasts the traditional concept of GPCR-stimulated PI3K activity generally thought to be mediated by Gβγ-subunits. One possible explanation for this discrepancy could be that in NG108-15 cells the DOR signals through $G\alpha_o$ [38], which in turn stimulates protein kinase C (PKC) activity [39]. Because PKC is also involved in stimulation of PI3K activity [40], PTX-sensitive Gα-subunits might account for DOR-stimulated Akt signaling in NG108-15 hybrid cells.

Opioid-induced stimulation of Akt involves transactivation of receptor tyrosine kinases

Very recently, the DOR has been shown to stimulate the nerve growth factor (NGF)-binding receptor tyrosine kinase Trk in NG108-15 cells by a PKC dependent mechanism [17]. Because in monocytes $G_{i/o}$-coupled VPAC-1 receptors are also involved in Trk-

mediation stimulation of Akt activity [14], we next examined the role of these receptor tyrosine kinases in DOR-mediated stimulation of the PI3K / Akt pathway. Trk receptors were inactivated by pre-treatment of NG108-15 hybrid cells with the Trk inhibitor AG879 [41], before DPDPE- and etorphine-stimulated Akt activation was determined. Whereas pre-treatment of the cells with AG879 had no effect on Akt abundance, it completely abolished basal as well as opioid-induced Akt phosphorylation (Fig. 5A). These results indicate that in NG108-15 hybrid cells Trk receptors are involved in opioid-induced PI3K / Akt signaling.

Trk receptors stimulate class IA PI3Ks by activation of the adaptor protein IRS-1, which represents a substrate for the insulin-like growth factor (IGF-1) receptor [11,12]. Because IGF-1 receptors may also be activated by other RTKs [42], we examined whether the NGF-binding receptor tyrosine kinase Trk might possibly mediate DOR-stimulated Akt phosphorylation by transactivation of IGF-1 receptors. As shown in Fig. 5A, pre-treatment of the cells with the IGF-1 receptor inhibitor AG1024 [43] completely abolished Akt stimulation by both DPDPE and etorphine. In addition, only AG1024 but not AG879 was able to interfere with IGF-induced Akt phosphorylation, whereas both inhibitors attenuated Akt signaling stimulated by NGF (Fig. 5B). These results demonstrate that in NG108-15 hybrid cells DOR-stimulated Akt signaling requires transactivation of Trk receptors, which in turn employ IGF-1 receptors to stimulate the PI3K /Akt pathway. Although the exact nature of the interaction between both receptor tyrosine kinases is currently unknown, it might involve Trk-stimulated Src kinase [44], which has been previously shown to directly mediate tyrosine phosphorylation and activation of IGF-1 receptors [45].

Opioid-induced Akt activation promotes its translocation to the nuclear membrane

Activated Akt redistributes from the cytosol to the plasma membrane [5]. Analysis of the subcellular localization of Akt in untreated NG108-15 hybrid cells by immunocytochemistry showed a diffuse distribution of Akt throughout the cytoplasm (Fig. 6). Upon stimulation of the cells with both DPDPE and etorphine (1 µM each; 5 min), Akt immunoreactivity redistributes towards the nuclear rather than the plasma membrane. A similar translocation of Akt to the nuclear membrane was found after stimulation of the cells with IGF-1 (10 ng/ml; 5 min), supporting the finding that opioid-induced activation of Akt involves IGF-1 receptor signaling (Fig. 6). A possible explanation for this somewhat surprising finding would be that Akt is activated by nuclear DORs present at high levels in

NG108-15 hybrid cells [46]. However, the finding that not only etorphine, but also the membrane impermeable peptide agonist DPDPE produces this effect argues against such a mechanism. An alternative explanation could be that incubation of the cells with opioids and IGF-1 might induce redistribution of PI3K to the nuclear membrane, where it could control nuclear phosphoinositide metabolism [47] and facilitate translocation of the Akt activator PDK-1 to the nucleus [48]. Indeed, such a mechanism appears plausible, because pre-treatment of the cells with the PI3K-inhibitor Wortmannin completely prevents opioid- and IGF-1-induced translocation of Akt to the nuclear membrane.

PI3K/Akt signaling mediates cytoprotective opioid effects

Akt is known to regulate a number of nucleus-arranged substrates. One of these is glycogen synthase kinase-3 (GSK3), a serine/threonine kinase that plays a critical role in stress induced cell death [49]. Consistent with its perinuclear accumulation, opioid-induced Akt activation results in phosphorylation of GSK3 (Fig. 7A), an effect that is largely prevented by pre-treatment of the cells with the PI3K inhibitor Wortmannin (1 µM, 30 min) and the Akt inhibitor VIII (2 µM, 30 min). Because Akt-mediated phosphorylation of GSK-3 results in inhibition of apoptosis [49], we next examined whether DOR-mediated PI3K/Akt signaling might protect the cells from stress-induced apoptosis. Prolonged culture of NG108-15 hybrid cells under serum-free conditions is associated with the initiation of apoptosis through activation of the dimeric cysteine protease caspase-3 [50]. Indeed, Western blot experiments revealed that serum deprivation for 2 h results in only barely detectable levels of cleaved caspase-3, which is indicative for a weak basal caspase-3 activity in this cell system (Fig. 7B). Serum-depletion for 24 h, however, significantly increased the amount of cleaved caspase-3, suggesting the initiation of stress-induced apoptosis [50]. Treatment of the cells with DPDPE and etorphine during the starvation period completely prevented caspase-3 cleavage. This finding suggests that DOR activation might also protect NG108-15 hybrid cells from entering the apoptosis pathway as recently described for DOR agonists in neocortical neurons [51]. Opioid-induced inactivation of caspase-3 was abolished by concomitant treatment of the cells with the PI3K-inhibitors Wortmannin (1 µM) and LY294002 (10 µM) as well as the Akt kinase inhibitor VIII (0.2 µM, 2 µM). These observations indicate that opioids indeed are able to prevent caspase-3 cleavage by stimulation of the PI3K/Akt pathway. Such a mechanism appears plausible because activated Akt is known to phosphorylate and thereby inactivate caspase-9, which in turn results in attenuation of caspase-3 activity [52].

Finally, we examined whether suppression of caspase-3 activity by opioids might counteract stress-induced apoptosis and possibly promotes cell survival. Annexin V-FITC binds to phosphatidylserine residues exposed to the cell surface at early stages of caspase-induced apoptosis [53]. PI is a marker of more advanced stages of apoptosis and cell necrosis and is only able to intercalate with nuclear DNA after cell membrane disintegration. Control cells grown in the presence of 10% FCS cells were negative for annexin V-FITC binding and nuclear incorporation of PI. In contrast, induction of cell stress by serum deprivation for 18 h resulted in strong annexin V-FITC binding to the cell membrane and PI incorporation into the nucleus (Fig 7C). Binding of both markers was prevented by co-incubation of the cells with 1 µM DPDPE during the starvation period, an effect that could be blocked by simultaneous treatment with Wortmannin (1 µM) and the Akt inhibitor VIII (2 µM). Similar results were obtained when cell viability was analyzed using the MTS assay. Again, the addition of DPDPE and etorphine (1 µM each) strongly enhanced cell viability after serum deprivation, an effect also prevented by Wortmannin and the Akt inhibitor VIII (Fig. 7D). These results demonstrate that DOR agonists protect NG108-15 hybrid cells from apoptosis and enhance cell survival by activating the PI3K/Akt signaling pathway. This finding is in line with the previous observation that DOR agonists may prevent apoptosis by up-regulating the caspase-3 inhibitor survivin [54]. Because the expression of survivin is under the control of IGF-1 receptor-stimulated PI3K/Akt kinase activity [55], it might be speculated hat the anti-apoptotic signal transduction pathway observed in the present study for NG108-15 hybrid cells might generally contribute to the neuroprotective effects of DOR agonists.

Conclusion

The present study delineates the signal transduction pathway and defines the functional significance of opioid-induced Akt activation in NG108-15 hybrid cells. Our results demonstrate that DOR activation results in $G\alpha_{i/o}$-dependent transactivation of a Trk/IGF-1 receptor complex that in turn stimulates Akt activation in a PI3K-dependent manner. Opioid-induced stimulation of the Akt signaling pathway blocks stress-induced cleavage of caspase-3 and, thus, prevents the cells from apoptosis. Because the insulin receptor-associated Akt signaling pathway has been recently suggested to play a critical role in opioid reward [56], DOR-mediated Akt activation might not only contribute to the

neuroprotective properties of opioids but also to the development of chronic opioid effects such as tolerance, dependence and addiction.

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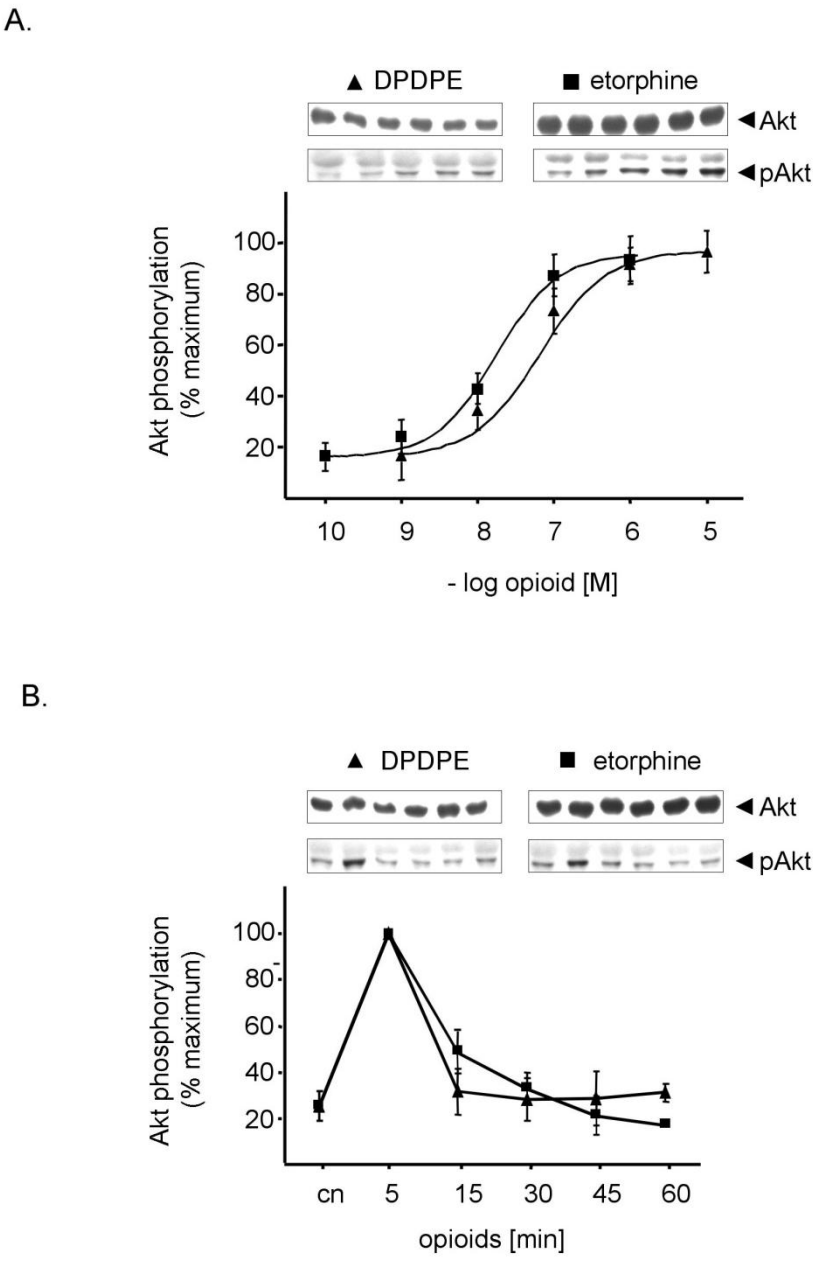
Figure 1:

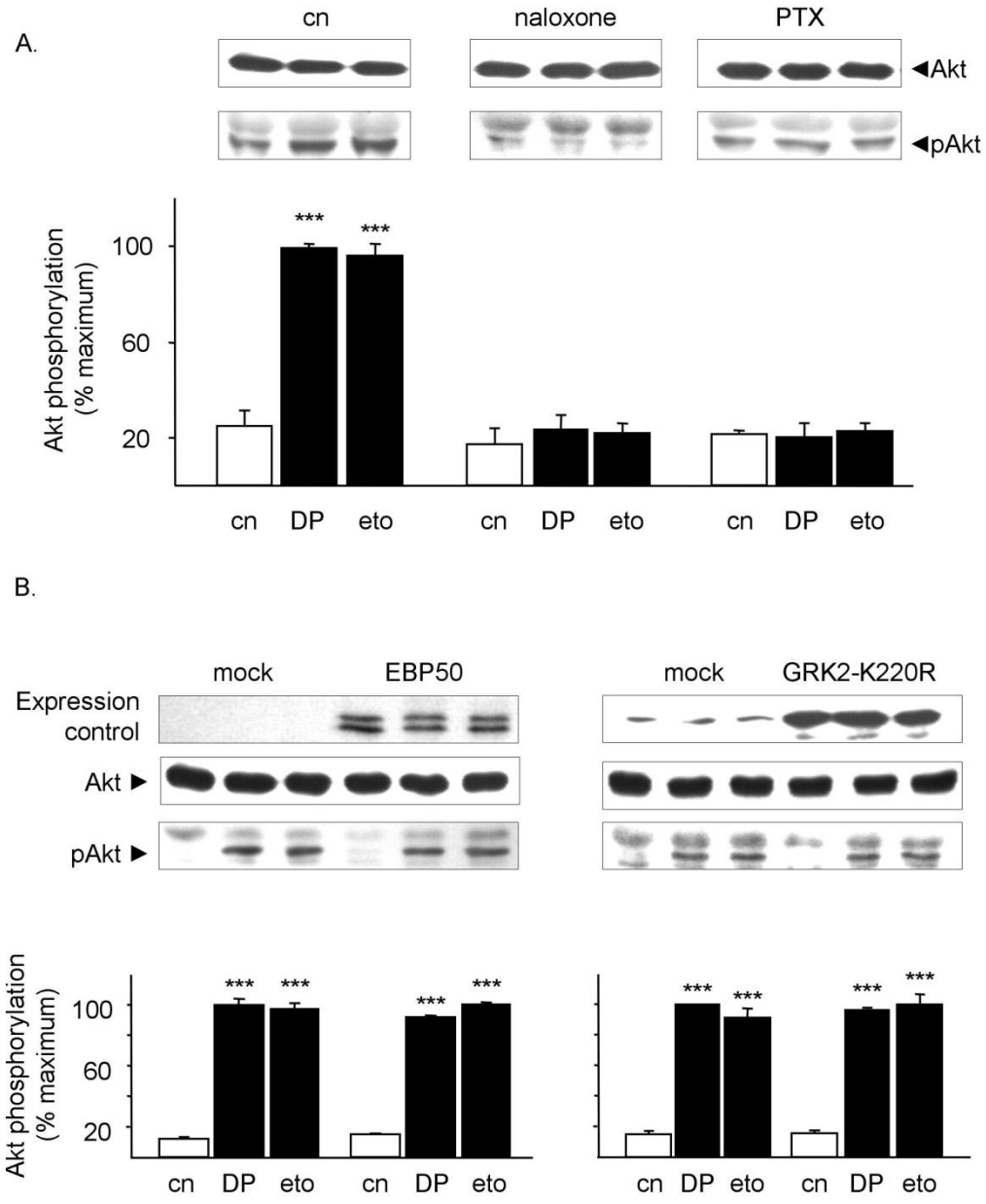


Kinetics of opioid-induced stimulation of Akt in NG108-15 hybrid cells.
(A) NG108-15 cells were exposed for 5 min to increasing concentrations of DPDPE and etorphine. Cells were lysed and activation of Akt was determined by Western blot using an antibody against phosphoserine-473. In NG108-15 hybrid cells, this antibody not only detects phosphorylated Akt (*pAkt;* prominent band at 60 kDa that is regulated by opioids), but also occasionally cross-reacts to another unrelated protein of about 80 kDa (weaker band). Overall abundance of Akt (*Akt*) was determined by analyzing the same samples with a pan-reactive anti-Akt antibody.

(B) The time-course of opioid-induced Akt stimulation was evaluated by treatment of the cells with DPDPE and etorphine (1 µM each) for increasing times. Overall abundance of

Akt (*Akt*) as well as opioid-stimulated activation of Akt (*pAkt*) was determined by Western blot using a pan-reactive anti-Akt and a phosphoserine-473 Akt antibody, respectively. Immunoreactivity was quantified by video densitometry. Akt phosphorylation is expressed in % of maximum stimulation. The data shown are the mean values ± S.D. of n > 3 independent experiments.

Figure 2:



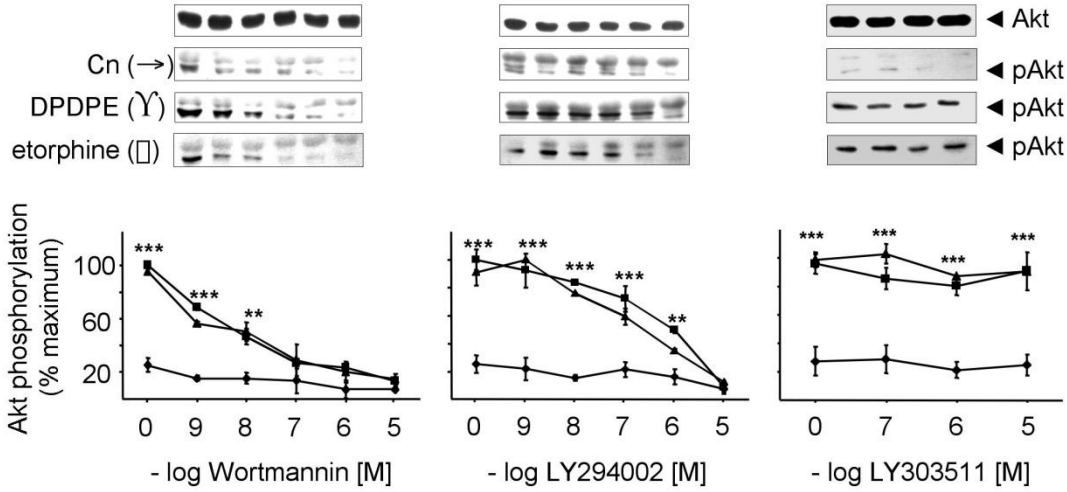
Activation of Akt is blocked by naloxone, PTX, but not by the $G_{q/11}$ scavengers EBP50 and GRK2-K220R.

(A) NG108-15 cells were pre-treated with naloxone (10 µM; 10 min) and pertussis toxin (PTX; 100 ng/ml; 18 h), before DPDPE (*DP; 1* µM and etorphine (*eto*; 1 µM) were added for 5 min. Thereafter, cells were lysed and subsequently assayed for total Akt (*Akt*) and Akt phosphorylation (*pAkt*).

(B) NG108-15 cells were transiently transfected with either HA-tagged EBP50 or GRK2-K220R, two different $G_{q/11}$ scavengers. Mock transfected cells of the same passage served as controls (*mock*). Cells were stimulated for 5 min with DPDPE (*DP;* 1 µM) or etorphine

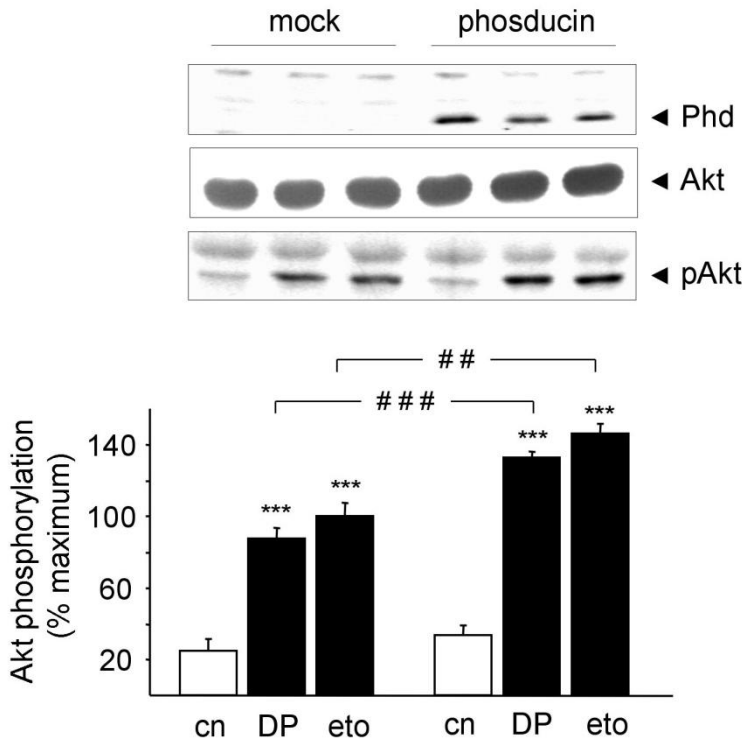
(*eto;* 1 µM), before cells were lysed and examined for total Akt (*Akt; middle panel*) and Akt phosphorylation (*pAkt, lower panel*). Expression of EBP50 and GRK2-K220R was verified using an anti-HA and GRK2 antibody, respectively (*expression control, upper panel*). Immunoreactivity was quantified by video densitometry. Opioid-induced Akt phosphorylation is expressed in % of maximum stimulation. The data shown are the mean values ± S.D. of $n > 3$ independent experiments. ***; Statistically significant at $p < 0.001$ as compared to controls.

Figure 3:



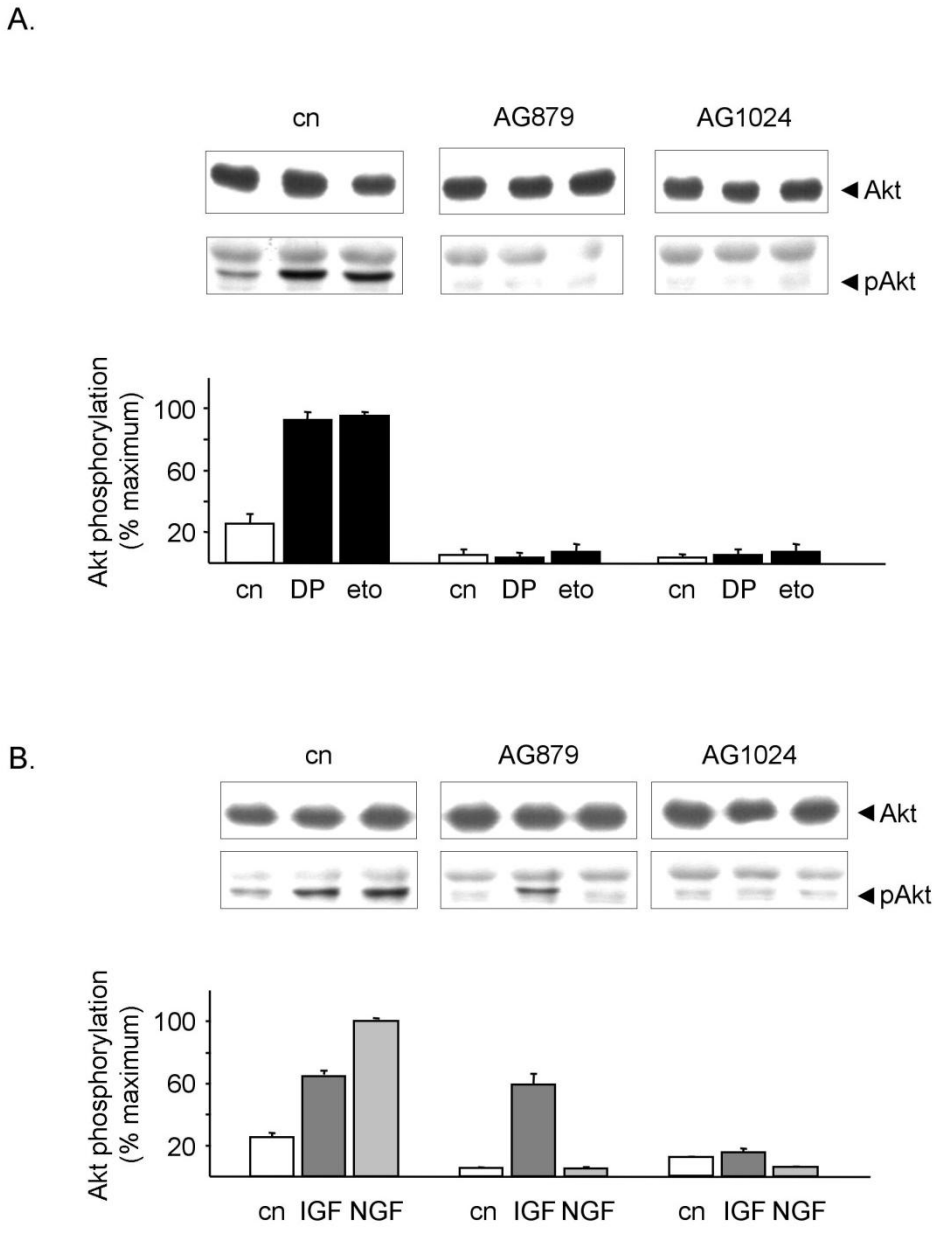
Opioid-induced stimulation of Akt is mediated by PI3K.
NG108-15 cells were cultured for 30 min in the absence or presence of increasing concentrations of Wortmannin (1 nM – 10 µM; *left panel*), LY294002 (1 nM- 10 µM; *middle panel*) and the PI3K-insensitive analog LY303511 (0.1 – 10 µM; *right panel*). Cells were stimulated for 5 min with 1 µM DPDPE (*DP*) or 1 µM etorphine (*eto*) to induce Akt phosphorylation. Cells were lysed and examined by Western blot using pan-reactive (*Akt*) and phosphoserine-473 specific Akt (*pAkt*) antibodies, respectively. The inserts show representative immunoblots. Quantified data are the mean values ± S.D. of n > 3 independent experiments. ***, $p < 0.001$; **, $p < 0.01$; Statistically different as compared to non-stimulated controls.

Figure 4:



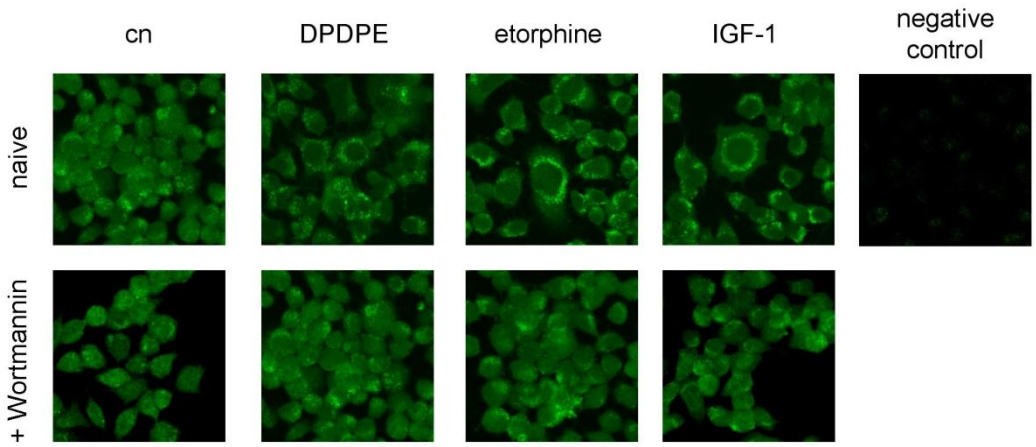
Phosducin fails to attenuate opioid-induced Akt stimulation.
NG108-15 cells were transiently transfected to express HA-tagged phosducin (*Phd*; *upper panel*). Mock transfected cells of parallel cultures served as controls (*mock*). On the day of experimentation, cells were washed and cultured in the absence of FCS for 2 h, before Akt stimulation was initiated by the addition of 1 µM DPDPE (*DP*) and 1 µM etorphine (*eto*) for 5 min. Phosducin expression (*Phd*) and activation of Akt (*pAkt*) were examined in cell lysates by Western blot using specific antibodies recognizing phosducin and phosphoserine-473 Akt, respectively. Total Akt was determined using a pan-reactive Akt antibody (*Akt; middle panel*). The data shown are the mean values ± S.D. of n > 3 independent experiments. ***, Statistically different at $p < 0.001$ as compared to non-opioid treated controls. ###, $p < 0.001$; ##, $p < 0.01$ versus phosducin-expressing cells.

Figure 5:



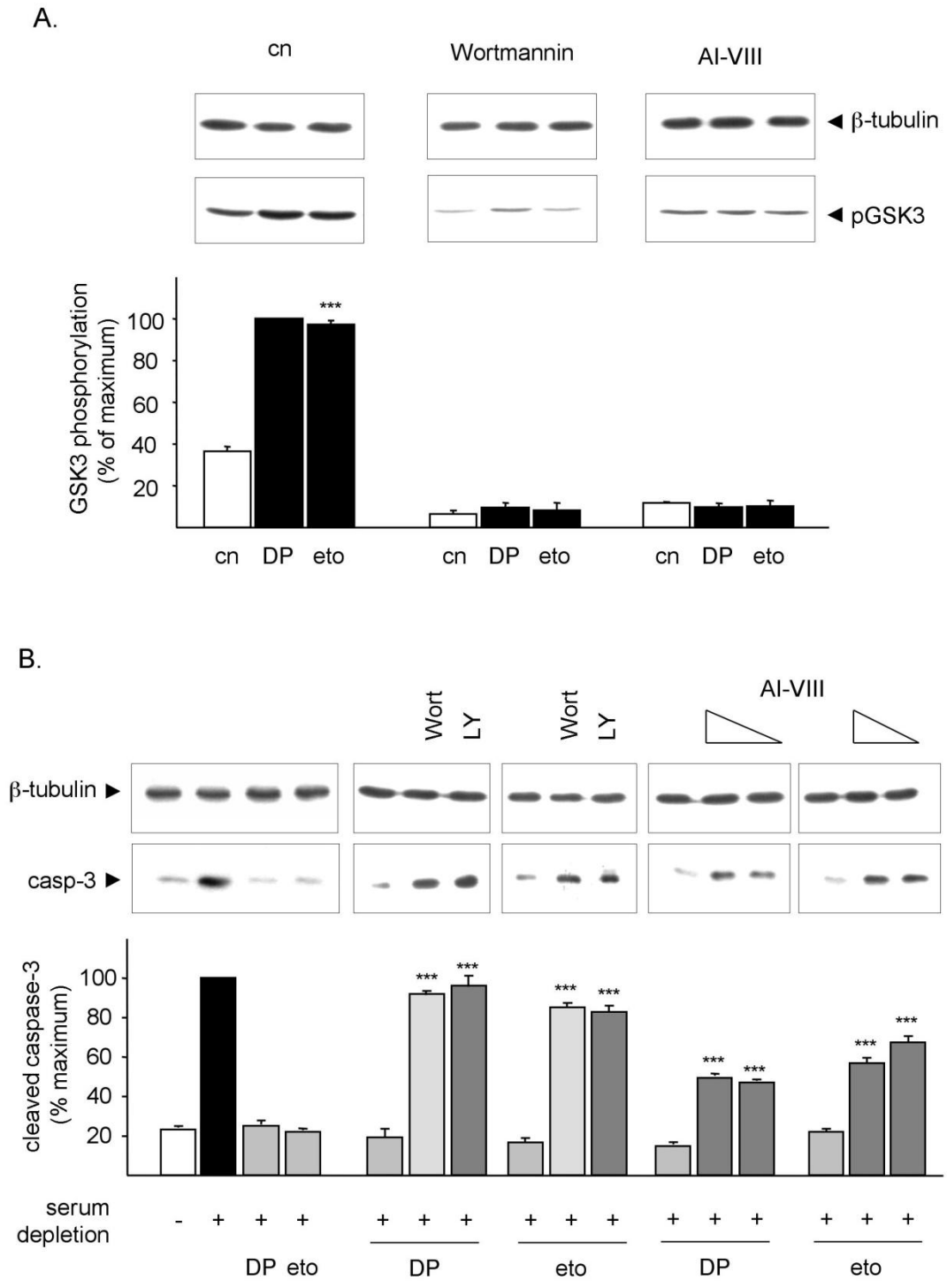
Opioid-induced Akt stimulation is mediated by Trk and IGF-1 receptors.
NG108-15 cells were cultured either in the absence (cn) or presence of 100 µM AG879 and 10 µM AG1024 for 30 min, before (A) DPDPE and etorphine (1 µM each) or (B) NGF (100 ng/ml) and IGF (10 ng/ml) was added for 5 min in order to activate Akt. Cell lysates were subjected to Western Blot analysis using an overall reactive Akt (*Akt*) and an antibody specific for phosphoserine-473 Akt (*p-Akt*). The data are the mean values ± S.D. of n > 3 independent experiments. ***, Statistically different at $p < 0.001$ as compared to opioid naive controls.

Figure 6:



DOR activation induces translocation of Akt to the nuclear membrane. Subcellular redistribution of activated Akt was examined by immunocytochemistry either in the absence or in the presence of 1 µM Wortmannin pre-treatment (30 min). Thereafter, Akt was stimulated for 5 min by the addition of 1 µM DPDPE, 1 µM etorphine, and 10 ng/ml IGF-1, before cells were fixed with paraformaldehyde and stained with an overall reactive Akt antibody followed by FITC-conjugated anti-rabbit IgG. Cells kept in the absence of Wortmannin and opioids served as the control (*cn*). For validation of Akt-specific antibody binding, cells were incubated with the secondary antibody alone (negative control). Fluorescence was visualized by laser scanning microscopy. The images shown are representative for at least 3 independent experiments.

Figure 7:


A.
cn
Wortmannin
AI-VIII
β-tubulin
pGSK3
GSK3 phosphorylation (% of maximum)
100
80
60
40
20
***
cn DP eto
cn DP eto
cn DP eto
B.
Wort
LY
Wort
LY
AI-VIII
β-tubulin
casp-3
cleaved caspase-3 (% maximum)
100
80
60
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***
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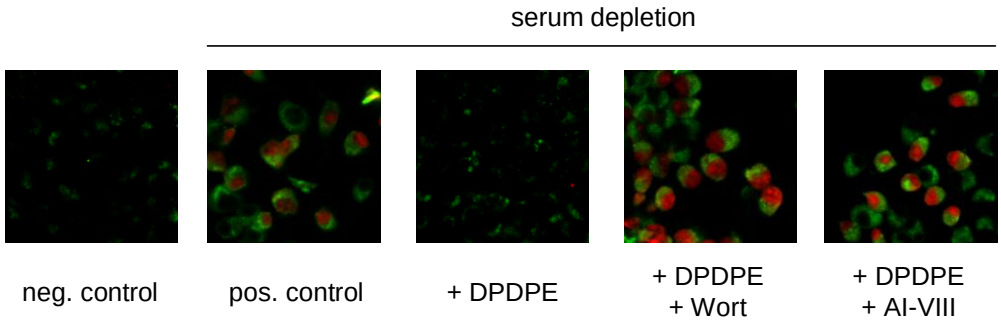
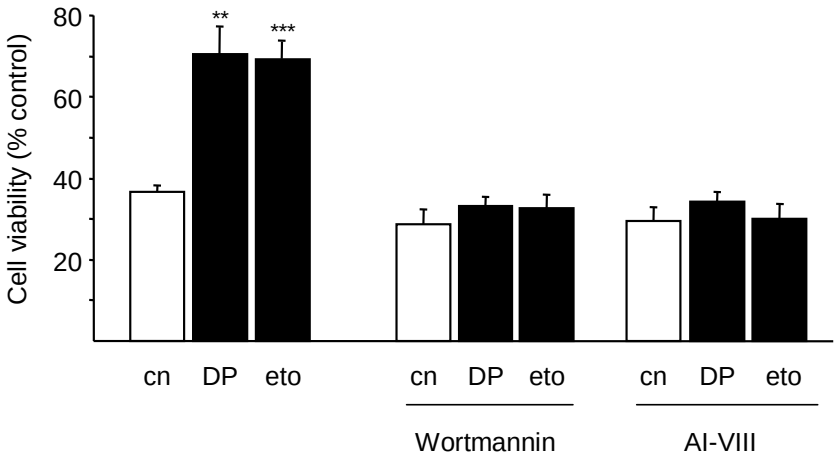


D.



DOR-mediated PI3K/Akt signaling prevents stress-induced apoptosis.
(A) Phosphorylation of GSK3 by opioids. NG108-15 cells were kept in the absence (*cn*) or presence of 1 µM Wortmannin (*Wort*) or 2 µM Akt inhibitor VIII (*AI-VIII*) for 30 min, before GSK3 phosphorylation was simulated for 5 min by the addition of 1 µM DPDPE and 1 µM etorphine. Thereafter, cells were lysed and examined for GSK3 phosphorylation (*pGSK3*) and β-tubulin (loading control) by Western blot.

(B) DOR-mediated Akt stimulation inhibits caspase-3 activation. NG108-15 cells were grown for 24 h under serum-free conditions either in the absence or presence of 1 µM DPDPE (*DP*) and 1 µM etorphine (*eto*). Where indicated, the PI3K inhibitors Wortmannin (*Wort;* 1 µM), LY294002 (*LY;* 10 µM) and the Akt kinase inhibitor VIII (*AI-VIII*; 0.2 and 2 µM) were added during the starvation period. Cells grown for 2 h in the absence of serum served as negative control. Reactions were stopped by addition of sample buffer and cell lysates were subjected to immunoblot analysis using an antibody specifically

recognizing cleaved caspase-3 (*casp-3; lower panel*). For loading control, blots were analyzed for β-tubulin (*upper panel*).

C) Annexin V / propidium iodide staining. NG108-15 cells were cultured for 18 h in the presence (*negative control*) or absence of FCS (*positive control*). During serum deprivation, cells were treated either with 1 µM DPDPE alone or together with 1 µM Wortmannin (*Wort*) or 2 µM Akt-I VIII (*AI-VIII*). After incubation, cells were stained with annexin V-FITC (*green*) and propidium iodide (*red*), and analyzed by confocal microscopy. Representative sections from 3 independent experiments are shown.

D) DOR-stimulated PI3K/Akt signaling promotes cell survival. NG108-15 hybrid cells were incubated for 24 h under serum-free conditions either in the absence or presence of 1 µM DPDPE (*DP*) 1 µM etorphine (*eto*), 1 µM Wortmannin (*Wort*), and 2 µM Akt-Inhibitor VIII (*AI-VIII*). Cell viability was assessed by the MTS assay and is expressed as the percentage of viable cells compared to cells grown in the presence of 10% FCS (set to 100%). The data shown are the mean values ± S.D. of n = 3 independent experiments. **, $p < 0.01$; ***, $p < 0.001$; Statistically different as compared to controls.

III Diskussion

Die vorliegende Studie hatte zum Ziel, den intrazellulären Signalweg der von δ-Opioidrezeptoren vermittelten Stimulation der Proteinkinase B / Akt zu charakterisieren. Darüber hinaus sollten die aus der Aktivierung der AKT resultierenden funktionellen Konsequenzen erarbeitet werden. Mit Hilfe von Neuroblastoma x Glioma (NG108-15) Hybridzellen konnten wir zeigen, dass die Aktivierung von δ-Opioidrezeptoren zu einer $G\alpha_{i/o}$-vermittelten Transaktivierung des Trk/IGF-1 Rezeptorkomplexes führt, der nachfolgend über einen PI3K-abhängigen Mechanismus die AKT stimuliert. Die von δ-Opioidrezeptoren vermittelte Aktivierung der AKT ist in der Lage, über eine Hemmung der Caspase-3 der Stress-induzierten Apoptose entgegen zu wirken. Die Ergebnisse tragen nicht nur wesentlich zur Aufklärung des intrazellulären Signalweges für die neuroprotektive Wirkung von Opioiden bei, aufgrund der vielfältigen zellulären Wirkungen der AKT eröffnen sie auch neue Perspektiven für den therapeutischen Einsatz von Opioiden als zytoprotektive Wirkstoffe [42].

Opioidrezeptoren werden nicht nur im zentralen und peripheren Nervensystem, sondern auch im Immunsystem [43] und vielen Tumorzellarten exprimiert [14]. Auch in diesen Zellen können Opioide über die Regulation der AKT Differenzierungs- und Wachstumsprozesse beeinflussen. So greifen Opioide in die T-Zellaktivierung ein und können auf diese Weise immunsuppressive Wirkungen vermitteln [43,44]. Bei Tumorpatienten kann es im Rahmen einer längerfristigen analgetischen Behandlung mit Opioiden zu einer AKT-vermittelten Steigerung der Zellproliferation und Verstärkung der Neoangiogenese kommen [36]. Darüber hinaus wird eine gesteigerte AKT Aktivität auch mit cancerogenen Mechanismen in Verbindung gebracht [33]. Da wir in der vorliegenden Studie zeigen konnten, dass Opioide ihre neuroprotektiven Wirkungen indirekt über eine IGF-1 Rezeptor-vermittelte Aktivierung der AKT ausüben, muss insbesondere bei allen Tumorarten mit einer Überexpression von IGF-1 Rezeptoren mit einer Auswirkung auf das Tumorwachstum durch Opioide gerechnet werden. Dies könnte vor allem bei verschiedenen Mammacarcinomen, beim Coloncarcinom und beim Pankreascarcinom des Menschen von Bedeutung sein [45,46].

Neben ihren zytoprotektiven Wirkungen besitzt die AKT auch regulatorischen Einfluss auf den Glucosestoffwechsel. Dieser wird durch eine IGF-1 Rezeptor-vermittelte Aktivierung

der GSK3β Isoform vermittelt [28]. In diesem Zusammenhang wurde beim Insulin-resistenten Diabetes mellitus eine verminderte Expression und Funktion von IGF-1 Rezeptoren gefunden, die nachfolgend in einer Abnahme der AKT-vermittelten Stimulation von GSK3β resultiert [32]. Beim Diabetes mellitus ist es jedoch fraglich, ob Opioide in der Tat therapeutisch zu einer Steigerung der AKT Aktivität eingesetzt werden könnten. Zum einen konnten bisher noch keine Opioidrezeptoren in Skelettmuskelzellen nachgewiesen werden, zum anderen konnten wir lediglich zeigen, dass δ-Opioidrezeptoren IGF-1 Rezeptoren transaktivieren können. Ob Opioidrezeptoren auch in der Lage sind die Funktion oder Expression von IGF-1 Rezeptoren zu steigern, muss noch geklärt werden. Unabhängig von diesen Vorbehalten konnte bei Typ 2 Diabetikern aber trotzdem eine vorübergehende Senkung der Blutglucosekonzentration, nicht aber der Menge an Glycohämoglobin (HbA1C), durch Opioide demonstriert werden [38]. Ob der dabei beobachtete schnelle Verlust der hypoglycämischen Opioidwirkung möglicherweise durch eine Toleranzentstehung vermittelt wird, ist nicht bekannt.

Aus Studien mit spontanen Tiermodellen für Diabetes mellitus Typ 1 und 2 ergibt sich ein weiteres mögliches Einsatzgebiet für die neuroprotektive Wirkung von Opioiden. Sowohl in BB/wor (Diabetes mellitus Typ 1) als auch BBZDR/wor (Diabetes mellitus Typ 2) Ratten geht die Entwicklung des Krankheitsbildes mit einer Abnahme der Expression von IGF-1 Rezeptoren, AKT und GSK3β einher. Gleichzeitig kommt es bei diesen Tieren zu einer Anreicherung von β-Amyloid und p-Tau im ZNS, wie sie für die Alzheimer-Krankheit typisch ist. In der Tat nimmt die GSK3, ein Substrat der AKT, eine zentrale Rolle in der Pathogenese der Alzheimer-Krankheit ein [47]. Da die Phosphorylierung der GSK3 mit einem Wirkungsverlust einhergeht, werden derzeit GSK3 Inhibitoren für die Behandlung der Alzheimer-Krankheit evaluiert [48]. Da Opioide über eine Aktivierung der AKT ebenfalls eine Hemmung der GSK3 Funktion vermitteln, könnten diese der Entstehung und Progredienz der Alzheimer-Krankheit entgegen wirken.

Neben der Alzheimer-Krankheit wurde bisher noch zwei weitere neuronale Erkrankungen mit einer verminderten AKT Aktivität in Verbindung gebracht. Aber sowohl bei Chorea Huntington [49] als auch bei Morbus Parkinson [50] fehlen bisher Hinweise über mögliche therapeutische Strategien für den Einsatz von AKT Aktivatoren.

Ein weiteres Ergebnis dieser Arbeit ist, dass die von δ-Opioidrezeptoren vermittelte Aktivierung der AKT die Stress-induzierte Apoptose hemmen kann. Die daraus resultierende neuroprotektive Opioidwirkung ist in der Lage, der hypoxischen und ischämischen Schädigung von Neuronen, zum Beispiel in Folge einer Apoplexie, entgegen zu wirken [41]. Ein weiteres mögliches Anwendungsgebiet für die zytoprotektive Wirkung von Opioiden stellen hypoxische Schäden beim Myocardinfarkt oder bei Organtransplantationen dar. Aufgrund der Expression von κ-Opioidrezeptoren im Myocard weisen insbesondere κ-Agonisten cardioprotektive Wirkungen auf, die am Tiermodell Reperfusionsschäden erfolgreich verhindern können [51]. Die cardioprotektive Wirkung von κ-Agonisten wird ebenfalls über eine AKT-vermittelte Phosphorylierung der GSK3β Isoform erreicht [49]. Da es wie oben erwähnt beim Insulin-resistenten Diabetes mellitus zu einer eingeschränkten IGF-1 Rezeptor-induzierten Aktivierung der AKT kommt, ist die cardioprotektive Wirkung von Opioiden bei diesem Krankheitsbild limitiert [52].

Abschießend kann gesagt werden, dass die an Neuroblastoma x Glioma (NG108-15) Hybridzellen erarbeiteten Mechanismen wesentlich zur Aufklärung der zellulären Grundlagen der neuroprotektiven Wirkung von Opioiden beitragen. Dabei kommt einer IGF-1 Rezeptor-vermittelten Aktivierung der AKT eine zentrale Rolle zu. Ob diese Mechanismen auch den oben genannten Krankheitsbildern zugrunde liegen, muss im Einzelfall in weiterführenden *in-vivo* Studien geklärt werden. Die Ergebnisse der vorliegenden Arbeit eröffnen zudem interessante Perspektiven, die physiologischen und pathophysiologischen Grundlagen verschiedener Krankheiten wie Diabetes mellitus, Alzheimer-Krankheit und Myocardinfarkt zu untersuchten. Im Bezug auf den möglichen therapeutischen Einsatz eventuell peripher wirkender Opioide bei Diabetes mellitus wäre beispielsweise interessant, ob der von Opioidrezeptoren induzierte Signalweg auch die Regulation des GLUT4-Transporters steuern kann. Ebenso könnte in einem zellulären Modell für Diabetes mellitus Typ 2 die Möglichkeit untersucht werden, die Insulin-vermittelte Aktivierung der AKT oder die Expression von IGF-1 Rezeptoren durch Opioide zu steigern.

IV Zusammenfassung

Die neuroprotektive Wirkung von Opioiden wird durch die Proteinkinase B / AKT vermittelt

Neben ihrer therapeutisch erwünschten analgetischen und antitussiven Wirkung, können Opioide auf vielfältige Weise in die Zellproliferation, -differenzierung und in die Überlebensmechanismen der Zelle eingreifen. So besitzen DOR-Agonisten zytoprotektive Eigenschaften, die mit einer Regulation der Proteinkinase B / AKT in Verbindung gebracht werden. Ziel der vorliegenden Arbeit war es daher, die intrazelluläre Signalkaskade der DOR-vermittelten Aktivierung von AKT aufzuklären und die physiologische Bedeutung dieses Signalwegs aufzuzeigen. Als Modellsystem hierfür wurden Neuroblastoma x Glioma (NG108-15) Hybridzellen verwendet, die den DOR endogen exprimieren und eine Vielzahl akuter und chronischer Opioideffekte ausbilden. Die Behandlung der Zellen mit DPDPE und Etorphin resultiert in der Tat in einer zeit- und konzentrationsabhängigen Aktivierung der AKT, die mit Hilfe eines phosphospezifischen Antikörpers gegen Serin-473 im Westernblot nachgewiesen wurde. Die DOR-vermittelte Aktivierung der AKT kann durch den Opioidantagonisten Naloxon blockiert werden, stellt also einen Rezeptor-vermittelten Effekt dar. Studien mit PTX und Bindeproteinen für $G_{q/11}$ (EBP50; GRK2-K220R) zeigten zudem, dass dieser Opioideffekt durch inhibitorische $G_{i/o}$-Proteine vermittelt wird. Die Stimulation der AKT durch Opioide kann durch Wortmannin und LY294002 blockiert werden, was auf eine Beteiligung der PI3K an der Signalvermittlung hinweist. Weiter Untersuchungen zeigten, dass Opioide die AKT über eine Transaktivierung von IGF-1 stimulieren, wohingegen die Rolle der NGF-Rezeptoren noch unklar ist. Eine Beteiligung von Gβγ-vermittelten intrazellulären Signalwegen konnte ausgeschlossen werden. Nach der DOR-vermittelten Aktivierung wandert die AKT zur Kernmembran, wo sie nachfolgend die Induktion der Apoptose durch Phosphorylierung der GSK3 und Inaktivierung der Caspase-3 hemmt. Die vorliegende Studie zeigt, dass DOR-Agonisten in NG108/15 Hybridzellen neuroprotektive Effekte entfalten, die über eine Transaktivierung des IGF-1 Rezeptor / PI3K / AKT Signalweges vermittelt werden. Die gewonnenen Ergebnisse tragen nicht nur zur Aufklärung der intrazellulären Mechanismen der neuroprotektiven Opioidwirkung bei, sondern liefern auch die Basis für einen möglichen therapeutischen Einsatz von Opioiden als zytoprotektive und anti-apoptotische Wirkstoffe.

V Summary

Protein kinase B / AKT mediates the neuroprotective effects of opioids

Besides their clinically desired antinociceptive and antitussive actions, opioids are also known to interfere with many aspects of cell proliferation, differentiation and cell survival. In particular, DOR agonists have been shown to possess cytoprotective properties associated with regulation of the "pro-survival" kinase AKT. Thus, aim of the present study was to delineate the signal transduction pathway by which opioids might stimulate AKT activity and to evaluate the physiological consequences of this effect. Neuroblastoma x Glioma (NG108-15) hybrid cells served as a model system, because they carry high amounts of endogenous DORs that induce a wide array of acute and chronic opioid actions. We found that exposure of the cells to both DPDPE and etorphine results in a time- and dose-dependent increase in AKT activity, as measured by means of an activation-specific antibody recognizing phosphoserine-473. DOR-mediated AKT signaling is blocked by the opioid antagonist naloxone and involves inhibitory $G_{i/o}$ proteins, because pre-treatment of the cells with PTX, but not over-expression of the $G_{q/11}$ scavengers EBP50 and GRK2-K220R, prevents this effect. Further studies with Wortmannin and LY294002 revealed that PI3K plays a central role in opioid-induced AKT activation. Opioids stimulate AKT activity through transactivation of receptor tyrosine kinases, because pre-treatment of the cells with inhibitors for neurotrophin receptor tyrosine kinases (AG879) and the insulin-like growth factor receptor IGF-1 (AG1024) abolished this effect. In contrast, over-expression of the Gβγ scavenger phosducin failed to interfere with opioid-induced AKT activation. Upon activation, we found that AKT translocates to the nuclear membrane, where it is able to promote phosphorylation of GSK3, and to prevent cleavage of caspase-3. These two key events ultimately result in inhibition of cell apoptosis and enhancement of cell survival. Taken together, the present study demonstrates that in NG108/15 hybrid cells DOR agonists possess neuroprotective properties, which are mediated by transactivation of the IGF-1 receptor / PI3K / Akt signaling pathway. These results not only contribute to the intracellular signal transduction mechanisms mediating neuroprotective opioid effects, they also provide the basis for the therapeutic use of opioids as cytoprotective and anti-apoptotic agents in a number of disease states.

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