Peptiderger Einfluss auf 3T3-L1 Adipozyten

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Bei der vorliegenden Arbeit handelt es sich um eine experimentelle Untersuchung zum Einfluss von zwei Ko-Transmittern des autonomen Nervensystems, Neuropeptid Y und dem Pituitary Adenylate Cyclase-activating Polypeptide (PACAP), auf den intrazellulären Kalziumspiegel und die Insulinsensitivität von 3T3-L1 Adipozyten. Mittels Polymerasekettenreaktion und Western Blot Analyse konnte die Expression des NPY-1 (Y1) Rezeptors als auch die der PACAP Rezeptoren PAC1 und VPAC2 nachgewiesen werden. Die Aktivierung des Y1 oder des PAC1 Rezeptors durch ihre Agonisten führte zur Erhöhung des intrazellulären Kalziumspiegels. Im Weiteren führte NPY nach Ko-Applikation mit Insulin zu einer abgeschwächten Insulinsensitivität der Adipozyten, da sowohl die insulin-stimulierte Translokation von Glukosetransporter 4 zur Zelloberfläche als auch die Glukoseaufnahme durch NPY abgeschwächt wurde. Dieser Effekt konnte als Y1 spezifisch beschrieben werden.

Diese Ergebnisse gewähren somit neue Einblicke über den peptidergen Einfluss auf den Adipozytenstoffwechsel und erlauben Rückschlüsse über die Rolle des autonomen Nervensystems in der Entwicklung von Adipositas und Diabetes mellitus Typ 2.

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[1] Seitenzahl insgesamt

[2] Zahl der im Literaturverzeichnis der Einleitung ausgewiesenen Literaturangaben

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1 Einleitung

1.1 Fettgewebe

Das Fettgewebe ist ein heterogenes Gewebe. Es besteht neben der vorherrschenden Zellpopulation der Adipozyten, aus Fibroblasten, Präadipozyten, Endothelzellen und Makrophagen. Diese Zellverbände werden durch feine Bindegewebssepten in Läppchen organisiert, die mit bloßem Auge sichtbar sind. Das Fettgewebe macht beim adulten Menschen durchschnittlich etwa 15-20% des Körpergewichts aus (Geneser 1990, Welsch 2006).

Traditionellerweise unterscheidet man die einzelnen Fettgewebsdepots von Säugetieren nach ihrer makroskopischen Farbe in weißes und braunes Fettgewebe. Weißes Fettgewebe besteht vorwiegend aus univakuolären Fettzellen und befindet sich beim Erwachsenen vorwiegend subkutan, im Omentum und im Mesenterium, sowie im Retroperitonealraum. Diese „weißen Adipozyten“ sind von spheroider Form und können weit über 100 µm im Durchmesser werden. Ihr Kern ist randständig im schmalen Zytoplasmasaum um den zentralen Lipidtropfen angeordnet. Weiterhin ist das weiße Fettgewebe mäßig stark vaskularisiert und von autonomen und sensiblen Nerven durchzogen (Mantovani 2006). Es dient in erster Linie der Aufnahme, Speicherung und Abgabe von Energie je nach Bedarf des Körpers. Neben dieser zentralen Aufgabe ist das Fettgewebe aber auch aufgrund seiner schlechten Wärmeleitfähigkeit als Isolierfett und teilweise als bloßes Baufett um innere Organe anzusehen (Geneser 1990). Zu diesen historisch bekannten Funktionen kam spätestens seit der Entdeckung des Sättigungshormons Leptin im Dezember 1994 von Friedman und Kollegen die bedeutende Rolle als endokrines Organ hinzu (Zhang et al. 1994). Seither wurden zahlreiche Hormone beschrieben, die von Adipozyten sezerniert werden, wie beispielsweise Adiponectin, Resistin und Visfatin (Waki und Tontonoz 2007, Rasouli und Kern 2008).

Das braune Fettgewebe erhält seine rotbraune bis goldene Farbe von einer starken Vaskularisierung. Die Adipozyten im braunen Fettgewebe sind vornehmlich plurivakuolär und kleiner als „weiße“ Adipozyten (30-50 µm). Ihre Hauptaufgabe besteht in der muskelunabhängigen Wärmeproduktion. Dies geschieht durch spezielle Proteine in den zahlreichen Mitochondrien der „braunen“ Adipozyten, die eine Entkopplung der

Atmungskette induzieren können (Mantovani 2006). Braunes Fettgewebe kommt beim Menschen nur bis zum Neugeborenenalter regelhaft vor. Hier sind vor allem die Region zwischen den Schulterblättern, die Achselhöhle und die Nackenregion zu nennen (Geneser 1990). Im Erwachsenalter findet man es nur sporadisch an wenigen Stellen, wie entlang der großen Blutgefäße, im Mediastinum, im Nierenhilus und der Achselhöhle (Truong et al. 2004, Farmer 2008). Allerdings findet man braune Adipozyten auch regelmäßig im weißen Fettgewebe (Cinti 2005).

Seit einigen Jahren ist bekannt, dass es unter bestimmten Bedingungen, wie beispielsweise nach Kälteexposition, zu einer reversiblen Transdifferenzierung von univakuolären in plurivakuoläre Adipozyten kommen kann. Diese plurivakuolären Fettzellen haben zwar nicht denselben Ursprung wie „braune“ Adipozyten, jedoch viele Ihrer charakteristischen Eigenschaften (de Matteis et al. 2009).

1.2 Adipozyten-Vorläuferzellen und Ihre Differenzierung

Das Fettgewebe entsteht aus dem Mesoderm, das sich während der Gastrulation zwischen Ektoderm und Entoderm als dritte Keimscheibe formiert. Weitere Zellreihen, die aus mesodermalem Gewebe entstehen sind Chondrozyten und Myozyten (Enerbäck 2009).

Obwohl bereits der Schweizer Arzt und Naturforscher Konrad Gessner, der im Jahre 1551 das braune Fettgewebe als erster beschrieb, es als “weder Fett noch Fleisch [nec pinguitudo, nec caro], doch irgendetwas dazwischen” einstufte, hielt sich bis vor wenigen Jahren die Vorstellung einer gemeinsamen adipogenen Vorläuferzelle, dem Adipoblasten (Cannon und Nedergaard 2008, Kahn 2008). Tatsächlich unterscheiden sich weiße und braune Adipozyten hinsichtlich Ihrer Funktion und Ihres Ursprungs beträchtlich. Der gemeinsame Terminus Adipozyt beruht lediglich auf dem morphologischen Kennzeichen der Lipidtropfen (Fantuzzi und Mazzone 2007). Braune, plurivakuoläre Fettzellen haben gemeinsame Vorläuferzellen mit Myozyten und entstehen aus Myf5 positiven Zellen des paraaxialen Mesoderms, während weiße, univakuoläre Adipozyten aus Myf5 negativen Zellen des Seitenplattenmesoderms entstehen (Seale et al. 2008, Enerbäck 2009).

Adipogene Vorläuferzellen befinden sich im Fettgewebe vornehmlich im perivaskulärem Kompartiment, auch genannt stroma-vaskuläre Fraktion (Tang et al. 2008). Ob adipogene

Vorläuferzellen nach der Migration aus dem Mesoderm zeitlebens im perivaskulären Kompartment residieren oder stetig aus dem Knochenmark rekrutiert werden, wie es für plurivakuoläre Fettzellen gezeigt wurde, ist unklar (Crossno et al. 2006).

Die Differenzierung von adipogenen Vorläuferzellen, den Präadipozyten, zu reifen Adipozyten, wird Adipogenese genannt. Die meisten Untersuchungen über die molekularen Mechanismen wurden in vitro erhoben. Hierfür dienen eine Fülle von klonalen Präadipozytenkulturen, wie beispielsweise die 3T3-F442A, Ob17, sowie ihr Subtyp Ob1771 und die hier verwendent 3T3-L1 Zelllinie (Gaillard et al. 1984, 1989a, 1989b, 1991), Zellkulturen aus embryonalen Stammzellen (Dani et al. 1989) und primäre Zellkulturen, die aus den isolierten Präadipozyten der stroma-vaskulären Fraktion gewonnen werden können (Perrini et al. 2009). Morphologisch lassen sich frühe Präadipozyten, späte Präadipozyten und reife Adipozyten unterscheiden. Die Unterscheidung von frühen Präadipozyten und echten Fibroblasten ist morphologisch kaum möglich, sie werden daher in der Literatur auch häufig als fibroblasten-ähnliche Präadipozyten bezeichnet. Diese frühen Vorläuferzellen proliferieren in vitro bis sie durch Kontaktinhibition die Proliferation und Teilung einstellen. Für die meisten Präadipozyten-Zelllinien und primären Zellkulturen ist dieser Wachstumsarrest Grundvorraussetzung für eine erfolgreiche Differenzierung in reife Adipozyten. Späte Präadipozyten sind eher spindelförmig bis oval. Nach Induktion der Adipogenese mittels Glukokortikoiden, Isobuthylmethylxanthinen oder Insulin teilen sich die post-konfluenten Präadipozyten ein letztes Mal und beginnen anschließend Lipidtropfen zu akkumulieren. In reifen Adipozytenkulturen sollten mindestens 95% der Zellen Lipidtropfen im Zytoplasma entwickelt haben (Gregoire 2001, Gericke et al. 2009).

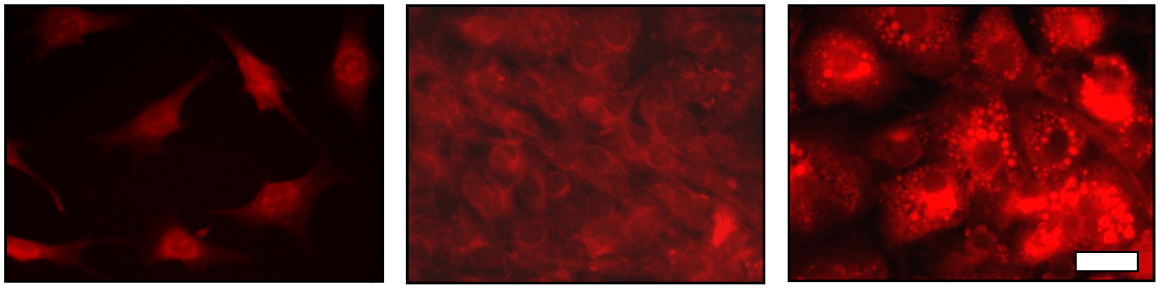

Abb.1 Charakterisierung von 3T3-L1 Zellen nach Fettfärbung mit Nile Red. 3 Tage nach Kultivierung zeigen frühe Präadipozyten einen fibroblasten-ähnlichen Phänotyp (links). Späte Präadipozyten sind spindelförmig und befinden sich im Wachstumsarrest (mitte). Reife 3T3-L1 Adipozyten zeigen typische plurivakuoläre Fetttropfen im Zytoplasma (rechts). Der Messbalken entspricht 40 µm.

Auf molekularbiologischer Ebene finden im Laufe der Adipogenese verschiedene Regulationsprozesse statt. So sind eine ganze Reihe von Transkriptionsfaktoren-Familien an der Differenzierung beteiligt, wie beispielsweise die CCAAT/enhancer binding proteins (C/EBP) und die Peroxisome proliferator activated receptors (PPAR). Nach Induktion der Adipogenese bei post-konfluenten Präadipozyten werden als erstes die frühen Transkriptionsfaktoren C/EBPβ und δ exprimiert. Diese wiederum aktivieren im Laufe der Differenzierung die späten Transkriptionsfaktoren C/EBPα und PPARγ (Wu et al. 1996, Gregoire 2001). Im Zuge der Adipogenese werden Präadipozyten-spezifische Proteine wie Pref-1 oder Necdin herunterreguliert, während typische Markerproteine reifer Adipozyten wie Glukosetransporter 4, Adiponectin oder Uncoupling Protein 1 verstärkt oder de novo exprimiert werden (Tseng et al. 2008, Vázquez-Vela et al. 2008). Interessanterweise hemmt Kalzium in der frühen Adipogenese die Proliferation und Differenzierung von adipogenen Vorläuferzellen (Miller et al. 1996, Ntambi und Takova 1996) während in den späten Phasen der Adipozytenentwicklung die Einlagerung von Lipiden und somit die Reifung der Fettzellen durch Kalzium begünstigt wird. Intrazelluläres Kalzium schein somit einen bi-phasischen Einfluss auf die Adipogenese zu haben (Shi et al. 2000).

1.2.1 Humorale Regulation des Fettgewebes

Als endokrines Organ ist das Fettgewebe stark vaskularisiert. So steht jeder Adipozyt in Verbindung mit wenigstens einer Kapillare (Geneser 1990). Neben der Sekretion von Adipokinen in die Blutbahn erhält das Fettgewebe hierdurch eine Fülle von humoralen Einflussen, die sich auf Zellproliferation, Differenzierung und Energiestoffwechsel auswirken. Das wohl bekannteste dieser Hormone ist das von pankreatischen β-Zellen produzierte Insulin. Insulin stimuliert Adipozytendifferenzierung und Fettdepotexpansion. In reifen Adipozyten hemmt es die Lipolyse, also den Abbau von Triglyzeriden, und fördert die de novo Synthese von freien Fettsäuren. Weiterhin wird die zelluläre Aufnahme von Glukose und Fettsäuren in Fettzellen unter Insulin gesteigert (Madsen und Kristiansen 2010). Die insulin-abhängige Aufnahme von Glukose geschieht vor allem durch den Glukosetransporter 4, der nach Stimulation aus zytosolischen Vesikeln ins Plasmalemm transloziert wird und somit entscheidend zur Senkung des Blutglukosespiegels nach Insulingabe beiträgt (Pattaranit et al. 2008).

Schilddrüdenhormone fördern ebenfalls die Adipogenese (Hausman et al. 2001). Trijodothyronin (T3) reguliert Schlüsselelemente der Adipozytenentwicklung, wie PPARγ oder Transkiptionsfaktoren der C/EBP Familie durch Thyroid Response Elements (TRE) oder direkt durch nukleäre Isoformen des T3 Rezeptors (Obregon 2008). Weiterhin spielen Schilddrüsenhormone eine wichtige Rolle bei der Thermogenese im braunen Fettgewebe (Leppäluoto et al. 2005).

Glukokortikoide sind entscheidend an der Regulation von Glukose- und Fettsäure-stoffwechsel beteiligt. In Adipozyten hemmt Kortisol die Lipidmobilisation und fördert die Akkumulation von Triglyzeriden (Roberge et al. 2007). Die Dichte von Glukokortikoid-Rezeptoren ist in viszeralen Fettdepots höher als in subkutanen. Überproduktion oder exzessive Zufuhr resultiert deshalb vorwiegend in viszeraler Adipositas (Björntorp und Rosmond 2000). Glukokortikoide führen zu peripherer Insulinresistenz und werden als pathogenetischer Link zwischen Adipositas und Diabetes mellitus Typ 2 diskutiert (Qi und Rodrigues 2007). Interessanterweise steigern Glukokortikoide die Expression von NPY in sympathischen Nervenfasern (Kuo et al. 2007).

Es wären noch zahlreiche Botenstoffe zu nennen, von denen bekannt ist, dass sie Regulationsvorgänge im Fettgewebe beeinflussen. Kurz erwähnt sei hier zirkulierendes Adrenalin, Glukagon, Insulin-like Growth Factor (IGF)-1 und -2 als auch Angiotensin. Auch parakrine Einflüsse durch Adipokine wie Adiponectin, Visfatin und Resistin oder inflammmatorische Zytokine wie TNF-α oder Interleukin-6 spielen bei der Insulinsensitivität von Fettzellen eine Schlüsselrolle (Stumvoll et al. 2005, Rasouli und Kern 2008).

1.2.2 Nervale Regulation des Fettgewebes

Seit der Beobachtung von Claude Bernard in der 1850er Jahren, dass die Punktion des vierten Ventrikels beim Hund zu einer transienten Glukosurie führt, wird das Gehirn in Überlegungen zur Regulation und Kontrolle des Fett- und Glukosestoffwechsels mit einbezogen (Nonogaki 2000). Interessanterweise enthält das Fettgewebe zahlreiche Nervenfasern, wobei es starke Unterschiede zwischen weißem und braunem Fettgewebe gibt. Das braune Fettgewebe ist deutlich stärker innerviert. Die Nervenfasern ziehen meist mit Blutgefäßen ins Fettgewebe und zweigen sich dann in den Fettlobuli in parenchymatöse

Fasern auf und treten direkt in Kontakt mit Adipozyten. Die meisten Autoren gehen hierbei von einer Synapses en passant Innervation aus (Kosacka et al. 2006).

Die sympathische Innervation des Fettgewebes konnte in mehreren Studien und mit unterschiedlichen Techniken wie der antero- und retrograden Markierung der Nervenfasern (Kreier et al. 2006) als auch mittels Fluoreszenzmarkern (Youngstrom und Bartness 1995) nachgewiesen werden. Der bekannteste Botenstoff postganglionärer sympathischer Nervenfasern ist Noradrenalin. Sie werden daher häufig als noradrenerg bezeichnet. Im Fettgewebe führt die Freisetzung von Noradrenalin nach Aktivierung G-Protein-gekoppelter β-Rezeptoren zu einer gesteigerten Lipolyse und dadurch zur Freisetzung von freien Fettsäuren. Der noradrenerge Einfluss auf die Lipolyse scheint im Adipozyten jedoch abhängig von der Balance zwischen den α- und β-Rezeptoren zu sein. So kann beim Überwiegen der α-Rezeptoren die Lipolyse gehemmt werden, da weniger des sekundären Messengers cAMP gebildet wird (Bartness und Bamshad 1998, Löffler und Petrides 2003).

Zusätzlich scheint der Sympathikus einen inhibitorischen Einfluss auf die Proliferation von adipogenen Vorläuferzellen auszuüben. Dies konnte nach chirurgischer als auch nach chemischer Sympathektomie gezeigt werden. (Bowers et al. 2004, Foster und Bartness 2006). Weiterhin weisen Nscl-2 knockout Mäuse, ein Modelltier für einen peripheren Nervenschaden mit verringertem Anteil autonomer Nervenfasern, eine erhöhte Anzahl von Präadipozyten auf (Ruschke et al. 2009). Die Datenlage zum Glukosestoffwechsel nach pharmakologischer, chemischer oder chirurgischer Alteration des sympathischen Nervensystems ist teilweise widersprüchlich. Einige Autoren berichten über eine gesteigerte periphere Glukoseaufnahme bei erhöhtem Sympathotonus unabhängig von Insulin (Nonogaki 2000, Young et al. 2004). Anderen Arbeiten zufolge führt eine erhöhte Sympathikusaktivität zur Abschwächung der peripheren Insulinwirkung, wie der Glukoseaufnahme (Kaaja und Pöyhönen-Alho 2006, Flaa et al. 2008). Allerdings sind auch das Inselorgan des Pankreas und die Leber stark sympathisch innerviert (Rosengren et al. 2010). Dies macht die differenzierte Unterscheidung der nervalen Regulation der Glukosehomöostase schwierig, da sowohl Pankreas, Muskelgewebe, Leber und Fettgewebe an der Glukoseverwertung in vivo beteiligt sind und sich teilweise gegenseitig kompensieren (Stumvoll et al. 2005) können. In vitro führt die Applikation von β-Rezeptoragonisten zu einer gehemmten Glukoseaufnahme von 3T3-L1 Zellen und primären Adipozyten nach

Stimulation mit Insulin (Lee et al. 1998, Mulder et al. 2005). Weiterhin scheint die humane Adipositas eng mit einer Überaktivität des sympathischen Nervensystems assoziiert zu sein (Seematter et al. 2000).

Eine parasympathische Innervation des Fettgewebes wurde in den letzten Jahren kontrovers diskutiert (Berthoud et al. 2006, Kreier und Buijs 2007). So konnte nach Injektion eines neuronalen Tracers in das subkutane und retroperitoneale Fettgewebe der Nucleus dorsalis nervi vagi angefärbt werden (Kreier et al. 2002, 2006). Immunhistochemisch konnten cholinerge Nervenfasern nur im braunen Fettgewebe des Mediastinums nachgewiesen werden (Giordano et al. 2004). Interessanterweise reduziert eine chirurgische Vagotomie die insulin-abhängige Aufnahme von Glukose und freien Fettsäuren in das weiße Fettgewebe (Kreier et al. 2002). Die Abgrenzung von Regulationseffekten im Pankreas oder der Leber ist auch hierbei schwierig.

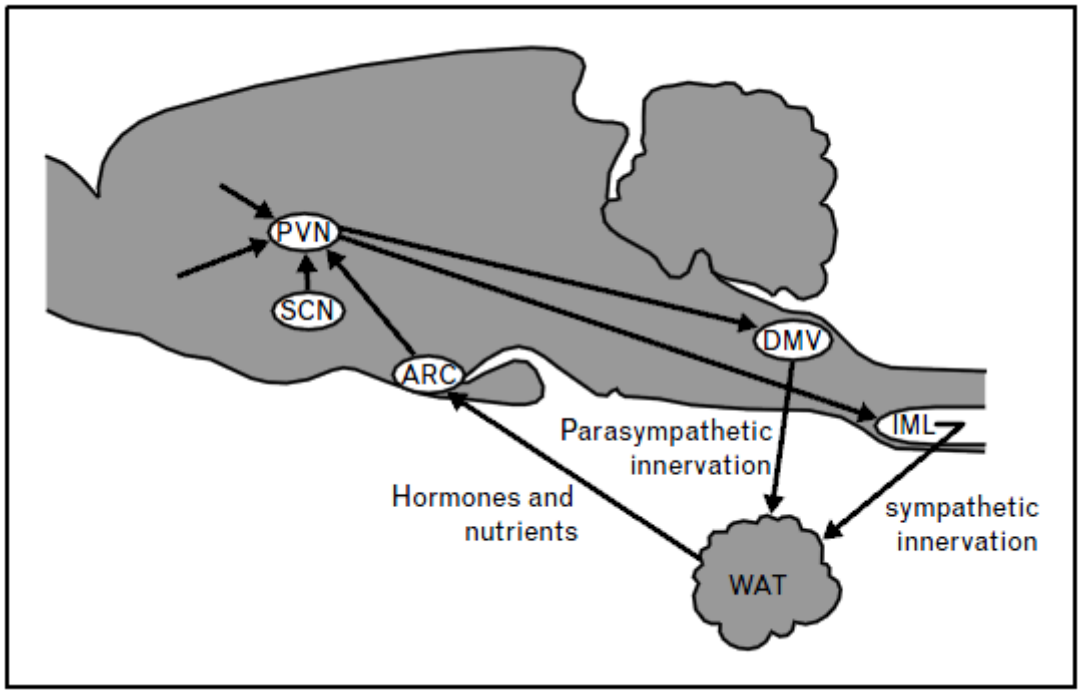

Abb. 2 Schematische Darstellung der Kommunikation zwischen Gehirn und Fettgewebe nach Fliers et al. 2003. Der Nucleus paraventricularis (PVN) im Hypothalamus erhält afferente Fasern aus dem Nucleus arcuatus (ARC), welcher durch Leptinrezeptoren über den Füllungsstand des weißen Fettgewebes (WAT) informiert wird. Die sensible afferente Innervation des weißen Fettgewebes über die Hinterstränge im Rückenmark wurde hier vernachlässigt. Der PVN sendet seinerseits efferente Projektionen in den Nucleus dorsalis nervi vagi (DMV) und anschließend über den Nervus vagus ins WAT. Weiterhin werden über die Nuclei intermediolaterales (IML) im Rückenmark die sympathischen Nervenfasern, die mit den Gefäßen ins Fettgewebe ziehen, erregt.

Bereits 1987 konnte mittels des anterograden Markers „True blue“ die Anfärbung von sensiblen Ganglienzellen aus dem Fettgewebe gezeigt und damit auf eine sensible

Innervation des Fettgewebes geschlossen werden (Fishman und Dark 1987). Immunhistochemisch lassen sich typische sensible Ko-Transmitter wie Substance P und Calcitonin Gene Related Peptide (CGRP) im Fettgewebe nachweisen (Giordano et al. 1996). Eine mögliche Funktion könnte die afferente Übermittlung des Lipidstatus an das zentrale Nervensystem sein (Bartness and Song 2007). Beispielsweise reagieren sensible Nervenfasern auf lokal sezerniertes Leptin oder Lipolyseprodukte wie Glyzerol und freie Fettsäuren (Niijima 1998, Bartness et al. 2010). Durch lokal appliziertes Capsaicin, einer neurotoxischen Substanz, welche über den TRPV1 (Transient Receptor Potential Vanilloid 1) wirkt, werden nicht myelinisierte sensible Nervenfasern selektiv zerstört (Caterina et al. 1997). Die selektive Zerstörung dieser Nervenfasern durch Capsaicin in einem speziellen Fettgewebsdepot führt kompensatorisch zu einer Vergrößerung von anderen Fettgewebsdepots im selben Tier (Shi et al. 2005). Hierdurch wird die Bedeutung der afferenten Signalübermittlung der Ernergiespeicher an das Zentralnervensystem sowie die selektive Einlagerung von Energiereserven in verschiedene Depots des Körpers deutlich.

1.3 Neuropeptide und ihr Einfluss auf das Fettgewebe

Neuropeptide sind Peptidhormone, die als Botenstoff von Neuronen produziert und freigesetzt werden. Sie werden entweder als neurosekretorische Peptidhormone in die Blutbahn sezerniert oder wirken direkt bei der synaptischen Erregung durch Nervenzellen als Kotransmitter. Solche Peptide wirken häufig nicht über schnelle Ionenkanal-gebundende Rezeptoren, sondern eher modulierend über metabotrope, G-Protein-gekoppelte Rezeptoren (Burgis 2008). Derzeit sind über 100 verschiedene Neuropeptide beschrieben. Bekannte Vertreter sind die Endorphine, Prolaktin oder Oxytocin.

1.3.1 Neuropeptide Y (NPY)

NPY ist ein 36-Aminosäure langes Peptid und gehört in die Gruppe der Pankreatischen Polypeptide mit ubiquitärem Vorkommen im Zentralnervensystem und den peripheren Organen. Im Hypothalamus spielt NPY eine wichtige Rolle bei der Leptin-abhängigen Hungerregulation. Unter Leptinwirkung wird NPY im Hypothalamus vermindert exprimiert und sezerniert (Erickson et al. 1996). In der Peripherie wird NPY bevorzugt von

sympathischen Nervenfasern produziert und weist zahlreiche Einflüsse auf Gefäßsystem und endokrine Organe auf (Zukowska et al. 2003, Kuo et al. 2007). NPY ist ein starker Vasokonstriktor, wirkt mitogen auf vaskuläre Vorläuferzellen und scheint über postsynaptische NPY-Rezeptoren eine Rolle in der Entwicklung von Atherosklerose zu spielen (Pons et al. 2004, Zukowska 2005).

Zurzeit sind fünf NPY-Rezeptoren bekannt: Y1, Y2, Y4, Y5, und Y6 (Ingenhoven und Beck-Sickinger 1999). Nach ihrer Aktivierung wird die Adenylatzyklase gehemmt und es resultiert ein verringerter intrazellulärer Spiegel an zyklischen Adenosinmonophosphat (cAMP). Für den Y1-Rezeptor sind zusätzlich Kopplungen zu anderen Second Messenger Systemen und direkter Kalziumeintrom nach Aktivierung beschrieben (Prieto et al. 2000). In primären humanen Adipozytenkulturen hemmt die Aktivierung des Y1-Rezeptors die Lipolyse (Serradeil-Le Gal et al. 2000). In 3T3-L1 Preadipozyten, einem Modell der adipogenen Differenzierung, das auch in der vorliegenden Arbeit verwendet wurde, verstärkt NPY deren Proliferation und Differenzierung (Kuo et al. 2007). Uneinigkeit herrscht in der Literatur in Bezug auf die Expression von NPY-Rezeptoren in 3T3-L1 Adipozyten. So wurde die exklusive Expression von Y1, Y2 oder Y5 beschrieben (Turtzo et al. 2001, Kuo et al. 2007, Yang et al. 2008). In vivo konnte der Zusammenhang zwischen NPY und stress-induzierter Adipositas, wie auch zwischen Überexpression von NPY in sympathischen Nervenzellen und gestörter Glukosetoleranz festgestellt werden (Kuo et al. 2007, Ruohonen et al. 2009).

1.3.2 Pituitary Adenylate Cyclase-activating Polypeptide (PACAP)

PACAP kommt ebenso wie NPY sowohl im Gehirn, als auch in peripheren Nervenfasern vor. Das Neuropeptid existiert in zwei biologisch aktiven Isoformen; eine Isoform ist 38 Aminosäuren lang (PACAP-38), während die andere Form N-terminal trunkiert ist und nur noch 27 Aminosäuren aufweist (PACAP-27; Miyata et al. 1990). In der vorliegenden Arbeit wurde ausschließlich mit PACAP-38 gearbeitet. Beide Formen von PACAP gehören der Vasoactive Intestinal Polypeptide (VIP)-Familie an, zu der auch VIP selbst gehört. Es sind derzeit drei PACAP-Rezeptoren bekannt, zwei davon weisen vergleichbare Affinitätswerte für PACAP und VIP auf. Sie werden als VPAC1 und VPAC2 bezeichnet. Ein weiterer Rezeptorsubtyp, PAC1 bindet PACAP exklusiv und hat somit eine sehr viel stärkere Affinität zu PACAP als zu VIP (Dautzenberg et al. 1999). Durch alternatives Splicing des PAC1 Genes

kommt es zu mindestens sechs Isoformen des PAC1 Rezeptors, die jeweils in der dritten intrazellulären Schleife der Peptidkette eine der drei bekannten, 28-Aminosäure langen Sequenzen HIP, HOP-1 oder HOP-2 beinhaltet. Bei der kurzen Isoform des Rezeptors ist an dieser Stelle die jeweilige Sequenz herausgeschnitten (Spengler et al. 1993). Für die vorliegende Arbeit ist vor allem interessant, dass die HOP Splicing-Variante des Rezeptors für die Kopplung an Second Messenger Systeme, wie dem Phospholipase C Signalweg, verantwortlich gemacht wird, die zu einem Kalziumeinstrom in die Zelle führt (Ushiyama et al. 2007, Mustafa et al. 2007). PACAP stellt eine große Hoffnung für die Diabetesforschung dar, da es das stärkste bekannte Insulinotropin ist und somit fast alle Insulinwirkungen verstärkt (Nakata und Yada 2007). Die intraperitoneale Gabe von PACAP verringert den Blutglukosespiegel bei Ratten und Mäusen (Yada et al. 2000), wahrscheinlich durch eine verstärkte Sekretion von Insulin aus den pankreatischen β-Zellen. Dieser Effekt konnte sowohl in vitro als auch in vivo belegt werden (Yamaguchi 2001, Jamen et al. 2002). Weiterhin interessant ist, dass PACAP die insulin-abhängige Glukoseaufnahme von primären Adipozyten und 3T3-L1 Zellen verstärkt (Nakata et al. 1999, Akesson et al. 2003). Aktivierung des VPAC2 Rezeptors in primären Adipozyten führt zu Lipolyse in den Fettzellen (Akesson et al. 2005).

1.4 Adipositas und adipositas-assoziierte Erkrankungen

Adipositas ist die krankhafte Vermehrung von Fettgewebe und ist sowohl durch Hyperplasie von adipogenen Vorläuferzellen, als auch durch Hypertrophie reifer Adipozyten bedingt. In den Staaten der westlichen Welt stellt Adipositas durch seine steigende Prävalenz von bis zu 20% in der Bevölkerung und dem Anstieg an adipositas-assoziierten Erkrankungen wie Diabetes mellitus Typ 2, Hypertonus, Atherosklerose, koronarer Herzkrankheit, Leberverfettung und verschiedenen Arten von Krebs ein gewaltiges Problem dar (Blüher 2009). Diese Problematik ist seit über 2500 Jahren bekannt, denn schon Hippokrates schrieb: „Korpulenz ist nicht nur selbst eine Krankheit, sondern auch ein Vorbote für andere!“ (Percik und Stumvoll 2009). Derzeit ist die Lebenserwartung von adipösen Patienten um ca. fünf Jahre verringert und die hohe Prävalenz in der Gesellschaft könnte zum ersten Mal in der jüngeren Geschichte zu einer Reduktion der Lebenserwartung insgesamt führen (Olshansky et al. 2005, van Baal et al. 2008).

Adipositas ist mit einer gestörten Insulinsensitivität und Glukosetoleranz assoziiert. Adipöse Patienten haben ein neunfach erhöhtes Risiko, an Diabetes mellitus zu erkranken (Weinstein et al. 2004). Die Pathogenese ist hierbei multifaktoriell und hängt sowohl vom Nahrungsverhalten, einer gestörter Adipokinsekretion und genetischen Faktoren ab.

Auf zellulärer Ebene konnte gezeigt werden, dass ein erhöhter globaler intrazellulärer Kalziumspiegel zu Resistenz gegenüber Insulinaktivierung führt und somit zu einer reduzierten Glukoseaufnahme von Adipozyten (Draznin et al. 1987, Kennedy et al. 2010). Andererseits ist Kalzium essentiell für eine funktionelle Antwort der Adipozyten auf Insulin, wie für die Translokation von Glukosetransportern und zur Glukoseaufnahme (Whitehead et al. 2001). Frühe Schritte in der Insulinsignalkaskade wie Insulin Receptor Substrate 1 Phosphorylierung werden durch Kalzium gehemmt, wobei spätere Schritte eher gefördert werden (Worrall und Olefsky 2002). Draznin und Kollegen konnten bereits 1987 nachweisen, dass es einen Optimalbereich für den Gehalt an intrazellulärem Kalzium in Bezug auf die Glukoseaufnahme von Adipozyten gibt (Draznin et al. 1987).

2 Fragestellung

Aus diesen Vorbefunden ergaben sich für uns verschiedene Fragestellungen:

1. Welche Rezeptoren für NPY bzw. PACAP exprimieren die 3T3-L1 Zellen und verändert sich das Expressionsmuster im Laufe der Adipogenese?
2. Sind diese Rezeptoren an Second Messenger Systeme gekoppelt, die zu einem Anstieg des intrazellulären Kalziumsspiegels führen?
3. Verändert sich durch die Ko-Stimulation von NPY mit Insulin die Insulinsensitivität von 3T3-L1 Adipozyten?

Diese Fragen wurden an dem Zellkulturmodell der 3T3-L1 Adipozyten untersucht. Methodisch kamen dabei die Polymerase Kettenreaktion, Western Blot Analysen von ganzen Zellen als auch von isolierten Plasmamembranen, histologische und Immunfluoreszenzfärbungen, Messungen der Glukoseaufnahme reifer Adipozyten wie auch live Imaging Experimente mittels eines Calcium Imaging Sytems zum Einsatz.

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4. Publikationen

4.1 Receptors for NPY and PACAP differ in expression and activity during adipogenesis of the murine 3T3 - L1 fibroblast cell line

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Running title: NPY and PACAP receptors in 3T3-L1 cells

No conflict of interest

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Abstract

Background and purpose: Neuropeptides are involved in the regulation of food-intake in the central nervous system, but they might also act on fat tissue *via* neuropeptide receptors.

Experimental approach: We investigated the receptor expression and activity of the pituitary adenylate cyclase-activating polypeptide (PACAP) and of the neuropeptide Y (NPY) in the 3T3-L1 fibroblast line during adipocyte differentiation at the mRNA and protein level. Intracellular calcium rise was measured by calcium imaging.

Key results: The PACAP-receptors PAC1 (PACAP-1) and VPAC2 (VIP-R2) as well as the NPY-1-receptor (Y1) were expressed at the mRNA level in fibroblasts, preadipocytes and adipocytes. The mRNA profile of the PAC1-isoforms showed the HOP sequence, whereas the HIP isoform was present in subconfluent 3T3-L1 fibroblasts only. At the protein level, the mature 3T3-L1 adipocytes produced the PAC1 and Y1 receptors. The mature adipocytes displayed an intracellular calcium rise in response to both neuropeptides, which was absent in the precursor cells. The findings were validated by specific agonist and antagonist treatment and declared as Y1- and PAC1-mediated.

Conclusions and implications: Considering that the PAC1-HOP variant seems to be responsible for PACAP-mediated calcium influx in many cell types, the HOP sequence might cause the intracellular calcium rise. Because a high calcium level is associated with lipogenesis, the peptidergic innervation of adipose tissue might be involved in stress-induced obesity.

Keywords*:* NPY, PACAP, neuropeptide receptor, adipogenesis, adipose tissue, peptidergic innervation

List of abbreviations

BIBP 3226	(*R*)-N^{α}-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226
C/EBP	CCAAT/enhancer binding protein family
CRB	cAMP-response-element
DMEM	Dulbecco`s modified Eagle medium
DRG	dorsal root ganglia
FCS	fetal calf serum
FURA2 AM	FURA2 acetoxymethyl ester
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IBMX	methylisobutylxanthine
NPY	neuropeptide Y
PAC1	PACAP type 1 receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate buffered saline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PNGase F	peptide: N-glycosidase F
PPAR-γ	peroxisome proliferator-activated receptor-γ
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
UΔR	arbitrary units of the delta ratio
VPAC1/2	$VIP_{1/2}$ / PACAP receptor
WGA-beads	wheat germ agglutinin-linked agarose beads
Y1-6	NPY-(1-6) receptor

Introduction

Adipocytes are the characteristic cells of adipose tissue. They undergo lipogenesis and lipolysis for the storage and release of energy to meet the needs of the body. Adipocytes are also endocrine cells due to the secretion of adipokines, which are highly influential on the immune system, blood vessels, and insulin sensitivity (Waki *et al*., 2007; Wang *et al*., 2008). Adipocyte-dependent physiological functions become pathophysiological effects when adipocytes develop excessively; it is a risk factor that may lead to diseases of the heart and circulatory system, such as diabetes and cancer (Visscher *et al*., 2001). Studies on adipose tissue have given insight into key transcription factors (CCAAT/enhancer binding protein family and peroxisome proliferator-activated receptor-γ) involved in adipocyte differentiation (Gregoire *et al*.,1998), the expression and function of new adipokines (Waki *et al*., 2007; Wang et al., 2008), and cross-talk with other tissues, such as nerves (Turtzo *et al*., 2001; Kosacka *et al*., 2006). Signals affecting adipocyte differentiation and function are currently of interest. According to a recent study, high extracellular Ca^{2+} levels impair adipogenesis by inhibiting the expression of key adipogenic genes in 3T3-L1 adipocytes (Jensen *et al*., 2004). Neuropeptide Y (NPY) and the pituitary adenylate cyclase-activating polypeptide (PACAP) act directly, though separately, on adipocytes; NPY has anti-lipolytic functions (Serradeil-Le Gal *et al*., 2000) and PACAP is lipogenic by potentiating insulin-dependent glucose uptake (Nakata *et al*., 1999) and lipolytic in the absence of insulin (Akesson *et al*., 2003; 2005). Both neuropeptides might be partners in the regulation of energy utilization because NPY-positive neurons in the hypothalamic arcuate nucleus display an intracellular calcium increase under the influence of PACAP (Nakata *et al*., 2004).

Neuropeptide Y is a 36-amino acid neuropeptide that belongs to the pancreatic polypeptide family with an ubiquitous occurrence in the brain and peripheral organs. Hypothalamic NPY increases appetite signaling in a leptin-dependent manner. Peripheral NPY, which is preferentially found in sympathetic fibers, has pleiotropic functions on the vascular and endocrine systems (Zukowska *et al*., 2003; Kuo *et al*., 2007). Neuropeptide Y acts as a vasoconstrictor and a vascular mitogen, which appears to be involved in the development of atherosclerosis via particular postsynaptic NPY receptors (Pons *et al*., 2004; Zukowska *et al*., 2005). Five NPY receptors have been classified: Y1, Y2, Y4, Y5, and Y6 (Ingenhoven and Beck-Sickinger, 1999). Their activation results in adenylate cyclase inhibition and a decrease in cAMP *via* pertussis toxin-sensitive G-proteins. The Y1 receptor can also couple to other

second messenger systems in control of direct Ca^{2+} influx (Prieto *et al*., 2000). In primary human adipocyte cultures, activation of the Y1 subtype inhibits lipolysis (Serradeil-Le Gal *et al.*, 2000). The anti-lipolytic effect may additionally be mediated by a Y2-dependent process, as suggested by the finding that abdominal obesity increases in mice in the context of Y2 up-regulation (Kuo *et al*., 2007). For 3T3-L1 preadipocytes, the NPY-activated proliferation and adipogenic differentiation was reported without further study of the NPY receptor profile (Kuo *et al*., 2007).

Similar to NPY, PACAP also occurs in the brain and peripheral nerve fibers. PACAP exists in two biologically active forms, the 38-amino acid residue long PACAP and the N-terminally truncated PACAP-27 (Miyata *et al*., 1990). Both PACAP isoforms belong to the vasoactive intestinal polypeptide (VIP) family. Two PACAP receptors with similar affinity for VIP and PACAP are VPAC1 and VPAC2, whereas the PAC1 receptor prefers PACAP exclusively (Dautzenberg *et al*., 1999). By alternative splicing of the PAC1 gene, six isoforms are generated with or without the inclusion of three 28 amino acid sequences/cassettes (HIP, HOP-1, and/ or HOP-2) in the third intracellular loop; the short form includes no cassettes (Spengler *et al*., 1993). The HOP splicing variant appears to be responsible for an intracellular Ca^{2+} increase and neurotransmitter release in chromaffin cells by coupling to the phospholipase C pathway (Ushiyama *et al*., 2007; Mustafa *et al*., 2007). The PACAP molecule could be beneficial in the treatment of diabetes type II, since this neuropeptide is one of the most effective insulinotropins known (Nakata and Yada, 2007). Intraperitoneal PACAP administration decreases blood glucose levels in rats and mice (Yada *et al*., 2000), probably by enhancing insulin secretion in pancreatic islets, which PACAP has been shown to accomplish *in vitro* as well as *in vivo* (Yamaguchi *et al*., 2001; Jamen *et al*., 2002). Furthermore, PACAP potentiates insulin-guided glucose-uptake in 3T3-L1 adipocytes and primary adipocytes (Nakata *et al*., 1999; Akesson *et al*., 2003). However, this lipogenic/anabolic effect turns into a lipolytic/catabolic process in the absence of insulin. It should be noted that the VPAC2 subtype is responsible for insulin-independent lipolysis resulting from PACAP application in primary adipocyte cultures (Akesson *et al*., 2005). It is not known whether the VPAC2 subtype is also present in 3T3-L1 adipocytes.

We recently shown that neurons from postnatal dorsal root ganglia considerably improve neurite outgrowth when co-cultured with 3T3-L1 adipocytes, and they even form synaptic contacts (Kosacka *et al*., 2005). Thus, the main aim of the current study was to clarify the

PACAP and NPY receptor mRNA profiles and activities during 3T3-L1 adipogenesis using a pharmacological approach in order to obtain new insights into signal transduction elicited in these cells by the two neuropeptides.

Methods

Cultures of 3T3-L1 fibroblasts, preadipocytes, and adipocytes

Mouse 3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco`s modified Eagle medium (DMEM) with 25 mM glucose (DMEM-H) and 10% fetal calf serum (FCS; all from Sigma, Deisenhofen, Germany). The cells were either grown as fibroblasts to subconfluence within 3 days or as preadipocytes to confluence within 5-6 days. On day 2 of confluence, preadipocytes were differentiated into adipocytes by DMEM supplemented with 1 µM insulin, 0.4 µg/ml dexamethasone, and 0.5 mM methylisobutylxanthine (IBMX). Three days later, the medium was switched to DMEM containing 1 µM insulin for 3 days. The daily medium replacement turned 95% of the preadipocytes into mature adipocytes as evident from accumulated fat droplets. The three different cell types were either plated onto round glass coverslips mounted into 24-well culture plates or small-sized Petri-dishes for morphological study and calcium imaging analysis, or plated into plastic flasks for Western blotting or PCR analysis.

Nile red staining

Cell cultures were rinsed with 0.1 M phosphate buffered saline (PBS) and fixed with 2% PBS-buffered formaldehyde containing 0.2% Triton X-100 and 5% sucrose at 37°C for 5 min. After a thorough PBS rinse (3 times, 5 min each), the cells were further permeabilized with 60% isopropanol solution in PBS (5 min) and incubated with 1:100 buffer diluted Nile red stock solution (20 µg ml^{-1} in acetone; ICN Biomedicals Inc., Aurora, Ohio, USA) for 5 min. Cultures were washed in PBS 3 times and the cover slips inverted and finally mounted on object slides with Glycergel® (Dako, Hamburg, Germany).

RT-PCR analysis

For receptor nomenclature, guidelines were followed as laid down by Alexander *et al*. (2008). Cultures developed at 250 ml flasks were scraped off in the stage of interest. The cells were homogenized in peqGold RNA-Pure^{TM} (PEQLAB Biotechnology, Erlangen, Germany), the RNA was deproteinized by phenol-chloroform and precipitated at 75% alcohol. The reverse transcription reaction was performed in a total volume of 20 µl and utilized 5 µg of RNA, 500 ng oligo $(dT)_{15}$ primers (Promega, Mannheim, Germany), 4 µl first strand buffer (Invitrogen, Karlsruhe, Germany), 2 µl 0.1M dithiothreitol, 1 µl of a dNTP-mix (10 mM each), 1 µl RNaseOUT (Invitrogen), and 200 units of SuperScript^{TM} II Reverse Transcriptase (Invitrogen). PCR reactions were performed in a total volume of 25 µl, using 1 µl of cDNA, 0.2 µM forward and reverse primers, 2.5 µl PCR-buffer (10x; Roche, Mannheim, Germany), 2 µl dNTP mix, and 2.5 U Taq DNA-Polymerase (Roche). Initial denaturation at 95°C for 5 min was followed by 35 cycles (25 cycles for the housekeeping gene GAPDH) of denaturing at 95°C for 30 s, annealing at either 55°C (PAC1, HIP, and HOP primers) or 60°C (Y1, Y5, VPAC1, and VPAC2) for 30 s, and extension at 72°C for 30 s. A final extension at 72°C for 10 min and subsequent cooling terminated the reaction. PCR products were electrophoresed on 1% agarose gel containing ethidium bromide and visualized by UV transillumination. To identify the PAC1, we used primers described by Jamen *et al.* (2002) that flank the HIP-HOP region, or the forward primer located in either the HIP or the HOP splicing variants for distinguishing isoforms. The VPAC1 and 2 primers were taken from Rawlings *et al*. (1995). Other primer sets and PCR conditions were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). The primer pairs are shown in table 1. Murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments were amplified with specific primers and served as an internal standard. Amplified cDNA fragments were cloned into pGEM-T (Promega). Inserts of the expected size were sequenced at the local core unit for DNA technology and identified by comparisons with GenBank sequences (BLAST search).

Table 1 Primers and details of the PCR analysis

Receptor	**mRNA Accession # at GenBank**	**Primers**
PAC1	NM_007407	*forward:* 5'-CAT CCT TGT GCA GAA GCT GC-3' *reverse:* 5'-GGTGCT TGA AGT CCA TAG TG-3'
HIP-cassette	NM_007407	*forward:* 5'-ACA AAT TTA AGA CTG AGA GT-3' *reverse:* 5'-GGT GCT TGA AGT CCA TAG TG-3'
HOP-cassette	NM_007407	*forward:* 5'-TCC ACC ATT ACT CTA CGG CT-3' *reverse:* 5'-GGT GCT TGA AGT CCA TAG TG-3'
VPAC1	NM_011703	*forward:* 5'-GGC CCC ATC CTC ATC TCC AT-3' *reverse:* 5'-CCG CCT GCA CCT CAC CAT TG-3'
VPAC2	NM_009511	*forward:* 5'-ATG GAC AGC AAC TCG CCT CTC TTT AG-3' *reverse:* 5'-GAA GGA ACC AAC ACA TAA CTC AAA CAG-3'
Y1	NM_010934	*forward:* 5'-TGA TTC GCT TGG TCT CAC TG-3' *reverse:* 5'-GTC CTT GCA GTG GCT TCT TC-3'
Y2	NM_008731	*forward:* 5'-CCA TCT TCC GGG AAT AC-3' *reverse:* 5'-CTG AGG AAC CAC GTC A-3'
Y5	NM_016708	*forward:* 5'-AGG CAG TGT TCC GAG CAG-3' *reverse:* 5'-AGA AGC GAC CGC ACT CAG-3'
GAPDH	XM_983502	*forward:* 5'-ATG GTG AAG GTC GGT GTG A-3' *reverse:* 5'-GGA AGC CCA TCA CCA TCT T-3'

Western blotting

For immunoblot analysis, cell cultures were lysed by ultrasonication in 60 mM Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 10% sucrose. The samples were then diluted 1:1 in sample buffer (250 mM TRIS-HCl at pH 6.8 and containing 4% SDS, 10% glycerin, and 2% β-mercaptoethanol) and denatured at 70°C for 10 min. Protein concentration was assessed using the BCA™ protein assay (Pierbo Science, Bonn, Germany). Proteins (50 µg whole cell amount or 1 µg of purified glycoproteins) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Nonspecific binding sites were blocked by preincubation with 5% nonfat milk for 60 min. The blots were incubated with polyclonal anti-PAC1 antiserum (diluted 1:5,000; obtained from Dr. S. Schulz; described in Schulz *et al*., 2004) or polyclonal anti-Y1 antiserum (1:2,000; Alpha Diagnostics, San Antonio, TX, USA) at 4°C overnight. Immunoreactions were detected with the appropriate peroxidase-conjugated anti-rabbit IgG secondary antibody (1:10,000; Vector Laboratories, Peterborough, UK) at room temperature (RT) for 2 h. Peroxidase activity was visualized with an enhanced chemiluminescence kit (Amersham, Pharmacia, Freiburg, Germany). In addition, blots were stripped and incubated with an anti-GAPDH monoclonal antibody (diluted 1:3,000, Research Diagnostics, Flanders, Netherlands) followed by the anti-mouse IgG secondary antibody (1:4,000; Vector Laboratories) to verify equal protein loading. To check specificity, antibodies were either preincubated with the corresponding blocking peptide (same source as the specific antibody) overnight at 4°C, or the protein extracts were deglycosylated with peptide N-glycosidase F (PNGase F; New England BioLabs, Beverly, MA, USA) between 1-4 h. We followed the manufacturer's protocol and additionally added 1µl of protease inhibitor cocktail (Sigma) to prevent degradation by endogenous proteases. Dorsal root ganglia (DRG) cells isolated from 3-day-old rat pups according to the protocol of Kosacka et al. (2005) were used as a positive control. Additionally, we performed a glycoprotein purification from the whole cell protein using wheat germ agglutinin-linked agarose beads (WGA-beads; Sigma). Separation of the glycoprotein fraction was performed as previously described (Schulz *et al*., 2004).

Measurement of intracellular calcium levels by calcium imaging

3T3-L1 cells were loaded with 10 µM FURA 2 acetoxymethyl ester (FURA2-AM; TEF Labs, Austin, Texas, USA) and 0.0125% Pluronic ® (TEF Labs) in 1 ml of standard Ringer-solution (125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 7.5 mM glucose; adjusted to pH 7.4 with NaOH) at 37°C for 30 min in the dark. The coverslips were placed in a superfusion chamber and continuously perfused at RT at a rate of 2 ml min^{-1}. Solutions were removed by a vacuum pump. After an initial 3 min rinsing, cells were superfused with either PACAP38, NPY (both AnaSpec Inc., San Jose, CA, USA), or specific agonists (Table 2) at a concentration of 100 nM for 2 min. Blocking experiments were performed in a 500 nM solution of the specific antagonist, including the specific Y1-antagonist BIBP 3226 (Sigma) and the specific PAC1-antagonist PACAP(6-38) (American Peptide Company, Sunnyvale, CA, USA). After a 5 min preincubation with the antagonist, the cells were superfused with a solution containing 500 nM of the antagonist and 100 nM of the agonist for another 2 min. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 µM) was used as a positive control.

Experiments were performed on a Zeiss Axiovert 135 microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with an Axiovert 135 UV transparent optic (Carl Zeiss). Dye-excitation illumination was provided by a dual-wavelength illuminator system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) consisting of a xenon arc lamp, variable speed reflective optic chopper, and two monochromators, both under computer control. The excitation wavelengths were 340 and 380 nm. Emitted fluorescence was filtered at 510 nm by a photomultiplier tube and a photon-counting photometer. Changes in the intracellular calcium level were expressed as arbitrary units of the delta ratio (UΔR) of dye fluorescence at 340 and 380 nm. Fluorescence intensities for both excitation wavelengths were acquired in 2 s intervals. Calcium measurements were performed using objective magnification of 20x on areas with 10 to 20 fibroblasts, 30 to 50 preadipocytes, or 20 to 30 mature adipocytes, respectively.

Table 2 Agonists and antagonists of neuropeptide Y and PACAP receptors

Reagent	Function	Reference	Source
NPY	Agonist for all NPY receptors		AnaSpec Inc., San Jose, CA, USA
[Phe(7),Pro(34)] porcine NPY (pNPY)	Agonist for Y1	Söll *et al*., 2001 Höfliger *et al*, 2003 Lecklin *et al*., 2003	Prof. Beck-Sickinger, Leipzig, Germany
BIBP 3226	Antagonist for Y1	Rudolf *et al*., 1994	SIGMA
Ahx[5-24] pNPY	Agonist for Y2	El Bahh *et al.,* 2002 Höfliger *et al*, 2003	Prof. Beck-Sickinger
[hPP1-17,A31, Aib32] pNPY	Agonist for Y5	Cabrele *et al.,* 2000 Höfliger *et al*, 2003 Lecklin *et al*., 2003	Prof. Beck-Sickinger
PACAP	Agonist for all PACAP receptors		AnaSpec Inc., San Jose, CA, USA
VIP	Agonist for VPAC1 & VPAC2	DeHaven and Cuevas, 2004	BioTrend Chemicals AG, Zurich, Switzerland
PACAP(6-38)	Antagonist for PAC1	Bergström *et al*., 2003	American Peptide Company Inc., Sunnyvale, CA, USA
[K15,R16,L27] VIP(1-7)/GRF (8-27)	Agonist for VPAC1	Gourlet *et al*., 1997	Prof. Gregoire, Brussel, Belgium

$PGF_{2\alpha}$	Positive control	Nakada *et al*., 1990 Miller *et al*., 1996	SIGMA

Statistical analysis

Data is presented as mean ± SEM of at least three independent experiments. The data from calcium imaging was quantified using Sigma Plot software and evaluated by the multiple comparison Holm-Sidak test (SigmaStat Software, Jandel Scientific, San Rafael, CA).

Results

Characterization of 3T3-L1 fibroblasts, preadipocytes, and adipocytes

Using Nile red staining, 3T3-L1 fibroblasts exhibited typical fibroblast-like morphology during subconfluence (Figure 1A). Due to the contact inhibition, cell growth stopped at the confluence (Gregoire, 2001). At this stage, we termed these cells preadipocytes (Figure 1B). The terminology concerning subconfluent 3T3-L1 cells is currently not precise. Also, the conversion of the 3T3-L1 fibroblast to the preadipocyte phenotype is insufficiently understood. Nevertheless, in the differentiation medium used, adipocytes developed over the course of the following seven days. The cells converted from a fibroblast-like to a spherical shape and increased in size with an abundant lipid droplet accumulation (Figure 1C). All cell stages maintained their typical morphology during the loading with FURA2-AM for the calcium imaging experiments (Figs. 1D-F).

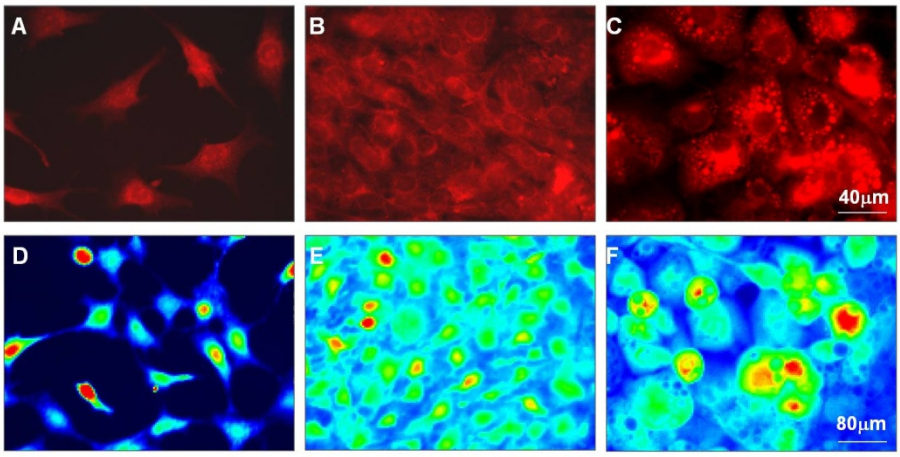

Figure 1 - Characterization of 3T3-L1 fibroblasts, preadipocytes, and adipocytes by staining. (A-C) Nile red staining of fibroblasts (A), preadipocytes (B), and adipocytes (C). (D-F) For comparison, the cell types are depicted by FURA2-AM loading. On culture day 3, 3T3-cells exhibited a fibroblast-like subconfluent morphology (A, D). On day 6, confluent preadipocytes display no lipid droplets (B), whereas adipocytes exhibit lipid droplets as signs of maturation (C). Scale bars represent 40 µm at a magnification of 40x (A-C) or 80 µm at a magnification of 20x (D-F).

Presence of the NPY and PACAP receptor mRNA during 3T3-L1 adipogenesis

In fibroblasts, preadipocytes, and mature adipocytes, the mRNA encoding NPY and PACAP receptor subtypes was detected by RT-PCR analysis. Because 3T3-L1 cells were derived from mice, whole mouse brain RNA was used as a positive control. Amplification of whole mouse brain RNA with primer pairs specific for Y1-R produced a band with the expected length of 295 bp (Figure 2A, left). Strong bands of the same length were found in fibroblasts, preadipocytes, and mature adipocytes (Figure 2B). To verify the presence of Y2-R and Y5-R mRNA, primers were used that had generated clear amplification products of the expected length in the mouse brain mRNA extract: 201 bp for Y2-R and 217 bp for Y5-R (Figure 2A, left). Also in the mouse brain RNA, the Y5-R primers produced an additional band around 300 bp, which had been found previously in the hypothalamus as a long isoform of Y5-R (Rodriguez *et al*., 2003). In contrast to the Y1 receptor, transcripts of the Y2 and Y5 receptors were absent from the three cell types (Figure 2B).

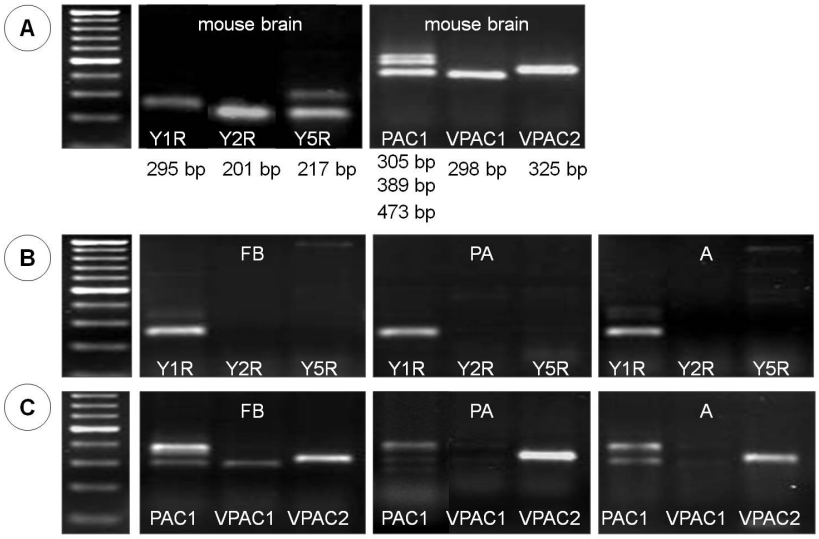

Figure 2 - The mRNA profile of receptors for neuropeptide Y and PACAP at different stages of 3T3-L1 cell adipogenesis as determined by RT-PCR. (A) Whole mouse brain RNA was used to validate the expected sizes of the receptor products. (B) Neuropeptide Y receptor Y1 was present in each stage of adipogenesis, whereas Y2 and Y5 were absent in 3T3-L1 cell extracts. (C) The PACAP receptors PAC1 and VPAC2 were detected in the three cell types of adipogenesis. The VPAC1 appeared only in fibroblasts. FB - fibroblasts; PA - preadipocytes; A - mature adipocytes

To detect the PAC1-mRNA, primers able to differentiate between a possible insertion of either one HIP or HOP splicing variants (386 or 389 bp), both cassettes (473 bp), or no cassette for the short receptor variant (305 bp) were used. The primers revealed two amplification products in the mouse brain mRNA, 305 and 390 bp (Figure 2A, right). These two bands indicated the presence of the short form of the PAC1-receptor and a variant with an insertion of one 28-amino acid cassette. The upper band was consistently seen throughout all adipogenic types, whereas the lower band was absent in preadipocytes (Figure 2C). The VPAC1 primers produced transcripts of 298 bp only in 3T3 fibroblasts and no products in preadipocytes and adipocytes. Primers specific for the VPAC2 generated strong bands corresponding to 325 bp that were consistently present throughout 3T3-L1 adipogenesis, which was similar to the expression of PAC1 (Figure 2C). All products seen in mature adipocytes were cloned and successfully sequenced. They were at least 99% identical with the GenBank sequences.

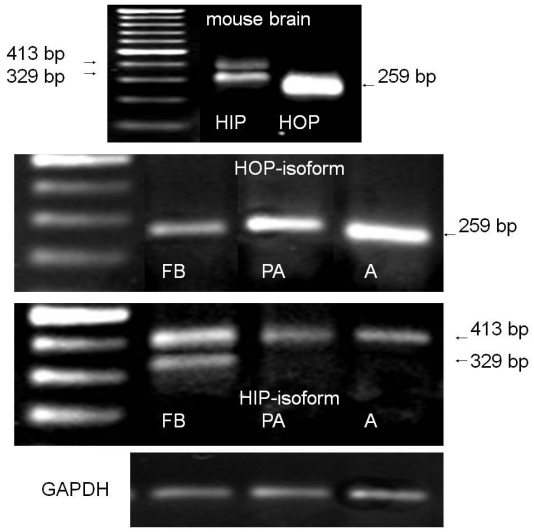

Figure 3 - RT-PCR products of mouse brain mRNA for the PAC1 variants. *Upper row:* Amplicons of 329 bp and 413 bp corresponding to the HIP-isoform of PAC1 (left lane) and 259 bp band generated when using HOP-specific primers. *Second row*: Primers for HOP-cDNA produced strong amplicons with the expected size of 259 bp in fibroblasts (FB), preadipocytes (PA), and mature adipocytes (A). *Third row*: A 413 bp band appeared in each cell stage of adipogenesis. The negative outcome of sequencing determined them to be unspecific. The HIP-cassette of 329 bp, which was only verified in 3T3-L1 fibroblasts, was confirmed by sequencing. *Lower row*: The GAPDH band indicates equal sample loading.

The PAC1 mRNA isoforms

To examine the predominant isoforms of the PAC1 receptor, we used mouse brain RNA to validate the success of our RT-PCR procedures. The transcript containing the HOP sequence resulted in the expected band with a length of 259 bp (Figure 3, upper row). Amplification products resulting from the primers containing the HOP sequence revealed a 259 bp band in fibroblasts, preadipocytes, and adipocytes (Figure 3, second row). The HOP-amplicons were also confirmed by sequencing; they were 100% identical with the GenBank sequences. Amplification of the transcripts produced two bands for the HIP-isoform, one of the expected size of 329 bp and another of 413 bp. The latter represented an unspecific amplification according to the sequencing outcome. The 413 bp transcript likely occurred as co-product during the partial reverse transcription and may not arise in the full-length transcription. The lower band of 329 bp was only found in fibroblasts and was identified successfully by sequencing (99% identity) as HIP-related. This indicated that the HIP-isoform of the PAC1-receptor was present only in 3T3-L1 fibroblasts (Figure 3, third row). GAPDH-amplification revealed equal sampling (Figure 3, lower row).

Presence of Y1 and of PAC1 proteins in mature adipocytes

Investigating the lysates of mature adipocytes for the presence of the Y1 protein by Western blotting a expected 45 kDa protein was found. Considering that both receptors as G-protein coupled receptors had putative N-glycosylation sides, deglycosylation with PNGase F treatment was performed. After enzymatic digestion up to 4 h immunodensity did not change. Specifity of the immunoband was assumed because the application of the preabsorbed antibody led to the nearly disappearance 45 kDa band. All findings were also validated with DRG cell cultures, used as positive control and displayed equal results. Further, the immunoglycoprotein fraction showed no Y1 protein-related band (not shown). Western blot analysis of cell lysates for PAC1 protein revealed two bands with 55 and 50 kDa. The 55 kDa band disappeared, whereas the 50 kDa protein remained after PNGase F treatment for 2 h (Figure 4B, left). Thus, the 55 kDa band represented the glycosylated form. In the DRG cell cultures this band convincingly increased in intensity (not shown). Blocking the PAC1-antibody with the specific immunopeptide caused a disappearance and decrease of the 55 kDa and the 50 kDa immunoband, respectively (Figure 4B, middle). The glycoprotein fraction contained only one strong band at 55 kDa (Figure 4B, right).

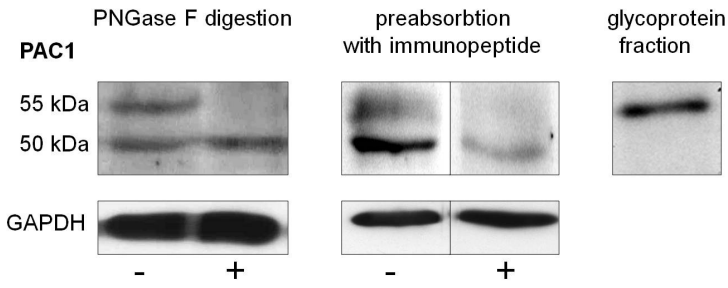

Figure 4 - Western blot analysis using the PAC1 antibody (1:5,000), two distinct bands with 50 kDa and 55 kDa were detected. Enzymatic deglycosylation led to the disappearance of the 55 kDa band (left). After blocking the antibody with the specific immunopeptide, the blot lacked the 55 kDa band, and the 50 kDa band seemed to diminish (middle).The glycoprotein fraction by separation of glycoproteins with wheat-germ agglutinin linked beads showed only the 55 kDa band (right).

NPY- and PACAP-mediated intracellular calcium increase in adipocytes

Pilot experiments for a positive control were performed with 30 or 60 mM KCl or 100 mM ATP. Neither solution affected intracellular calcium levels (data not shown). Prostaglandin $F_{2\alpha}$, which is known to be responsible for calcium mobilization in 3T3-L1 preadipocytes but not in fibroblasts and mature adipocytes (Nakada *et al*., 1990; Miller *et al*., 1996), was then successfully used as a positive control; stimulation with 1µM $PGF_{2\alpha}$ resulted in a transient rise of intracellular calcium levels in all stages of 3T3-adipogenesis. In comparison, we observed a notably lower calcium peak after preincubation with PACAP.

The extent of the calcium response was also examined in the presence of 100 nM NPY, which was applied for 2 min. Fibroblasts exhibited a moderate calcium increase, whereas confluent preadipocytes did not respond (Figure 5A, left and middle graph). In mature

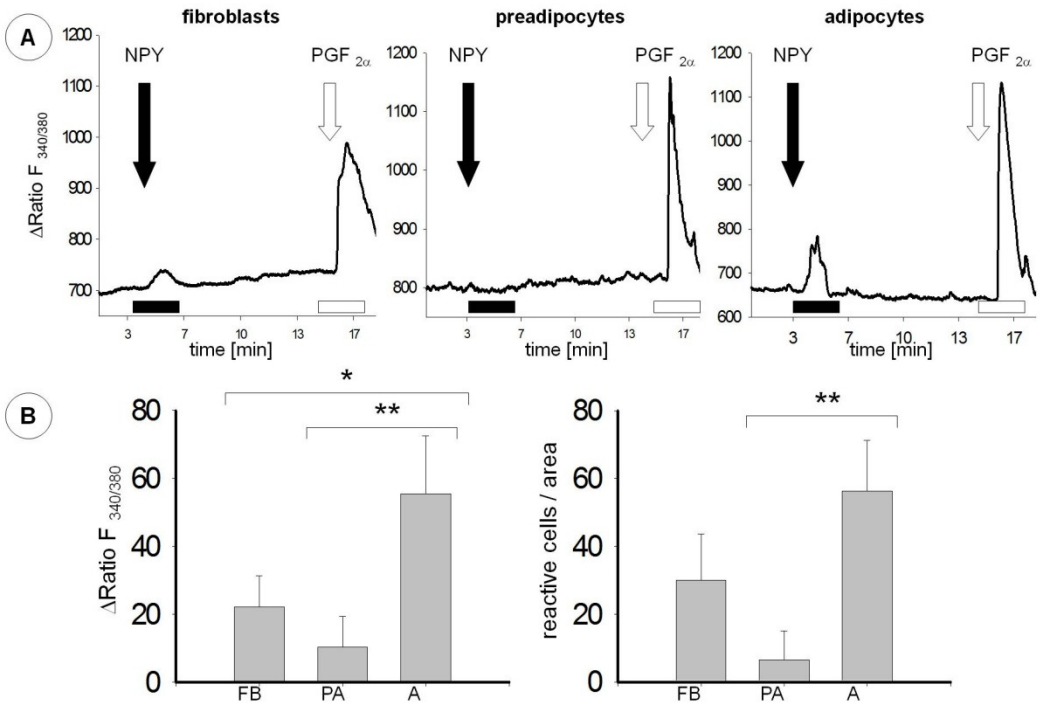

Figure 5 - Calcium response of 3T3-L1 cells under neuropeptide Y or prostaglandin stimulation. (A) Low calcium responses were seen in subconfluent cultures of fibroblasts (left) and preadipocytes (middle) and moderate calcium mobilization was seen in subconfluent cultures of mature adipocytes (right) after stimulation with 100 nM NPY for 2 min. Stimulation with 1 µM $PGF_{2\alpha}$ was used to check the viability of cells after a 10 min buffer wash to remove neuropeptides. (B) Measuring the extension of the calcium peak (left) and the amount of reactive cells per defined area (right) revealed statistically significant differences between adipocytes and preadipocytes and between fibroblasts and adipocytes. Data from three independent experiments is represented as mean ± SEM. FB - fibroblasts; PA - preadipocytes; A - mature adipocytes. * $p<0.05$¸ ** $p<0.01$

adipocytes, NPY stimulation led to a conspicuous calcium peak (Figure 5A, right graph). By measuring the peak extension for the intracellular calcium increase, a significantly higher response to NPY was noted for mature adipocytes (55.5 ± 9.9 UΔR) compared to 22.2 ± 5.3 UΔR in fibroblasts ($p < 0.05$) and 10.4 ± 5.2 UΔR in preadipocytes ($p < 0.01$; Figure 5B, left graph). The statistically significant difference was confirmed by the multiple comparison Holm-Sidak test. Comparing the number of reactive cells per area, mature adipocytes were more responsive (56.3% ± 8.6) than preadipocytes (6.4% ± 5.0; $p < 0.01$). Fibroblast cultures with nearly 30% ± 7.9 reacting cells lacked a statistically significant difference from preadipocytes and adipocytes (Figure 5B, right panel).

Stimulation with a 100 nM solution of PACAP led to a moderate calcium response in mature adipocytes measuring 27.4 ± 5.0 UΔR (Figure 6A, right graph). This PACAP-mediated response was significantly greater than the preadipocyte response (3.2 ± 0.8 UΔR; $p < 0.01$), but not significantly different from the response in fibroblasts (12.8 ± 3.8 UΔR; Figure 6B, left graph). This was supported by much more reacting cells in the adipocyte cultures (47.2% ± 5.0) compared to fibroblasts or preadipocytes in which 7.6% ± 0.5 and 5.9% ± 2.4 cells responded (Figure 6B, right graph).

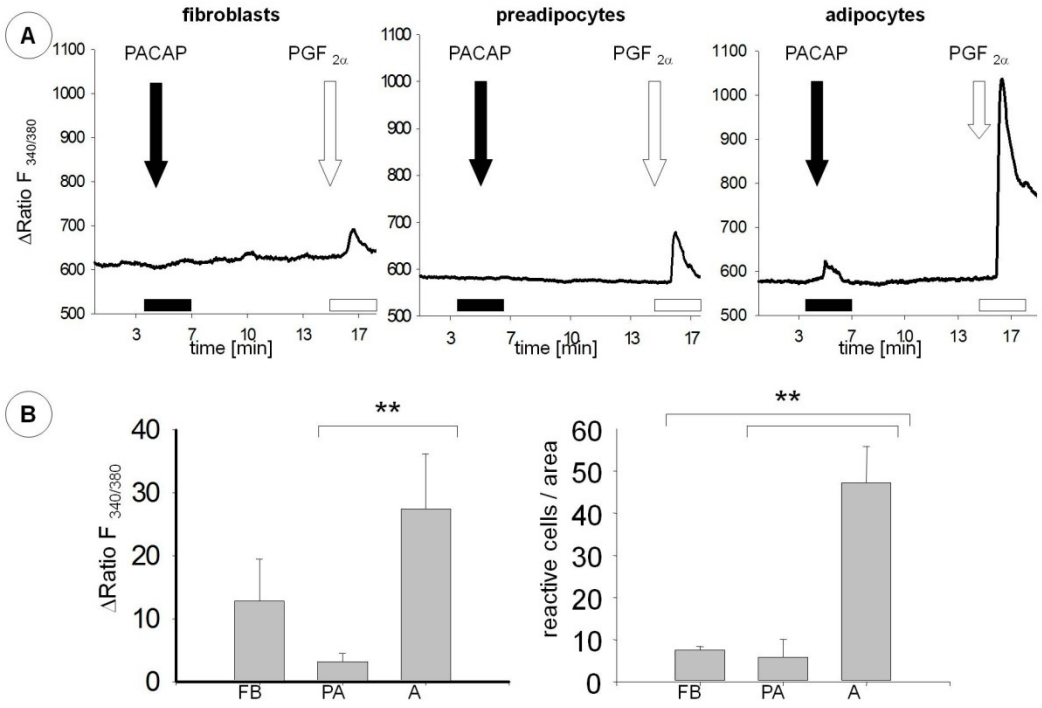

Figure 6 – Calcium response of 3T3-L1 cells under PACAP or prostaglandin stimulation. (A) Fibroblasts (left), preadipocytes (middle), and mature adipocytes (right) were stimulated with 100 nM PACAP for 2 min. The treatment resulted in an increase of cell calcium only in mature adipocytes. The cells were treated with 1 µM $PGF_{2\alpha}$ as a positive control after a 10 min buffer wash. (B) Statistically significant differences between adipocytes and preadipocytes were detected by measuring the extent of the calcium elevation. In addition, significantly more adipocytes were responsive compared to subconfluent fibroblasts and preadipocytes. Data from three independent experiments is represented as mean ± SEM. FB - fibroblasts; PA - preadipocytes; A - mature adipocytes. * $p<0.05$¸ ** $p<0.01$

NPY-triggered calcium peak as the Y1R-mediated pathway in adipocytes

To obtain pharmacological evidence of NPY-mediated calcium mobilization, we used specific agonists for the Y1, Y2, and Y5 NPY receptors, verified at the mRNA level (see above). The value of calcium mobilization by 100 nM NPY (55.7 ± 9.9 UΔR) was defined as 100%. Equal molar doses of the Y1 receptor-agonist [Phe(7),Pro(34)] pNPY imitated the NPY-induced calcium elevation and was not notably different (100% vs. 89.1%; Figure 7A). On the other hand, neither the Y2R-agonist Ahx[5-24] pNPY nor the Y5R-agonist [hPP1-17,A31,Aib32] pNPY led to comparable responses (6.0% and 4.7%, respectively; Figure 7A). Differences between the NPY and Y1R-agonist responses and the Y2R or Y5R agonists were highly significant ($p < 0.01$ for all). For blocking experiments with the Y1R-antagonist, BIBP 3226, we removed the first NPY dose with a 15-min buffer wash. The second NPY application resulted in a less effective, but distinct, intracellular calcium elevation (62.9%; Figure 7A). Preincubation of 500 nM BIBP 3226 for 5 min with an additional co-application of the Y1R-antagonist during the final 2 min completely blocked the NPY-induced calcium elevation in mature adipocytes (4.9%), compared to the unblocked second NPY-mediated calcium peak ($p < 0.01$; Figs. 7A and B).

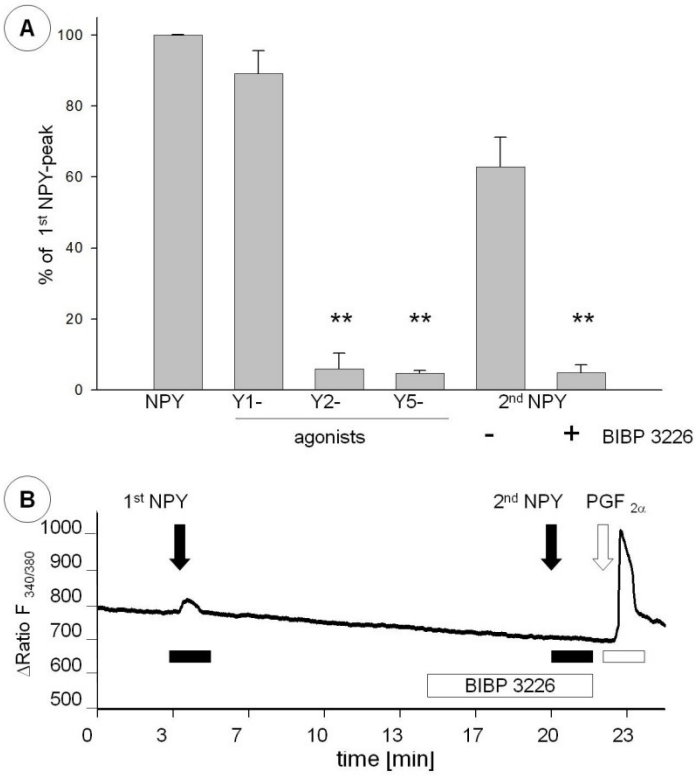

Figure 7 - The neuropeptide Y-mediated calcium peak in mature adipocytes depends on its Y1 receptor. (A) An equal dose of the Y1R agonist [Phe(7),Pro(34)] pNPY mimicked the effects of NPY, whereas the Y2R agonist Ahx[5-24] pNPY and Y5R agonist [hPP1-17,A31,Aib32] pNPY did not influence calcium levels, as revealed by a measurement of the calcium peak extension. Additionally, the NPY-triggered peak was reproducible 15 min after the 1st NPY application. (B) The 2nd NPY-mediated calcium increase was blocked completely by the Y1R-antagonist BIBP 3226 in mature adipocytes. Treatment with $PGF_{2\alpha}$ was used as a positive control. Data from three independent experiments is represented as mean ± SEM. FB - fibroblasts; PA - preadipocytes; A - mature adipocytes. *p<0.05¸ ** p<0.01

PAC1 is implicated in the PACAP-induced calcium elevation in adipocytes

To ascertain the receptors involved in PACAP-dependent intracellular calcium elevation in mature adipocytes, we used VIP, which binds to the VPAC1 and VPAC2 with an affinity similar to PACAP. Stimulation with 100 nM VIP resulted in no comparable changes in intracellular calcium (21.7% of the first PACAP-peak; Figs. 8A and B). Additionally, application of the VPAC1-agonist [K15,R16,L27]VIP / GRF(8-27) did not affect intracellular calcium (20.6%), whereas the 100 nM PACAP application resulted in the expected calcium mobilization (31.7 ± 3.3 UΔR: further defined as 100%; Figure 8A). Differences between PACAP, VIP, and the VPAC1 agonist were significant (p < 0.01).

A 15 min buffer wash was used to eliminate the first PACAP before applying a second dose of PACAP between 15 to 60 min. The calcium responses (21.7% and less) were far below that of the first PACAP dose. Because of the lack of a second PACAP-triggered calcium increase, we had to inhibit the first PACAP-mediated calcium peak. After 5 min of preincubation with 500 nM PACAP(6-38), a specific PAC1-antagonist, PACAP was added to 100 nM. This cotreatment with the antagonist severely reduced the normal calcium mobilization to 5.9% (1.7 ± 0.4 UΔR; $p < 0.01$; Figs. 8A and C).

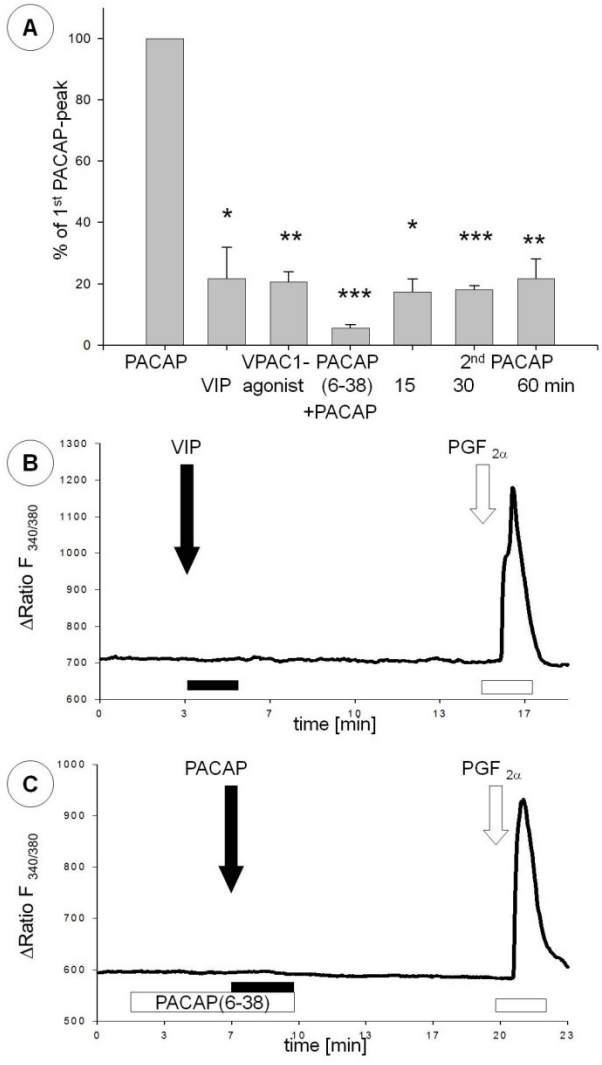

Figure 8 – The PAC1-receptor dependence on the PACAP-induced calcium mobilization. (A, B) The PACAP-mediated calcium increase was not mimicked by VIP (binds to VPAC1 and 2) or the VPAC1 agonist [K15,R16,L27]VIP/GRF(8-27). Notably, the 1st PACAP-mediated intracellular calcium increase was not reproducible with a 2nd PACAP application after a 60 min buffer wash to remove neuropeptides. (C) The PACAP-mediated calcium increase was completely abolished by preincubation with the PAC1-specific antagonist PACAP(6-38) . $PGF_{2\alpha}$ was used as a positive control. Data from three independent experiments is presented as mean ± SEM. FB - fibroblasts; PA - preadipocytes; A - mature adipocytes. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Discussion and conclusions

This study presents a comprehensive analysis of the NYP and PACAP receptor mRNA during the differentiation of 3T3-L1 fibroblasts into mature adipocytes. This new knowledge is helpful for future research on *in vitro* adipocyte function. In addition, the receptor activity was pharmacologically examined in the presence of 100 nM NPY/PACAP to observe the calcium profiles of FURA2-AM loaded 3T3-L1 cell types. Although the dose was beyond physiological serum levels, it might be close to the local concentration of NPY and PACAP at the postsynaptic site *in vivo*. Evidence is also provided by selective agonist and antagonist treatments that the Y1 receptor and PAC1 receptor are coupled to G_q-type subunits in mature adipocytes and being engaged in phospholipase effectors. Yet the G-proteins are absent in their precursor cells, because fibroblasts and preadipocytes also expressed the receptor mRNA, but did not exhibit an intracellular Ca^{2+} increase upon ligand exposure.

In respect to the PAC1 protein, an unglycosylated and glycosylated form was here found in mature adipocytes. Binding and activity of specific agonists is improved by the glycosylated receptors (Rengifo *et al*., 2007). Thus, the glycosylated PAC1 receptor might be more active in mature adipocytes than the unglycosylated one. The presently observed absence of intracellular calcium rise after re-stimulation with PACAP points to desensitization of the PACAP receptors as is reported for chromaffin cells and for cortex slices (Taupenot *et al.*, 1999; Niewiadomski *et al*., 2002). In contrast to PAC1, no glycosylation was detected for the Y1 protein.

In addition, intra- and extracellular calcium changes strongly affect the energy homeostasis and dysregulation may result in obesity. In preadipocytes, high extra- and intracellular calcium levels attenuate proliferation and differentiation (Miller *et al*., 1996; Ntambi *et al*., 1996; Shi *et al*., 2000). Our data support recent findings that NPY stimulates preadipocyte proliferation in the absence of a NPY-mediated Ca^{2+} increase (Kuo *et al*., 2007). This finding is recently described as an Y1-mediated effect (Yang *et al*., 2008). On the other hand, in 3T3-L1 adipocytes, NPY application leads to lipid accumulation and cell differentiation. In the present study we observed Ca^{2+} elevating effects through NPY stimulation more for mature adipocytes than for fibroblasts and preadipocytes. There is consensus that the intracellular calcium pools are modified via phospholipase C and that Y1-dependent signaling transduces

phospholipase C activation in myocytes (Heredia Mdel *et al.*, 2005). Notably, an intracellular Ca^{2+} increase promotes triglyceride accumulation and lipid storage in mature adipocytes by upregulating fatty acid synthase, a key enzyme of *de novo* lipogenesis (Kim *et al*., 1996), and by elevated glucose uptake via insulin-responsive glucose transporter translocation (Whitehead *et al*., 2001; Worrall and Olefsky, 2002). Considering the pathophysiological relevance for humans, long-term exposure of adipocytes to PACAP and NPY might result in obesity, which actually has been shown in mice under stress-induced NPY upregulation (Kuo *et al*., 2007).

A discrepancy between mRNA expression and the function of NPY receptors seems to exist between *in vitro* and *in vivo* studies. According to the literature, primary human adipocytes derived from abdominal adipose tissue express three NPY receptors at the mRNA level: Y1, Y2, and Y5. However, the binding profile of selective radioactive ligands is only typical for the Y1, which has an anti-lipolytic effect (Serradeil-Le Gal *et al*., 2000). We demonstrated that the Y1 gene is expressed in all stages of the adipogenesis of 3T3-L1 cells, but it was associated with an intracellular Ca^{2+} increase only in NPY-stimulated adipocytes.

PACAP-dependent signaling occurs via two principal routes (Alexander et *al*., 2008). In the $G_{s\alpha}$-linked transduction, there is the activation of the receptor and of the adenylate cyclase system. With increase of cAMP and thus without significant influence on the intracellular calcium pools the protein kinase A is stimulated, which activates the hormone-sensitive lipase. This appears to be the pathway for PACAP-stimulated insulin-independent lipolysis in primary rat adipocytes derived from epididymal fat pads. It is exclusively mediated by the VPAC2 (Akesson *et al*., 2005). Interestingly, when rat pancreatic islets are stimulated by PACAP, insulin is released by adenylate cyclase signaling, which is coupled to the PAC1 and VPAC1 receptors (Jamen *et al*., 2002). The insulinotropic activity of PAC1 has also been documented for PAC1 gene-deficient mice with impaired glucose tolerance (Jamen *et al*., 2000). In the current study, the absence of calcium mobilization is found for the HIP isoform of PAC1 as well as for VPAC2.

In the $G_{q/12}$-linked transduction, the HOP isoform of the PAC1 receptor appears to interact with the G_q-α subunit, which induces a phospholipase C-dependent inositol triphosphate

(IP_3) elevation with an intracellular Ca^{2+} increase (Mustafa *et al*., 2007). Also produced is diacylglycerol, which triggers different isoforms of protein kinase C. This pathway is associated with a lipogenetic effect in 3T3-L1 adipocytes (Nakata *et al*., 1999; Akesson *et al*., 2003). Yet in the present study, repeated stimulation with PACAP consistently failed to increase the accumulation of lipid droplets.

Though only the VPAC2 receptor is expressed in pancreas and skeletal muscle, all three PACAP receptor types are present in the human heart and adipose tissue (Wei and Mojsov, 1996). In the present study, 3T3-L1 preadipocytes and adipocytes expressed PAC1 in addition to VPAC2. All three receptor types were expressed only in 3T3-L1 fibroblasts. This finding could indicate a low amount of the VPAC2 mRNA not being functionally significant, or reflect a minor contribution of $G_{s\alpha}$-linked cAMP generating activity of the PACAP receptors. We assume that all three receptors are required to induce the transcription of specific genes via increased levels of cAMP, which is known to bind to the cAMP-response-element (CRB) in specific promoters. The cAMP-dependent increase of CAAT/enhancer binding protein (C/EBP)-β and C/EBP-δ in the early stages of adipocyte conversion induces an elevated expression of C/EBP-α, which is, together with the peroxisome proliferator-activated receptor Y, one of the most clearly identified transcription factors of adipogenesis (Gregoire *et al*.1998; Wu *et al*., 1996). Notably, in astrocyte cultures, PACAP induces the C/EBP transcription factors, including C/EBP-β and C/EBP-δ (Cardinaux and Magistretti, 1996). In sympathetic neurons, which express the PAC1- HOP, the PACAP-dependent transcripts are related to peptide plasticity and nerve regeneration (Braas *et al*., 2007). Furthermore, preadipocytes from breast adipose tissue transiently increase prolactin expression and function *via* cAMP activating ligands (McFarland-Mancini *et al*., 2006). We are presently studying the production and release of neurotropic and angiogenic factors by 3T3-L1 cells as related to the treatment with NPY and PACAP.

In conclusion, the 3T3-L1 adipocyte cell line expresses the Y1 receptors, the PAC1-HOP isoform, and VPAC2 in fibroblasts, preadipocytes, and mature adipocytes. However, only mature adipocytes exhibit an intracellular Ca^{2+} increase, which could result from activation of both Y1 and PAC1-HOP receptors.

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Conflict of interest

The authors state no conflict of interest.

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4.2 Neuropeptide Y impaires insulin-stimulated translocation of glucose transporter 4 in 3T3-L1 adipocytes through the Y1 receptor

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Running title: **NPY impaires GLUT4 translocation**

No conflict of interest

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Abstract

Adipose tissue metabolism is regulated by the sympathetic system. Its physiological influence can become a pathophysiological dimension in case of obesity, insulin resistance and type 2 diabetes mellitus (T2DM) might develop. We here focused on neuropeptide Y (NPY), a sympathetic co-transmitter, and its influence on insulin-stimulated translocation of the glucose transporter 4 (GLUT4) from intracellular stores to the cell surface. We analysed separated fractions of plasma membranes of 3T3-L1 adipocytes for the GLUT4 content, as well as in cell cultures by immunofluorescence. The phosphorylation of the Akt protein and [^{3}H]-deoxyglucose uptake were also investigated for the effect of NPY on insulin action in adipocyte cultures. NPY inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes in a GLUT4-dependent manner. This inhibition was mediated by the Y1 receptor, because the insulin-guided GLUT4 translocation was attenuated by the Y1-receptor antagonist BIBP 3226. Further, NPY impaired Akt phosphorylation dose-dependently.

Because chronic stress leads to increased NPY release of sympathetic nerve fibers in the adipose tissue, the inhibition of insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes by NPY could contribute to the influence of the sympathetic system in developing peripheral insulin resistance and T2DM.

Keywords*:* NPY, neuropeptide Y, Y-1 receptor, insulin resistance, 3T3-L1

adipocytes, adipose tissue, peptidergic innervation

Abbreviations

BIBP 3226, (*R*)-N^{α}-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226; DMEM, Dulbecco`s modified Eagle medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, glucose transporter 4; IBMX, methylisobutylxanthine; NPY, neuropeptide Y; PBS, phosphate buffered Saline; SDS, sodium dodecyl sulfate; T2DM, type 2 diabetes mellitus; Y1-6, NPY-(1-6) receptor

1. Introduction

Adipocytes store and release energy to meet the need of the body. Obesity, the excessive accumulation of adipocytes, is a major risk factor for diabetes mellitus, artherosclerosis, hypertension, coronary heart disease and cancer. (Visscher et al., 2001).

Novel knowledge on the regulation of adipocyte endocrinology might modify the treatment of the peripheral insulin resistance occurring in type 2 diabetes mellitus (T2DM). Beside hormonal input via the blood stream, afferent and efferent nerve fibers affect adipocytes as convincingly shown for the sympathetic and sensory system (Bartness et al., 2010). Especially the sympathetic nervous system impacts on peripheral glucose utilization and insulin sensitivity (Nonogaki, 2000; Flaa et al., 2008). However, human obesity seems to be linked to sympathetic overactivity (Seematter et al*.,* 2000).

Neuropeptides can act as co-transmitters like the pituitary adenylate cyclase-activating polypeptide (PACAP) in parasympathetic fibers, substance P (SP) in sensory fibers or Neuropeptide Y (NPY) in sympathetic fibers. Co-transmitters can influence insulin sensitivity of adipocytes, as it was shown for PACAP and SP, respectively (Nakata et al., 1999; Karagiannides, 2008). The effect of NPY on glucose metabolism in adipocytes is unknown.

Neuropeptide Y is a 36-amino acid neuropeptide and a member of the pancreatic polypeptide family with an ubiquitous occurrence in the brain and peripheral organs. Presently, five NPY receptors have been cloned: Y1, Y2, Y4, Y5, and Y6 (Ingenhoven and Beck-Sickinger, 1999). Their activation results in adenylate cyclase inhibition with subsequent decrease of cyclic adenosin monophosphate (cAMP) levels or coupling to other second messenger systems like calcium influx, preferentially through the Y1 receptor (Gericke et al., 2009). In addition, Y1 activation results in a reduced lipolysis in human adipocyte cultures (Serradeil-Le Gal et al., 2000). Hypothalamic NPY acts orexigenic by increasing food intake (Chee and Colmers, 2008). Peripherally, NPY stimulates proliferation of adipocyte precursor cells as well as preadipocyte differentiation *in vitro* (Kuo et al., 2007; Yang et al., 2008). Interestingly, chronic stress leads to increased levels of NPY with subsequent development of obesity, hyperinsulinemia and impaired glucose tolerance. The metabolic syndrome-like conditions can be prevented by inhibition of Y2 (Kuo et al., 2007).

We hypothesized that the *in vivo* data indicate a potential impact of NPY on peripheral

insulin resistance and T2DM. For this reason, we focused on the neuromodulatory influence of NPY on insulin-stimulated translocation of glucose transporters 4 (GLUT4) from cytosolic stores to the cell surface as well as glucose uptake. We found, that NPY impaires insulin sensitivity of 3T3-L1 adipocytes by a reduced translocation rate of GLUT4 to cell surface.

2. Methods

2.1. Cultures of 3T3-L1 adipocytes

Mouse 3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were either plated on round glass coverslips mounted into 24-well-culture plates for immunofluorescence study, into 12-well-plates for deoxyglucose uptake measurement or into 6-well-culture-plates for cell fractioning with subsequent Western blot analysis. Maintenance and differentiation were performed in Dulbecco`s modified Eagle medium (DMEM; Invitrogen GmbH, Darmstadt, Germany) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) as described previously (Gericke et al., 2009). Cultures were used between days 10 to 15 after induction of adipogenesis, depending on maturity. Mature cells were checked for insulin sensitivity as an internal standart during the experimental procedures.

2.2. Experimental procedure and preparation of subcellular fractions

One day prior to the experiment, mature adipocytes were serum starved overnight by changing medium to DMEM with 0.25 % FCS. Next day and 2 h prior to the experiment, cells were switched to serum-free DMEM. To translocate GLUT4 from intracellular stores to the cell surface adipocytes were stimulated with 10 nM of NPY, 10 nM of the specific Y1-agonist [Phe(7),Pro(34)] pNPY (kindly provided by Prof. A. Beck-Sickinger, Leipzig, Germany) and/or insulin (0.1 nM, 1 nM, 10 nM), diluted in warmed-up DMEM for 15 min at 37 °C. Blocking experiments were performed with preincubation (15 min) and co-application of 500 nM Y1-antagonist BIBP 3226 (Sigma). After a 15 min preincubation with the antagonist, the cells were stimulated with 500 nM of the antagonist and 10 nM NPY for another 15 min. Then,

adipocytes were rinsed with ice-cold phosphate buffered saline (PBS) to stop reaction. To investigate insulin signaling, cells were stimulated for 5 min.

Separation of membranes and cytosol was performed by a protocol modified from Nishiumi et al. (2007). Briefly, cells were collected in buffer A (50 mmol*l^{-1} TRIS, 0.5 mmol*l^{-1} dithiothreitol, adjusted to pH 8.0 and 1 % PMSF, 10 mM sodium orthovanadate and 1 % Sigma protease inhibitor cocktail freshly added) supplemented with 0.1 % NP-40 (New England BioLabs, Beverly, MA, USA). Cells kept on ice and were homogenized with a Dounce Homogenisator (Schütt HOMOGEN; Schütt Labortechnik GmbH; Göttingen, Germany) with continuous up and down movements at 300 rpm for 2 min. Nuclear fractions were pelleted by spinning suspensions at 2,000 rpm for 10 min. Pellets were discarded. Supernatants were collected and centrifuged at 13,000 rpm for 20 min. The pellet was washed once again with buffer A without NP-40 and the final membrane pellets were obtained at 13,000 rpm for 20 min, resuspended in 30 µl buffer A supplemented with 1.0 % NP-40 and incubated for 1 h to solubilize membrane proteins. Afterwards, lysate was centrifuged at 13,000 rpm for 30 min.

For chronic NPY exposure, cells were stimulated with either 10 or 100 nM NPY every second day for 14 days, starting from the day of adipogenesis induction. Whole cell protein was collected in 60 mM Tris-HCl buffer, pH 6.8, containing 2 % sodium dodecyl sulfate (SDS) and 10 % sucrose with subsequent ultrasonication.

2.3. Western blotting

Western blot analysis was performed as described earlier (Gericke *et al.* 2009). Blots were incubated with polyclonal antisera against GLUT4 (diluted 1:10,000; Abcam, Cambridge, UK), phospho-Akt (1:2000; Biosource, Camarillo, CA, USA) or pan-Akt (1:1000; Cell Signaling, Boston, MA, USA) at 4 °C overnight. Immunoreactions were detected with the appropriate peroxidase-conjugated anti-rabbit IgG secondary antibody (1:20,000 for GLUT4 detection and 1:5,000 for phospho-specific antibodies; Vector Laboratories, Peterborough, UK) at room temperature for 2 h. Peroxidase activity was visualized with an enhanced chemiluminescence kit (Amersham, Pharmacia, Freiburg, Germany). In addition, blots were stripped and incubated with anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (diluted 1:100,000; Research Diagnostics, Flanders, Netherlands)

followed by the anti-mouse IgG secondary antibody (1:10,000; Vector Laboratories) to check purity. Semiquantitative evaluation of arbitrary unit was performed with the gel analyzer software (Medio Cypernetics, Bethesda, MD, USA).

2.4. Immunofluorescence

3T3-L1 adipocyte cultures on glass coverslips were rinsed with PBS and fixed with 2 % formaldehyde in 0.1 M PBS containing 0.2 % Triton X-100 and 5 % sucrose at 37 °C for 15 min. For blocking unspecific binding sides, samples were incubated with 10 % normal goat serum (DakoCytomation, Hamburg, Germany) for 1 h and subsequently with GLUT4 antiserum (1:250; Abcam) at 4 °C overnight. Next day after buffer rinse, CY3-conjugated donkey anti-rabbit IgG (Dianova, Hamburg, Germany) were applied at a 1: 1,000 dilution at RT for another hour. Coverslips were mounted with Glycergel (DakoCytomation) containing 10 $\mu g*mg^{-1}$ DAPI (Serva, Heidelberg, Germany) for nuclear staining and 25 $\mu g*ml^{-1}$ DABCO (Sigma) to prevent photobleaching. Digitized images were taken by confocal microscopy using an LSM 510 Meta (Zeiss, Jena, Germany).

2.5. Glucose uptake by determination of [^{3}H]-deoxyglucose incorporation

Basal and stimulated glucose uptake was studied in differentiated monolayers. They had been starved as described above and were subsequently incubated with 10 nM insulin and/or 10 nM NPY in KRH buffer (20 nM HEPES, 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, adjusted to pH 7.4) at 37 °C, for 30 min. Afterwards, [^{3}H] –deoxyglucose was added at a final concentration of 0.5 $\mu Ci*ml^{-1}$ for another 4 min. The stop solution contained 100 mM 2-deoxyglucose (Sigma, St. Louis, MO, USA). After two buffer rinses with ice-cold PBS, adipocytes were scraped off in 0.1% SDS. The incorporated radioactivity per well was determined by a liquid scintillation counter.

2.6. Statistical analysis

Data presented as means ± SEM of at least three independent experiments evaluated by the Student-Newman-Keuls method for multiple comparison (SigmaStat Software, Jandel Scientific, San Rafael, CA, USA).

3. Results

3.1. Influence of NPY on insulin-stimulated GLUT4-translocation

Mature adipocytes were stimulated with various concentrations of insulin to verify translocation of GLUT4 to the plasma membranes. Further, the effect of NPY on the insulin-stimulated GLUT4 translocation was investigated.

Insulin increased the GLUT4 protein content in adipocytes membranes dose-dependently (0.1 - 10 nM) and the effects of 1 nM and 10 nM were significant ($p<0.05$ and $p<0.001$, respectively). NPY counteracted the effect of 10 nM insulin by 50% significantly ($p<0.05$, all Fig.1). Taken together, co-stimulation of NPY attenuated the insulin-stimulated GLUT4 increase in the cell membranes.

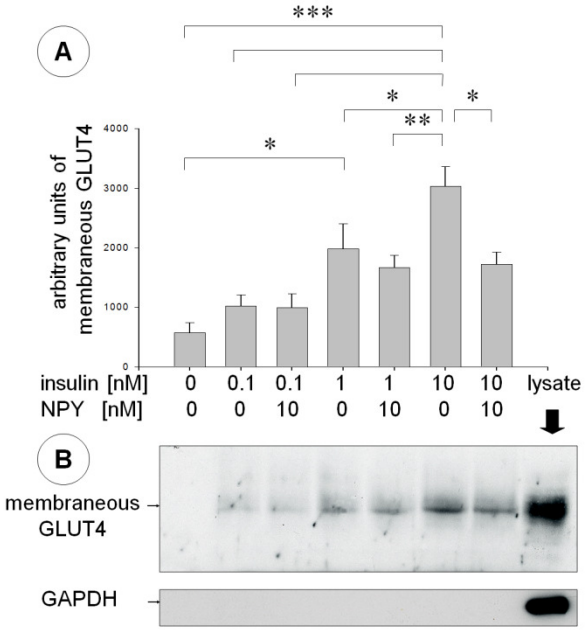

Figure 1 Mature 3T3-L1 adipocyte cultures were incubated with different concentrations of insulin with our without 10 nM NPY for 15 min. (A) Semiquantitative analysis of GLUT4 in isolated membranes reveals less GLUT4 upon stimulation with insulin together with NPY than without. This indicated an inhibition of the insulin-induced GLUT4 translocation by NPY. (B) A representative Western blot is shown for GLUT4 detection in isolated membranes. The GAPDH control exhibits high purity of the membrane fraction. Whole cell lysate was used as positive control. Data from four independent experiments are represented as means ± SEM and checked for statistical significance by the Student-Newman-Keuls multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$

3.2. NPY-mediated inhibition of GLUT4 translocation acts through Y1 receptors

Next, the Y1 was studied as potential receptor to reduce insulin-stimulated GLUT4 translocation to the cell surface upon NPY co-application, because Y1 is the only NPY receptor expressed in our 3T3-L1 cells as shown earlier (Gericke et al., 2009). Thus, we investigated the response to the specific Y1-agonist [Phe(7),Pro(34)] pNPY. The co-application of insulin and the Y1-agonist led to an approximately 40 % reduced GLUT4 content in the plasma membranes compared to insulin treatment only ($p < 0.05$). NPY decreased the GLUT4 content equally (Fig. 2, left).

In addition, the inhibitory impact of NPY on insulin-stimulated GLUT4 increase in adipocyte membranes could be blocked significantly by the specific Y1-receptor inhibitor BIBP 3226 (Fig. 2, right).

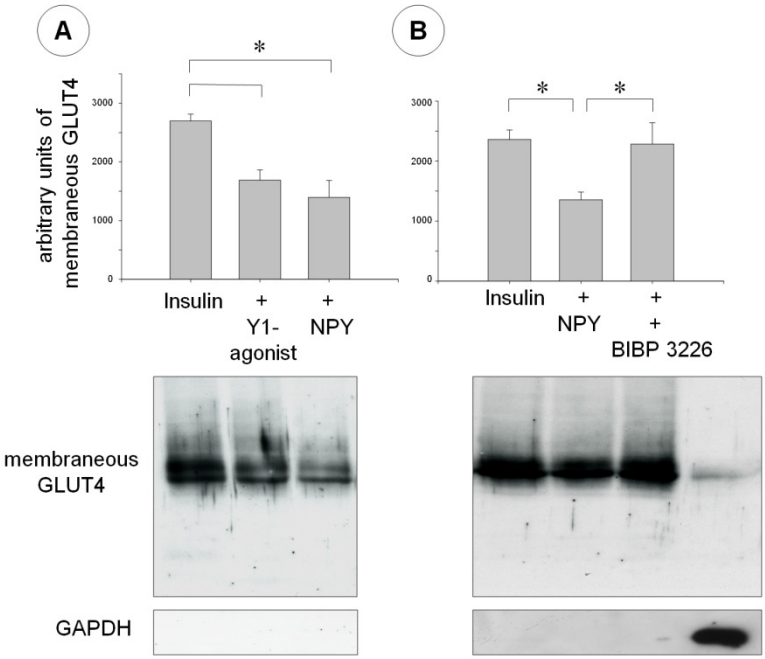

Figure 2 Mature 3T3-L1 adipocyte cultures were stimulated with insulin and the effect of either the specific Y1-agonist [Phe(7),Pro(34)] pNPY or NPY on GLUT4 translocation was tested (A). Both of them reduce insulin-stimulated GLUT4 translocation significantly. Pretreatment and co-application with the Y1-blocker BIBP 3226 nullifies the inhibitory influence of NPY (B). GAPDH control reveales high purity of the membrane isolation. Data from three independent experiments each are means ± SEM and checked for statistical significance by the Student-Newman-Keuls multiple comparison test. * $p \leq 0.05$

3.3. Immunofluorescence staining reveals the inhibitory effect of NPY on insulin-stimulated GLUT4 translocation

3T3-L1 adipocytes without additional stimulation revealed GLUT4-positive vesicles with predominant perinuclear localization (red arrow) and no staining of the cell edges (white arrow) as a sign of unstimulated adipocytes (Fig. 3; left panel). Insulin treatment of 3T3-L1 cells appeared to shift the GLUT4 protein from the perinuclear compartment to all over the cytoplasm and the cell edges (Fig. 3; red arrow, second panel). After co-application of insulin and NPY, GLUT4 protein was exclusively detected in perinuclear location (red arrow) again. Cell edges were not stained, suggesting an inhibitory effect of NPY on GLUT4 spread (Fig.3; white arrow, third panel). Inhibition of the Y1-signaling by the Y1-antagonist BIBP 3226 led to a diffuse staining of the whole cell with detectable GLUT4 at the cell edges, similar to cultures treated with insulin only (Fig.3; red arrow, fourth panel).

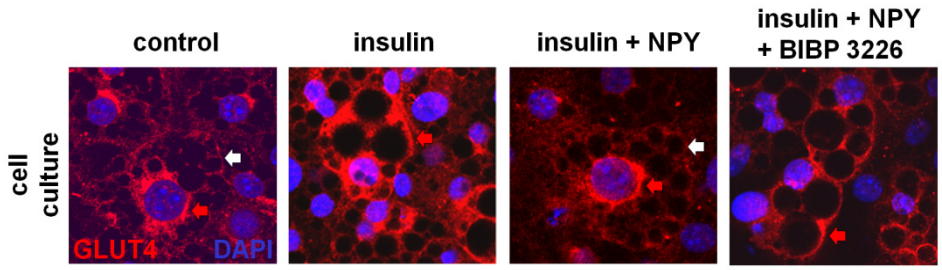

Figure 3 Mature 3T3-L1 adipocyte cultures were differently stimulated with 10 nM insulin, 10 nM NPY and 500 nM Y1 antagonist BIBP 3226 and GLUT4 immunofluorescence staining was conducted for the adipocyte cultures. Control adipocytes show strong perinuclear immunofluorescence (red arrow) compared to the cell edges (white arrow), indicating GLUT4 vesicles in the perinuclear compartment (first panel). Insulin treatment leads to a diffuse immunostaining of the cytoplasm and the cell edges (red arrow, second panel). NPY co-application reveals detectable GLUT4 in the perinuclear compartment only (red arrow), with no staining of the cell edges (white arrow, third panel). The application of BIBP 3226 generates a diffuse response and GLUT4-positive cell edges (red arrow, fourth panel) similar to the insulin treatment. All pictures made by the confocal microscopy.

3.4. NPY attenuates insulin-stimulated GLUT4 translocation in an Akt-dependent manner

Phosphorylation of the Akt protein seems to be an important step in insulin signaling after insulin receptor activation (Choi and Kim, 2010). Thus, we wanted to investigate Akt phosphorylation in 3T3-L1 cells. Incubation of 3T3-L1 adipocytes with insulin showed the expected Akt phosphorylation. NPY reduced insulin-stimulated Akt phosphorylation significantly and this inhibition was sensitive to pretreatment with the Y1-blocker BIBP 3226 (Fig. 4A). Further, the inhibitory effect of NPY was dose-dependent (Fig. 4B).

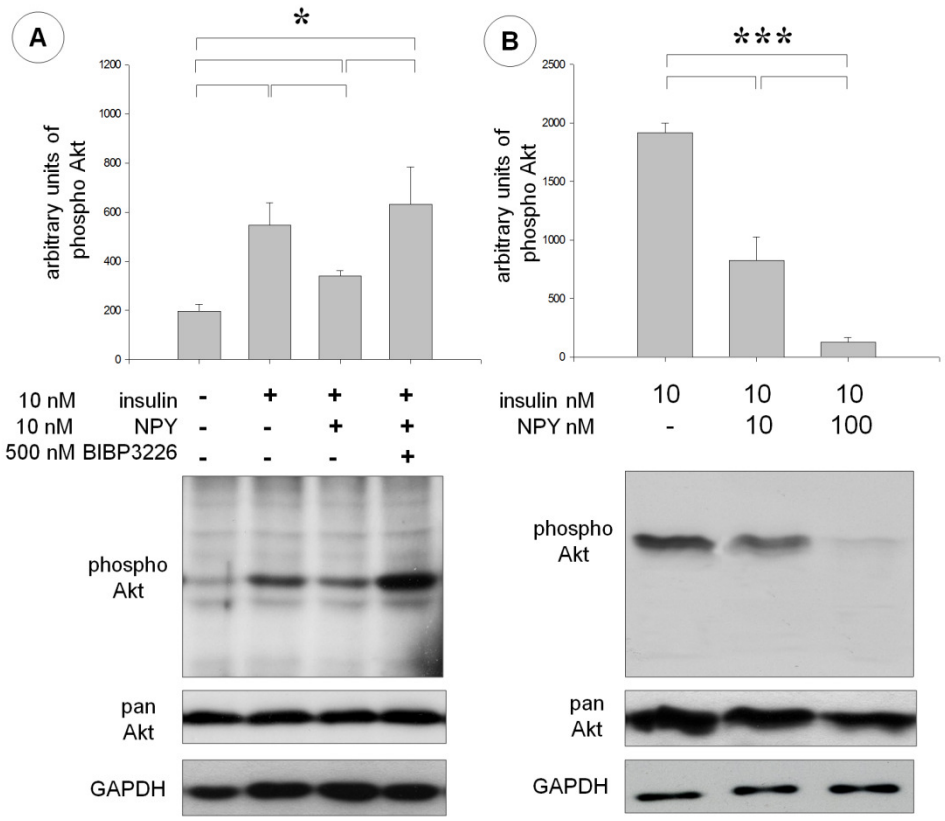

Figure 4 NPY and the Y1-antagonist were investigated in mature 3T3-L1 adipocytes for their impact on phoshorylation of Akt, a key step in insulin signaling. (A) 5 min of insulin incubation leads to an increase of phosphorylated Akt. The co-application of NPY inhibits insulin-stimulated Akt phosphorylation and this inhibition is sensitive to pretreatment with the Y1-antagonist BIBP 3226. (B) shows that NPY impaires Akt phosphorylation dose-dependently. Pan Akt and GAPDH control reveals equal protein loading. Data from four independent experiments each are means ± SEM and checked for statistical significance by the Student-Newman-Keuls multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$

3.5. Insulin-stimulated [^{3}H]-deoxyglucose uptake is inhibited by NPY

Next, either basal or insulin-stimulated deoxyglucose uptake of 3T3-L1 cultures was measured and investigated for the influence of NPY.

Stimulation of 10 nM NPY alone did not change basal levels of deoxyglucose uptake (Fig. 5 left). Co-application of NPY and insulin resulted in an impaired insulin-stimulated incorporation of [^{3}H]-deoxyglucose into 3T3-L1 cells, compared to insulin stimulation only ($p<0.05$; Fig.5 right).

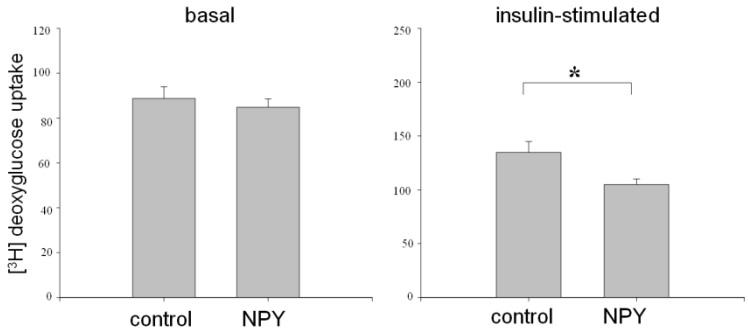

Figure 5 Determination of glucose uptake by mature 3T3-L1 adipocytes was studied with [^{3}H]-deoxyglucose incorporation. NPY only has no influence on basal glucose uptake. The co-stimulation of insulin with NPY impaires the insulin-stimulated glucose uptake. Data from four independent experiments performed in a triplicate are means ± SEM and analysed for statistical significance by the Student-Newman-Keuls multiple comparison test. * $p \leq 0.05$

3.6. Chronic NPY exposure leads to an increased GLUT4 expression

Chronic application of both, 10 or 100 nM NPY resulted in a significant increase in GLUT4 protein expression by 3-fold in cultured 3T3-L1 adipocytes and there was no difference between both concentrations (Fig. 6).

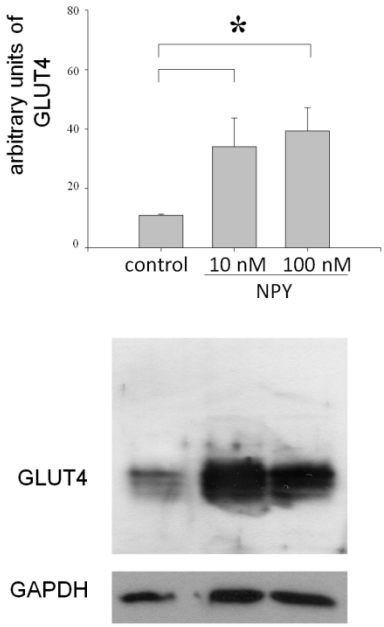

Figure 6 Chronic application of either 10 or 100 nM NPY for 14 days resulted in a 3-fold increase in expression of the GLUT4 protein. Data from five independent experiments are means ± SEM and checked for statistical significance by the Student-Newman-Keuls multiple comparison test. *p≤0.05

4. Discussion and conclusions

We here report a NPY-dependent inhibition of insulin-related GLUT4 increase at the cell surface associated with impaired glucose uptake by 3T3-L1 cells. This inhibitory effect is explained by a reduced translocation of GLUT4 from the cytosol to the cell surface. The NPY action could be mimicked by the specific Y1 agonist [Phe(7),Pro(34)] porcine NPY (Söll et al., 2001; Lecklin et al., 2003).

The NPY-dependent inhibition is most effective at 10 nM insulin that is close to the optimal insulin receptor binding and autophosphorylation (Frasca et al., 1999). This impaired insulin action involves activated Y1 receptor, as was evident by blocked NPY influence by the NPY inhibitor BIBP 3226, which is a highly selective Y1 antagonist (Rudolf et al., 1994). The Y1 receptor occurs in the 3T3-L1 cells as we and others have shown at mRNA and protein level (Yang et al., 2008; Gericke et al., 2009).

Whereas Y2 and Y4 double knockout mice are protected against diet-induced obesity, genetic Y1 deficiency results in an obese phenotype probably not due to glucose uptake of

muscle tissue but by adipose tissue. (Burcelin et al., 2001; Sainsbury et al., 2006). Noteworthy, mice with selective overexpression of NPY in noradrenergic neurons, excluding the appetite regulating areas in the hypothalamus, develop obese phenotypes and an impaired glucose tolerance as well (Ruohonen et al., 2008). Because NPY is co-expressed in sympathetic neurons the peripheral NPY actions on adipose tissue likely occurs *via* the sympathetic nervous systeme. Of note, chronic stress leads to increased secretion of NPY in the peripheral sympathetic neurons with subsequent impaired glucose tolerance (Kuo et al., 2007). Further, the Leu7Pro polymorphism of the NPY gene is associated with high processing of preproNPY into mature NPY, high NPY levels in plasma and cerebrospinal fluid leading to impaired glucose tolerance and T2DM (Nordman et al., 2005). The NPY influence on pancreatic islets and insulin secretion through the sympathetic nervous system is conceivable (Adeghate, 1999). NPY inhibits insulin secretion of insulinoma cells via the Y1 receptor *in vitro*, which explains why Y1 knockout mice develop glucose intolerance and hyperinsulinemia (Kushi et al., 1998 Morgan et al., 1998; Burcelin et al., 2001). Leptin-deficient mice (ob/ob) develop obesity, mild diabetes and strikingly increased insulin secretion after downregulation of pancreatic NPY and the Y1 (Imai et al., 2007).

We could show that NPY inhibition on insulin-stimulated glucose uptake is GLUT4-dependent and most likely due to an impaired Akt phosphorylation. Phosphorylation of Akt is a key step in insulin signaling and its central role in glucose uptake and GLUT4 translocation has been reported with overwhelming evidence. NPY mediated inhibition of the stimulated Akt phosphorylation acts via the Y1 receptor and was dose-dependent. Of note, central resistin application induces hepatic insulin resistance, which is mediated by the sympathetic nervous system and leads to a reduced Akt phosphorylation. Further, this inhibition is associated with activation of the Y1 receptor (Singhal et al., 2007; van den Hoek et al., 2008).

Chronic NPY application increases GLUT4 expression in 3T3-L1 adipocytes, probably to counteract the NPY mediated insulin resistance. Accordingly, GLUT4 content increases in the adipose tissue upon chronic NPY administration in lateral brain ventricles, whereas muscle GLUT4 content was not altered by NPY (Zarjevski et al., 1994). In conclusion, NPY inhibits insulin-stimulated glucose uptake by impaired GLUT4 translocation in an Akt-dependent manner in 3T3-L1 cells. The molecular mechanism of NPY-mediated insulin resistance in adipocytes could explain similar finding with mice gene-deficient for Y1 or transgene NPY

overexpression. Other studies on pancreatic NPY influence support the diabetogenic potential of NPY and Y1. Thus, NPY as well as Y1 could be targets for anti-diabetic drugs in the future.

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Conflict of interest

The authors state no conflict of interest.

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5 Zusammenfassung

Dissertation zur Erlangung des akademischen Grades Dr. med.

Titel:	Peptiderger Einfluss auf 3T3-L1-Adipozyten
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Angefertigt am:	Institut für Anatomie der Medizinischen Fakultät der Universität Leipzig
Betreuerin:	Frau Prof. Dr. med. Spanel-Borowski
Eingereicht im:	April 2011

Das Fettgewebe ist durch die drastisch steigende Prävalenz der Adipositas in der Bevölkerung der Westlichen Welt und den daraus resultierenden Herausforderungen für die Gesundheitssysteme immer stärker in den Fokus der medizinischen Forschung gerückt. Vor allem die Regulation des Fettgewebestoffwechsels ist hierbei von großer Bedeutung. Da das Fettgewebe stark durch Hormone, wie Insulin, Glukokortikoide oder Schilddrüsenhormone, beeinflusst wird, wurden nervale Regulationsmechanismen lange Zeit vernachlässigt. Neue Erkenntnisse weisen nun dem autonomen Nervensystem eine Schlüsselfunktion bei der Regulation von Glukose- und Fettstoffwechsel zu. Eine parasympathische Innervation des Fettgewebes wird derzeit kontrovers diskutiert. Unstrittig jedoch ist der starke Einfluss, den das sympathische Nervensystem auf die Differenzierung und Proliferation von adipogenen Vorläuferzellen sowie die Regulation der Lipolyse ausübt. Die Datenlage zum sympathischen Einfluss auf den Glukosestoffwechsel ist teilweise widersprüchlich.

Neuropeptide sind Ko-Transmitter bei der nervalen Transmission. Zwei Neuropeptide, bei denen bisher verschiedene Wirkungen auf Adipozyten beschrieben sind, wurden in der vorliegenden Arbeit untersucht. Das Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) wird im peripheren Nervensystem vor allem von parasympathischen Nervenfasern

exprimiert. Sein Einfluss auf die Insulinsensitivität und die Glukoseaufnahme von 3T3-L1 Adipozyten, eine etablierte Adipozytenzelllinie, die auch in der vorliegenden Arbeit verwendet wurde, ist vielfach belegt. PACAP steigert die peripheren Insulinwirkungen und gilt als das stärkste Insulinotropin. Ein weiteres Peptid, das in der Peripherie vorwiegend von sympathischen Nervenzellen sezerniert wird, ist das Neuropeptid Y (NPY). NPY spielt im Hypothalamus eine entscheidende Rolle bei der Hungerregulation. Im Fettgewebe wurde eine fördernde Wirkung auf Proliferation und Differenzierung von Adipozytenvorläuferzellen beschrieben. NPY wird von einigen Autoren als ursächlich für stress-induzierte Adipositas angesehen.

Obwohl ein intrazellulärer Kalziumanstieg entscheidenden Einfluss sowohl auf Differenzierung von Präadipozyten als auch auf die Insulinsensitivität reifer Fettzellen hat, liegen hierfür bei Fettzellen nach PACAP oder NPY Stimulation keine Daten vor. Wir haben aus diesem Grund im ersten Schritt mittels Polymerase Kettenreaktion die Rezeptorenexpression untersucht und zeigen können, dass sowohl frühe und späte Präadipozyten, als auch reife Adipozyten Y1 für NPY, als auch PAC1 und VPAC2 für PACAP exprimieren. Der PACAP-Rezeptor VPAC1 wird nur von frühen, fibroblasten-ähnlichen Präadipozyten exprimiert. Da nach Aktivierung des Y1, als auch des PAC1-Rezeptor jeweils Kalziumanstiege in anderen Zellen beschrieben sind, wurden diese genauer untersucht. Für die kalziummobilisierende Wirkung des PAC1-Rezeptors wird vor allem eine Isoform des Rezeptors verantwortlich gemacht, die in der dritten intrazellulären Schleife des Rezeptors eine HOP-Kassette trägt. Diesen Isotyp konnten wir auch in 3T3-L1 Adipozyten nachweisen. Weiterhin konnten beide Rezeptoren auf Proteinebene durch Western Blot Analysen gezeigt werden. In live-Imaging Versuchen mittels eines Calcium-Imaging Systems konnten wir anschließend die kalziummobilisierende Wirkung von PACAP und NPY in reifen Adipozyten nachweisen, wobei die intrazellulären Kalziumanstiege in frühen oder späten Präadipozyten deutlich geringer waren. Die Rezeptorspezifität für den Y1- bzw. den PAC1-Rezeptor wurde im Anschluß mit verschiedenen A- und Antagonisten für verschieden NPY- und PACAP-Rezeptoren bestätigt.

Da für PACAP bereits Daten zur Glukoseaufnahme und zur Wirkung auf die Insulinsensitivität von Adipozyten vorlagen, fokussierten wir uns im Weiteren auf NPY. Nach Aufreinigung von 3T3-L1 Adipozytenmembranen konnte ein Anstieg des insulin-sensitiven

Glukosetransporters 4 (GLUT4) in der Plasmamembran nach Insulinstimulation gezeigt werden. Hierdurch konnte die Translokation von GLUT4 von intrazellulären Speichern zur Zelloberfläche nach Insulinstimulation bestätigt werden. Ko-Applikation mit NPY führte zu einer deutlichen Reduktion des GLUT4 Gehalts in der Plasmamembran, was auf eine verringerte Insulinsensitivität hindeutete. Die Spezifität des NPY Signals für den Y1-Rezeptor konnte hierbei erneut bestätigt werden. Die Ergebnisse wurden anschließend in Immunfluoreszenzfärbungen gegen das GLUT4 Protein von reifen Adipozyten nachvollzogen. Untersuchungen zur Insulin-Signalkaskade zeigten einen inhibitorischen Einfluss von NPY auf die Phosphorylierung des Akt Proteins, einem Schlüsselschritt der Insulinkaskade. Diese Hemmung der Phosphorylierung von Akt war konzentrationsabhängig und durch den Y1-Rezeptor vermittelt. Aufgrund der verminderten Präsenz von Glukosetransportern in der Plasmamembran, haben wir die Glukoseaufnahme von 3T3-L1 Adipozyten untersucht. Wir konnten zeigen, dass NPY zwar keinen Einfluss auf die basale Glukoseaufnahme dieser Zellen hat, jedoch die insulin-stimulierte Glukoseaufnahme der Adipoyzten hemmt, höchstwahrscheinlich durch eine reduzierte Translokation von GLUT4 aus intrazellulären Speichern an die Zelloberfläche.

Diese Ergebnisse belegen den Einfluss von PACAP und NPY auf 3T3-L1 Adipozyten in vitro. Obwohl ein direkter Zusammenhang zwischen erhöhtem intrazellulärem Kalziumspiegel und der Insulinsensitivität von Adipozyten in der Literatur beschrieben wurde, kann auf der Basis derzeitiger Daten über eine molekulare Verbindung nur spekuliert werden. Kalzium ist in geringen Mengen essentiell für die physiologische Insulinantwort und kann darum nicht komplett entzogen werden. Da NPY den Kalziumspiegel von 3T3-L1 Adipoyzten erhöht und gleichzeitig die Insulinsensitivität herabsetzt, müssen hier weitere Daten erhoben werden, um einen möglichen kausalen Zusammenhang einer kalzium-abhängigen Hemmung der Akt Phosphorylierung auf molekularer Ebene aufzuklären. Trotzdem stellen sowohl NPY, als auch PACAP interessante Angriffspunkte für mögliche pharmakologische Interventionen dar und könnten in Zukunft helfen, Patienten mit Adipositas und adipositas-assoziierten Erkrankungen wie Diabetes mellitus Typ 2 zu behandeln.

6 Summary

The adipose tissue is in focus of medical research since prevalence of obesity increases in the societies of the Western world and thus challenges health systems in these societies. Particularly, regulation of the adipose tissue metabolism becomes more and more important in regard to obesity and impaired glucose tolerance. Since the adipose tissue is highly influenced by hormones such as insulin, glucocorticoids and thyroid hormones, the regulation by peripheral nerve fibers has been neglected for many years. New insights into regulation of glucose and lipid metabolism point to a strong influence of the autonomic nervous system. Although a parasympathetic innervation of the adipose tissue is discussed controversially, the strong influence of the sympathetic nervous system on differentiation and proliferation of adipogenic precursor cells as well as on regulation of the lipolyse has been shown beyond doubt. Data regarding the sympathetic influence on glucose metabolism are contradictory.

Neuropetides are co-transmitters in the synaptic transmission by nerve fibers. Two of them are described to affect adipocytes metabolism and thus are further investigated in this work. Pituitary adenylate cyclase-activating polypeptide (PACAP) is found in peripheral nerve fibers preferentially in parasympathetic nerves. Its Influence on insulin sensitivity and glucose uptake on 3T3-L1 adipocytes, an established adipocytes cell line, which is used in this work as well, is well-known. PACAP enhances peripheral insulin actions and is considered to be the most effective insulinotropin. Another neuropeptide, which is found in peripheral organs preferentially in sympathetic nerve fibers, is Neuropeptide Y (NPY). NPY is a key player in hunger regulation in the hypothalamus. Further, a promoting influence on adipocyte precursor cell proliferation and differentiation was shown in the adipose tissue. Thus, some authors suggest NPY to be responsible for stress-induced obesity.

Although an increase of intracellular calcium has a crucial influence on differentiation of adipocytes precursors as well as on insulin sensitivity of mature fat cells, data focusing this issue upon NPY or PACAP stimulation are missing. For this reason, we first studied expression of NPY and PACAP receptors using the polymerase chain reaction technique and found the NPY-1-receptor (Y1) for NPY and PAC1 and VPAC2 for PACAP expressed in early and late preadipocytes, as well as in mature adipocytes, respectively. The PACAP receptor VPAC1 was expressed in early, fibroblastic preadipocytes, exclusively. We further studied Y1

and PAC1, because of their well-known calcium mobilizing effects in other cells. For PAC1, an isoform with an inset of a HOP cassette (PAC1-HOP) in the third intracellular loop of the receptor is described to be responsible for coupling to calcium signaling. We could confirm the PAC1-HOP expression in 3T3-L1 adipocytes. Further, both receptor proteins (Y1 and PAC1) were shown by Western blot analysis. The calcium mobilizing effect of NPY and PACAP on mature adipocytes was shown by live-imaging experiments in a calcium imaging system, whereas early and late preadipocyte exhibited minor calcium increases, only. Receptor specificity for Y1 and PAC1, respectively, was confirmed by treatment with several agonists or antagonists.

Because PACAP is well-known to affect glucose metabolism and insulin sensitivity of adipocytes, we further focused on NPY. After purification of 3T3-L1 adipocyte membranes, we could show an increase of the insulin-sensitive glucose transporter 4 (GLUT4) protein in plasma membranes of adipocytes upon insulin stimulation. Hence, translocation of GLUT4 from intracellular stores to the cell surface upon insulin stimulation was confirmed. Co-application with NPY resulted in a reduced GLUT4 level in plasma membranes of adipocytes, suggesting a decrease in insulin sensitivity. Specificity of the NPY signal as Y1-mediated was also confirmed. These results were verified by immunofluorescence staining against the GLUT4 protein of mature adipocytes. Studying the insulin signaling cascade revealed a NPY mediated influence on phorsphorylation of the Akt protein, a key step in insulin signaling. This inhibitory effect acted dose-dependently and was mediated via the Y1 receptor. Because of the reduced content of glucose transporters in the plasma membranes of 3T3-L1 adipocytes upon NPY co-treatment, we measured glucose uptake of these cells. Although NPY stimulation did not change basal glucose uptake, insulin-stimulated glucose uptake was reduced by NPY co-application, most likely due to an impaired GLUT4 translocation from intracellular stores to the cell surface.

These results confirm the influence of PACAP and NPY on 3T3-L1 adipocytes in vitro. Although according to the literature, a direct correlation of the intracellular calcium level and insulin sensitivity of adipocytes is described, on the basis of our current data we can speculate only. Because calcium is essential for physiological insulin signaling, we could not deplete cellular calcium completely. Since NPY enhances the intracellular calcium level of 3T3-L1 adipocytes and reduces insulin sensitivity, more data are needed to verify a causal

connection of a calcium-dependent inhibition of the Akt phosphorylation. Nevertheless, NPY and PACAP are highly interesting peptides for pharmacological intervention and thus could help to treat patients, suffering from obesity and obesity-associated diseases, like type 2 diabetes mellitus, in the future.

7 Danksagung

Die vorliegende Promotionsarbeit entstand am Institut für Anatomie und am Institut für Pharmazie der Universität Leipzig.

Ich möchte mich hiermit vor allem bei Frau Prof. Dr. med. Katharina Spanel-Borowski für die Überlassung des Themas bedanken. Durch ihre stete Ansprechbarkeit einerseits und die Ermutigung zum selbstständigen Denken andererseits, fand sie ein optimales Mittelmaß zwischen Führung und Individualität. Dies führte dazu, dass mir wissenschaftliches Arbeiten so großen Spaß machte und macht. Weiterhin möchte ich Frau Prof. Dr. rer. nat. Karen Nieber vom Institut für Pharmazie recht herzlich für Ihre Unterstützung danken. Herrn Prof. Dr. med. Ingo Bechmann danke ich dafür, dass er das kontinuierliche Arbeiten an diesem Thema möglich machte.

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8 Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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