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Der Einfluss von 5-Hydroxytryptamin und Glucose auf die CREB/CRE-vermittelte Genexpression in murinen Hypothalamuszellen

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Ich, Lisa Sophie Lauffer, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

„Der Einfluss von 5-Hydroxytryptamin und Glucose auf die CREB/CRE-vermittelte Genexpression in murinen Hypothalamuszellen“

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München, 14. Mai 2020 Lisa Sophie Lauffer

Inhaltsverzeichnis

Abkürzungsverzeichnis

5-HT	5-Hydroxytryptamin
5-HTR	5-HT-Rezeptor
AC	Adenylylcyclase
ACTH	*adrenocorticotropic hormone*
AgRP	*agouti-related protein*
AMP	Adenosinmonophosphat
AMPK	Adenosinmonophosphat-aktivierte Proteinkinase
ARC	Nucleus arcuatus
ATP	Adenosintriphosphat
BMI	*Body Mass Index*
bZIP	*carboxy-terminal basic Leu zipper*
Ca^{2+}	Calcium
CaMK	*Ca^{2+}-calmodulin-dependent protein kinase*
cAMP	cyclisches Adenosinmonophosphat
CART	*cocaine and amphetamine regulated transcript*
CBD	CREB-Bindedomäne
CBP	CREB-*binding protein*
CRE	*cAMP response element*
CREB	*cAMP response element-binding protein*
CRH	*corticotropin-releasing hormone*
CRTC	*CREB-regulated transcriptional co-activator*
DAG	Diacylglycerin
DMH	dorsomedialer Hypothalamus
DNA	*deoxyribonucleic acid*
EPAC	*exchange proteins directly activated by cAMP*
ERK	*extracellular-regulated kinase*
FDA	*Food and Drug Administration*
GEF	*guanine nucleotide exchange factor*
GTP	Guanosintriphosphat
GABA	γ-Aminobuttersäure
GPCR	*G protein coupled receptor*

G-Protein	Guanosinnukleotid-bindendes Protein
HDL	*high density lipoprotein*
IP_3	Inositol-1,4,5-triphosphat
Irs2	*insulin receptor substrate 2*
JNK	*jun amino-terminal kinase*
K^+	Kalium
K_{ATP}	ATP-sensitive K^+-Kanäle
kg	Kilogramm
l	Liter
LH	lateraler Hypothalamus
m	Meter
MAPK	*mitogen-activated protein kinase*
MAPKK	*MAPK kinase*
MAPKKK	*MAPK kinase kinase*
MEK	*MAPK/ERK kinase*
MCR	Melanocortin-Rezeptor
mmol	Millimol
MSH	Melanozyten-stimulierendes Hormon
Na^+	Natrium
NPY	Neuropeptid Y
PIP_2	Phosphatidylinositol-4,5-bisphosphat
PKA	Proteinkinase A
PKC	Proteinkinase C
PLC	Phospholipase C
POMC	Proopiomelanocortin
PVN	Nucleus paraventricularis
Pyk2	*proline-rich tyrosine kinase* 2
Raf	*rat fibrosarcoma*
Rap	*ras-related protein*
Ras	*rat sarcoma*
Rho	*ras homologue*
RNA	*ribonucleic acid*

RTK	Rezeptor-Tyrosinkinase
SAPK	*stress-activated protein kinase*
Src	*sarcoma*
T_3	Triiodthyronin
T_4	Thyroxin
TAS	*taste*
TAS-R	*taste-receptor*
TRH	*thyreotropin-releasing hormone*
TSH	Thyreoidea-stimulierendes Hormon, Thyreotropin
VMH	ventromedialer Hypothalamus
WHO	*World Health Organisation*
YR	Y-Rezeptoren
ZNS	zentrales Nervensystem

Publikationsliste

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Einleitung

Adipositas als Krankheitsbild

Fettleibigkeit gilt im 21. Jahrhundert als eine der größten Herausforderungen für unser Gesundheitswesen [1]. In den entwickelten Nationen der Welt hat das Krankheitsbild bereits epidemische Ausmaße erreicht und stellt ein erhebliches gesundheitliches sowie sozioökonomisches Problem dar [2]. Insbesondere die zentrale Adipositas ist neben arterieller Hypertonie, Hypertriglyceridämie, verringerter HDL (*high density lipoprotein*)-Konzentration und beeinträchtigter Glucosetoleranz Teil einer Gruppe koinzidenter Risikofaktoren, welche als „metabolisches Syndrom“ bekannt ist [3, 4]. Sie begünstigt das Auftreten kardiovaskulärer Erkrankungen wie der koronaren Herzkrankheit und der zerebrovaskulären Insuffizienz. Erkrankungen des Herz-Kreislauf-Systems gelten derzeit als führende Todesursache weltweit (WHO 2017). Fettleibigkeit ist ferner mit der Entstehung von Diabetes mellitus Typ 2, einigen Malignomen und neuropsychiatrischen Erkrankungen assoziiert [5, 6]. Aufgrund der bedeutenden Morbidität und Mortalität, welche mit Adipositas einhergehen, ist es unerlässlich, therapeutische Ansätze zu schaffen und weiterzuentwickeln.

Definition und Klassifikation

Adipositas ist definiert als eine Vermehrung des Körperfetts, welche über das Normalmaß hinaus geht. Der Körpermasseindex (*Body Mass Index*, BMI) ist die Berechnungsgrundlage für die Klassifikation des Körpergewichts [7]. Er lässt sich aus dem Gewicht eines Menschen [kg] dividiert durch seine Größe zum Quadrat $[m]^2$ bestimmen. Als untergewichtig gelten hiernach Personen mit einem BMI kleiner 18,5 kg/m^2, von Normalgewicht ist bei einem BMI zwischen 18,5 und 24,9 kg/m^2 auszugehen. Ab einem BMI von 25,0 kg/m^2 spricht man von Übergewicht bzw. Präadipositas, während Menschen mit einem BMI ab 30,0 kg/m^2 als fettleibig ergo adipös eingestuft werden. Darüber hinaus wird Adipositas anhand des BMI in verschiedene Schweregrade eingeteilt. So definieren Werte zwischen 30,0 und 34,9 kg/m^2 den Grad I, 35,0 bis 39,9 kg/m^2 den Grad II und größer als 40,0 kg/m^2 den Grad III [8].

Ätiologie

Die Ätiologie der Adipositas ist multifaktoriell bedingt. Sie wird zurückgeführt auf genetische, verhaltens- bzw. umweltbedingte, psychologische, soziale und wirtschaftliche Umstände [9, 10]. Während bei einigen Individuen eine Tendenz zu Fettspeicherung und erniedrigter

Stoffwechselaktivität beobachtet werden kann, sind bei anderen eine unausgewogene Ernährung und Bewegungsmangel ursächlich für das Übergewicht [11]. Weitere Faktoren wie endokrine Dysfunktionen, Schlafstörungen oder die Einnahme bestimmter Medikamente spielen ebenfalls eine Rolle [9, 12, 13].

Therapeutische Strategien

Eine Therapieindikation besteht aus medizinischer Sicht ab einem BMI größer als 30,0 kg/m^2 bzw. bei Übergewicht mit einem BMI zwischen 25 und 30 kg/m^2 und gleichzeitigem Vorliegen übergewichtsbedingter Gesundheitsstörungen wie z. B. arterieller Hypertonie und Diabetes mellitus Typ 2, abdominaler Adipositas, durch Adipositas aggravierter Erkrankungen oder bei Bestehen eines hohen psychosozialen Leidensdruckes [7]. Grundlage jedes Gewichtsmanagements, welches sowohl die Reduktion als auch eine langfristige Stabilisierung des Körpergewichts umfasst, sollte eine Kombination aus Ernährungs-, Bewegungs- und Verhaltenstherapie sein [7]. Wird durch Änderungen des Lebensstils keine oder eine lediglich unzureichende Gewichtsabnahme erzielt, kann zusätzlich eine medikamentöse Therapie erfolgen [7]. Diese sollte ausschließlich mit Orlistat, einem die Fettresorption hemmenden Inhibitor der gastrointestinalen Lipase [14], erfolgen und nur dann fortgesetzt werden, wenn innerhalb von vier Wochen eine Gewichtsabnahme von mindestens 2 kg verzeichnet werden kann [7]. In den Vereinigten Staaten von Amerika ist ferner Lorcaserin, ein selektiver Agonist am 5-Hydroxytryptamin $(5\text{-HT})_{2C}$-Rezeptor, zur Adipositastherapie zugelassen. Die Sicherheit und Wirksamkeit des Medikaments wurde anhand dreier randomisierter, doppelblinder, multizentrischer Studien bei übergewichtigen Patienten mit und ohne Diabetes mellitus Typ 2 untersucht [15]. Bei Letzteren wurde nach einem Beobachtungszeitraum von 52 Wochen ein mittlerer Gewichtsverlust von 5,8 % in Relation zum Ausgangswert mit Lorcaserin, verglichen mit 2,5 % bei Verabreichung eines Placebo ermittelt [16]. Doppelt so viele mit Lorcaserin behandelte Patienten erzielten einen Gewichtsverlust ≥ 5 %, signifikant öfter wurde auch eine Gewichtsabnahme ≥ 10 % im Vergleich zu Placebo beobachtet [17]. Hinsichtlich der Gewichtsreduktion erfüllten die primären klinischen Studienergebnisse zwar die Zulassungskriterien der FDA, blieben jedoch bei begrenzter Wirksamkeit des Medikaments hinter dem durchschnittlichen Maßstab der Institution zurück [18].

Die Energiehomöostase

Adipositas ist das Ergebnis einer dysbalancierten Energiehomöostase [19]. Zu deren physiologischen Aufrechterhaltung integriert das zentrale Nervensystem (ZNS) verschiedenartige Informationen, um den Energiebedarf des Körpers zu ermitteln und hiernach Hungergefühl und Essverhalten zu modifizieren [20, 21]. Hierzu gehören, neben neuronalen Signalen, auch Hormone wie Leptin, Insulin und Ghrelin sowie Nährstoffe bzw. Metabolite wie Glucose, Aminosäuren, Lipide oder Laktat. Ist die Energieaufnahme höher als der -verbrauch, liegt eine positive Energiebilanz vor und der Organismus lagert überschüssige Kalorien als Fett ein [22].

Das hypothalamische Melanocortin-System

Dem Hypothalamus kommt bei der Regulation von Körpergewicht und Nahrungsaufnahme eine Schlüsselrolle zu. Hypothalamische Neurone weisen sowohl umfangreiche intra- und internukleäre Verbindungen, als auch Vernetzungen in andere Regionen des ZNS und in die Körperperipherie auf [23]. Die Kerngebiete des *Nucleus arcuatus* (ARC), des ventromedialen (VMH) und dorsomedialen Hypothalamus (DMH), des *Nucleus paraventricularis* (PVN) und des lateralen Hypothalamus (LH) sind hierbei von besonderer Bedeutung [24]. Der ARC ist dank seiner Nähe zum dritten Ventrikel sowie einer modifizierten Blut-Hirn-Schranke empfindlich gegenüber systemisch zirkulierenden, den Energiestatus des Organismus anzeigenden Signalstoffen [25]. Diese vermitteln an Rezeptoren dort ansässiger Neurone erster Ordnung ihre Effekte [26, 27]. Der ARC beheimatet zwei verschiedene Neuronenpopulationen; solche, welche die anorexigenen Neuropeptide Proopiomelanocortin (POMC) und *cocaine and amphetamine-regulated transcript* (CART) synthetisieren und jene, welche die orexigenen Neuropeptide *Agouti-related protein* (AgRP) und Neuropeptid Y (NPY) exprimieren [28, 29]. An präsynaptischen Enden der POMC/CART-Neurone wird das aus POMC prozessierte, appetithemmende α-Melanozyten-stimulierende Hormon (α-MSH) freigesetzt [30]. Nach Bindung an Melanocortin-Rezeptoren (MCR) wie den MC3R bzw. MC4R an Neuronen zweiter Ordnung aktiviert α-MSH katabole Stoffwechselwege, die eine verringerte Nahrungsaufnahme und einen erhöhten Energieaufwand zur Folge haben [26]. Der endogene MC3R/MC4R-Antagonist AgRP wird aus NPY/AgRP-Neuronen freigesetzt, konkurriert mit α-MSH an MCR und antagonisiert dort dessen Effekt [31]. Das ebenfalls orexigene NPY regt die Nahrungsaufnahme über Y1- und Y5-Rezeptoren an [32-34]. Ferner sind NPY/AgRP-Neurone in der Lage, POMC-

Neurone durch Freisetzung des inhibitorischen Neurotransmitters γ-Aminobuttersäure (GABA) oder NPY direkt in ihrer Aktivität zu hemmen [25, 35, 36].

Integration peripherer Signale

Die Kommunikation hypothalamischer Neurone mit der Körperperipherie wird über entsprechende Rezeptoren für zirkulierende Moleküle aus u. a. Pankreas, Fettgewebe und Gastrointestinaltrakt ermöglicht [37]. Das von pankreatischen β-Zellen sezernierte Insulin wirkt in der Peripherie über die Aufstockung von Energiereserven anabol, während es zentral eine katabole Wirkung ausübt, indem Nahrungsaufnahme und folglich Körpergewicht reduziert werden [38, 39]. Leptin, ein hauptsächlich dem weißen Fettgewebe entstammendes Hormon, dessen Konzentration mit der Menge an Körperfett korreliert und den Energiestatus des Organismus widerspiegelt [40], inhibiert die Aktivität der NPY/AgRP-Neurone, während POMC-Neurone stimuliert werden [36]. Das im Gastrointestinaltrakt freigesetzte Ghrelin hingegen erzielt über Aktivierung der NPY/AgRP-Neurone [41, 42] und Hemmung der POMC-Neurone [43] eine orexigene Wirkung.

Intrahypothalamische Verbindungen

Neurone des ARC vermitteln appetitregulierende Effekte über die Projektion zu nachgeschalteten Neuronen weiterer hypothalamischer Kerngebiete. So erreichen AgRP/NPY-Neurone sowohl den LH als auch den PVN, während POMC/CART-Neurone neben dem PVN auch den LH, VMH, DMH und andere Regionen innervieren [25]. Der im Gehirn weit verbreitete MC4R besiedelt Regionen, welche an der Kontrolle des Ernährungsverhaltens beteiligt sind, wie PVN, DMH und LH, besonders dicht [44-46]. Auch verschiedene Y-Rezeptoren werden in ARC, PVN und LH co-exprimiert [47, 48]. PVN-Neurone synthetisieren und sezernieren ihrerseits Neuropeptide mit katabolem Stoffwechseleffekt, darunter das *corticotropin-* (CRH) und *thyreotropin-releasing hormone* (TRH) [26]. Ihre Aktivität wird durch POMC/CART-Neurone stimuliert, während NPY/AgRP-Neurone sie inhibieren. Je nach Stimulus durch den ARC werden TRH oder CRH im PVN freigesetzt. Zusätzlich zu dem indirekten Weg über die Neurone des ARC ist neben α-MSH auch Leptin dazu in der Lage, die TRH-Expression an ortsansässigen Rezeptoren des PVN direkt zu stimulieren [49].

Thyreotroper und corticotroper Regelkreis

Die Aktivität der Hypothalamus-Hypophysen-Schilddrüsen-Achse (HHA) wird durch hypophysiotrope TRH-Neurone des PVN koordiniert. TRH kontrolliert die Synthese und Freisetzung von hypophysärem Thyreotropin (Thyreoidea-stimulierendes Hormon, TSH), welches seinerseits die Produktion und Sekretion von Triiodthyronin (T_3) und Thyroxin (T_4) in bzw. aus der Schilddrüse reguliert [50]. Die peripheren Schilddrüsenhormone wirken an metabolisch aktiven Zielgeweben wie weißem und braunem Fettgewebe, Leber, Herz und Skelettmuskulatur, wo sie Stoffwechselumsatz und Thermogenese steigern [51-53].

Analog zu TRH wird die Stressantwort des Körpers durch CRH-Neurone des PVN initiiert. Stressoren bewirken die Ausschüttung von CRH in das hypophysäre Pfortadersystem, welches Peptide zum Hypophysenvorderlappen transportiert. Stimulierte corticotrope Neurone setzen dort das *adrenocorticotropic hormone* (ACTH) in die systemische Zirkulation frei. ACTH stimuliert so die Synthese und Sekretion von Glukokortikoiden in der Nebennierenrinde [54]. Hierüber werden Energiereserven mobilisiert und der Energieverbrauch erhöht.

Hypothalamische Genexpression am Beispiel von 5-Hydroxytryptamin

Signaltransduktion an G-Protein-gekoppelten Rezeptoren (GPCR)

Die ligandeninduzierte Aktivierung G-Protein-gekoppelter Rezeptoren (GPCR) führt über zelluläre Signaltransduktionswege via *second messenger* zur Regulation bestimmter Zielgene. GPCR bilden die größte Familie membrangebundener Rezeptoren und die Zielstruktur für vielerlei Pharmaka [55]. Gemäß ihrer α-Untereinheit, welche die Grundeigenschaften der G-Proteine definiert, werden diese im Allgemeinen in vier Gruppen unterteilt; $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ und $G\alpha_{12/13}$.

5-Hydroxytryptamin-Rezeptoren (5-HTR) sind, bis auf den 5-HT_3R als ligandengesteuerten Na^+/K^+-Ionenkanal, den GPCR zuzuordnen [56, 57]. Sie binden sowohl an $G\alpha_s$-, als auch an $G\alpha_{i/o}$- und $G\alpha_q$-Proteine. 5-HT_4R gehören wie die 5-HT_6R und 5-HT_7R zu den $G\alpha_s$-Protein-gekoppelten Rezeptoren [58]. $G\alpha_s$ aktiviert die Adenylylcyclase (AC), welche die Reaktion von Adenosintriphosphat (ATP) zu cyclischem Adenosinmonophosphat (cAMP) katalysiert [59]. Hierdurch werden cAMP-abhängige Proteine, wie die Proteinkinase A (PKA), cyclische Nucleotid-gesteuerte Kanäle und die *exchange proteins directly activated by cAMP* (EPAC)

aktiviert [60]. MCR gehören ebenfalls zur Familie der GPCR und koppeln an $G\alpha_s$-Proteine [61]. 5-HT_1R- und 5-HT_5R-Subtypen inhibieren über assoziierte $G\alpha_{i/o}$-Proteine die AC und senken so den intrazellulären cAMP-Spiegel [62]. Auch YR aktivieren inhibitorische G-Proteine [63]. 5-HT_2R koppeln an $G\alpha_{q/11}$-Proteine [64, 65]. Diese aktivieren die β-Isoform der Phospholipase C (PLC) [66], welche Phosphatidylinositol-4,5-bisphosphat (PIP_2) in Inositol-3,4,5-triphosphat (IP_3) und membrangebundenes Diacylglycerol (DAG) spaltet. Während IP_3 den Ca^{2+}-Kanal IP_3-Rezeptor am endoplasmatischen Retikulum öffnet und somit zu einer Erhöhung der intrazellulären Ca^{2+}-Konzentration beiträgt, aktiviert DAG die Proteinkinase C (PKC) [67]. $G_{12/13}$-Proteine koppeln an die Rho-*Guanin nucleotide exchange factors* (GEF) [68]. Die $G_{\beta\gamma}$-Dimere nehmen auf eine Vielzahl von Effektoren Einfluss, darunter Ionenkanäle, Phospholipasen, Phosphoinositidkinasen und der *rat sarcoma* (Ras)/*rat fibrosarcoma* (Raf)/*extracellular signal-regulated kinase* (ERK)-Signalweg [69].

Der Transkriptionsfaktor *cAMP response element-binding protein* (CREB)

Der Aktivierung von GPCR durch extrazelluläre Stimuli folgt über Konzentrationsänderungen der *second messenger* wie cAMP oder Ca^{2+} nach dem Anstoß intrazellulärer Proteinkinasekaskaden die Modulation von Transkriptionsfaktoren [70, 71]. Diese binden im Zellkern an spezifische DNA-Sequenzen in der Promotorregion bestimmter Zielgene und beeinflussen so die Initiation der Transkription; sie sind demnach essenziell für die Regulation der Genexpression [72]. Neben bekannten allgemeinen Transkriptions- und Co-Faktoren, welche in sämtlichen Körperzellen exprimiert werden, existieren spezifische Transkriptionsfaktoren und Faktoren ohne DNA-bindende Domäne, welche ihre regulatorische Rolle durch Interaktionen mit anderen Proteinen des Transkriptionskomplexes erfüllen [73]. Der spezifische Transkriptionsfaktor *cAMP response element-binding protein* (CREB) verdankt seinen Namen der Aktivierung durch erhöhte intrazelluläre cAMP-Spiegel [74]. Entscheidend für die Funktion von CREB sind seine DNA-Bindungsspezifität und selektive Dimerisierung, welche durch die Basisregion bzw. den Leucin-Zipper (bZip) vermittelt werden [75]. CREB bindet als Dimer an das *cAMP response element* (CRE), einen DNA-Abschnitt in der regulatorischen Region zahlreicher Zielgene, welcher durch die Nukleotidsequenz TGACGTCA gekennzeichnet ist [76]. Die CREB-Phosphorylierung, welche hauptsächlich an der Position Serin 133 stattfindet, ermöglicht die Rekrutierung der Co-Aktivatoren *CREB-binding protein* (CBP) sowie dessen Paralog p-300 und stimuliert die CREB-abhängige Transkription [77]. Die

CREB-Aktivität wird reguliert durch eine Vielzahl an Proteinkinasen, welche an unterschiedlichsten Signalkaskaden beteiligt sind, sowie posttranslationale Modifikationen wie Acetylierung, Ubiquitinylierung oder Glykosylierung [74].

Die *CREB-regulated transcriptional coactivators*

Die *CREB-regulated transcriptional coactivators* (CRTC), welche auch als *transducer of regulated CREB activity* (TORC) bezeichnet werden, gehören zu den sogenannten Co-Faktoren von CREB [78]. Sie schließen die CRTC1, CRTC2 und CRTC3 mit ein, welche unter basalen Bedingungen über eine phosphorylierungsabhängige Wechselwirkung mit 14-3-3 Proteinen im Zytoplasma gebunden sind [79]. Steigende cAMP- und Ca^{2+}-Konzentrationen forcieren die Dephosphorylierung von CRTC und deren Freisetzung aus 14-3-3-Komplexen, woraufhin die Co-Faktoren in den Nukleus translozieren und die CREB-abhängige Transkription potenzieren [78].

CREB-Phosphorylierung und CRE-Aktivierung

Zu jenen Stimuli, welche eine Phosphorylierung von CREB am Serin 133 induzieren, gehören neben Wachstumsfaktoren auch Hormone, (Neuro-)Peptide, Zytokine, Phospholipide, verschiedene Stressfaktoren, Erregerkomponenten und Bestandteile intrazellulärer Ca^{2+}-assoziierter Signalwege [74]. CREB dient als Substrat für Kinasen wie die PKA, PKC, *mitogen activated protein kinases* (MAPK) bzw. deren Kinasen (MAPKK), AKT sowie die *Ca^{2+}/calmodulin-dependent protein kinases* (CaMK) II und -IV [80]. Grundsätzlich muss zwischen einer Phosphorylierung und der Aktivierung von CREB unterschieden werden [81], ferner ist neben einer CREB-Aktivierung nicht zwangsläufig eine CRE-abhängige Genexpression nachweisbar.

Extracellular signal-regulated kinases (ERK)

ERK werden neben *jun amino-terminal kinases* (JNK) und *stress-activated protein kinases* (p38/SAPK) den MAPK zugeordnet [82]. Jede dieser Kaskaden besteht in ihrem Kernmodul aus drei Reihen von Proteinkinasen, die als MAPK, MAPKK (MAPK-Kinase) und MAPKKK (MAPKK-Kinase) bezeichnet werden. Die Übertragung des Signals wird durch sequenzielle Phosphorylierung und Aktivierung der Komponenten in den aufeinanderfolgenden Stufen vermittelt [83]. Die ERK-Familie setzt sich aus den Subtypen ERK1 bis ERK8 zusammen. Aufgrund ihrer weitestgehenden strukturellen und funktionellen Übereinstimmung werden die ERK1 und ERK2 als ERK1/2 zusammengefasst [84]. Sie sprechen vorwiegend auf Mitogene wie

Wachstumsfaktoren und Zytokine an, um Zellproliferation und -differenzierung sowie Lernen und Gedächtnis in Nervenzellen zu regulieren [85]. Die Signaltransduktion erfolgt über Zelloberflächenrezeptoren wie GPCR, Rezeptortyrosinkinasen (RTK), Integrine oder die kleinen GTPasen der Ras-Familie bzw. der *ras-related proteins* (Rap). MAPKKs für die ERK1/2 sind bspw. MEK1/2, zu den MAPKKK gehört unter anderem die Raf-Familie [82].

Grundsätzlich können sowohl $G\alpha_s$- als auch $G\alpha_{i/o}$- und $G\alpha_q$-Proteine eine Phosphorylierung der ERK1/2 induzieren [86]. Eine Möglichkeit, ERK1/2 über $G\alpha_q$ zu aktivieren, ist die direkte Phosphorylierung von C-Raf durch die PKC [87, 88]. Einen weiteren Mechanismus stellt die Ca^{2+}/CaM-vermittelte Aktivierung der Tyrosinkinasen Pyk2 und Src dar, welche zur Phosphorylierung von Ras führt [89]. Die Aktivierung von Ras zieht die Stimulation der Raf-, MEK- und ERK1/2-Kinase-Kaskade nach sich [71].

$G\alpha_s$

Sind $G\alpha_s$-gekoppelte Rezeptoren wie die 5-HT_4R, 5-HT_6R und 5-HT_7R dazu in der Lage, CREB zu aktivieren, erfolgt dies klassischerweise über die cAMP-vermittelte Aktivierung der PKA [81, 90]. Agonisten am 5-HT_4R induzieren über Aktivierung der PKA die Phosphorylierung und nukleare Translokation von CREB in enterischen Neuronen [91]. Auch in murinen, pluripotenten Stammzellen wurde eine PKA-abhängige CREB-Phosphorylierung via 5-HT_4R beschrieben [92]. Alternativ stellen EPAC1/2 eine mögliche Verbindung zwischen $G\alpha_s$-Proteinen und CREB/CRE-abhängigen Gentranskription dar [93]. EPAC fungieren als GEF für Rap 1 und Rap 2 [94], welche bei ihrer Aktivierung den Ras/MAPK-Signalweg anstoßen.

$G\alpha_q$

$G\alpha_q$-gekoppelte Rezeptoren wie die 5-HT_2R aktivieren die PKC über IP_3 oder DAG direkt oder indirekt über die Freisetzung von intrazellulärem Ca^{2+}. Zusätzlich zu diesem $G\alpha$-vermittelten Weg kann auch die $\beta\gamma$-Untereinheit die PLCβ aktivieren und dadurch die PKC stimulieren [95, 96]. Ca^{2+} stößt als pleiotroper *second messenger* eine Vielzahl an Signaltransduktionswegen an. Intrazellulär bildet es mit dem Protein Calmodulin (CaM) einen Komplex, welcher über die Aktivierung Ca^{2+}-sensitiver Isoformen der AC direkt den PKA-Signalweg oder auch *Ca^{2+}/calmodulin-dependent protein kinases* (CaMK) wie die CaMKI, -II und -IV aktiviert [71]. Ca^{2+}/CaM aktiviert ferner den Ras/MAPK-Signalweg. In nichthypothalamischen Zellen wurde

eine CREB-Phosphorylierung über $G\alpha_q$-assoziierte Kinasen wie PKC, CaMKII und auch *extracellular-signal-regulated kinases* (ERK) beobachtet, jedoch konnte lediglich bei Phosphorylierung durch die CaMKII auch eine CRE-Aktivierung festgestellt werden [97]. In Neuronen des VMH wurde infolge einer 5-HT_{2C}R-Aktivierung eine CaMKIV-vermittelte Phosphorylierung von CREB an der Position Serin 133 beschrieben [98]. In hypothalamischen Neuronen des PVN hingegen wurde bislang keine $G\alpha_q$-vermittelte CRE-Aktivierung beobachtet, ebenso wenig konnte eine 5-HT-induzierte CREB-Phosphorylierung nachgewiesen werden [99].

5-HT und die Energiehomöostase

Der Neurotransmitter 5-HT vermittelt seine zahlreichen physiologischen Effekte über 14 verschiedene Rezeptorsubtypen, die alle im ZNS präsent und unter anderem über Frontalhirn, Hippocampus, Amygdala, Striatum, Hypothalamus und das Hinterhorn des Rückenmarks verteilt sind [100-103]. Der Einfluss von 5-HT auf die Energiehomöostase wird durch die Induktion von Hyperphagie und Adipositas bei 5-HT-Depletion [104, 105] sowie die Verminderung von Appetit und Körpergewicht bei Erhöhung der zentralen 5-HT-Bioverfügbarkeit deutlich [106-108]. 5-HT ist eines der anorexigenen Schlüsselsignale, welche das zentrale Melanocortin-System aktivieren [28]. Serotonerge Neurone der Raphe-Kerne projizieren zu hypothalamischen Regionen, einschließlich ARC und PVN [109, 110]. Sowohl 5-HT_{1B}R als auch 5-HT_{2C}R werden im ARC exprimiert [111-114]. Die dort situierten POMC-Neurone co-exprimieren 5-HT_{2C}R und werden durch Bindung von Agonisten an den $G\alpha_{q/11}$-Protein-gekoppelten Rezeptor depolarisiert und somit aktiviert [113]. Umgekehrt werden NPY/AgRP-Neurone des ARC durch sowohl 5-HT als auch 5-HT_{1B}R-Agonisten hyperpolarisiert [115], wodurch der orexigene Signalweg direkt gehemmt und die anorexigenen POMC-Neurone indirekt durch Disinhibition aktiviert werden [106]. Neben der serotonergen Modulation von POMC- bzw. NPY/AgRP-Neuronen beeinflusst 5-HT direkt die Aktivität MC4R-exprimierender Zellen [116]. So kann durch ortsgerichtete Injektion von 5-HT in den PVN bei Ratten eine Reduktion der Nahrungsaufnahme beobachtet werden [116, 117]. Über die zugrunde liegenden molekularen Mechanismen der direkten anorexigenen Wirkung von 5-HT auf PVN-Neurone ist jedoch nur wenig bekannt. 5-HT ist somit via 5-HT_{2C}R und 5-HT_{1B}R über unterschiedliche, komplementäre Mechanismen in die Kontrolle der Nahrungsaufnahme durch das Melanocortin-System involviert.

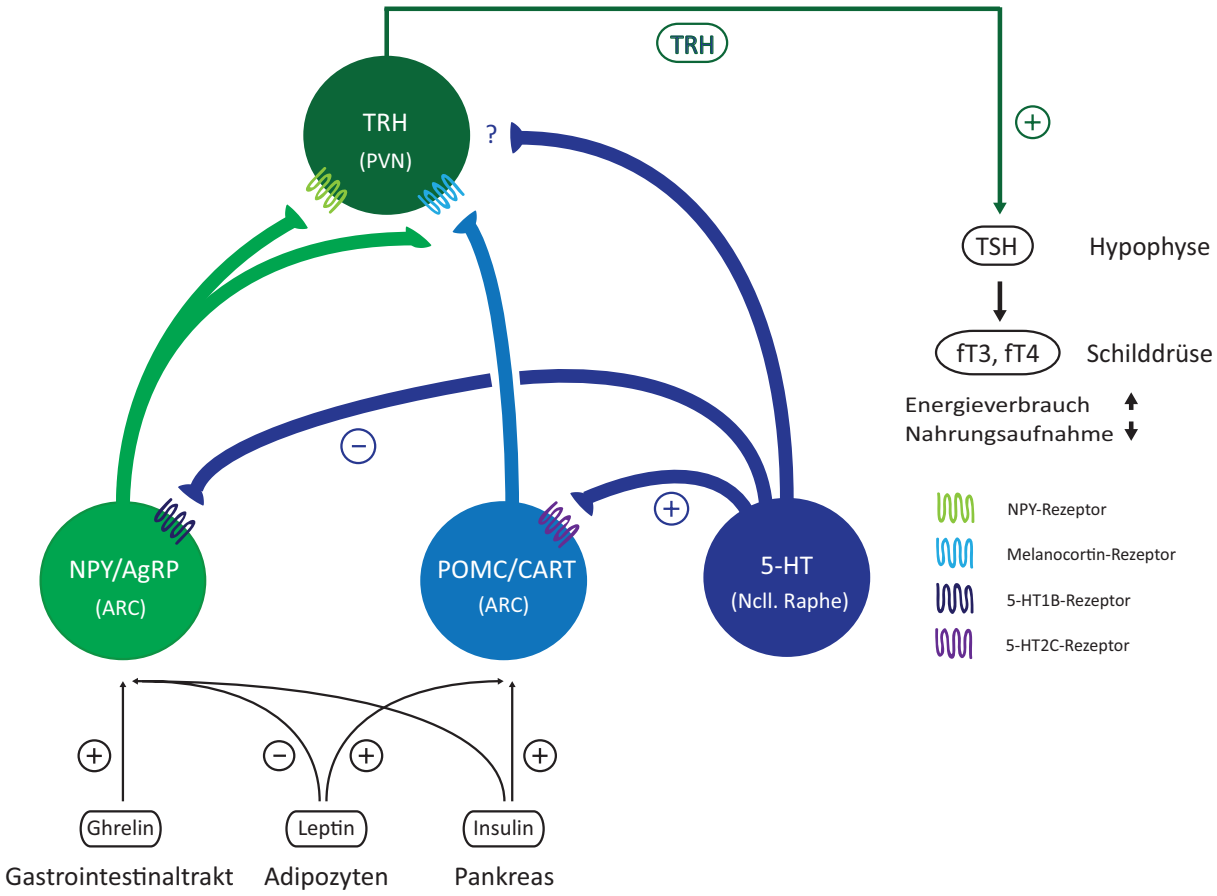

Abbildung 1 Die Energiehomöostase

Dargestellt sind die Kerngebiete der Nuclei arcuatus, paraventricularis und Raphe mit stimulierenden (+) und hemmenden (-) internukleären Verbindungen und peripheren Signalen sowie die thyreotrope Achse

Appetitregulation durch Glucose

Glucose wirkt auf hypothalamische Neurone des ARC, PVN, VMH und LH [118]. Dort variieren interstitielle Glucosekonzentrationen abhängig von Nahrungsaufnahme und Insulinspiegel zwischen 0.1 und 5 mmol/l [119]. Ähnlich wie das anorexigene Leptin stimuliert Glucose direkt POMC- und hemmt umgekehrt AgRP-Neurone des ARC [120]. Neben seiner zentralen Funktion als Energieträger ist aus Glucose gewonnenes Adenosintriphosphat (ATP) ein wichtiges Glied intrazellulärer Signaltransduktionsketten. Analog zu pankreatischen β-Zellen inhibiert ATP ATP-sensitive K^+-Kanäle (K_{ATP}), wodurch hypothalamische Neurone depolarisiert werden [121]. Des Weiteren drosselt ATP die Aktivität der *adenosine monophosphate-activated protein kinase* (AMPK). Die AMPK-Aktivität in ARC und PVN wird durch Leptin sowie in mehreren hypothalamischen Regionen durch Insulin und auch hohe Glucosekonzentrationen inhibiert [122]. Eine Abnahme der hypothalamischen AMPK-Aktivität ist mit vermindertem Appetit assoziiert, während eine AMPK-Aktivierung die Nahrungsaufnahme steigert [123]. Es wurde gezeigt, dass Glucose die hypothalamische Aktivität des CREB-Cofaktors CRTC2 über dessen AMPK-vermittelte Phosphorylierung und somit die Expression des CRE-Zielgens *insulin receptor substrate 2* (Irs2) reguliert [124]. Hiernach konnte erstmals eine Verbindung zwischen hypothalamischem Glucosespiegel und CREB/CRTC2-abhängiger Genregulation hergestellt werden. Ob Glucose auch einen direkten Effekt auf die CRE-Aktivität in hypothalamischen Neuronen hat, ist bislang unklar.

Pharmakotherapeutische Ansätze

Eine kalorienarme Diät und körperliche Betätigung sind essentielle Bestandteile einer erfolgreichen Adipositastherapie, jedoch reichen diese Maßnahmen zur angestrebten Gewichtsreduktion oft nicht aus. Eine Alternative zu operativen Interventionen wie der bariatrischen Chirurgie ist die Entwicklung pharmakologischer Wirkstoffe, welche entweder eine Drosselung der Nahrungsaufnahme oder Erhöhung des Energieverbrauchs bewirken [125]. Pharmaka, deren Wirkmechanismus auf der Steigerung serotonerger Neurotransmission beruht, sind seit vielen Jahren Bestandteil der Adipositastherapie. Hierzu gehören D-Fenfluramin, ein Amphetamin-Analogon und Sibutramin, ein 5-HT- und Noradrenalin-Wiederaufnahmehemmer, welche beide aufgrund kardiovaskulärer Nebenwirkungen zurückgezogen wurden [126]. Während die bei der Anwendung von Fenfluramin beobachtete Valvulopathie am ehesten durch die Aktivierung von $5\text{-}HT_{2B}R$ an Herzklappen durch den

Metaboliten Norfenfluramin bedingt ist [127], beruht das kardiovaskuläre Risiko von Sibutramin auf dessen adrenergen Eigenschaften [111]. Auf der Basis experimenteller und humaner Studien identifizierte man unter den 5-HTR den 5-HT_{2C}R, dessen Verteilungsmuster auf das ZNS beschränkt ist, als mögliche Zielstruktur neuer Pharmaka zur Behandlung von Fettleibigkeit [128]. Das therapeutische Potential der selektiven Aktivierung von 5-HT_{2C}R wurde 2012 mit der Genehmigung des 5-HT_{2C}R-Agonisten Lorcaserin durch die *Food and Drug Administration* (FDA) realisiert [106]. Dieser bindet mit 18- bis 104-facher Selektivität an 5-HT_{2C}R im Vergleich zu den 5-HT_{2A}R und 5-HT_{2B}R, senkt das Körpergewicht adipöser Patienten und wurde als vielversprechendes Medikament gegen Fettleibigkeit gehandelt [129]. Die klinische Anwendbarkeit ist jedoch aufgrund möglicher bedeutender Nebenwirkungen an anderen 5-HTR wie Valvulopathien, systolischer pulmonalarterieller Hypertonie, Depression und Erhöhung des kardiovaskulären Risikos limitiert [130]; Langzeitergebnisse gilt es abzuwarten. Die auf Lorcaserin basierende Pharmakotherapie könnte dementsprechend durch höhere Selektivität bzgl. des 5-HT_{2C}R sowie die umfassende Darlegung zellulärer Signaltransduktionsmechanismen optimiert werden.

Übergeordnete Fragestellung

Die CREB-vermittelte CRE-Aktivierung spielt bei der hypothalamischen Gentranskription eine bedeutende Rolle. Sowohl das TRH- als auch das CRH-Gen beinhalten ein Motiv in ihrer Promotorregion, welches die Bindung von CREB erlaubt [131]. 5-HT wirkt anorexigen an MSH- und NPY-sensitiven Neuronen des PVN via 5-HT_{2C}R und 5-HT_{1B}R [99, 132]. Aufgrund des Zusammenhangs zwischen hypothalamischer Glucosekonzentration und CREB/CRTC2-abhängiger Genregulation lässt sich ferner eine appetithemmende Wirkung von Glucose vermuten, die molekularen Mechanismen sind jedoch noch weitestgehend ungeklärt. Bislang stellt das anorexigene Peptidhormon α-MSH den einzigen bekannten Stimulus dar, welcher die Genexpression von TRH in Neuronen des PVN via CREB-vermittelter CRE-Aktivierung reguliert [133, 134]. Angesichts der postulierten appetithemmenden Wirkung der untersuchten Substanzen auf hypothalamische Neurone des PVN sind die 5-HT- bzw. Glucose-vermittelte CREB/CRE-Aktivierung sowie die Charakterisierung der zugrunde liegenden Signaltransduktionsmechanismen im Hinblick auf therapeutische Strategien zur Behandlung von Adipositas als übergeordnete Fragestellung zu verstehen, welche beide Publikationen miteinander verbindet.

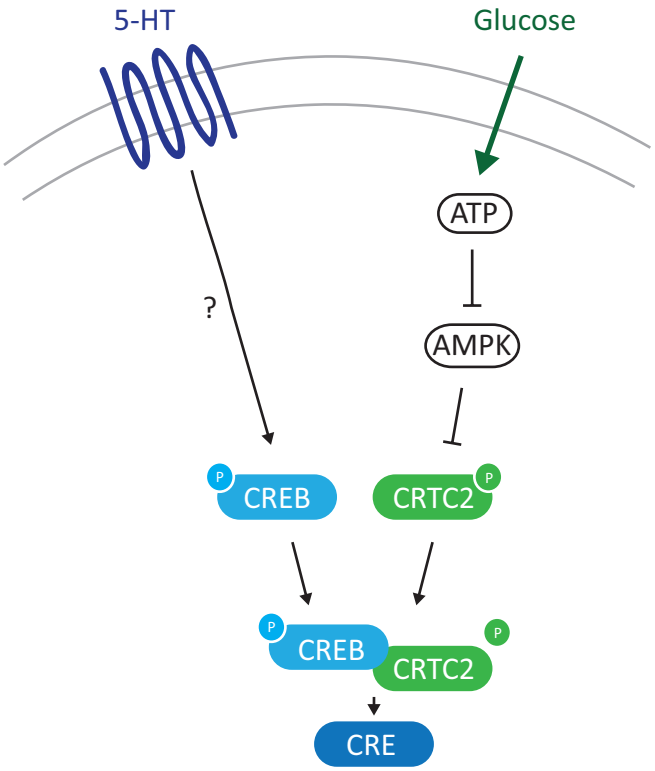

Abbildung 2 5-HT- bzw. Glucose-induzierte CREB-vermittelte CRE-Aktivierung in hypothalamischen Zellen

Der Eigenanteil der Arbeit an der Publikation II bestand in der Untersuchung möglicher zusätzlicher, für die Glucose-induzierte CRTC2-Aktivierung ursächlicher Mechanismen wie bspw. die Wirkung an Glucose-sensitiven *taste* (TAS) 1-Rezeptoren. Eine neuronale Expression solcher Geschmacksgene wurde in verschiedenen nährstoffsensitiven Arealen des Gehirns nachgewiesen, einschließlich der hypothalamischen Kerngebiete ARC und PVN [135]. Hierzu wurden einzelne mHypoA-2/10-CRE Zellen mittels Ca^{2+}-Imaging nach Stimulation mit Glucose auf eine Erhöhung der intrazellulären Ca^{2+}-Konzentration untersucht. Eine solche blieb aus, sodass eine für die CRE-Aktivierung möglicherweise ursächliche Wechselwirkung von Glucose mit dem TAS1-assoziierten *second messenger* Ca^{2+} ausgeschlossen werden konnte.

Zusammenfassung

Adipositas gilt als eine der bedeutendsten Herausforderungen für das Gesundheitswesen. Mit Fettleibigkeit assoziierte kardiovaskuläre Erkrankungen stellen derzeit die führende Todesursache weltweit dar. Die hohe Morbidität und Mortalität adipöser Patienten fordern die medizinische Forschung hinsichtlich der Kreation und Weiterentwicklung therapeutischer Ansätze. Vielversprechende Zielstrukturen stellen im zentralen Nervensystem exprimierte 5-Hydroxytryptamin (5-HT)$_{2C}$-Rezeptoren dar, anhand derer anorexigene Einflüsse vermittelt werden. Appetithemmende Pharmaka, welche auf der Aktivierung des 5-HT$_{2C}$R basieren, erwiesen sich bislang jedoch als nebenwirkungsreich oder wenig effizient. Während die Wirkungsweise von 5-HT auf hypothalamische Neurone des *Nucleus arcuatus* (ARC) profund dargelegt wurde, ist über die molekularen Signaltransduktionsmechanismen der direkten serotonergen Wirkung auf nachgeschaltete, *thyreotropin-releasing hormone* (TRH)-produzierende *Nucleus paraventricularis* (PVN)-Neurone nur wenig bekannt. Ein DNA-Motiv in der Promotorregion des TRH-Gens erlaubt die Bindung des *cAMP response element-binding protein* (CREB), eines Transkriptionsfaktors, dessen tragende Rolle bei der hypothalamischen Genexpression anhand seiner Aktivierung durch das anorexigene Peptidhormon α-Melanozyten-stimulierendes Hormon (MSH) etabliert wurde. In hypothalamischen, als PVN-Neurone charakterisierten mHypoA-2/10-Zellen aus der Maus wurde im Rahmen der Dissertation erstmals die endogene Expression des 5-HT$_{2C}$R nachgewiesen. Ferner konnte gezeigt werden, dass 5-HT$_{2C}$R die *cAMP response element* (CRE)-abhängige Genexpression über die Proteinkinase C (PKC)-vermittelte, *extracellular-signal-regulated kinases* (ERK)1/2-assoziierte Phosphorylierung von CREB induzieren. Des Weiteren konnte in stabil mit dem CRE-Reportergenkonstrukt ausgestatteten mHypoA-2/10-CRE-Zellen eine vermehrte sowohl basale als auch α-MSH-bedingte CRE-Aktivität sowie TRH-Expression bei Stimulation mit steigenden extrazellulären Glucosekonzentrationen beobachtet werden. Glucose induziert über die Inhibition der Adenosinmonophosphat-aktivierten Proteinkinase (AMPK) die nukleäre Translokation des *CREB-regulated transcriptional co-activator* 2 (CRTC2) und fördert so die CREB-abhängige Gentranskription. Sowohl 5-HT als auch Glucose nehmen hiernach über verschiedene komplementäre Mechanismen Einfluss auf die CREB/CRE-vermittelte hypothalamische Genexpression. Dies könnte sich auch im Hinblick auf die Entwicklung effizienterer, 5-HT$_{2C}$R-agonistischer Wirkstoffe zur Adipositastherapie als synergistisch wertvoll erweisen.

Summary

Obesity is considered as one of the major challenges regarding health care. Cardiovascular diseases, which are associated with obesity, currently represent the leading cause of death worldwide. The high morbidity and mortality of obese patients requires creation and further development of therapeutical approaches by medical research. Promising target structures are 5-Hydroxytryptamin (5-HT)$_{2C}$-receptors expressed in the central nervous system, by whom anorexigenic influence is conveyed. So far, appetite suppressant pharmaceuticals based on increased serotonergic neurotransmission turned out to be inefficient or associated with severe side effects. While the mode of action of 5-HT at 5-HT$_{2C}$R and 5-HT$_{1B}$R via stimulation of α-melanocyte-stimulating hormone (MSH)-producing and inhibition of neuropeptide Y (NPY)-secreting, hypothalamic neurons of the *nucleus arcuatus* (ARC) has been profoundly described, there is still little known about the molecular signalling pathways of the direct serotonergic action on *thyreotropin-releasing hormone* (TRH)-producing neurons of the *nucleus paraventricularis* (PVN). A DNA-motive in the promotor region of the TRH-gene allows binding of the *cAMP response element-binding protein* (CREB), a transcription factor, whose crucial role in hypothalamic gene expression has been established according to its activation by the anorexigenic peptid hormone α-MSH. In murine hypothalamic mHypoA-2/10-cells, which have been characterized as PVN-neurons, the presented work revealed endogenic expression of the 5-HT$_{2C}$R for the first time. Furthermore, it could be shown, that 5-HT$_{2C}$R induce *cAMP response element* (CRE)-dependent gene expression via *Proteinkinase C* (PKC)-promoted activation of *extracellular-signal-regulated kinases* (ERK)1/2 leading to phosphorylation of CREB. Moreover, in mHypoA-2/10-cells stably expressing the CRE reporter gene construct, enhanced basal and MSH-induced CRE-activity as well as TRH-expression have been observed in reaction to increased extracellular glucose concentration. Via inhibition of the adenosinmonophosphate-activated proteinkinase (AMPK) glucose induces the nuclear translocation of the CREB-co-factor *CREB-regulated transcriptional co-activator* 2 (CRTC2) and thereby stimulates CREB-dependent gene transcription. Hereafter both 5-HT and Glucose influence the CREB/CRE-imparted hypothalamic gene expression via different complementary mechanisms. This could turn out to be synergistically valuable with regard to the development of more efficient, 5-HT$_{2C}$R-agonistic substances for the treatment of obesity.

Publikation I

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Endogenous 5-HT2C receptors phosphorylate the cAMP response element binding protein via protein kinase C-promoted activation of extracellular-regulated kinases-1/2 in hypothalamic mHypoA-2/10 cells.

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Endogenous 5-HT_{2C} Receptors Phosphorylate the cAMP Response Element Binding Protein via Protein Kinase C-Promoted Activation of Extracellular-Regulated Kinases-1/2 in Hypothalamic mHypoA-2/10 Cells

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ABSTRACT

Serotonin 5-HT_{2C} receptors (5-HT_{2C}R) activate G_q proteins and are expressed in the central nervous system (CNS). 5-HT_{2C}R regulate emotion, feeding, reward, or cognition and may serve as promising drug targets to treat psychiatric disorders or obesity. Owing to technical difficulties in isolating cells from the CNS and the lack of suitable cell lines endogenously expressing 5-HT_{2C}R, our knowledge about this receptor subtype in native environments is rather limited. The hypothalamic mHypoA-2/10 cell line was recently established and resembles appetite-regulating hypothalamic neurons of the *paraventricular nucleus* (*PVN*), where 5-HT_{2C}R have been detected in vivo. Therefore, we tested mHypoA-2/10 cells for endogenous 5-HT_{2C}R expression. Serotonin or the 5-HT_{2C}R preferential agonist WAY-161,503 initiated cAMP response element (CRE)-dependent gene transcription with EC_{50} values of 15.5 ± 9.8 and 1.1 ± 0.9 nM, respectively. Both responses were blocked by two unrelated 5-HT_{2C}R-selective antagonists (SB-242,084, RS-102,221) but not by a 5-HT_{2A}R (EMD-281,014) or 5-HT_{2B}R (RS-127,455) antagonists. By single-cell calcium imaging, we found that serotonin and WAY-161,503 induced robust calcium transients, which were also blunted by both 5-HT_{2C}R antagonists. Additionally we revealed, first, that 5-HT_{2C}R induced CRE activation via protein kinase C (PKC)-mediated engagement of extracellular-regulated kinases-1/2 and, second, that intrinsic activity of WAY-161,503 was in the range of 0.3–0.5 compared with serotonin, defining the frequently used 5-HT_{2C}R agonist as a partial agonist of endogenous 5-HT_{2C}R. In conclusion, we have shown that hypothalamic mHypoA-2/10 cells endogenously express 5-HT_{2C}R and thus are the first cell line in which to analyze 5-HT_{2C}R pharmacology, signaling, and regulation in its natural environment.

Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT) or serotonin exerts its physiologic effects via at least 14 distinct receptor subtypes, including 5-HT_1 receptors that couple to G proteins of the $G_{i/o}$ family and 5-HT_2 receptors coupling to G_q proteins (Hoyer et al., 1994; Chang et al., 2000). The 5-HT_{2C} receptor (5-HT_{2C}R) subtype is predominantly expressed in the central nervous system (CNS), mostly in brain regions associated with emotion, feeding, reward, or cognition, and thus might serve as a drug target for the treatment of CNS disorders (Bhatnagar et al., 2001; Di Giovanni and De Deurwaerdere, 2016). In fact, 5-HT_{2C}R activity–controlling substances have been suggested as therapeutics against schizophrenia, depression, Parkinson's disease, anxiety, sleeping disorders, drug abuse, and obesity (Tecott et al., 1995; Frank et al., 2002; Rocha et al., 2002; Meltzer et al., 2003; Isaac, 2005; Millan, 2005; Di Giovanni et al., 2006; Thomsen et al., 2008). A major obstacle hampering the development of 5-HT_{2C}R-targeting drugs are technical difficulties in isolating significant numbers of primary cells from the CNS and the current lack of suitable CNS-derived cell lines that endogenously express 5-HT_{2C}R. Hence, discovering reliable CNS-derived cell lines that endogenously express the functional 5-HT_{2C}R protein will be an important asset for 5-HT_{2C}R-related research in general and for the development 5-HT_{2C}R-targeting drugs in particular.

5-HT-producing neurons of the *raphe nuclei* project to different hypothalamic regions, including the *nucleus arcuatus*

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ABBREVIATIONS: ANOVA, analysis of variance; *ARC*, *nucleus arcuatus*; BIM-X, bisindolylmaleimide X; BSA, bovine serum albumin; CNS, central nervous system; CP-94,253, 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine; CRE, cAMP response element; CREB, CRE binding protein; Df, degree of freedom; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; EMD-281,014, 7-[[4-[2-(4-fluorophenyl)ethyl]-1-piperazinyl]carbonyl]-1*H*-indole-3-carbonitrile hydrochloride; ERK-1/2, extracellular-regulated kinases-1/2; 5-HT, 5-Hydroxytryptamine; 5-HTR, 5-HT receptor(s); IBMX, 3-isobutyl-1-methylxanthine; MSH, melanocyte-stimulating hormone; NFAT, nuclear factor of activated T cells; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PD-184,352, 2-(2-chloro-4-iodophenylamino)-N-cyclopropylmethoxy-3,4-difluorobenzamide; PKC, protein kinase C; *PVN*, *paraventricular nucleus*; RS-102,221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione; RS-127,455, 1′-[2-[4-(trifluoromethyl) phenyl]ethyl]-spiro[4H-3,1-benzoxazine-4,4′-peperidin]-2(1H)-one; RT, room temperature; SB-242,084, 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxyamide; STAT-3, signal transducer and activator of transcription-3; WAY-161,503, 8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-*a*]quinoxalin-5(6H)-one.

(*ARC*) and the *paraventricular nucleus (PVN)* (Lam et al., 2010). 5-HT released to hypothalamic neurons reduces food intake by at least three distinct mechanisms: 1) 5-HT_{2C}R-induced excitation of the anorexigenic melanocyte-stimulating hormone (MSH)–producing *ARC* neurons, 2) 5-HT_{1B}R-mediated inhibition of the orexigenic neuropeptide Y (NPY)–producing *ARC* neurons, and 3) action upon MSH- and NPY-sensitive *PVN* neurons via 5-HT_{1B}R and 5-HT_{2C}R (Currie et al., 1999, 2002; Sohn et al., 2011; Doslikova et al., 2013; Burke et al., 2014). Consequently, mice deficient for the 5-HT_{2C}R protein exhibit severe hyperphagia leading to diabetes and concomitant hyperglycemia and insulin resistance (Tecott et al., 1995; Heisler et al., 1998; Tecott and Abdallah, 2003). Furthermore, administration of a 5-HT_{2C}R (WAY-161,503) or a 5-HT_{1B}R (CP-94,253) preferential agonists inhibited food intake by reducing meal size and duration (Halford and Blundell, 1996; Lee and Simansky, 1997; Rosenzweig-Lipson et al., 2006; Thomsen et al., 2008; Fletcher et al., 2009; Doslikova et al., 2013). Overall, it appears that *PVN* neurons play an important role in the central effects of 5-HT on appetite control and express 5-HT_{1B}R and HT_{2C}R subtypes.

The hypothalamic cell line mHypoA-2/10 has recently been introduced and characterized as a suitable *PVN*-derived cell model with regard to sensitivity toward MSH and NPY, but its sensitivity toward 5-HT has not been investigated yet (Belsham et al., 2009; Dalvi et al., 2012; Nazarians-Armavil et al., 2013; Breit et al., 2015). In the present study we dissected the effects of 5-HT, WAY-161,503, and CP-94,253 on classic G protein-signaling cascades that involve cAMP, calcium, extracellular-regulated kinases-1/2 (ERK-1/2), and CRE binding protein (CREB)/cAMP response element (CRE). We found that 5-HT and CP-94,253 inhibited forskolin-induced cAMP production, indicating expression of $G_{i/o}$-coupled 5-HT_{1B}R in mHypoA-2/10 cells. 5-HT and WAY-161,503 elicited calcium transients, extracellular-regulated kinases-1/2 phosphorylation, and CREB/CRE activation, which was blocked by two distinct selective 5-HT_{2C}R but not by a 5-HT_{2A}R or 5-HT_{2B}R antagonist, providing strong pharmacological evidence of functional 5-HT_{2C}R expression in mHypoA-2/10 cells. Hence, mHypoA-2/10 cells are a unique cell line that now allows the analysis of the 5-HT_{2C}R protein in its natural environment and that could serve as a 5-HT-sensitive *PVN*-derived cell model permitting the characterization of HT_{2C}R- and 5-HT_{1B}R-induced signaling when activated individually or in concert.

Materials and Methods

Materials

Invitrogen cell culture reagents were obtained from ThermoFisher Scientific (Darmstadt, Germany) and TurboFect from ThermoScientific Fermentas (Schwerte, Germany). The anti-pERK-1/2 (E-4) antiserum was from Santa Cruz Biotechnology (Heidelberg, Germany). The pCREB-Ser-133 antibody was purchased from Cell Signaling Technology (Leiden, The Netherlands), and RS-127,455 or the peroxidase-conjugated anti-mouse or anti-rabbit antibody, both raised in goat, from Sigma-Aldrich (Deisenhofen, Germany). The firefly luciferase substrate was from Promega (Mannheim, Germany). BIM-X, 5-HT, WAY-161,503, CP-94,253, EMD-281,014, and NPY were from Tocris Bioscience (Wiesbaden-Nordenstadt, Germany), and MSH from Biotrend Chemicals (Cologne, Germany). PD-184,352 was from Enzo Life Sciences (Lörrach, Germany). The signal transducer and activator of transcription-3 (STAT-3) and the nuclear factor of activated T cells (NFAT) reporter genes were purchased from Promega. The CRE reporter, containing six 5′-TGACCTCAC-3′sites, has been reported previously (Damm et al., 2012).

Cell Culture and Transfections

The adult mouse hypothalamic cell line mHypoA-2/10 (Clu-176) was purchased from Cedarlane Labs (Burlington, Canada) and cultured in Dulbecco's modified Eagle's medium (DMEM; 10% fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin) at 37°C and 5% CO_2. To obtain mHypoA-2/10 cells, hypothalamic neurons from 2-month-old male C57Bl/6 mice were isolated, ciliary neurotrophic factor was used to trigger neurogenesis of hypothalamic primary cultures, and immortalization was induced by retroviral transfer of SV40 T-Ag (Belsham et al., 2009). To obtain mHypoA-2/10 cells stably expressing the CRE reporter, cells were cotransfected with the CRE-reporter construct and an empty pcDNA4 vector containing the resistance gene for zeocin (Breit et al., 2015). Transfected cells were then selected with 600 μg/ml zeocin for 4 weeks and positively tested cell pools were used for further studies.

Fura-2-Based Single-Cell Calcium Imaging

Seventy-two hours prior to the experiment mHypoA-2/10 cells were seeded at a density of 2–4 $\times$ 10^4 cells on glass cover slips in six-well plates coated with 0.1% poly-L-lysine. Twenty-four hours before the experiment cells were serum-starved and at the day of the experiment loaded with 10 μM Fura 2-AM in HEPES-buffered Iscove's modified Dulbecco's medium (without phenol red) supplemented with 0.02% pluronic F-127. Cover slips were washed twice with Iscove's modified Dulbecco's medium and placed in a recording chamber. Cells were analyzed using a Polychrome 5000 monochromator (Till-Photonics, Gräfelfing, Germany) and an Andor charge-coupled device camera coupled to an inverted microscope (IX71, Olympus, Hamburg, Germany). Calcium binding to Fura-2 was determined by alternate excitation of the cells with 340 nm or 380 nm every 0.5 seconds and concomitant detection of fluorescence emission at 520 nm. Fura-2 ratios (340/380) were normalized by defining the first ratio measured as 100%.

Western Blot

Cells (~100,000/well) were seeded on six-well plates, cultured for 1 day, serum-starved for 24 hours the next day, stimulated for the indicated period of time with 5-HT, WAY-161,503, or MSH, and then lysed in Laemmli buffer. Lysates were subjected to SDS-PAGE and protein transferred to nitrocellulose by Western blotting. For detection of ERK-1/2 or CREB, phosphorylation blots were separated by a horizontal cut at 25 kDa. The upper part was used to analyze ERK-1/2 or CREB phosphorylation with phospho-specific antisera and the lower part to detect total histone proteins as a loading control. Immune-reactivity was quantified by densitometry, ratios between p-CREB or p-ERK-1/2 and histone signals were calculated, and ligand-induced protein phosphorylation normalized to not-stimulated cells.

ERK-1/2 pThr202/Tyr204 Enzyme-Linked Immunosorbent Assay

Phosphorylated ERK-1/2 was also detected using the PhosphoTracer ELISA Kit from abcam (ab119659) according to the manufacturer's protocol.

Whole-Cell ELISA. Cells were seeded in 24-well plates (~50,000/well) 2 days prior to the experiment. After 20 hours of serum-starvation, cells were stimulated with increasing concentrations of 5-HT or WAY-161,503 for 3 minutes, placed on ice, and washed with ice-cold phosphate-buffered saline (PBS). After fixing the cells for 15 minutes with 4% paraformaldehyde, they were permeabilized with ice-cold methanol/acetone (50:50) for 5 minutes. Cells were then

washed with PBS and then incubated with 1.0% of bovine serum albumin (BSA) in PBS for 15 minutes at room temperature (RT) to block unspecific binding sites. After an additional washing step, cells were incubated for 30 minutes at RT with anti-p-ERK-1/2 antiserum (1:2500) in PBS/BSA. First, antibodies were removed, cells washed, and incubated with the HRP-conjugated goat anti-rabbit secondary antibody (1:4,000) in PBS/BSA for 30 minutes at RT. After several washing steps 200 μl of the 1-Step-Ultra-TMB-ELISA Substrate (ThermoFisher Scientific, Waltham, MA) was added to the cells and incubated for 15 minutes at RT. The reaction was stopped by adding 50 ml of 1 M H_2SO_4, and 200 μl transferred to 96-well plates with clear bottom and OD_{450} measured in a FLUOstar Omega plate reader (BMG LABTECH, Inc., Cary, NC).

Reporter Gene Assays

mHypoA-2/10 cells (~20,000/well) were seeded on 12-well plates and 24 hours later transfected with various luciferase reporter gene constructs using the TurboFect reagent from ThermoFisher Scientific according to the manufacturer's protocol. mHypoA-2/10-CRE cells (~20,000/well) were seeded on 24-well plates. After serum-starvation of 24 hours, cells were stimulated the following day for 4–6 hours in serum-free medium. After stimulation cells were lysed (25 mM Tris/HCl, pH 7.4, 4 mM EGTA, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 1% Triton-X-100), and luciferase activity measured in white-bottom 96-well plates after automatic injection of luciferase substrate. Resulting total light emission was detected every second for 10 seconds post-injection in a FLUOstar Omega plate reader. Average light emission during this period was recorded and is indicated relative to unstimulated cells.

cAMP Accumulation

To determine agonist-induced cAMP accumulation, ~100,000 cells were seeded in 12-well dishes 48 hours prior to the experiment and labeled in serum-free DMEM containing 2 μCi/ml of [^{3}H]adenine for 24 hours. Cells were stimulated for 30 minutes at 37°C in DMEM containing 1 μM IBMX with MSH alone or along with forskolin and NPY, 5-HT, CP-94,253, or WAY-161,503. The reaction was terminated by removal of the medium and addition of ice-cold 5% trichloroacetic acid to the cells. [^{3}H]ATP and [^{3}H]cAMP were then purified by sequential chromatography (DOWEX-resin/aluminum oxide columns), and the accumulation of [^{3}H]cAMP was expressed as the ratio of [^{3}H]cAMP/([^{3}H]cAMP + [^{3}H]ATP).

Data Analysis

Data were analyzed using Prism5.0 (GraphPad Software Inc., San Diego, CA). Statistical significance of differences was assessed by the one-test indicated by hash signs ($^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$) or by a two-sample Student's t test indicated by asterisks ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). In Fig. 1B Kolmogorov-Smirnov test was first used to ensure that the data follow a normal distribution. Next, one-way two-way analysis of variance (ANOVA) was performed to analyze the variance of the data. Results were indicated as F[degree of freedom (Df) of the sample/Df of the residual (error)] = F-value, and the P value. Afterward Tukey's multiple-comparison test was used to indicate a significant difference to forskolin-treated cells by dagger signs ($^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$). In Figs. 3C and 6D, Kolmogorov-Smirnov test was also used to ensure that the data follow a normal distribution. Next, ANOVA was performed to analyze the variance of the data. Results were indicated as F[Df of the sample/Df of the residual (error)] = F-value, and the P value. Afterward Bonferroni post-test was used to indicate a significant differences to basal by dagger signs ($^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$).

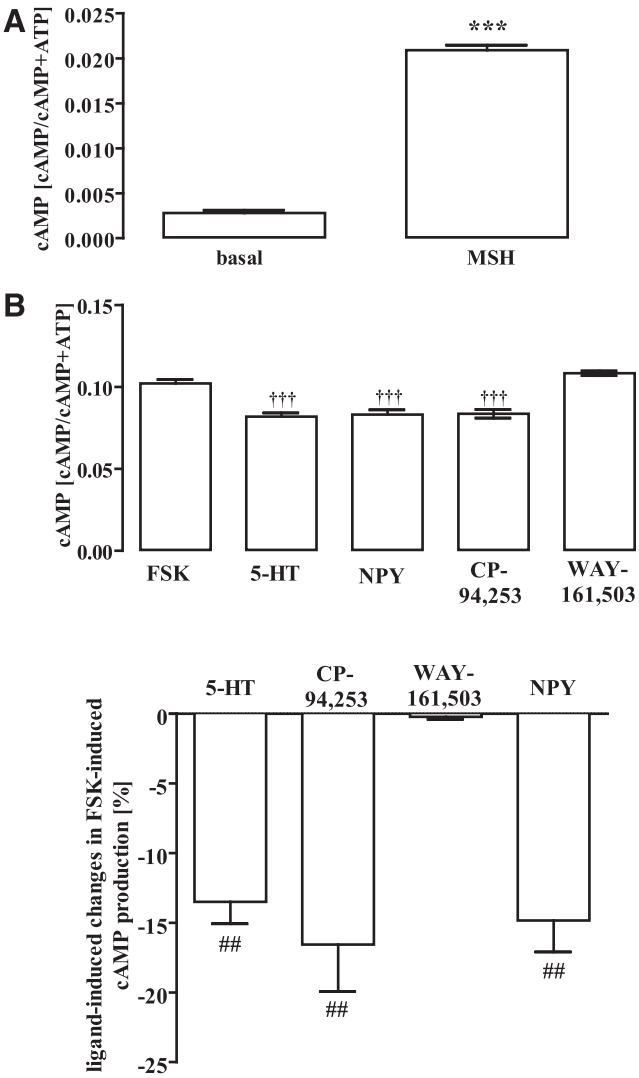

Fig. 1. mHypoA-2/10 cells respond to MSH, NPY, and 5-HT on the level of cAMP. (A, B) cAMP accumulation was assessed after labeling of serum-starved (24 hours) mHypoA-2/10 cells with [^{3}H]adenine followed by the purification of [^{3}H]cAMP by chromatography. (A) Cells were stimulated with 1 μM MSH and (B) with 1 μM forskolin (FSK) alone or along with 1 μM 5-HT, 500 nM CP-94,253, 500 nM WAY-161,503, or 100 nM NPY for 30 minutes at 37°C. (A) Data of five independent experiments performed in quadruplicate are presented as the mean ± S.E.M. Asterisks indicate a significant difference between basal and MSH (two-sample t test). (B) Upper panel, one representative experiment performed in quadruplicate. Lower panel, data of five independent experiments were normalized and presented as the mean ± S.E.M. Hash signs (one-sample t test) indicate a significant difference to zero. One-way ANOVA test results are F(4/18) = 7.891, $P < 0.01$. Dagger signs indicate significant differences (Tukey's test) to FSK alone.

Results

mHypoA-2/10 Cells Respond to 5-HT with Decreased cAMP Production. mHypoA-2/10 cells express a wide range of hypothalamic markers and, as with to *PVN* neurons, have been shown to respond to MSH and NPY (Belsham et al., 2009; Dalvi et al., 2012; Nazarians-Armavil et al., 2013; Breit et al., 2015). In line with these previous reports, mHypoA-2/10 cells used herein strongly reacted to MSH with cAMP accumulation, indicating expression of G_s-coupled MSH receptors (Fig. 1A). NPY significantly inhibited forskolin-induced cAMP production (Fig. 1B), illustrating that mHypoA-2/10 cells respond

to MSH and NPY on the level of cAMP. It has been shown that *PVN* neurons respond to 5-HT either via the $G_{i/o}$-coupled 5-HT_{1B}R or the G_q-coupled 5-HT_{2C}R subtype. In line with these observations, 5-HT- and the 5-HT_{1B}R-selective agonist CP-94,253 inhibited forskolin-induced cAMP production to the same extent as did NPY, indicating expression of the 5-HT_{1B}R subtype in mHypoA-2/10 cells.

5-HT_{2C}R Activate CREB-Dependent Gene Expression in mHypoA-2/10 Cells. Having shown that mHypoA-2/10 cells respond to 5-HT on the level of cAMP, we next sought to analyze putative effects of 5-HT on gene expression in hypothalamic cells. To this end, we stimulated mHypoA-2/10 cells expressing either a SRE/TCF-, STAT-, CREB-, or NFAT-dependent reporter gene construct with 5-HT for 6 hours. As shown in Fig. 2, 5-HT enhanced CREB-dependent gene expression, providing further evidence for endogenous 5-HTR expression in mHypoA-2/10 cells. Thus, we took advantage of previously established mHypoA-2/10-CRE cells, which stably express the CREB reporter, to determine which 5-HTR subtype is involved in 5-HT-induced CRE activation. The 5-HT and the 5-HT_{2C}R preferential agonist WAY-161,503, but not the 5-HT_{1B}R agonist CP-94,253, activated the CREB reporter (Fig. 3A), suggesting that endogenous 5-HT_{2C}R enhance CRE-controlled gene expression in the *PVN*-derived cell model. However, WAY-161,503 has been reported to activate 5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}R, but only the latter with a potency in the low nanomolar range. Therefore, we determined concentration-response curves of 5-HT and WAY-161,503 in mHypoA-2/10-CRE cells (Di Giovanni and De Deurwaerdere, 2016). As shown in Fig. 3B, WAY-161,503 induced CREB-dependent reporter activity with a potency of 1.1 ± 0.9 nM, strongly suggesting that the 5-HT_{2C}R subtype is the target of WAY-161,503 in mHypoA-2/10 cells. Furthermore, the efficacy of WAY-161,503 to activate the CRE reporter was significantly lower compared with 5-HT (~45%), suggesting that WAY-161,503 is a partial 5-HT_{2C}R agonist or that 5-HT

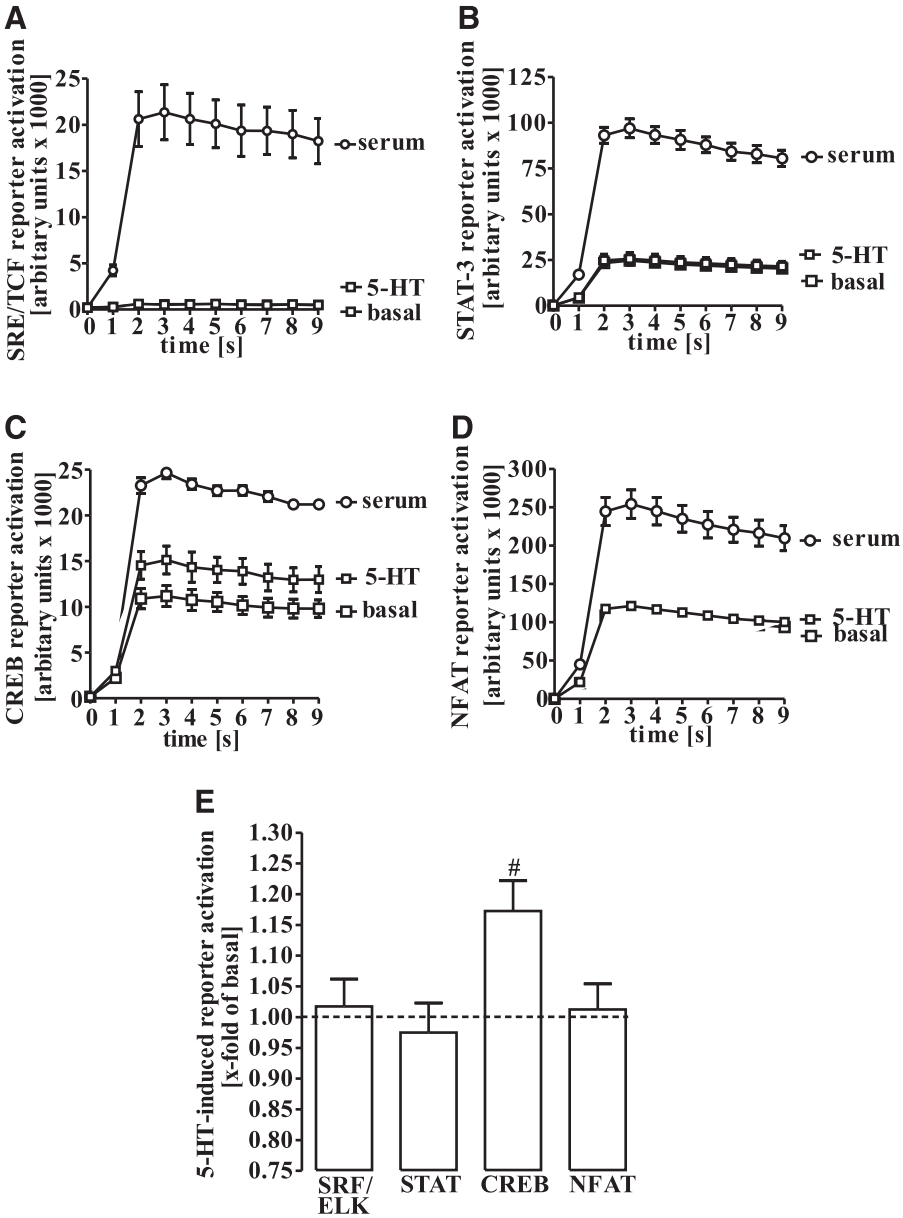

Fig. 2. 5-HT activates a CREB-dependent reporter in mHypoA-2/10 cells. mHypoA-2/10 cells were transfected with (A) serum response factor/ternary complex factor (SRF/TCF-), (B) STAT-3-, (C) CREB-, or (D) NFAT-dependent reporter constructs, serum-starved for 24 hours, stimulated with 5-HT (1 μM) for 6 hours, and then luciferase expression in cell lysates determined after injection of luciferin at time point 0 seconds. One representative experiment performed in quadruplicate is shown. Data of five independent experiments are compiled in (E) by setting the corresponding basal reporter activation to 1.0 and are given as the mean ± S.E.M. Hash sign (one-sample *t* test) indicates a significant difference to 1.0.

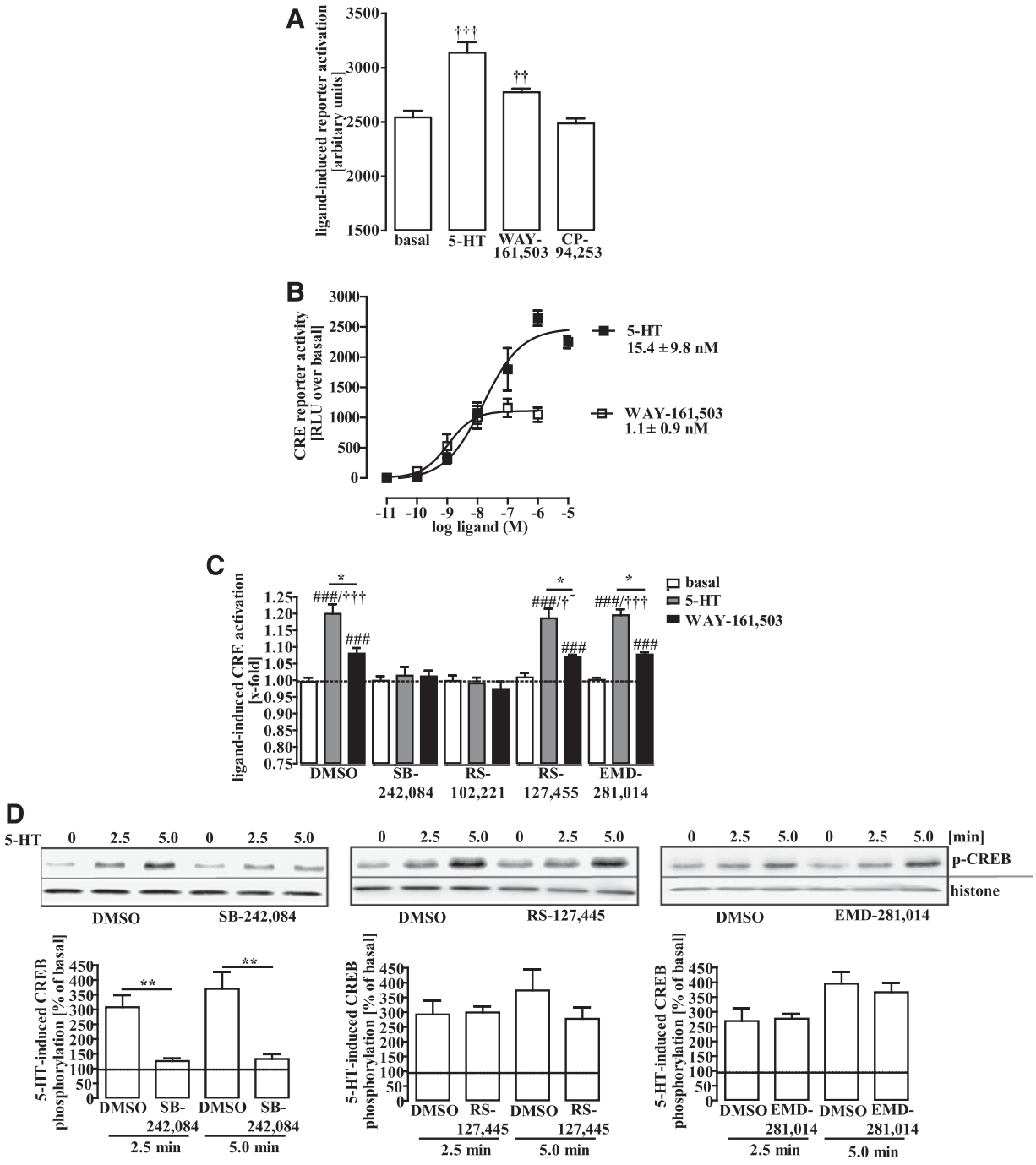

Fig. 3. 5-HT$_{2C}$R phosphorylate CREB and activate CRE in mHypoA-2/10 cells. Ligand-induced reporter activation was measured in serum-starved (24 hours) mHypoA-2/10-CRE cells. (A) Cells were stimulated with 1 μM 5-HT, 500 nM WAY-161,053, or 500 nM CP-94,253 for 6 hours and luciferase expression determined. Data from two representative experiments performed in quadruplicate were compiled and expressed as the mean ± S.E.M. One-way ANOVA test results are $F(2/12) = 4.623$, $P < 0.01$. Dagger signs indicate significant differences (Tukey's test) to basal. (B) Cells were stimulated with increasing concentrations of 5-HT or WAY-161,053. Compared with (A), the substrate concentrations were increased to enhance the signal. Data of four independent experiments performed in quadruplicate were compiled by subtracting the corresponding basal value and expressed as the mean ± S.E.M. (C) dimethyl sulfoxide (DMSO) (0.01%), SB-242,084 (100 nM), RS-102,221 (500 nM), RS-127,455 (500 nM), or EMD-281,014 (500 nM) pre-treated cells were stimulated or not with 1 μM 5-HT or 500 nM WAY-161,503. No significant differences between DMSO and cells treated with any antagonist were observed. Data from 10–20 experiments performed in quadruplicate were compiled by setting basal (DMSO) to 1.0 and expressed as the mean ± S.E.M. Hash signs indicate a significant difference (one-sample t test) to 1.0, asterisks (two-sample t test) between 5-HT and WAY-161,503. Two-way ANOVA test results are $F(2/133) = 7.501$, $P < 0.01$. Dagger signs indicate significant differences (Bonferroni post-test) to basal (DMSO). (D) 5-HT-induced CREB phosphorylation was measured in serum-starved mHypoA-2/10 cells stimulated for the indicated period of time with 5-HT (1 μM). Cells were pretreated with DMSO (0.01%), SB-242,084 (100 nM; left), RS-127,455 (500 nM; middle), or EMD-281,014 (500 nM; right) and lysates subjected to Western analysis using either a phospho-specific antiserum against CREB (p-CREB) or against the total histone protein to control for the total protein amount. One representative blot is shown. Data of 8–12 lysates were quantified by densitometry, ratios between p-CREB and histone signals calculated, and 5-HT-induced CREB phosphorylation normalized to not-stimulated cells and expressed as the mean ± S.E.M. Asterisks (two-sample t test) indicate a significant difference between DMSO and SB-242,084 pre-treated cells.

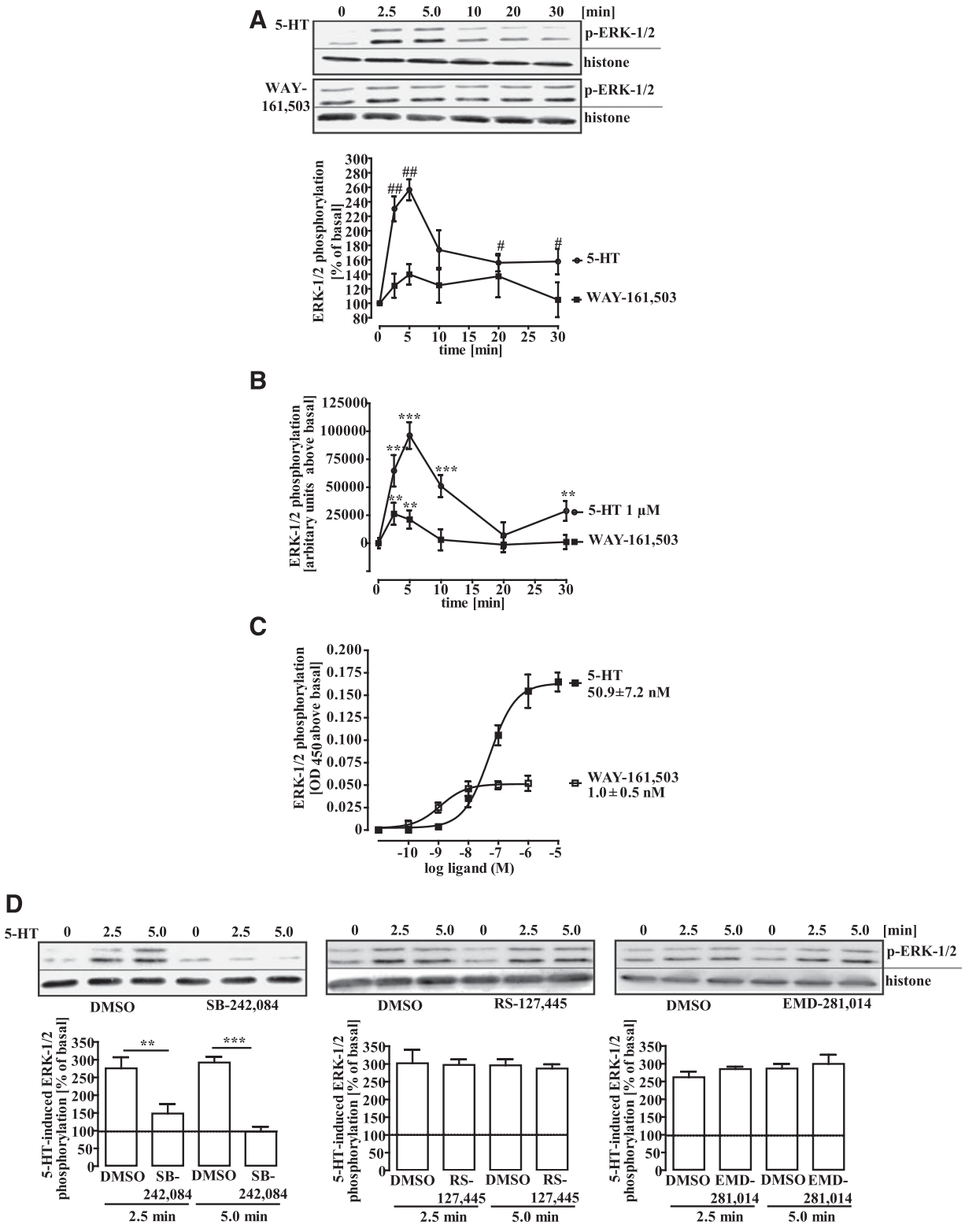

Fig. 4. 5-HT_{2C}R phosphorylate ERK-1/2 in mHypoA-2/10 cells. (A) Ligand-induced ERK-1/2 phosphorylation was measured in serum-starved mHypoA-2/10 cells stimulated for the indicated period of time with 5-HT (1 μM) or WAY-161,503 (500 nM). Cell lysates were subjected to Western analysis using either a phospho-specific antiserum against ERK-1/2 (p-ERK-1/2) or against the total histone protein to control for the total protein amount. One representative blot is shown. Data of six lysates were quantified by densitometry, ratios between p-ERK-1/2 and histone signals calculated, ligand-induced ERK-1/2 phosphorylation normalized to not-stimulated cells and expressed as the mean ± S.E.M. Hash signs indicate a significant difference (one-sample *t* test) to 100. (B) Ligand-induced ERK-1/2 phosphorylation was measured in serum-starved mHypoA-2/10 cells stimulated for the indicated period of time with 5-HT (1 μM) or WAY-161,503 (500 nM). Cell lysates were subjected to ELISA analysis using a phospho Tracer ELISA Kit. Data of four (5-HT) or six (WAY-163,503) independent experiments are shown after subtracting the corresponding basal. Asterisks indicate a significant (two-sample *t* test) difference between ligand stimulation and time point zero. (C) ERK-1/2 phosphorylation in mHypoA-2/10 cells was measured by whole-cell ELISA after stimulation with increasing concentration of 5-HT or WAY-161,503 for 3 minutes. Data of four experiments performed in quadruplicate were

interacts with other CRE-activating 5-HTRs besides HT$_{2C}$R. To further evaluate the role of distinct 5-HTR in hypothalamic CRE activation, we took advantage of the 5-HT$_{2C}$R antagonists SB-242,084 or RS-102,221, the 5-HT$_{2A}$R antagonist EMD-281,014, and the 5-HT$_{2B}$R antagonist RS-127,455 (Cussac et al., 2000; Walker et al., 2007). Both 5-HT$_{2C}$R, but none of the other antagonists, blunted WAY-161,503-induced CRE-dependent gene expression (Fig. 3C), indicating that 5-HT$_{2C}$R are indeed activated by WAY-161,503 in mHypoA-2/10 cells. 5-HT-induced CRE activation (Fig. 3C) and CREB phosphorylation (Fig. 3D) was also fully inhibited by 5-HT$_{2C}$R, but not by 5-HT$_{2A}$R or 5-HT$_{2B}$R antagonists, providing further evidence for 5-HT$_{2C}$R expression and suggesting that WAY-161,503 and 5-HT engage the same receptor to activate CRE in mHypoA-2/10 cells, albeit with distinct intrinsic activities.

5-HT$_{2C}$R Phosphorylate ERK-1/2 in mHypoA-2/10 Cells. In nonhypothalamic cells, 5-HT phosphorylates ERK-1/2 via 5-HT$_{2C}$R and almost all other 5-HTR subtypes. Hence, we next tested whether 5-HT would induce ERK-1/2 phosphorylation in mHypoA-2/10 cells. 5-HT stimulated significant ERK-1/2 phosphorylation after 2.5 and 5.0 minutes of stimulation, but as with our results in the CRE assay, the effects of WAY-161,503 were much weaker and did not reach statistical differences with unstimulated cells (Fig. 4A). Therefore, we additionally analyzed ERK-1/2 phosphorylation with a highly sensitive enzyme-linked immunosorbent assay (ELISA) (Fig. 4B) and a whole-cell ELISA (Fig. 4C). By both approaches, we detected significant increases in ERK-1/2 phosphorylation in response to 5-HT and WAY-161,503. The 5-HT$_{2C}$R preferential agonist exhibited a potency in the low nanomolar range (EC$_{50}$ value: 1.0 ± 0.5 nM) and significantly reduced efficacy compared with 5-HT (~47%), further supporting our findings that mHypoA-2/10 cells endogenously express 5-HT$_{2C}$R and that WAY-161,503 acts as a partial agonist. In line with this notion and as with results obtained on the level of CREB and CRE, 5-HT-induced ERK-1/2 phosphorylation was fully inhibited by a 5-HT$_{2C}$R antagonist and not by 5-HT$_{2A}$R or 5-HT$_{2B}$R antagonists (Fig. 4D).

5-HT$_{2C}$R Induce Calcium Signaling in mHypoA-2/10 Cells. To further characterize 5-HT$_{2C}$R signaling in mHypoA-2/10 cells, we next tested 5-HT- and WAY-161,503-induced calcium signaling by performing Fura-2-based single-cell calcium imaging. As seen in Fig. 5A, 5-HT induced robust calcium signals that peaked at ~20% above basal. Effects of 5-HT on calcium signaling were significantly reduced by SB-242,084 (~83%) and RS-102,222 (~86%), suggesting a significant role for the 5-HT$_{2C}$R subtype in 5-HT-induced calcium signaling. WAY-161,503 also significantly induced calcium signals, which were strongly inhibited by both 5-HT$_{2C}$R antagonists and, in line with our data obtained on the level of CRE and ERK-1/2, exhibited reduced efficacy (~5% above basal) compared with 5-HT (Fig. 5B).

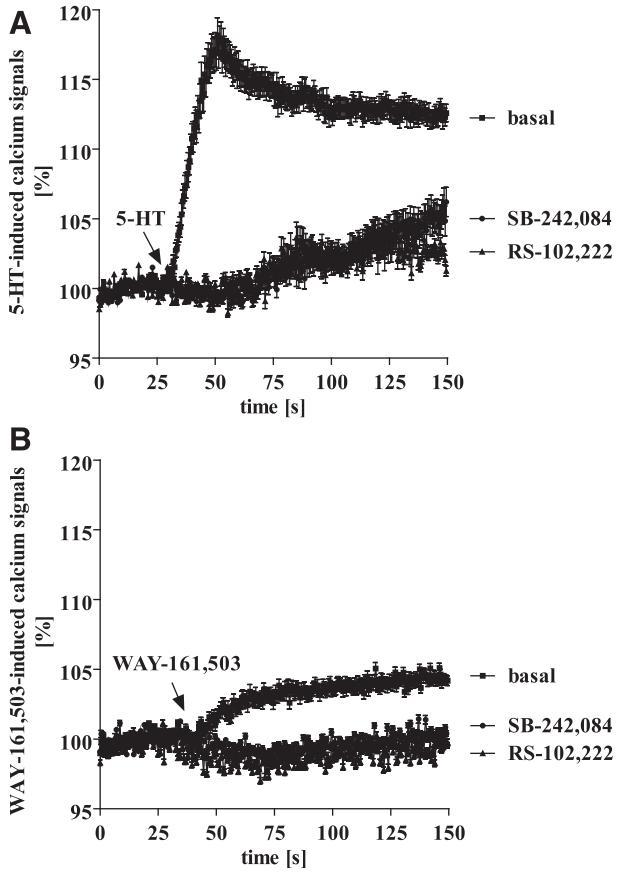

Fig. 5. Ligand-induced calcium signals in single, Fura-2-loaded, serum-starved (24 hours) mHypoA-2/10 cells were monitored. (A) 5-HT (1 μM) was manually added at time point 25 seconds either to DMSO (0.01%), SB-242,084 (100 nM), or RS-102,221 (500 nM) pretreated (5.0 minutes) cells. Data of 1037 (DMSO), 401 (SB-242,084), or 249 (RS-102,221) cells were compiled and expressed as the mean ± S.E.M. (B) WAY-161,503 (500 nM) was manually added at time point 25 seconds either to DMSO (0.01%), SB-242,084 (100 nM), or RS-102,221 (500 nM) pretreated (5.0 minutes) cells. Data of 1044 (DMSO), 410 (SB-242,084), or 423 (RS-102,221) cells were compiled and expressed as the mean ± S.E.M

5-HT$_{2C}$R Activate CRE-Dependent Reporter Activity via Protein Kinase C and ERK-1/2 in mHypoA-2/10 Cells. Having established endogenous 5-HT$_{2C}$R expression in mHypoA-2/10 cells, we next sought to define the signaling components leading to ERK-1/2 and CREB phosphorylation. Because G$_q$-coupled receptors have frequently been shown to phosphorylate ERK-1/2 via protein kinase C (PKC), we used the PKC inhibitor BIM-X (Fig. 6A). BIM-X blunted 5-HT-induced ERK-1/2 phosphorylation but did not affect phosphorylation by MSH. Thus, we provide evidence that 5-HT$_{2C}$R expressed in hypothalamic cells activate the ERK-1/2 pathway via PKC. Finally, we tested a putative link between PKC, ERK-1/2, and CREB by analyzing effects of BIM-X and the ERK-1/2

compiled by subtracting the corresponding basal and expressed as the mean ± S.E.M. (D) ERK-1/2 phosphorylation was measured in serum-starved mHypoA-2/10 cells pretreated with DMSO (0.01%) or SB-242,084 (100 nM; left), RS-127,455 (500 nM; center), or EMD-281,014 (500 nM; right) and stimulated for the indicated period of time with 5-HT (1 μM). Cell lysates were subjected to Western analysis using either a phospho-specific antiserum against ERK-1/2 (p-ERK-1/2) or against the total histone protein to control for the total protein amount. One representative blot is shown. Data of 8–12 lysates were quantified by densitometry, ratios between p-ERK-1/2 and histone signals calculated, and 5-HT-induced ERK-1/2 phosphorylation normalized to unstimulated cells and expressed as the mean ± S.E.M. Asterisks indicate a significant difference (two-sample *t* test) between DMSO and SB-242,084 pretreated cells.

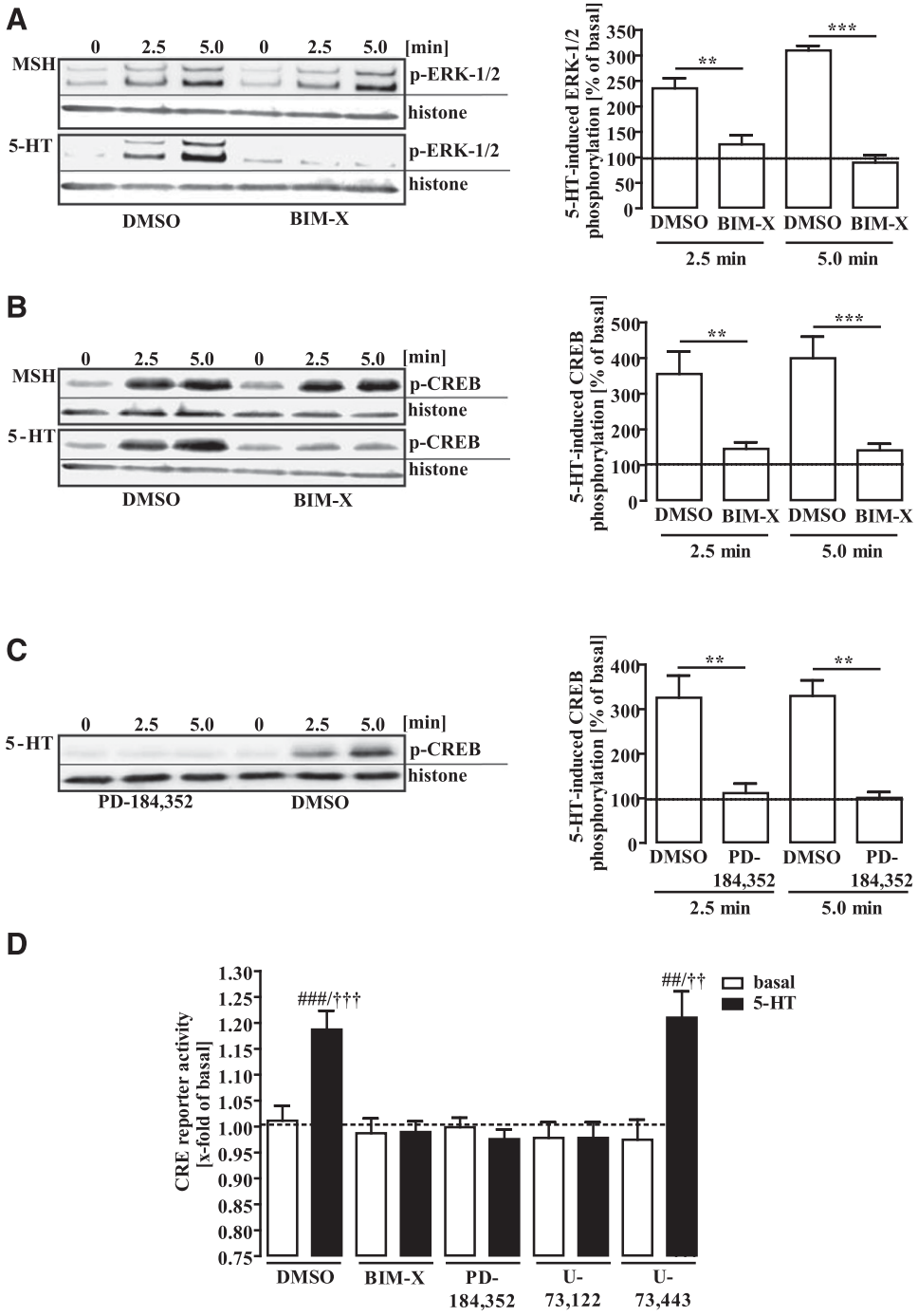

Fig. 6. 5-HT_{2C}R phosphorylate CREB via PKC and ERK-1/2 in mHypoA-2/10 cells. (A) ERK-1/2 phosphorylation was measured in serum-starved mHypoA-2/10 cells pretreated with DMSO (0.2%) or BIM-X (5 μM) and stimulated for the indicated period of time with 5-HT (1 μM) or MSH (1 μM). Cell lysates were subjected to Western analysis using either a phospho-specific antiserum against ERK-1/2 (p-ERK-1/2) or against the total histone protein to control for the total protein amount. One representative blot is shown. 5-HT data of six lysates were quantified by densitometry, ratios between p-ERK-1/2 and histone signals calculated, 5-HT-induced ERK-1/2 phosphorylation normalized to not-stimulated cells and expressed as the mean ± S.E.M. Asterisks indicate a significant difference (two-sample *t* test) between DMSO and BIM-X pretreated cells. Ligand-induced CREB phosphorylation was measured in serum-starved mHypoA-2/10 cells pretreated with DMSO (0.2%) or BIM-X (5 μM) in (B) or with DMSO (0.1%) or PD-184,352 (10 μM) in (C) and stimulated for the indicated period of time with 5-HT (1 μM) or MSH (1 μM) in (A) or only with 5-HT (1 μM) in (B). Cell lysates were subjected to Western analysis using either a phospho-specific antiserum against CREB (p-CREB) or against the total histone protein to control for the total protein amount. One representative blot is shown. Data of six independent experiments were quantified by densitometry, ratios between p-CREB and histone signals calculated, 5-HT-induced CREB phosphorylation normalized to not-stimulated cells and expressed as the mean ± S.E.M. Asterisks indicate a significant difference (two-sample *t* test) between DMSO- or inhibitor-pretreated cells. (D) 5-HT-induced reporter activation was measured in serum-starved (24 hours) mHypoA-2/10-CRE cells stably expressing the CRE-dependent reporter. DMSO (0.2%), BIM-X (5 μM), PD-184,352 (10 μM), U-73122 (10 μM), or its inactive analog U-73433 (10 μM) pretreated cells were stimulated or not with 1 μM 5-HT. No significant differences between DMSO and inhibitor-treated cells were observed. Data from four to six experiments performed in quadruplicate were compiled by setting basal to 1.0 and expressed as the mean ± S.E.M. Two-way ANOVA test results are $F(4/27) = 7.535$, $P < 0.01$. Dagger signs indicate significant differences (Bonferroni post-test) to basal (DMSO) and hash signs (one-sample *t* test) to 1.0.

inhibitor PD-184,352 on 5-HT-induced phosphorylation of CREB. Both inhibitors completely diminished 5-HT-induced CREB phosphorylation, indicating that endogenous 5-HT$_{2C}$R activate CREB/CRE via PKC-induced ERK-1/2 activation in a *PVN*-derived cell model (Fig. 6, B and C). To support this model we also tested effects of BIM-X, PD-184,352, and the phospholipase C (PLC) inhibitor U-73122 in the reporter assay. All inhibitors blunted 5-HT-induced CRE reporter activation, providing the first evidence that 5-HT$_{2C}$R mediate CREB/CRE signaling via PLC, PKC, and ERK-1/2 in hypothalamic cells.

Discussion

In this work we took advantage of the recently introduced mHypoA-2/10 cell line that has been shown to be sensitive toward MSH, NPY, and T3 hormone and thus resembles 5-HT$_{1B}$R- and 5-HT$_{2C}$R-expressing neurons of the hypothalamic *PVN* (Belsham et al., 2009; Dalvi et al., 2012; Nazarians-Armavil et al., 2013; Breit et al., 2015). We provide compelling pharmacological evidence on the basis of selective 5-HTR antagonists and agonists that mHypoA-2/10 cells express functional 5-HT$_{1B}$R and, of particular interest, 5-HT$_{2C}$R. Thus mHypoA-2/10 cells represent the first CNS-derived cell model known so far that allows investigation of 5-HT$_{2C}$R biology in its natural environment. Because of the outstanding role of 5-HT$_{2C}$R in many CNS disorders or pathophysiological states, mHypoA-2/10 cells represent a novel experimental tool for several 5-HT$_{2C}$R-based research areas, such as emotion, cognition, reward, and feeding.

It has been established that 5-HT exerts central anorexigenic effects by excitation of MSH-producing and inhibition of NPY-releasing neurons of the *ARC* (Currie et al., 1999; Currie et al., 2002; Sohn et al., 2011; Doslikova et al., 2013; Burke et al., 2014). Furthermore, activation of the 5-HT$_{2C}$R in the *PVN* has been shown to decrease food-intake and to enhance the hypothalamic-pituitary-adrenal axis by releasing corticotrophin-releasing hormone and adrenocorticotropic hormone (Heisler et al., 2007). However, the underlying signaling events have remained elusive. Analyzing mHypoA-2/10 cells, we first obtained data about gene expression–regulating signaling pathways induced by 5-HT$_{2C}$R in a *PVN*-like cell line. We showed that 5-HT$_{2C}$R activated CRE-dependent gene expression via PKC-induced and ERK-1/2-mediated phosphorylation of CREB. These findings are of particular interest because: 1) they provide the first piece of evidence for hypothalamic CRE activation by a G$_q$-coupled receptor in general and by the 5-HT$_{2C}$R in particular; 2) the corticotrophin-releasing hormone -promoter contains a binding site for CREB, suggesting that the PKC/ERK-1/2/CREB pathway might be responsible for the effects of the 5-HT$_{2C}$R on the hypothalamic-pituitary-adrenal axis; and 3) on the basis of data obtained in overexpression systems, it has been posited that 5-HT$_{2C}$R-induced ERK-1/2 activation is independent of PKC, thus highlighting the fact that endogenous 5-HT$_{2C}$R engage distinct signaling pathways compared with recombinant 5-HT$_{2C}$R (Legradi et al., 1997; Labasque et al., 2008).

The transcription factor CREB has traditionally been assigned to the PKA pathway and so far CREB/CRE activation in hypothalamic cells has been attributed to α-MSH-induced G$_s$ activation (Shaywitz and Greenberg, 1999; Harris et al., 2001). In nonhypothalamic cells G$_q$-controlled kinases such as calcium/calmodulin-dependent kinase II (CamKII), PKC, and ERK-1/2 have also been proposed to phosphorylate CREB, but the subsequent effects on CRE activation are not clear. G$_q$ activation via the gastrin-releasing peptide leads to CREB phosphorylation via PKC/ERK-1/2 in overexpression systems without subsequent CRE activation (Fitzgerald et al., 1999). Carbachol-induced activation of the muscarinic 3 receptor has been shown to phosphorylate CREB via CamKII and ERK-1/2, but only CamKII-induced CREB phosphorylation led to CRE activation (Chan et al., 2005; Rosethorne et al., 2008). Likewise, PKC-mediated ERK-1/2 activation has been shown to phosphorylate CREB via bradykinin-B2 receptors, calcium-sensing receptors, and orexin-2 receptors, but no CRE activation was found (Rosethorne et al., 2008; Guo and Feng, 2012; Avlani et al., 2013). Furthermore, CREB phosphorylation by the gonadotropin-releasing hormone required epidermal growth-factor receptor transactivation in hypothalamic cells (Neithardt et al., 2006). Interestingly, as with our results showing that 5-HT$_{2C}$R-induced signaling differs between recombinant and hypothalamic cell models, orexin-2 receptor–induced CREB phosphorylation varied in Chinese hamster ovary and hypothalamic cells (Guo and Feng, 2012). It appears that G$_q$-induced CREB/CRE activation is regulated by multilayer signaling networks strongly dependent on the stimulus and the cellular background. Thus, it will be important to analyze whether PKC/ERK-1/2-mediated CREB phosphorylation and subsequent CRE activation in hypothalamic cells is restricted to the 5-HT$_{2C}$R or also applies to other G$_q$-activation stimuli.

Because of the G$_q$ coupling of the 5-HT$_{2C}$R, one might also expect 5-HT-induced NFAT activation. Activation of PLC via G$_q$ leads to a transient release of calcium from intracellular stores, which is not necessarily sufficient to activate NFAT, because calcium influx through calcium release–activated calcium channels is required to activate NFAT (Serafini et al., 1995; Crabtree and Olson, 2002). Enhanced calcium signaling by this "inside-out" pathway might be particularly important for G$_q$-induced NFAT activation in endogenous

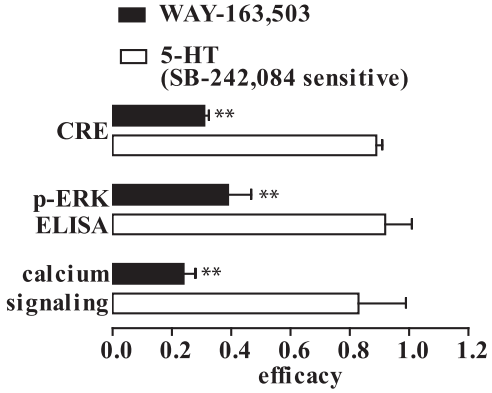

Fig. 7. WAY-163,503 is a partial agonist of endogenous 5-HT$_{2C}$R in mHypoA-2/10 cells. Data from Figs. 3C, 4B, and 5 were analyzed by setting the average signal induced by 5-HT (1 μM) in the absence of any antagonist to 1.0. Intrinsic activity of WAY-163,503 (500 nM), or of the SB-242,084-sensitive portion of the 5-HT signal, were calculated for each experiment. Asterisks indicate a significant difference between WAY-163,503 and 5-HT.

expression systems, when the initial calcium signaling from intracellular stores is rather low.

Interestingly, compared with WAY-161,503, 5-HT signaling on the level of CRE, ERK-1/2, and calcium was increased, suggesting that WAY-161,503 might be a partial 5-HT_{2C}R agonist or that 5-HT interacts with other 5-HTR besides the HT_{2C}R to activate CRE, ERK-1/2, or calcium signaling in mHypoA-2/10 cells. We can exclude a role for the 5-HT_{1B}R in 5-HT-induced CRE, ERK-1/2, or calcium signaling because the 5-HT_{1B}R agonist CP-94,253 did not activate either pathway (Fig. 3A, data not shown). We can further exclude the 5-HT_{2A}R or 5-HT_{2B}R subtypes, because antagonists of either receptor did not affect 5-HT-induced CREB and ERK-1/2 phosphorylation or CRE activation. In clear contrast, both 5-HT_{2C}R antagonists blunted 5-HT signaling, indicating that 5-HT exclusively employs the 5-HT_{2C}R to activate CREB/CRE and ERK-1/2, thus showing that WAY-161,503 and 5-HT engage the same receptor to activate CRE in mHypoA-2/10 cells, albeit with distinct intrinsic activities (Fig. 7). Previous reports using overexpression systems reported that WAY-161,503 reached maximal receptor activation in Chinese hamster ovary cells and ~80% activation levels in human embryonic kidney-293 cells, indicating that the efficacy of WAY-161,503 is generally overestimated in overexpression systems, probably owing to spare receptors (Schlag et al., 2004; Rosenzweig-Lipson et al., 2006). Partial 5-HT_{2C}R activation by WAY-161,503 in endogenous cells suggests that the anorexigenic effects of WAY-161,503 observed in animal studies might not reflect the full therapeutic potential of 5-HT_{2C}R activation on feeding. It is noteworthy in this context that the 5-HT_{2C}R-selective agonist lorcaserin (Belvique) decreases body weight in obese individuals and was touted as one of the most promising drugs available to treat obesity (Thomsen et al., 2008). However, the clinical use of lorcaserin has been limited because of severe cardiac side effects, most probably because of its binding to the 5-HT_{2A}R subtype expressed in cardiomyocytes (Miller and Wacker, 2010; Miller, 2013; Comerma-Steffensen et al., 2014). Thus, lorcaserin-based therapy could be improved either by increasing the selectivity toward the 5-HT_{2C}R subtype or by identifying the exact signaling events induced by lorcaserin via 5-HT_{2C}R in appetite-regulating neurons. mHypoA-2/10 cells might be quite useful in this regard: first, to determine the intrinsic activity of lorcaserin to activate endogenous 5-HT_{2C}R, and second, to identify pertinent signaling pathways in a hypothalamic cell model.

Herein, we not only provide data about 5-HT_{2C}R expression in mHypoA-2/10 cells, we also report that these cells endogenously express the 5-HT_{1B}R subtype. 5-HT_{1B}R are expressed in the hypothalamus and are involved in 5-HT-induced regulation of feeding. Furthermore, in vivo experiments strongly suggested that simultaneous 5-HT_{1B}R and 5-HT_{2C}R activation depressed food intake in a synergistic fashion. However, it still remains unclear whether both receptor subtypes cooperate in the same cell and, if so, which signaling pathways might be involved (Doslikova et al., 2013). Thus, mHypoA-2/10 cells may be a useful cell model to decipher complex functional interactions between 5-HT_{1B}R and 5-HT_{2C}R in a native environment.

In conclusion, we report endogenous 5-HT_{2C}R receptor expression in hypothalamic mHypoA-2/10 cells. Analysis of 5-HT_{2C}R signaling in an endogenous cell system rapidly revealed so-far unknown features of the hypothalamic 5-HT system, such as partial agonism of a selective 5-HT_{2C}R agonist and PKC/ERK-1/2-promoted CREB/CRE activation, implying that detailed dissection of the 5-HT_{2C}R biology in mHypoA-2/10 cells could significantly increase our knowledge about this intriguing receptor subtype.

Authorship Contributions

Participated in research design: Lauffer, Gudermann, Breit.
Conducted experiments: Lauffer, Glas, Breit.
Contributed new reagents or analytic tools: Breit.
Performed data analysis: Lauffer, Breit.
Wrote or contributed to the writing of the manuscript: Gudermann, Breit.

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Publikation II

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Glucose enhances basal or melanocortin-induced cAMP response element activity in hypothalamic cells.

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Glucose Enhances Basal or Melanocortin-Induced cAMP-Response Element Activity in Hypothalamic Cells

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Melanocyte-stimulating hormone (MSH)-induced activation of the cAMP-response element (CRE) via the CRE-binding protein in hypothalamic cells promotes expression of TRH and thereby restricts food intake and increases energy expenditure. Glucose also induces central anorexigenic effects by acting on hypothalamic neurons, but the underlying mechanisms are not completely understood. It has been proposed that glucose activates the CRE-binding protein-regulated transcriptional coactivator 2 (CRTC-2) in hypothalamic neurons by inhibition of AMP-activated protein kinases (AMPKs), but whether glucose directly affects hypothalamic CRE activity has not yet been shown. Hence, we dissected effects of glucose on basal and MSH-induced CRE activation in terms of kinetics, affinity, and desensitization in murine, hypothalamic mHypoA-2/10-CRE cells that stably express a CRE-dependent reporter gene construct. Physiologically relevant increases in extracellular glucose enhanced basal or MSH-induced CRE-dependent gene transcription, whereas prolonged elevated glucose concentrations reduced the sensitivity of mHypoA-2/10-CRE cells towards glucose. Glucose also induced CRCT-2 translocation into the nucleus and the AMPK activator metformin decreased basal and glucose-induced CRE activity, suggesting a role for AMPK/CRTC-2 in glucose-induced CRE activation. Accordingly, small interfering RNA-induced down-regulation of CRTC-2 expression decreased glucose-induced CRE-dependent reporter activation. Of note, glucose also induced expression of TRH, suggesting that glucose might affect the hypothalamic-pituitary-thyroid axis via the regulation of hypothalamic CRE activity. These findings significantly advance our knowledge about the impact of glucose on hypothalamic signaling and suggest that TRH release might account for the central anorexigenic effects of glucose and could represent a new molecular link between hyperglycaemia and thyroid dysfunction. **(*Molecular Endocrinology* 30: 748–762, 2016)**

The hypothalamic-pituitary-thyroid (HPT) axis is of prime importance for the central regulation of appetite and energy metabolism (1, 2). The hypothalamic nucleus arcuatus receives peripheral satiety signals such as leptin or insulin and reacts by releasing melanocyte-stimulating hormone (MSH). MSH activates cAMP-response element (CRE)-binding protein (CREB) in neurons of the paraventricular nucleus (PVN) and thereby controls the expression of TRH, resulting in TSH secretion from the pituitary gland. Thus, dysfunction of hypothalamic MSH signaling in humans or mice causes a severe obesity-diabetes syndrome (3–11). Regulation of transcriptional activity is a key control mechanism within the HPT axis and CREB-mediated CRE activation has been shown to play a major role in hypothalamic gene transcription (12–14). Because of its impact on energy metabolism, dysregulation of the HPT axis has frequently been implicated in severe metabolic disorders including type 2 diabetes mellitus (T2DM) (15–18).

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Abbreviations: AMPK, AMP-activated protein kinase; Cp, crossing point; CRE, cAMP-response element; CREB, CRE-binding protein; CRTC-2, CREB-regulated transcriptional coactivator 2; 2-DG, 2-deoxy-D-glucose; F-1,6-BP, fructose-1,6-bisphosphate; HPT, hypothalamic-pituitary-thyroid; K_{ATP}, ATP-sensitive K^+; MC4R, melanocortin-4-receptor; MSH, melanocyte-stimulating hormone; NFAT, nuclear factor of activated t cells; NO, nitrogen monoxide; p, probability; PBST, PBS with 0.02% Tween 20; PVN, paraventricular nucleus; qRT-PCR, quantitative RT-PCR; RT, room temperature; STAT, signal transducers and activator of transcription; T2DM, type 2 diabetes mellitus; TAS1, taste receptor type 1.

Glucose is the key player in diabetes and obesity and, similar to MSH, induces central anorexigenic effects by acting on hypothalamic neurons, but the underlying mechanisms are not completely understood. Besides being a pivotal energy carrier, glucose-derived ATP is an important regulator of intracellular signaling in hypothalamic neurons. In analogy to pancreatic β-cells, ATP inhibits ATP-sensitive K^+ (K_{ATP}) channels and thus depolarizes hypothalamic neurons (19–24). Furthermore, ATP dampens AMP-activated protein kinase (AMPK) activity in hypothalamic neurons, thereby relieving CREB-regulated transcriptional coactivator 2 (CRTC-2), an important transcriptional coactivator of CREB, from tonic phosphorylation and inhibition by AMPK (25). Elevating extracellular glucose concentrations from 0.5mM to 15.0mM was found to enhance CRTC-2 activity due to AMPK inhibition and expression of the insulin receptor substrate-2 in hypothalamic cells (25), thus establishing the first link between hypothalamic glucose sensing and CREB/CRTC-2-dependent gene regulation. Beyond this possible association of glucose and hypothalamic insulin signaling (25), there is also some evidence suggesting that glucose affects leptin-induced signal transduction in hypothalamic cells (26). Thus, at present, it appears that in hypothalamic cells glucose promotes gene transcription via CRTC-2 and affects key players of the HPT axis, such as leptin and insulin. However, direct effects of physiological relevant fluctuations in brain glucose levels on basal or MSH-induced CRE activity or hypothalamic TRH expression have not been shown yet.

In the present study, we investigated mHypoA-2/10-CRE cells that express a wide range of hypothalamic markers and resemble PVN neurons with regard to melanocortin sensitivity and TRH production and in addition, stable express a CRE-dependent reporter construct (27–33). We dissected effects of glucose on basal and MSH-induced CRE activation in terms of kinetics, affinity, and desensitization. Physiologically relevant increases in extracellular glucose enhanced basal or MSH-induced CRE-dependent gene transcription and hypothalamic TRH expression, whereas prolonged elevated glucose concentrations reduced the sensitivity of mHypoA-2/10-CRE cells towards glucose. These findings significantly advance our knowledge of the impact of glucose on hypothalamic signaling and might reveal a new association between hyperglycaemia and hyperthyroidism.

Materials and Methods

Materials

Cell culture reagents were obtained from Invitrogen and TurboFect from Fermentas. The anti-pro-TRH (M-166) antiserum was from Santa Cruz Biotechnology. The p-AMPK (2535s) and p-CREB (9198s) antiserum were purchased from Cell Signaling, the CRTC-2 (sc-46272) or CREB (sc-240) antiserum and fructose-1,6-bisphosphate (F-1,6-BP) was from Santa Cruz Biotechnology. The peroxidase-conjugated antimouse or antirabbit antibody, both raised in goat, the histone-3 antiserum and D-glucose, 2-deoxy-D-glucose (2-DG), and tolbutamide from Sigma-Aldrich. 2-[1,2-^{3}H(N)]-2-DG was from PerkinElmer. The firefly luciferase substrate, the signal transducers and activator of transcription (STAT), and nuclear factor of activated t cells (NFAT) reporter gene construct pGL4.47 were purchased from Promega. MSH and bradykinin were purchased from Biotrend. Dorsomorphin and metformin were purchased from Tocris. The CRE-dependent reporter, containing 6 5′-TGACCTCAC-3′ sites, has been reported previously (34).

Cell culture and transfections

The adult mouse hypothalamic cell lines mHypoA-2/10 (Clu^{176}) were purchased from Cedarlane and cultured in DMEM (10% fetal bovine serum, 2mM L-glutamine, penicillin/streptomycin, 1mM pyruvate, and 25mM glucose) at 37°C and 5% CO_2. To obtain mHypoA-2/10 cells stably expressing the CRE-dependent reporter, cells were cotransfected with the CRE reporter construct and an empty pcDNA4 vector containing the resistance gene for zeocin (27). Transfected cells were then selected with 600-μg/mL zeocin for 4 weeks and positively tested cell pools were used for further studies. To analyze effects of glucose on CRE activity DMEM without FBS, pyruvate and glucose was used. The indicated glucose concentrations were achieved by removing DMEM containing 25mM glucose, washing cells once without glucose and adding fresh DMEM containing the indicated D-glucose or 2-DG before the experiments. In order to down-regulate CRCT-2 expression, a cocktail of 3 specific siRNAs (catalog number sc-45833) and a random small interference RNA were purchased from Santa Cruz Biotechnology. To increase transfection efficacy, siRNAs were introduced into mHypoA-2/10-CRE cells via electroporation using the Neon transfection system from Invitrogen according to the manufacturer's protocol. Briefly, for 1 pulse 500 000 cells together with 10nM or 40nM the corresponding siRNA were challenged with 1450 V for 30 milliseconds and then place on a 48-well cavity for ELISA experiments or 96-well plates for reporter assays.

Whole-cell ELISA

Electroporated cells were seeded in 48-well plates (~15 000 per well) 2 days before the experiment. After 20 hours of serum starvation, total histone-3, CRTC-2, and CREB expression was analyzed at a glucose concentration of 0.1mM. After fixation for 15 minutes with 4% paraformaldehyde, cells were permeabilized with ice-cold methanol/acetone (50:50) for 5 minutes. Next, cells were washed with PBS and then incubated with 1.0% of BSA in PBS for 15 minutes at RT to block unspecific binding sites. After an additional washing step cells were incubated for 30 minutes at RT with the anti-CREB antiserum (1:2000), the anti-CRTC-2 serum (1:2000), or the antihistone serum (1:5000) in PBS/BSA. First, antibodies were removed, cells washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:4000) in PBS/BSA for 30 minutes at RT. After several washing steps, 200 μL of the 1-Step-Ultra-

TMB-ELISA substrate (Thermo Scientific) was added to the cells and incubated for 15 minutes at RT. The reaction was stopped by adding 50 mL of 1M H_2SO_4, 200 μL transferred to 96-well plates with clear bottom, and OD_{450} measured in a FLUOstar Omega plate reader.

Calcium

Calcium transients in mHypoA-2/10-CRE cells were measured with a FLUOstar Omega plate reader (BMG Labtech) as described previously (35) using the fluorescent calcium indicator fura 2.

Glucose uptake

Cells were seeded on 24-well plates (~20 000 per well) 48 hours before the experiment. After various glucose treatments, cells were cultured for 30 minutes without glucose and then glucose uptake measured by adding 0,5 μCi 2-[1,2-^{3}H(N)]-2-DG in the presence of 100μM glucose. Afterwards, cells were washed with 1 ml PBS, lysed with 0.5% sodium dodecyl sulfate and 0.1N NaOH and total radioactivity was measured.

Western blotting

Cells were seeded on 6-well plates (~100 000 per well), cultured for 1 day, serum starved for 20 hours the next day, stimulated for the indicated period of time with glucose, and then lysed in Laemmli buffer. Lysates were subjected to SDS-PAGE, and protein was transferred to nitrocellulose by Western blotting. For detection of TRH expression, blots were separated in 2 parts, by a horizontal cut at 25 kDa. The upper part was used to analyze TRH expression (30 kDa) with a TRH-specific antiserum (1:2000) and the lower part to detect total histone-3 (17 kDa) proteins (1:10 000) as a loading control. To detect AMPK phosphorylation, blots were separated by a horizontal cut at 50 kDa. The upper part was used to analyze AMPK (68 kDa) phosphorylation with phospho-specific AMPK (Thr^{172}; 1:5000 antisera and the lower part to detect total ERK-2 (42 kDa) proteins (1:10 000) as a loading control. For detection of CREB phosphorylation, blots were separated by a horizontal cut at 25 kDa. The upper part was used to analyze CREB (43 kDa) phosphorylation with phospho-specific CREB antisera (Ser^{133}; 1:5000) and the lower part to detect total histone-3 (17 kDa) proteins (1:10 000) as a loading control. Immuno-reactivity was quantified by densitometry using ImageJ, ratios between TRH and histone-3 or p-AMPK and total-ERK-2 signals were calculated, and ligand-induced TRH expression or AMPK phosphorylation normalized to unstimulated cells.

Reporter gene assays

mHypoA-2/10-CRE cells were seeded in white 96-well plates with clear bottom (~5000 per well) for the experiments shown in figures 1, 2, 4, or 5 below and in 24-well plates (~20 000 per well) for the experiments shown in figures 3 or 4 below. For the experiments shown in figure 1 below, mHypoA-2/10 cells were seeded in 12-well plates (~20 000 per well) 24 hours before the experiment and transfected with various luciferase reporter genes constructs using the Turbofect reagent (R0531) from Thermo Scientific according to the manufacturers' protocol the next day. After stimulation, cells were lysed (25mM Tris-HCl [pH 7.4], 4mM EGTA, 8mM $MgCl_2$, 1mM dithiothreitol, and 1% Triton X-100) and luciferase activity measured in white bottom 96-well plates after automatically injecting luciferase substrate. Resulting total light emission was detected every s for 10 seconds after injection in a FLUOstar Omega plate reader. Maximal light emission during this period was recorded and is indicated relative to unstimulated cells (0mM or 0.1mM glucose).

cAMP accumulation

To determine agonist-induced cAMP accumulation, 200 000 cells were seeded in 12-well dishes 24 hours before the experiment and labeled in serum-free DMEM containing 2 μCi/mL of [^{3}H]adenine for 4 hours. Cells were stimulated for 30 minutes at 37°C in DMEM containing 1μM 3-isobutyl-1-methylxanthine and MSH. The reaction was terminated by removing the medium and adding ice-cold 5% trichloroacetic acid. [^{3}H]ATP and [^{3}H]cAMP were then purified by sequential chromatography (dowex-resin/aluminum oxide columns), and the accumulation of [^{3}H]cAMP was expressed as the ratio of [^{3}H]cAMP to ([^{3}H]cAMP + [^{3}H]ATP).

Immunostaining

Approximately 4000 mHypoA-2/10-CRE cells per well were seeded on 0.1% poly-L-lysine-coated black 96-well plates with clear bottom. Twenty-four hours later, cells were cultured with distinct glucose concentrations and, after additional 24 hours, fixed with 4.0% paraformaldehyde for 10 minutes, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and unspecific binding sites blocked with 2.0% BSA in PBS with 0.02% Tween 20 (PBST) for 45 minutes. A monoclonal anti-CRTC-2 antiserum (clone number 628430 from R&D Systems) was incubated for 60 minutes at 37°C in PBST with a final concentration of 100 μg/mL and detected by an Alexa Fluor 488-conjugated goat antiserum raised against mouse IgG also for 60 minutes at 37°C in PBST with a final concentration of 4 μg/mL. Nuclei were stained using 4′,6-diamidino-2-phenylindole (0.1 μg/mL) for 30 minutes at 37°C. After intense washing cells were conserved with a drop of Ibidi mounting medium and analyzed using an Olympus IX 71 microscope. Alexa Fluor 488 immunostaining in nuclei and background areas was quantified by densitometry using ImageJ.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from mHypoA-2/10-CRE cells using the TriFast reagent (Invitrogen) according to the manufacturer's instructions. First strand synthesis was carried out with oligo(dT)$_{18}$ primer using 2 μg of total RNA and the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). qRT-PCR was done using the LightCycler 480 SybrGreen I Master Mix (Roche), intron-spanning primer pairs at a final concentration of 1μM each and 0.08 μL of the first strand synthesis reaction in a LightCycler 480 (Roche) using the following conditions: initial denaturation for 15 minutes at 94°C, 55 cycles of 94°C for 10 seconds, 55°C for 10 seconds, and 72°C for 10 seconds. Crossing points (Cps) were determined by the software supplied with the LightCycler 480. Sequences of primer pairs were 5′-ccaaccgcgagaagatga-3′ and 5′-ccagaggcgtacagggatag-3′ for β-actin, 5′-tgcagagtctccaccttgc-3′ and 5′-ggggataccagttagcacga-3′ for TRH. Data were analyzed by $2^{-\Delta\Delta Cp}$ calculations: Cp values measured for TRH under stimulated conditions (either increased glucose concentrations or MSH)

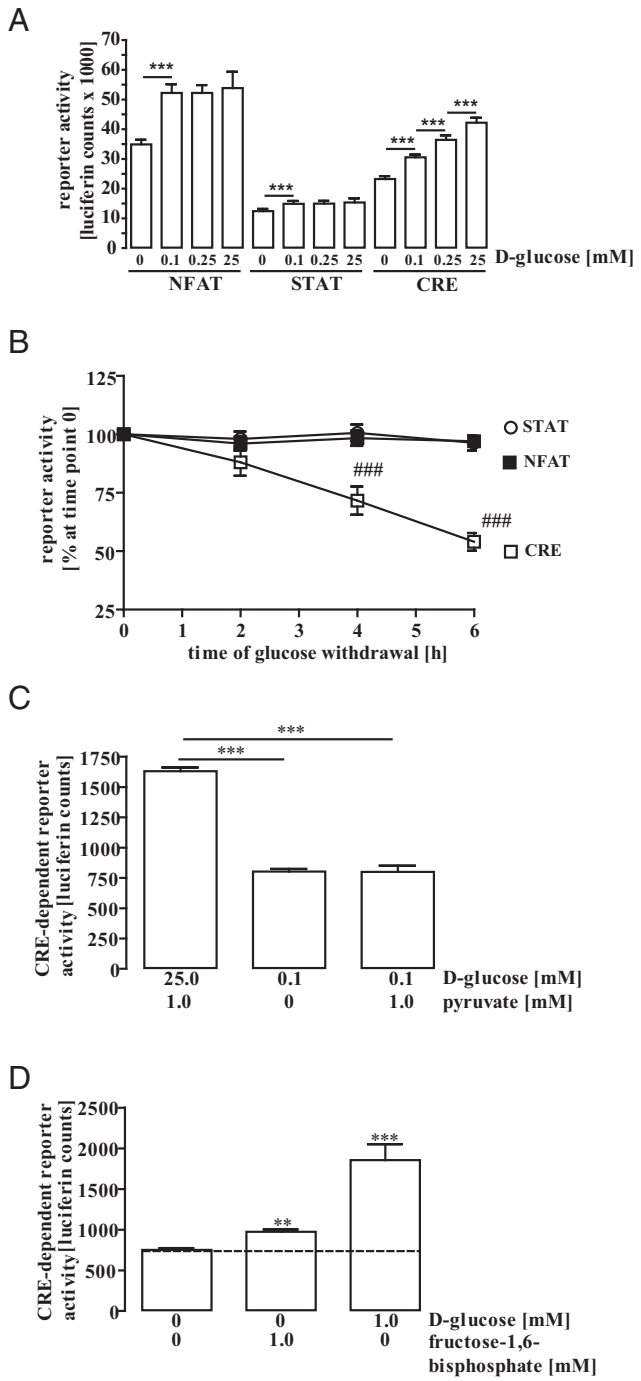

Figure 1. Glucose exerts specific effects on CRE-dependent reporter gene activity in mHypoA-2/10 cells. A and B, Reporter activity was measured after transient expression of a CRE-, NFAT-, or STAT-dependent reporter construct. A, Cells were cultured with the indicated glucose concentration for 24 hours. Data of 5 independent experiments performed in quadruplicates are presented as the mean ± SEM. Asterisks indicate a significant difference to the next lower glucose concentration. B, Cells were cultured without glucose for the indicated period of time. Data of 3 independent experiments performed in quadruplicates were compiled by setting the corresponding reporter activity measured at time point 0 to 100% and expressed as the mean ± SEM. Hash signs indicate a significant difference to 100%. C, mHypoA-2/10-CRE cells stably expressing the CRE-dependent reporter were cultured with the indicated glucose or pyruvate concentration for 24 hours. Data of 15 independent experiments performed in quadruplicates are presented as the mean ± SEM. Asterisks indicate a significant difference to the values of 25mM glucose. D, mHypoA-2/10-CRE cells were cultured with 1.0mM glucose or fructose-1,6-bisphophate for 24 hours. Data of 5 independent experiments performed in quadruplicates are presented as the mean ± SEM. Asterisks indicate a significant difference to the values of 25mM glucose.

were subtracted from those measured for actin under the same conditions (ΔCp stimulated). Cp values measured for TRH under basal conditions were subtracted from those measured for actin under the same conditions (ΔCp basal). Afterwards, ΔCp-basal values were subtracted from ΔCp-stimulated values.

Data analysis

Data were analyzed using Prism4.0 (GraphPad Software, Inc). Statistical significance of differences was assessed by 1- or 2-sample Student's *t* test. Asterisks (***, $P < .001$; **, $P < .01$; *, $P < .05$) in the case of the 2-sample and hash signs (###, $P < .001$; ##, $P < .01$; #, $P < .05$) in the case of the 1-sample test were used to indicate significant differences.

Results

Glucose exerts specific effects on CRE-dependent reporter gene activity in murine hypothalamic mHypoA-2/10 cells

In vivo brain glucose concentrations vary between 0.2mM and 2.5mM under physiological conditions (19, 36–40). mHypoA-2/10 cells are cultured under high-glucose conditions (25.0mM), clearly exceeding physiological extracellular brain glucose concentrations. To investigate the effects of extracellular glucose on gene expression in hypothalamic cells, we transfected NFAT, STAT, or CREB-dependent reporter gene constructs into mHypoA-2/10 cells and cultured these cells with reduced glucose concentrations for 24 hours. As shown in Figure 1A, complete removal of glucose decreased reporter activity independently from the promoter region tested, indicating that a minimal glucose concentration is required to maintain salient cell functions such as transcription and translation. In contrast, no differences in STAT- or NFAT-dependent reporter activity were observed when glucose concentrations were varied between 0.1mM and 25.0mM, indicating that the activity of both promoters is glucose insensitive. However, the activity of the CRE reporter construct increased incrementally from 0.1mM to 25.0mM glucose, providing first evidence that glucose specifically affects CRE-dependent gene expression in mHypoA-2/10 cells. Next, we analyzed how the different reporter gene constructs would react to complete glucose withdrawal over time. As opposed to NFAT or STAT reporters, we observed decreased CRE reporter activity after 4 and 6 hours (Figure 1B), confirming the selective

impact of glucose on CRE-dependent promoter activity and further illustrating that glucose withdrawal for up to 6 hours does not compromise protein biosynthesis in general. In order to gain deeper insight into glucose-induced regulation of CRE-dependent reporter activity, we used a previously established mHypoA-2/10 cell pool that stably expresses the CRE reporter construct (mHypoA-2/10-CRE cells). After growing mHypoA-2/10-CRE cells in 0.1mM instead of 25.0mM glucose for 24 hours, an approximately 50% reduction in basal CRE activity was observed that could not be recovered by addition of 1.0mM pyruvate (Figure 1C). These data suggest that cytosolic, but not mitochondrial, glucose metabolism affects CRE-dependent reporter activity. Thus, we next tested effects of the cytosolic glucose metabolite F-1,6-BP on CRE activity. As shown in Figure 1D, F-1,6-BP significantly enhanced the CRE reporter, albeit with reduced efficacy compared with glucose most probably due to less efficient uptake into cells. However, because glucose and F-1,6-BP, but not pyruvate, affects the CRE-dependent reporter, we assume that cytosolic products of glucose metabolism are required to activate CRE-dependent gene expression.

Physiologically relevant changes in extracellular glucose concentrations enhance basal CRE-dependent gene expression in mHypoA-2/10-CRE cells

So far, we had established that glucose withdrawal from 25.0mM to 0.1mM for 24 hours selectively reduced CRE-dependent promoter activity. Next, we sought to explore how mHypoA-2/10-CRE cells that are adapted to low-glucose concentrations, would react when stimulated with increasing physiologically relevant glucose concentrations. To this end, we weaned cells off in 0.1mM glucose for 18 hours and then stimulated these cells with increasing glucose concentrations (0mM–2.0mM) for an additional 6-hour period (Figure 2, A and C). An incremental increase in extracellular glucose concentration dependently enhanced CRE-dependent reporter activity in low-glucose-adapted cells with an EC_{50} value of 0.4 ± 0.1mM. In parallel, we administered the nonmetabolizable glucose derivative 2-DG that had no impact on CRE reporter activity, providing further evidence that the effects of glucose depend on metabolism. In a second approach, we tested how glucose might affect basal CRE-dependent activity if extracellular glucose was adapted to physiologically relevant levels over a longer time period. Thus, we cultured cells with glucose concentrations between 0.1mM and 2.0mM for 24 hours without a previous weaning period (Figure 2, B and C). We also observed dose-dependent changes in CRE-dependent activity, al-

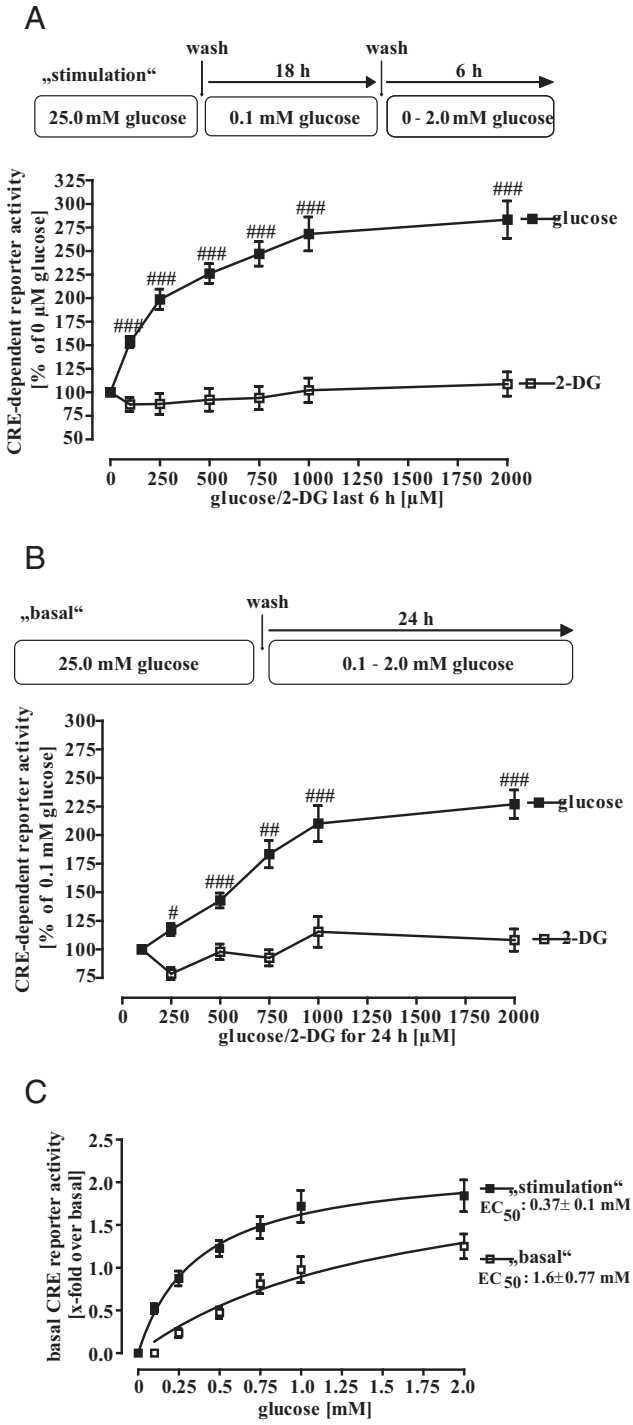

Figure 2. Physiological-relevant changes in glucose concentrations enhance basal CRE-dependent reporter activity in mHypoA-2/10-CRE cells. A, Cells were cultured for 18 hours with 0.1mM glucose and afterwards with 0mM, 0.1mM, 0.25mM, 0.5mM, 1.0mM, or 2.0mM glucose for additional 6 hours. Data of 10 independent experiments performed in quadruplicates were compiled by setting CRE activity of no glucose condition to 100% and expressed as the mean ± SEM. Hash signs indicate a significant difference to 100%. B, Cells were cultured with 0.1mM, 0.25mM, 0.5mM, 1.0mM, or 2.0mM glucose for 24 hours. Data of 10 independent experiments performed in quadruplicates were compiled by setting CRE activity of 0.1mM glucose to 100% and expressed as the mean ± SEM. Hash signs indicate a significant difference to 100%. C, Data obtained in A and B are presented as x-fold over basal and fitted to a 1-site saturation curve to calculate corresponding EC_{50} values.

beit with a slightly increased EC_{50} value of 1.6 ± 0.8mM compared with "stimulation" conditions. Similarly, 2-DG did not mimic the effects of glucose on the CRE-dependent reporter under "basal" conditions.

Prolonged elevated glucose levels reduce the sensitivity of hypothalamic cells towards glucose

Based on data shown in Figure 2, it appeared that low-glucose-adapted mHypoA-2/10-CRE cells displayed

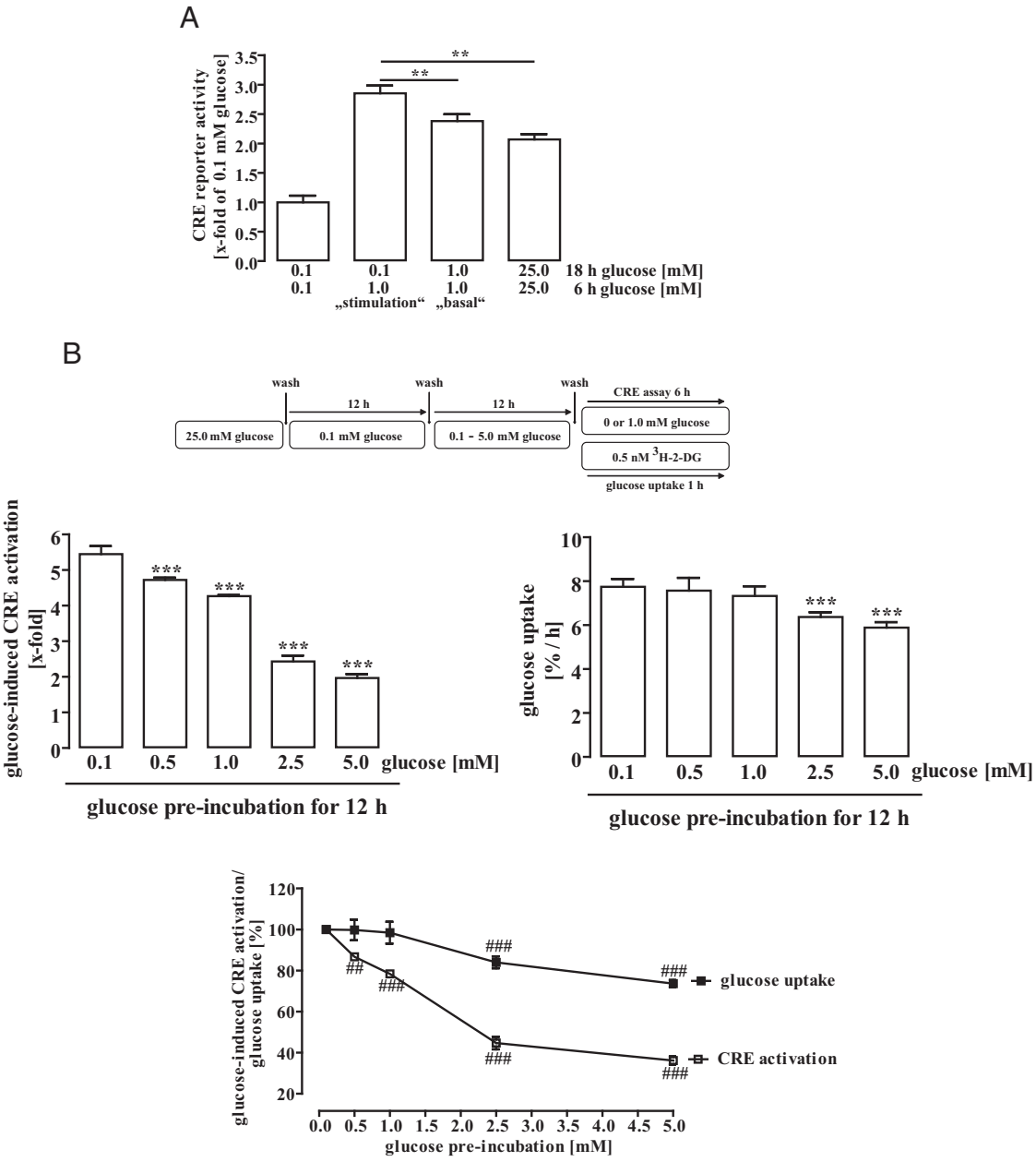

Figure 3. Prolonged elevated glucose levels reduced sensitivity of hypothalamic cells towards glucose. A, mHypoA-2/10-CRE cells were incubated with the indicated glucose concentration for 24 hours. Data of 5 independent experiments performed in quadruplicates were compiled by calculating the x-fold over the values obtained for 0.1mM glucose (24 h) and expressed as the mean ± SEM. Asterisks indicate a significant difference to the values obtained for 0.1 (18 h) and 1.0mM (6 h) glucose. B, mHypoA-2/10-CRE cells were incubated with 0.1mM glucose for 12 hours. Afterwards, cells were incubated with 0.1mM, 0.5mM, 1.0mM, 2.5mM, or 5.0mM glucose for additional 12 hours. Next, cells were cultured for 30 minutes without glucose, stimulated or not with 1.0mM glucose for 6 hours, and CRE-dependent reporter activity measured (left panel) or glucose uptake monitored by measuring ^{3}H-2-DG uptake for 1 hour (right panel). Left panel, Data of 4 independent experiments performed in quadruplicates were compiled by calculating the x-fold over basal of 1.0mM glucose for each condition and expressed as the mean ± SEM. Asterisks indicate a significant difference to the values obtained for 0.1mM glucose preincubation. Right panel, Data of 4 independent experiments performed in quadruplicates were compiled by calculating glucose uptake as percentage per hour for each condition and expressed as the mean ± SEM. Asterisks indicate a significant difference to the values obtained for 0.1mM glucose preincubation. Lower panel, Data of both experiments was normalized by setting values obtained with 0.1mM glucose as 100%. Hash signs indicate a significant difference to 100%.

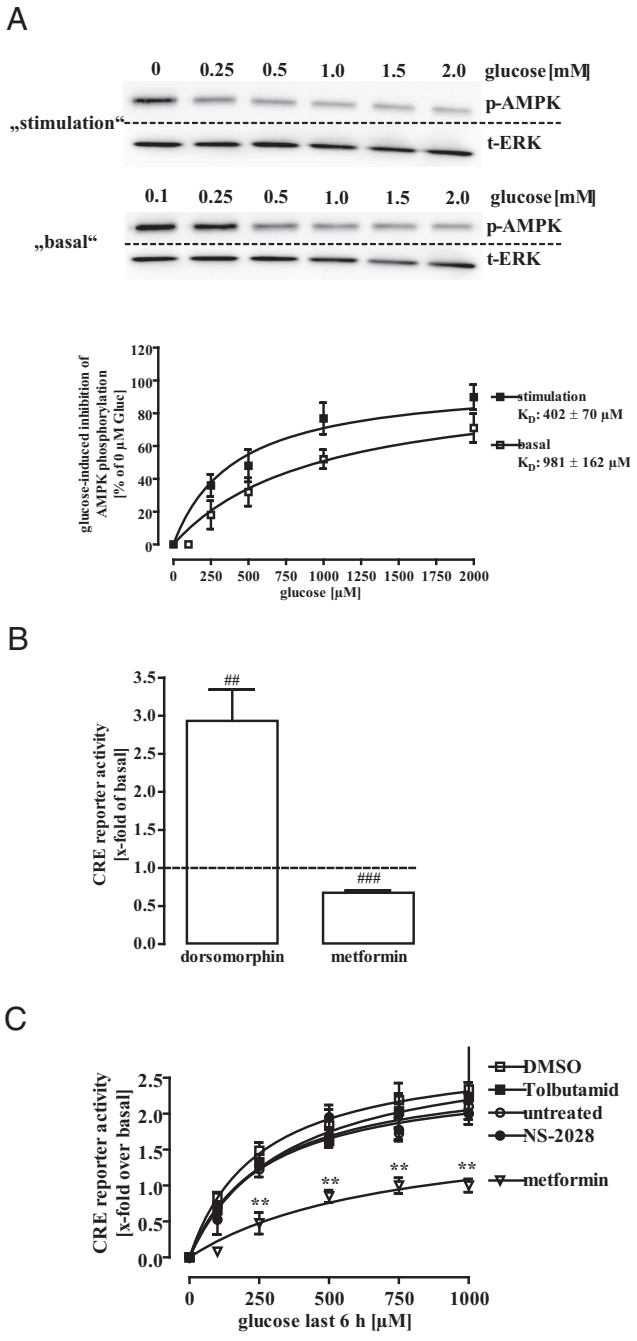

Figure 4. Role for AMPK in glucose-induced CRE-dependent reporter activation. A, Lysates of mHypoA-2/10-CRE cells, either cultured for 18 hours with 0.1mM glucose and then stimulated with 0mM, 0.25mM, 0.5mM, 1.0mM, 1.5mM, or 2.0mM glucose for additional 6 hours or with 0.1mM, 0.25mM, 0.5mM, 1.0mM, 1.5mM, or 2.0mM glucose for 24 hours were subjected to Western blot analysis using either a phospho-specific antiserum against p-AMPK or against the total ERK-2 protein to control for the total protein amount. One representative blot is shown. Data of 5 independent experiments were quantified by densitometry, ratios between p-AMPK and t-ERK-2 signals calculated, glucose-induced AMPK dephosphorylation normalized to not stimulated cells (no glucose), and data fitted to a 1-site saturation curve to calculate corresponding EC_{50} values. B, mHypoA-2/10-CRE cells were cultured under high-glucose conditions (25.0mM) and either stimulated with 10μM dorsomorphin or 100μM metformin. Data of 5 independent experiments performed in quadruplicates were compiled by calculation the x-fold of basal expressed as the mean ± SEM. Hash signs indicate a significant difference to 1.0. C, mHypoA-2/10-CRE cells were cultured for 18 hours with 0.1mM glucose and then with 0mM, 0.25mM, 0.5mM, 1.0mM, or 2.0mM glucose for additional 6 hours. Before glucose stimulation, cells were treated with 500μM tolbutamide, 10μM NS-2028, 100μM metformin, or the carrier control (0.1% DMSO). Data of 4 independent experiments performed in quadruplicates were compiled and expressed as the mean ± SEM. Asterisks indicate a significant difference between the control and metformin-treated cells.

higher CRE-dependent reporter activity when stimulated with 1.0mM glucose for 6 hours compared with cells that had been cultured in 1.0mM or even 25.0mM glucose for 24 hours (Figure 1C), suggesting that sustained elevated glucose concentrations desensitize glucose-induced CRE signaling. To rigorously test this hypothesis, we performed the basal and stimulation experiment with 1.0mM glucose in parallel (Figure 3A). Under stimulation conditions, 1.0mM glucose promoted significantly more CRE-dependent reporter activity compared with the basal experiment and even more compared with cells permanently cultured in 25.0mM glucose. In order to analyze whether reduced sensitivity of mHypoA-2/10 cells towards glucose is a dynamic process, we adapted cells to low-glucose conditions (0.1mM) for 12 hours, elevated glucose concentrations afterwards for additional 12 hours (0.5mM to 5.0mM), and then measured CRE-dependent reporter activity induced by 1.0mM glucose in parallel with time-dependent ^{3}H-2-DG uptake (Figure 3B). Preincubation of glucose-starved cells with physiologically relevant brain glucose concentrations resulted in a stepwise decrease of glucose-induced CRE activation with a maximal desensitization of approximately 63% at 5.0mM. Interestingly, concentrations higher than 1.0mM also reduced glucose uptake with a maximal reduction of approximately 24%.

Potential role of AMPK in glucose-induced CRE-dependent reporter activation

So far, we discovered that glucose and F-1,6-BP, but not 2-DG or pyruvate, enhance CRE-dependent reporter activity, suggesting that cytosolic products of glucose metabolism are involved. Metabolized glucose decreases the cytosolic AMP to ATP ratio and thus inhibits AMPK activity. In line with this notion, we observed decreased AMPK phosphorylation under both glucose stimulation and basal conditions with EC_{50} values of 0.4 ± 0.07mM and 1.0 ± 0.2mM, respectively (Figure 4A). To further evaluate a possible role of AMPK for glucose-induced CRE-dependent reporter activation, we applied the AMPK inhibitor dorsomorphin or the activator metformin and monitored CRE-dependent reporter activity (Figure 4B). Dorsomorphin enhanced and metformin in-

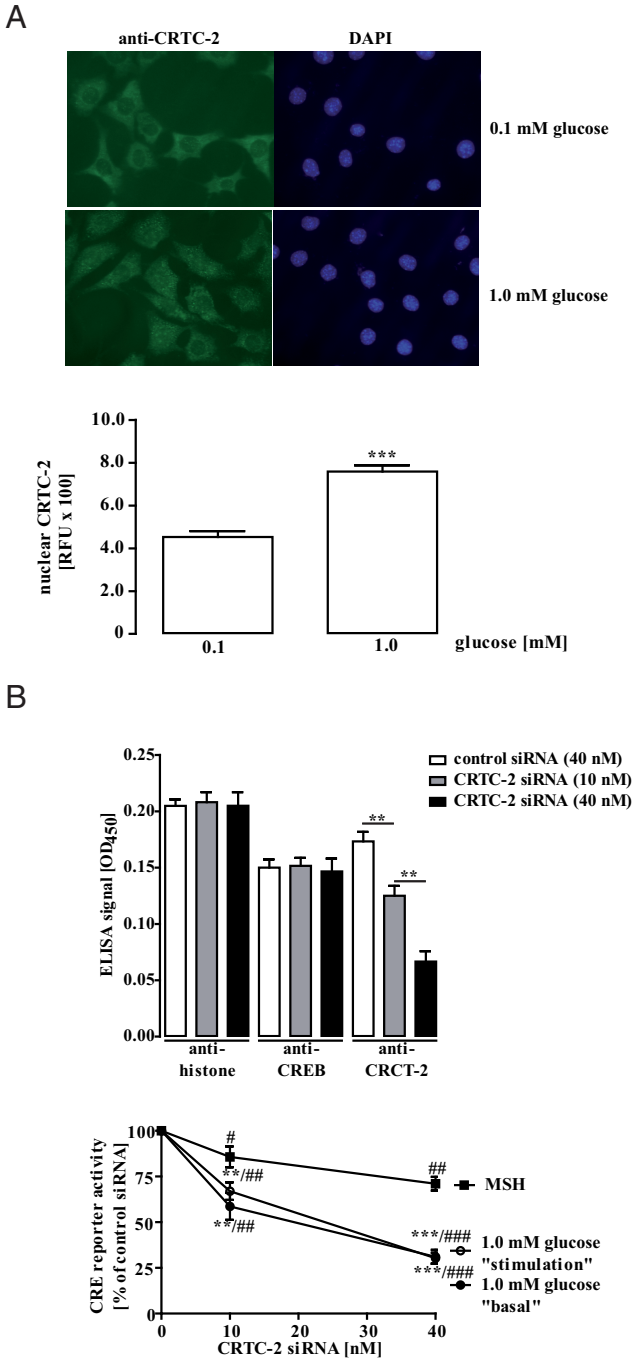

Figure 5. Role for CRTC-2 in glucose-induced CRE-dependent reporter activation. A, Immunostaining of mHypoA-2/10-CRE cells cultured with 0.1mM or 1.0mM glucose for 24 hours was performed using anti-CRCT-2/ Alexa Fluor 488-conjugated antimouse antibodies or DAPI. Immunostaining of cells cultured in 0.1mM (78 cells) or 1.0mM (82 cells) glucose was quantified and the corresponding background values subtracted. Random fluorescence units (RFUs) are expressed as the mean ± SEM, and asterisks indicate a significant difference between both glucose concentrations. B, mHypoA-2/10-CRE cells were transfected with 10nM or 40nM a cocktail of 3 distinct siRNAs against the murine CRCT-2 protein or with 40nM a random control siRNA. Cells were seeded on 48-well plates to monitor CRTC-2, CREB, or histone expression by whole cell ELISA (upper panel) and on 96-well plates to determine CRE-dependent reporter activation (lower panel). Upper panel, Cells were cultured for 24 hours with 0.1mM glucose. One representative experiment performed in quadruplicates is shown as the ± SEM. Background values of nonpermeabilized cells were subtracted. Lower panel, Cells were cultured either for 24 with 0.1mM or 1.0mM glucose (basal), for 18 hours with 0.1mM glucose and then 6 hours with no glucose or 1.0mM glucose (stimulation) or for 24 hours with 1.0mM and treated or not with 1μM MSH for the last 6 hours. Data of 5 independent experiments performed in quadruplicates were normalized by setting CRE-dependent reporter activation after expression of the control siRNA to 100% and are expressed as the mean ± SEM. Hash signs indicate a significant difference between MSH and glucose-treated cells. Asterisks indicate a significant difference between MSH and glucose-treated cells.

hibited CRE-dependent gene transcription in mHypoA-2/10-CRE cells, underscoring the role of AMPK as a negative regulator of CRE activity in hypothalamic cells. Furthermore, although metformin inhibited glucose-induced CRE-dependent reporter activation, blocking K_{ATP} channels by the sulfonylurea tolbutamide or neuronal NO synthase activity by NS-2028 had no effect (Figure 4C), strongly suggesting a role for AMPK in glucose-induced CRE activation. Tonic phosphorylation of hypothalamic CRTC-2 by AMPK has been reported to retain CRTC-2 in the cytosol (25). In line with these data, CRTC-2 was almost exclusively located in the cytosol of mHypoA-2/10-CRE cells cultured with 0.1mM glucose but translocated to the nucleus when cells were cultured with 1.0mM glucose (Figure 5A), suggesting that CRTC-2 is a target of glucose in hypothalamic cells. To further assess the role of CRCT-2 in glucose-induced CRE-dependent reporter activation, we aimed at downregulating CRTC-2 protein expression by specific siRNAs. A cocktail of 3 distinct siRNAs against CRTC-2 dose dependently decreased CRCT-2, but not CREB expression, when compared with cells receiving control siRNA (Figure 5B, upper panel). Of note, decreased CRTC-2 expression inhibited CRE-dependent reporter activation induced by glucose and to a lesser extent by MSH activating CRE via CREB (Figure 5B, lower panel). Hence, we provide first experimental data to support the concept that enhancing effects of glucose on CRE-dependent gene expression require activation of the CRTC-2 transcription factor, most probably via inhibition of AMPK.

Physiologically relevant increases in extracellular glucose concentrations enhance MSH-induced CRE activation

MSH is a key player within the HPT axis and the only known physiological enhancer of hypothalamic CRE activity so far. Because data shown in Figure 5B propose that glucose and MSH induce CRE-dependent reporter activation via distinct cellular mechanisms, we next assessed putative effects of glucose on MSH-induced CRE

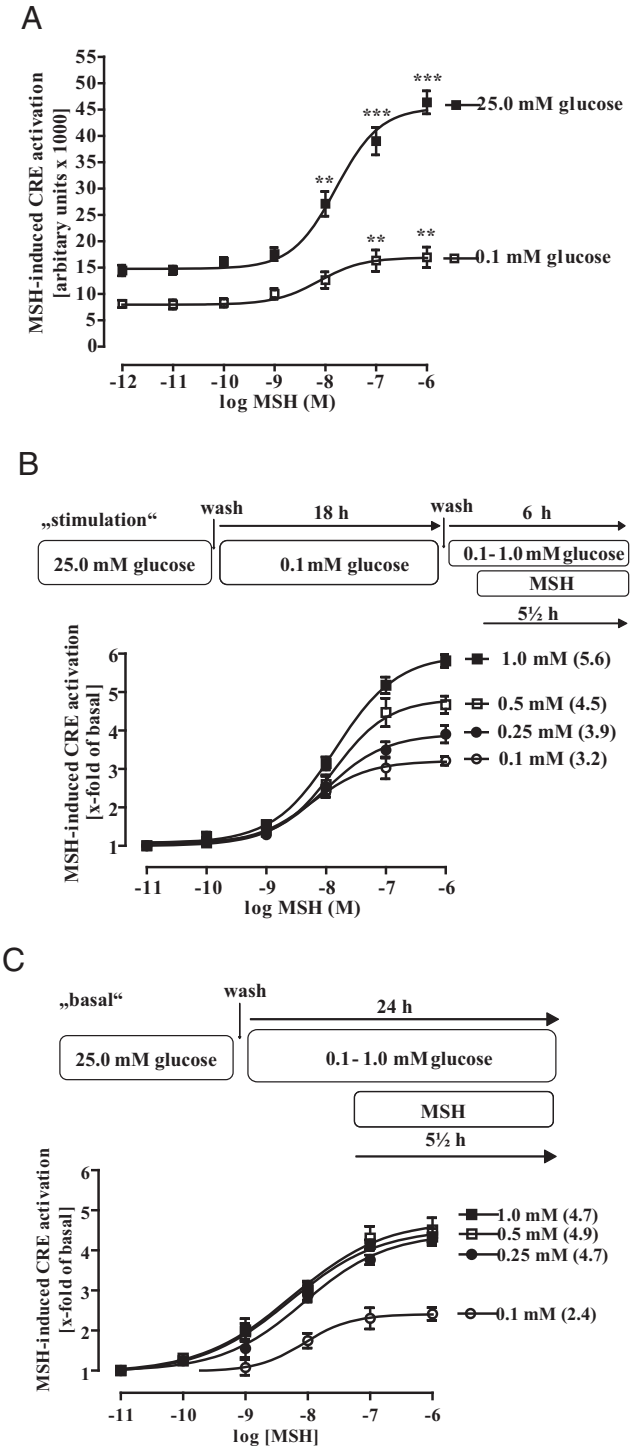

Figure 6. Physiological-relevant changes in glucose concentrations enhance MSH-induced CRE-dependent reporter activation. A, mHypoA-2/12-CRE cells were either cultured with 25.0mM or 0.1mM glucose for 18 and then stimulated with various MSH concentrations for additional 6 hours without changing the glucose level. Data of 5 independent experiments performed in quadruplicates were compiled, expressed as the mean ± SEM, and fitted to dose-response curves with fixed slopes. Asterisks indicate a significant difference to the basal values obtained with no MSH. B, Cells were cultured for 18 hours with 0.1mM glucose and then with 0.1mM, 0.25mM, 0.5mM, or 1.0mM glucose (as indicated) for additional 6 hours to obtain basal CRE activity. To measure MSH-induced CRE-dependent reporter activation distinct pools of cells were also stimulated with the indicated MSH concentration for the last 5.5 hours without changing the glucose level. Data of 5 independent experiments performed in quadruplicates were compiled, by calculating the corresponding x-fold of basal value, expressed as the mean ± SEM, and fitted to dose-response curves with fixed slopes. Numbers in parenthesis indicate the x-fold over basal for 1.0μM MSH. C, Cells were cultured with 0.1mM, 0.25mM, 0.5mM, or 1.0mM glucose (as indicated) for 24 hours to obtain basal CRE activity. To measure MSH-induced CRE-dependent reporter activation, distinct pools of cells were stimulated or not with the indicated MSH concentrations for the last 5.5 hours without changing the glucose concentration. Data of 5 independent experiments performed in quadruplicates were compiled, by calculating the corresponding x-fold of basal value, expressed as the mean ± SEM, and fitted to dose-response curves with fixed slopes. Numbers in parenthesis indicate the x-fold over basal for 1.0μM MSH.

activation. To this end we first constructed concentration-response curves in mHypoA-2/10-CRE cells cultured in 25.0mM or 0.1mM glucose for 24 hours (Figure 6A). Reducing extracellular glucose concentrations had no effect on the potency of MSH to activate CRE but significantly reduced its efficacy. Thus, we determined concentration-response relationships of MSH-induced CRE activation under stimulation conditions, applying glucose concentrations between 0.1mM and 1.0mM (Figure 6B). Increasing extracellular glucose gradually improved the efficacy of MSH-induced CRE activation without affecting potency. Under basal conditions, 0.25mM glucose was sufficient to yield the maximal MSH effect on the CRE promoter (Figure 6C).

MSH acts via G protein-coupled receptors dependent on the nucleoside triphosphate GTP whose metabolism is closely linked to glucose and ATP. Therefore, we wondered whether glucose deprivation would affect G protein-dependent signaling in general and measured Gq-dependent calcium signaling induced by bradykinin (Figure 7A) and Gs-dependent cAMP accumulation by MSH (Figure 7B). Both signaling pathways were unaltered when extracellular glucose concentrations were reduced to 0.1mM for 24 hours, indicating that the stimulatory effects of glucose on MSH-induced CRE activation are not caused by impaired G protein signaling. To garner further evidence that the potentiation of MSH-induced CRE activation by glucose is not due to direct effects on MSH signaling, we measured MSH-induced phosphorylation of CREB. As shown in Figure 7C, MSH-induced CREB phosphorylation was undistinguishable, when cells were cultured either with 0.1mM or 25.0mM glucose for 24 hours.

Glucose-induced TRH expression in hypothalamic cells

After having established that glucose enhances CRE-dependent reporter activity in mHypoA-2/10-CRE cells,

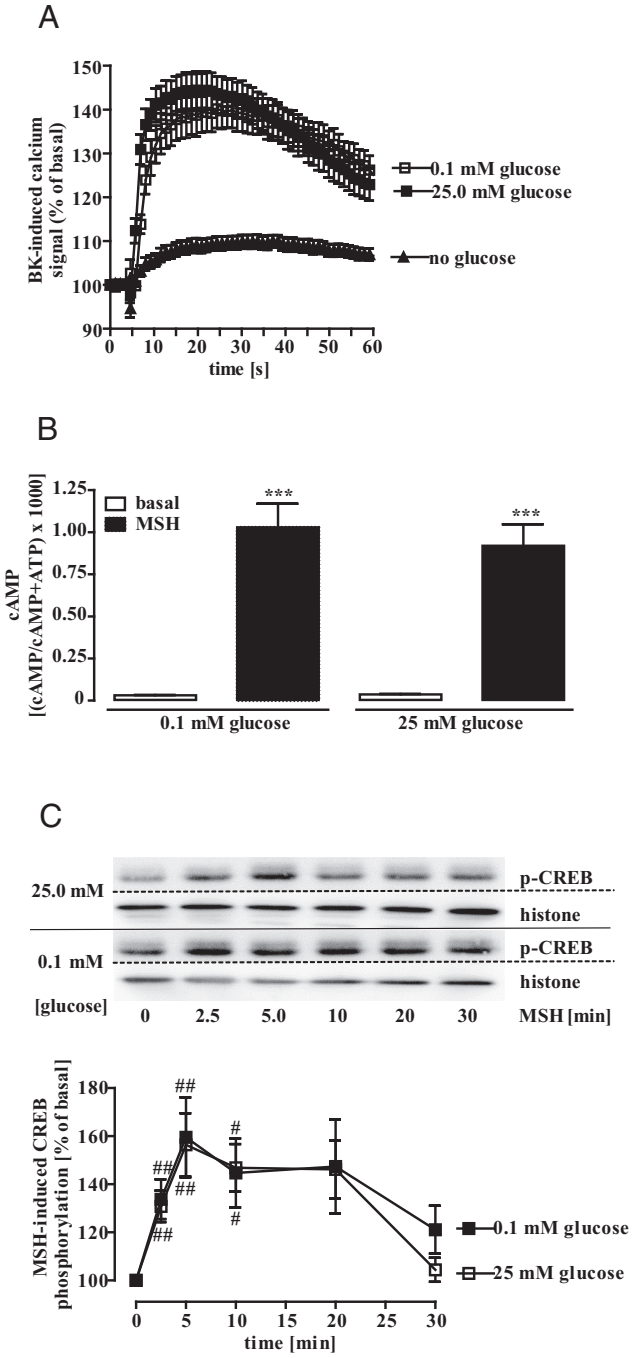

Figure 7. Effects of glucose on MSH-induced CRE-dependent reporter activation are not due to direct effects on MSH signaling. A, Bradykinin-induced calcium signaling (1.0μM) was analyzed in fura 2-loaded mHypoA-2/12-CRE cells cultured with 25.0mM, 0.1mM, or no glucose for 24 hours. Data of 3 independent experiments performed in quadruplicates were compiled by setting the first value measured to 100% and expressed as the mean ± SEM. B, MSH-induced cAMP accumulation was measured in mHypoA-2/12-CRE cells cultured with 25.0mM or 0.1mM glucose for 24 hours. Data of 4 independent experiments performed in quadruplicates were compiled and expressed as the mean ± SEM. Asterisks indicate a significant difference to basal. C, Lysates of mHypoA-2/10-CRE cells, cultured for 24 hours with 0.1mM or 25mM glucose and then stimulated with 1.0μM MSH for the indicated period of time were subjected to Western blot analysis using either a phospho-specific antiserum against p-CREB or against the total histone-3 protein to control for the total protein amount. One representative blot is shown. Data of 5 independent experiments were quantified by densitometry, ratios between p-AMPK and histone signals calculated, MSH-induced CREB phosphorylation normalized to not stimulated cells, and expressed as the mean ± SEM. Hash signs indicate a significant difference to 100%.

we next investigated the effect of glucose on the expression of the CRE-controlled gene TRH. To this end, we monitored TRH protein expression by Western blot analysis under basal and glucose stimulation conditions (Figure 8). Glucose induced TRH expression under both conditions in a concentration-dependent manner with an EC_{50} value of 699 ± 342μM and 1203 ± 877μM, indicating that changes in glucose levels that affect CRE-dependent reporter activity also affect TRH protein expression. In order to investigate whether these effects occur on the transcriptional or translational level, we additionally analyzed the effects of glucose on TRH mRNA levels. As shown in Figure 9A, glucose significantly increased TRH mRNA levels under basal and stimulation conditions, clearly demonstrating that glucose affects TRH expression on the transcriptional level. Glucose also enhanced effects of MSH on TRH mRNA levels (Figure 9B), highlighting the important role of glucose for TRH expression and providing first evidence that glucose may engage the HPT axis by fostering hypothalamic TRH expression.

Discussion

Glucose has been reported to act on hypothalamic nuclei such as the nucleus arcuatus, the PVN and the ventromedial or lateral hypothalamus to affect the central regulation of food intake (19–23, 41–45). Interstitial hypothalamic glucose concentrations vary depending on food intake or insulin levels suggesting that naturally occurring fluctuations in hypothalamic glucose concentrations may directly induce anorexigenic signaling or modulate the impact of food intake-regulating hormones like leptin and insulin (19, 36, 37, 39, 40). However, little is known about the cellular events underlying the glucose-induced regulation of food intake.

Effects of glucose on central appetite control have frequently been attributed to the same mechanisms mediating glucose effects in pancreatic β-cells. Extracellular glucose is transported into the cell, metabolized to ATP that inhibits K_{ATP} channels and thereby depolarizes the plasma membrane leading to reduced insulin secretion (19–24). Besides this well-established effect of glucose on K_{ATP} channels, glucose-derived ATP also activates the CREB coactivator CRTC-2 in hypothalamic neurons (25). So far, however, it has remained elusive whether

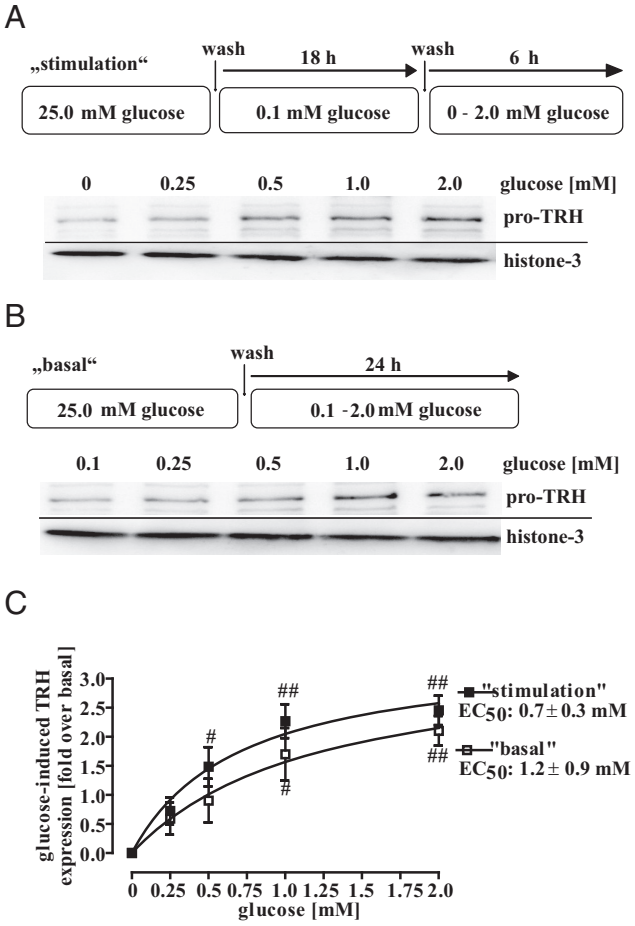

Figure 8. Glucose-induced TRH protein expression in hypothalamic cells. A, Cells were cultured for 18 hours with 0.1mM glucose and afterwards with 0mM, 0.25mM, 0.5mM, 1.0mM, or 2.0mM glucose for additional 6 hours. B, Cells were cultured with 0mM, 0.25mM, 0.5mM, 1.0mM, or 2.0mM glucose for 24 hours. Lysates were subjected to Western blot analysis using either a specific antiserum against pro-TRH or against the total histone-3 protein to control for the total protein amount. One representative blot is shown. C, Data of 5 independent experiments were quantified by densitometry, ratios between TRH and histone signals calculated, glucose-induced TRH expression normalized to not stimulated cells (no glucose), and data fitted to a 1-site saturation curve to calculate the EC_{50} value. Hash signs indicate a significant difference to 0 fold over basal.

physiologically relevant fluctuations of extracellular glucose concentrations would activate CREB via CRTC-2 and whether glucose-induced CRTC-2/CREB activation would subsequently lead to CRE-controlled gene expression. Here, we conclusively show that physiologically relevant variations of extracellular brain glucose concentrations 1) fine-tune basal and MSH-induced CRE activity, 2) induce CRTC-2 translocation, 3) reduce glucose sensitivity of hypothalamic cells because of desensitization and diminished uptake, and 4) control hypothalamic TRH expression.

Our study shows that glucose selectively induces CRE-dependent reporter activity in hypothalamic cells; because incremental changes in the extracellular glucose concentration selectively enhance CRE but not NFAT- or STAT-dependent reporter activity. Thus, the effects of glucose on reporter activity cannot simply be explained by altered protein biosynthesis, but appear to reflect an intrinsic property of the CRE-dependent reporter. In vivo, brain glucose concentrations vary between 0.2mM and 2.5mM under physiological and between 0.1mM and 5.0mM under pathophysiological conditions (19, 36–40). It has been shown that interstitial hypothalamic glucose concentrations of freely feed rats were 1.42mM and of food-deprived (12 h) animals 0.84mM (36). Thus, EC_{50} values for glucose-induced CRE activation obtained in mHypoA-2/10-CRE cells (0.4mM and 1.6mM) indicate that physiological changes of extracellular glucose concentration may affect hypothalamic CRE activity. The same study showed that refeeding of food-deprived animals, led to a profound increase (5- to 10-fold) in hypothalamic glucose levels 2 hours after feeding lasting for an additional 2 hours. Thus, the kinetics of glucose-induced CRE activation in mHypoA-2/10-CRE cells mirrors that of food-induced changes in hypothalamic glucose levels observed in vivo.

Intracerebroventricular administration of glucose, but not of 2-DG, depressed food intake in mice (45). Concordant with these in vivo data, we show that glucose and its cytosolic metabolite F-1,6-BP, but not 2-DG, regulates CRE-dependent reporter activity in mHypoA-2/10-CRE cells. Hence, the effects of glucose on the CRE-dependent reporter depend on its metabolism, most probably due to the increase of cytosolic ATP levels. This observation highlights AMPK as a potential molecular target mediating glucose-induced CRE-dependent reporter activation. In accord with this assumption, we demonstrated that glucose induces AMPK dephosphorylation at Thr^{172}, pertinent to its catalytic activity, with EC_{50} values (0.4mM and 1.0mM) similar to glucose-promoted CRE activation (46). Furthermore, we discovered that the AMPK inhibitor dorsomorphin enhances and the activator metformin decreases CRE-dependent reporter activity, illustrating an inverse correlation between AMPK and CRE activity in hypothalamic cells and suggesting that inhibition of AMPK activity is responsible for glucose-induced CRE activation. This notion is supported by our findings that 1) metformin inhibited glucose-induced CRE-dependent reporter activation, 2) glucose induces translocation of CRCT-2 to the nucleus, and 3) down-regulation of the CRTC-2 protein diminishes glucose-induced CRE-dependent reporter activation. However, at this stage, additional mechanisms underlying glucose-induced CRTC-2 activation cannot be ruled out. Alternative glucose targets could be K_{ATP} channels, neuronal NO synthase or glucose-sensitive TAS1 receptors, which were recently found

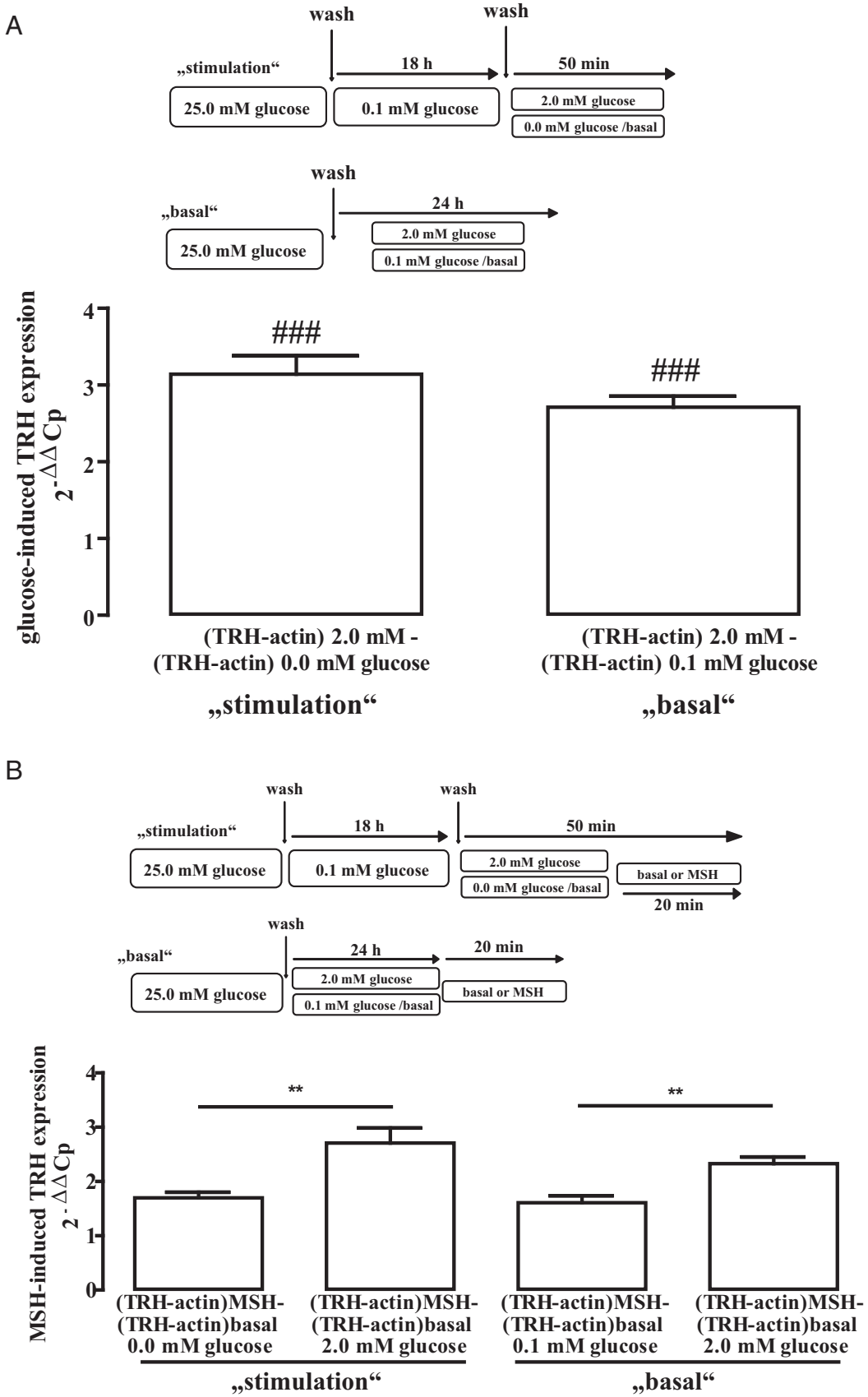

Figure 9. Glucose-induced TRH mRNA expression in hypothalamic cells. A, Cells were either cultured for 18 hours with 0.1mM glucose and afterwards with 0.0mM or 2.0mM glucose for 50 minutes (stimulation) or for 24 hours with 0.1mM or 2.0mM glucose (basal). Total mRNA was isolated and TRH mRNA specifically detected by qRT-PCR. Cp values measured for TRH after increasing glucose concentrations to 2.0mM were

in hypothalamic neurons (47). A K_{ATP} channel blocker (tolbutamide) or a NO synthase inhibitor (NS-2028) did not affect basal or glucose-induced CRE activity, excluding K_{ATP} channels and NO synthase as potential targets for glucose-induced CRE activation. At present, we cannot rigorously rule out a role for TAS1 receptors, but glucose did not interfere with known TAS1-induced second messengers such as calcium or cAMP in mHypoA-2/10 cells (data not shown).

Activation of melanocortin-4-receptors (MC4Rs) by MSH induces the expression of TRH via CRE activation in PVN neurons (48, 49). This sequel of events is of prime importance for proper functioning of the HPT axis and the regulation of human metabolism, as highlighted by the finding that mutations in the MC4R gene are the most frequent monogenic cause of severe obesity (3–9). Accordingly, targeted disruption of the MC4R or the MSH gene in mice causes an obesity-diabetes syndrome characterized by hyperphagia, hyperinsulinemia and hyperglycemia (10, 11). Here, we complement the overall picture by revealing that glucose and the melanocortin system functionally interact on the level of hypothalamic cells. It appears that under stimulation conditions, mimicking a high caloric meal after a longer fasting period, the efficacy of MSH-induced signaling is closely linked to the glucose concentration. Hence we propose that anorexigenic actions of MSH are controlled by glucose in that high glucose concentrations enhance MSH-mediated depression of food intake. Enhanced anorexigenic signaling by MSH in the presence of elevated glucose concentrations appears to be reasonable in the physiological context, but in the case of dysregulation or malfunction, might also contribute to pathophysiological consequences such as obesity. However, further in vivo studies are required to analyze the physiological outcome of the functional interactions between glucose and MSH in the hypothalamus.

With regard to putative signaling events responsible for the effects of glucose on MSH, we provide data indicating that glucose does not directly affect MSH signaling on the level of G protein activation or CREB phosphorylation. However, our study supports a model in which CRE activity in hypothalamic cells is controlled by CREB and CRTC-2, thus coactivation of both transcription factors might be required for full CRE activation. In the framework of such a model, glucose coadministration may support MSH-promoted CREB/CRE activation by inducing significant nuclear CRTC-2 translocation due to its strong inhibitory effects on AMPK.

With this report we provide new insight into the actions of glucose on cellular signaling that affect hypothalamic gene expression. Assuming that our conclusions can be extended to the physiological level, some of them may be of clinical interest (1). Several studies reported that metformin reduces serum TSH levels in patients with T2DM, a phenomenon not observed for sulfonylureas, such as tolbutamide (50–56). If hypothalamic CRE activity is correlated with serum TSH levels, as shown for MSH, differential effects of metformin and tolbutamide in hypothalamic cells may contribute to the distinct actions of the 2 drug classes in T2DM patients (2). The present paper provides first experimental evidence that physiologically relevant glucose fluctuations enhance TRH expression on the mRNA and protein levels in hypothalamic cells. Interestingly, glucose concentrations above 5mM have also been reported to depress TRH release from rat hypothalamic nuclei, suggesting a negative feed-back loop (57). Further, glucose also stimulates the release of pancreatic TRH in rats, indicating that glucose affects central and peripheral TRH production/release (58). Because of the established anorexigenic actions of TRH, our data contribute to a better understanding of glucose-induced appetite control and thus may pave the way for new strategies to treat obesity. Furthermore, it has been recognized that T2DM severely disturbs the regulation of the HPT axis and that suboptimally controlled patients show increased serum TSH levels (17, 18). In line with this notion, it has been recommended that the diagnosis of thyroid function in T2DM patients be delayed until improvement of the metabolic status (15). Thus, glucose-induced hypothalamic TRH expression could likely be responsible for the association between hyperglycaemia and hyperthyroidism (3). We show that incubation of cells, which have been adapted to low-glucose concentrations, with increasing glucose concentrations exhibited a significant loss of sensitivity towards glucose. At this point we can only speculate about the molecular

Figure 9 (Continued). subtracted from those measured for actin (ΔCp stimulated). Cp values measured for TRH under basal conditions (0.0mM glucose for stimulation and 0.1mM glucose for basal) were subtracted from those measured for actin under the same conditions (ΔCp basal). Afterwards, ΔCp-basal values were subtracted from ΔCp-stimulated values and presented as $2^{-\Delta\Delta Cp}$. B, Cells were first cultured for 18 hours with 0.1mM glucose, then for 30 minutes with 0.0mM or 2.0mM glucose (stimulation) and afterwards treated or not with 1μM MSH for 20 minutes or for 24 hours with 0.1mM or 2.0mM glucose (basal) and then treated or not with 1μM MSH for 20 minutes. Total mRNA was isolated and TRH mRNA specifically detected by qRT-PCR. Cp values measured for TRH after MSH stimulation were subtracted from those measured for actin under the same conditions (ΔCp stimulated). Cp values measured for TRH under basal conditions (no MSH) were subtracted from those measured for actin under the same conditions (ΔCp basal). Afterwards, ΔCp-basal values were subtracted from ΔCp-stimulated values and presented as $2^{-\Delta\Delta Cp}$. Data of 4 independent experiments performed in triplicates were compiled and expressed as the mean ± SEM. Hash signs indicate a significant difference to 0; asterisks between basal and MSH-stimulated cells.

basis of glucose-induced desensitization. Of note, AMPK has been shown to enhance glucose uptake into skeletal muscle cells (59), thus sustained inhibition of AMPK activity due to high glucose levels could reduce glucose uptake into hypothalamic cells and thus decrease glucose-induced CRE activation. Indeed, we observed reduced glucose uptake when cells were incubated with glucose concentrations above 1.0mM, but these effects were small and concentrations below 1.0mM, also eliciting CRE desensitization, had no effect on glucose uptake. Therefore, we assume that reduced uptake is not sufficient to fully account for glucose desensitization implicating uptake-independent additional mechanisms. However, these data suggest that prolonged hyperglycaemic conditions, for instance due to uncontrolled eating behavior, weaken the central anorexigenic effects of glucose and thus may provide a new association between T2DM and obesity. Hence, it will be enlightening in future studies to see whether prolonged elevations of hypothalamic glucose concentrations impair glucose sensitivity of food intake-controlling neurons in vivo and thereby interfere with central appetite control.

Acknowledgments

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