Orexin/Hypocretin Activates mTOR Complex 1 (mTORC1) via an Erk/Akt-independent and Calcium-stimulated Lysosome v-ATPase Pathway*

Received for publication, July 25, 2014, and in revised form, September 30, 2014. Published, JBC Papers in Press, October 2, 2014, DOI 10.1074/jbc.M114.600015

Zhiqiang Wang\(^3\), Shimeng Liu\(^{1,2}\), Miyo Kakizaki\(^{\dagger}\), Yuuki Hirose\(^\ddagger\), Yukiko Ishikawa\(^1\), Hiromasa Funato\(^{11}\), Masashi Yanagisawa\(^1\), Yonghao Yu\(^1\), and Qinghua Liu\(^{1,2}\)

From the \(^{\ddagger}\)Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390, the \(^{\dagger}\)College of Life Sciences, Wuhan University, Wuhan 430072, China, the \(^{\ddagger}\)International Institute of Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, Ibaraki 305-8575, Japan, and the \(^{\dagger}\)Department of Anatomy, Toho University School of Medicine, Tokyo 143-8540, Japan

**Background:** Narcolepsy is caused by deficiency of neuropeptide orexin/hypocretin, of which downstream signaling pathways are unclear.

**Results:** Orexin activates the mTOR pathway in the mouse brain and multiple cell lines expressing OX1R or OX2R.

**Conclusion:** Orexin activates mTOR complex 1 (mTORC1) via calcium-stimulated lysosome v-ATPase pathway.

**Significance:** mTORC1 may play a key role in the functions of orexin in physiology and metabolism.

The lack of the neuropeptide orexin, also known as hypocretin, results in narcolepsy, a chronic sleep disorder characterized by frequent sleep/cataplexy attacks and rapid eye movement sleep abnormalities. However, the downstream pathways of orexin signaling are not clearly understood. Here, we show that orexin activates the mTOR pathway, a central regulator of cell growth and metabolism, in the mouse brain and multiple recombinant cell lines that express the G protein-coupled receptors (GPCRs), orexin 1 receptor (OX1R) or orexin 2 receptor (OX2R). This orexin/GPCR-stimulated mTOR activation is sensitive to rapamycin, an inhibitor of mTOR complex 1 (mTORC1) but is independent of two well known mTORC1 activators, Erk and Akt. Rather, our studies indicate that orexin activates mTORC1 via extracellular calcium influx and the lysosome pathway involving v-ATPase and Rag GTPases. Moreover, a cytoplasmic calcium transient is sufficient to mimic orexin/GPCR signaling to mTORC1 activation in a v-ATPase-dependent manner. Together, our studies suggest that the mTORC1 pathway functions downstream of orexin/GPCR signaling, which plays a crucial role in many physiological and metabolic processes.

Narcolepsy, a chronic sleep disorder that affects 1/600 to 1/2,000 individuals, is characterized by excessive daytime sleepiness and sleep attacks, as well as abnormal transition from wake to rapid eye movement sleep, manifested by cataplexy attacks, sleep paralysis, and hypnagogic hallucinations (1). Deficiencies in signaling by the neuropeptide orexin/hypocretin have been linked to narcolepsy in humans, dogs, and mice (2–4). Two orexin peptides, orexin-A and orexin-B, which are derived from the same prepropeptide, are produced exclusively by a small number (~50,000) of neurons in the lateral hypothalamus of human brain (5, 6). The unexplained loss of these orexin neurons is the most common cause for human narcolepsy. The orexin neurons spread projections throughout the whole brain and regulate a variety of important physiological processes such as sleep/wake cycle, reproduction, brown fat and bone development, feeding, and energy metabolism (7–10).

At the cellular level, orexin-A and B exert their effects by binding and activating either of two related GPCRs, OX1R and OX2R (11). Whereas orexin-A binds to OX1R and OX2R non-selectively, orexin-B preferentially binds OX2R with much higher affinity (6, 12). A well studied cellular response to orexin is a dose-dependent transient increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). It is thought that this [Ca\(^{2+}\)]\(_i\) surge is a result of extracellular Ca\(^{2+}\) influx through TRPC3 and L-type Ca\(^{2+}\) channels following the activation of phospholipase C by orexin/GPCR signaling (12–15).

Furthermore, orexin has been shown to activate multiple protein kinases such as PKA, PKC, mitogen-activated protein kinase (MAPK)/Erk, and PDK1 in various cell contexts (16). Activation of a particular signaling pathway depends on the combination of the peptide (orexin-A or B), the receptor sub-
type (OX1R or OX2R), and the cellular context. Because it is difficult to obtain cell lines naturally expressing the orexin receptors, the majority of these studies use recombinant cell lines engineered to express either OX1R or OX2R (16). Despite the intense series of genetic and biochemical studies in the last 16 years, the downstream signaling pathways of orexin/GPCR are still not clearly understood. It is also uncertain which pathway plays a crucial role in mediating the many important functions of orexin in physiology and metabolism.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and a master regulator of cell growth and metabolism (17, 18). mTOR exists in two distinct complexes: complex 1 (mTORC1) and complex 2 (mTORC2) (17). mTORC1, which consists of mTOR, Raptor, mLST8, PRAS40, and DEPTOR, is much better studied than mTORC2 in terms of both upstream regulators and downstream substrates (17). mTORC1 regulates cellular growth by increasing anabolic processes such as the macromolecular synthesis and nutrient storage and suppressing catabolic processes such as autophagy. For example, mTORC1 enhances the efficiency of protein synthesis by phosphorylation of the eukaryotic translation initiation factor 4E (eIF4E) binding proteins (e.g. 4E-BP1) and the S6 kinases (e.g. S6K1) (19–21). Activation of S6Ks promotes mRNA translation through several substrates such as the ribosome protein S6 (21), eIF4B (20), and eukaryotic elongation factor 2 kinase (22).

mTORC1 can be activated in response to a wide range of upstream signals such as nutrient, oxygen, and energy availability (18, 23–26) as well as growth factors (e.g. IGF-1) signaling through the receptor tyrosine kinases (27). Two major downstream signaling pathways, phosphatidylinositol-3 kinase (PI3K)-Akt and Ras-Erk, are involved in the activation of mTORC1 (24, 28). The activity of mTORC1 is negatively controlled by the tumor suppressors tuberous sclerosis complex 1 (TSC1, also known as hamartin) and TSC2 (also known as tuberin) (29–31). TSC2 functions as a GTPase-activation protein for the small GTPase Rheb, a direct activator for mTORC1. TSC2 can be phosphorylated by Akt (32), Erk (33, 34), and p90 RSK1 (35). Phosphorylation of TSC2 suppresses its ability to inhibit Rheb, allowing Rheb-GTP to accumulate and activate mTORC1 on the surface of lysosome (34, 35). Alternatively, Erk and RSK1 can phosphorylate the Raptor, which binds mTOR and recruits substrate to mTOR, to promote the mTORC1 activation (36, 37).

Amino acids can activate mTORC1 by signaling to the vacuolar proton (H+) -translocating ATPases (v-ATPase) from the lysosome lumen via an undefined “inside-out” mechanism (26). The primary function of v-ATPase is to hydrolyze ATP and pump H+ into the lysosome to maintain its acidic environment (38, 39). The v-ATPase physically interacts with Ragulator and controls the binding between the Ragulator and RAG complexes. As a result, v-ATPase regulates the guanine exchange factor activity of Ragulator to facilitate the exchange of GTP onto RagA and RagB GTPases (25, 26). Activated Rag complex (RagA/BGTP-RagC/DGDPP) recruits mTORC1 to the lysosome surface for activation by Rheb (23, 40).

There are a few reports of GPCR signaling to mTORC1 activation in the literature. A GPCR taste receptor T1R1/T1R3 plays a critical role in amino acid-stimulated mTORC1 activation (24). It has been proposed that TIR1/TIR3 may function as a direct sensor for extracellular amino acids to rapidly and transiently activate Erk1/2 activity. Inhibition of Erk1/2 activation by U0126 diminishes the amino acids-stimulated mTORC1 activation (24). Similarly, the GPCR ligand prostaglandin F2α-stimulated mTORC1 activation is sensitive to U0126, suggesting that MAPK/Erk is an important signal transducer from GPCR signaling to mTORC1 (28).

Here, we show that the neuropeptide orexin can activate the mTORC1 pathway in the mouse brain and three recombinant cell lines expressing either OX1R or OX2R. Moreover, our studies suggest that the orexin-stimulated mTORC1 activation was independent of Erk and Akt in the hypothalamic N41 neuronal cell model. Rather, orexin/GPCR signaling activates mTORC1 via cytoplasmic calcium transient that triggers the lysosome v-ATPase pathway. These studies identified the mTORC1 pathway as a key component of the downstream signaling network of orexin/GPCR. The discovery of this orexin-mTORC1 signaling axis may have important mechanistic implications for the various functions of orexin in physiology and metabolism.

**EXPERIMENTAL PROCEDURES**

**General Reagents and Antibodies—** Orexin-A/B were obtained from Peptide Institute, Inc. (Osaka, Japan), and IGF-1 was from ProSpec (Rehovot, Israel). MK-2206, rapamycin, and BI-D1870 were purchased from Selleck Chemicals (Houston, TX). AZD-6244, bafilomycin A1, and ionomycin were purchased from LC Laboratories (Woburn, MA). EBSS, Fura-2/AM, and Lipofectamine 2000 were from Invitrogen. EGTA, BAPTA/AM, CaCl2, W7, and thapsigargin were purchased from Sigma-Aldrich. Universal nuclease was purchased Thermo Scientific Pierce (Rockford, IL). Saliphenylandalimide (saliPhe) was a generous gift from Dr. Jef DeBrabander at University of Texas Southwestern Medical Center.

Anti-ribosomal S6 kinase (p70 S6K), anti-phospho-Thr-389 p70 S6K, anti-phospho-Ser-422 eIF4B, anti-phospho-Ser-235/236 S6 ribosomal protein, anti-ERK1/2, anti-phospho-Thr-202/Tyr-204 ERK1/2, anti-Akt, anti-phospho-Thr-308 Akt, anti-phospho-Ser-473 Akt, anti-RagC, anti-Raptor, and U0126 were all purchased from Cell Signaling Technologies (Danvers, MA).

**Cell Lines and Tissue Culture—** Stably transected HEK-293T cell lines expressing the OX1R or OX2R were described previously by Sakurai et al. (6). The mouse embryonic hypothalamus N41 neuronal cell line (mHypoE-N41) was obtained from Cedarlane Laboratories, Ltd. (Burlington, NC), which were stably infected with retroviruses expressing OX1R (N41/OX1R) or OX2R (N41/OX2R). Mouse embryonic fibroblasts expressing OX1R (MEF/OX1R) was similarly generated. Transgenic cell lines were selected with puromycin (2 mg/ml) and cultured in growth media consisting of high glucose DMEM supplemented with 10% FBS (Sigma).

**Serum Starvation and Stimulation of Cells—** Cultured cells were plated on six-well dishes at 40–60% confluence. After 24 h, the cells were washed once with PBS and incubated in starvation medium (DMEM supplemented with 20 μg/ml HEPES)
Orexin Activates mTOR

(pH 7.0)) for 24 h before IGF-1 (10 ng/ml), orexin-A/B (50 nM), ionomycin (1 μM), or thapsigargin (5 μM) treatment for 1 h in the serum-starved condition. All of the compounds such as rapamycin, EGTA, BAPTA/AM, saliPhe, and bafilomycin A1 as well as the various kinase inhibitors were added 10 min prior to addition of IGF-1 or orexin to the growth media.

Western Blot Analysis—Cells were rinsed once with ice-cold PBS and lysed in lysis buffer (20 mM HEPES (pH 7.4), 2 mM MgCl₂, 1% SDS, and universal nuclease). After centrifugation at 13,000 rpm for 10 min, the soluble fractions of cell lysates were saved for further analysis. Equal amounts of protein samples were resolved by SDS-PAGE and transferred to PVDF membrane, and Western blotting was performed according to standard procedures using the corresponding antibodies.

RNAi in Mammalian Cells—The control GFP shRNA (shGFP) and the shRNAs targeting RagC (shRagC) were described previously (41, 42). The oligonucleotides of lentiviral shRNAs were obtained from Sigma and cloned into plasmid pLKO.1-Hygro. The shRNA target sequence is as follows: mRagC-1, TGGGCCATTATCAAGCTGAATA; mRagC-2, GTGGATATGCAATAGTCTTATGAA; and hRagC-1, TGGCAA-TTATCAAGCTGAATA; hRagC-2, GTGGATATGCAGTCTTATGAA.

The shRNA-encoding plasmids were cotransfected with the pCMV-dR8.2 dvpr envelope and pCMV-VSVG packaging plasmids into actively growing HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Virus-containing supernatants were collected 48 h after transfection and passed through a 0.45-mm filter. Target cells in 10-cm culture dishes were infected in the presence of 8 μg/ml polybrene. After 24 h, cells were selected with puromycin for 3 days and then plated on six-well dishes at a density of 2.5 × 10⁵ cells/ml and incubated overnight before serum starvation. Cells were serum starved in parallel incubated with the 50 nM orexin-A or -B treatment for 24 h before serum starvation. Cells were serum starved 293T/OX1R and 293T/OX2R cells (Fig. 1A). All of these phosphorylation events are characteristic downstream markers for activation of the mTOR pathway. In contrast, the same orexin-A/B treatment could not induce any of these phosphorylation events in empty vector control HEK-293T cells (Fig. 1B). Furthermore, rapamycin, a potent inhibitor of mTORC1, abolished the orexinA/B-induced S6k and S6 phosphorylation in the 293T/OX1R and 293T/OX2R cells (Fig. 1C, D), suggesting that orexin/GPCR signaling specifically activates the mTORC1 pathway in human HEK-293T cells expressing either OX1R or OX2R.

RESULTS

Orexin Activates mTORC1 in HEK293T Cells Expressing OX1R or OX2R—To investigate downstream signaling pathways of orexin, we used synthetic orexin peptides to treat human embryonic kidney (HEK)-293T cells that express either of two orexin receptors, OX1R and OX2R. These cell lines were previously used to biochemically purify orexin-A and B from rat brain extract as specific neuropeptide ligands for OX1R and OX2R (then as orphan receptors) (6). We showed that orexin-A/B treatment induced the rapid phosphorylation of elf4B at serine 422, the S6 kinase p70S6K at Thr-389, and ribosomal protein S6 at Ser-235/236 and Ser-240/244 in the serum-starved 293T/OX1R and 293T/OX2R cells (Fig. 2A). Overexpression of Orexin Causes Hyperactivation of mTOR in the Mouse Brain—Previous studies showed that the CAG/orexin transgene expressed severalfold higher levels of orexin-A and B in the mouse brain and could rescue the narcolepsy and cataplexy phenotype of mice lacking the endogenous orexin-producing neurons (45). To study whether orexin activated the mTOR pathway in vivo, we examined the effect of orexin overexpression on the mTOR activity in whole brain extracts of wild-type and CAG/orexin mice fed on high fat diet.

At the indicated times, the animals were euthanized, and brain tissues were removed and frozen in liquid N2.
In three of four mice, we observed significantly higher levels of phosphorylations of S6K, S6, and Erk1/2 in the CAG/orexin brain extracts than wild-type brain extracts (Fig. 2F). This in vivo experiment suggests that overexpression of orexin can cause hyperactivation of the mTOR pathway in the mouse brain.

Orexin Activates mTORC1 Independent of Erk and Akt—As reported previously (47), we observed that orexin-A treatment induced the phosphorylation and activation of the MAPK kinase/MEK1/2, MAPK/Erk, and RSK in the N41/OX1R neurons (Fig. 3A). Pretreatment of N41/OX1R cells with the MEK1/2 inhibitor, U0126 or AZD6244, increased phosphorylation of MEK1/2, but inhibited the phosphorylation of the downstream substrates Erk1/2 and RSK (Fig. 3B). At 10 μM concentration, both U0126 and AZD6244 abolished the orexin-induced Erk1/2 and RSK phosphorylation (Fig. 3B). However, neither treatment had any noticeable effect on the orexin-induced mTORC1 activation as measured by the level of S6K and S6 phosphorylation (Fig. 3B). These results suggest that the MAPK/Erk and RSK does not play a critical role in the orexin-induced mTORC1 activation.

However, IGF-1, but not orexin-A, could induce rapid and robust activation of Akt kinase in the N41/OX1R neurons (Fig. 3D). Pretreatment of N41/OX1R cells with the Akt inhibitor, MK-2206, abolished the IGF-1-induced activation of Akt as well as the mTORC1-mediated S6K and S6 phosphorylation (Fig. 3E). In contrast, the same MK-2206 treatment had no effect on the orexin-induced S6K and S6 phosphorylation (Fig. 3E). Furthermore, inhibition of both Erk and Akt activation did not affect orexin-stimulated mTORC1 activation either (Fig. 3F). Therefore, we concluded that orexin could activate mTORC1 via an Erk- and Akt-independent pathway.

Orexin-induced mTORC1 Activation Is Dependent on Extracellular Calcium Influx—In the N41/OX1R and N41/OX2R neurons, orexin-A/B treatment could induce rapid increase in [Ca^{2+}], and mTORC1 activation in a corresponding dose-dependent manner (Fig. 4, A and B). To determine whether extracellular Ca^{2+} is essential for orexin signaling to mTORC1, we cultured N41/OX1R neurons in media that contained or lacked Ca^{2+} ion. The results showed that extracellular Ca^{2+} greatly enhanced both orexin-A and IGF-1-induced S6K and S6 phosphorylation (Fig. 4C). Addition of EGTA, an extracellular Ca^{2+} chelator, to the growth media diminished orexin-induced S6K and S6 phosphorylation, consistent with that Ca^{2+} influx from extracellular space is the primary response of orexin signaling (Fig. 4D). In contrast, EGTA partially reduced IGF-1-induced S6K and S6 phosphorylation (Fig. 4D). This is because IGF-1 could trigger both extracellular Ca^{2+} influx and release of Ca^{2+} from intracellular stores (48, 49). Accordingly, treatment of BAPTA/AM, a cell permeable Ca^{2+} chelator, abolished both orexin-A and IGF-1-induced S6K and S6 phosphorylation (Fig. 4E). A similar result was obtained with orexin-B treatment of N41/OX2R cells (Fig. 4F). Moreover, extracellular Ca^{2+} was essential for the orexin-induced Erk1/2 phosphorylation, but not the IGF-1-mediated activation of Akt (Fig. 4, C and D). Taken together, these results indicate that extracellular Ca^{2+} influx is required for orexin-induced mTORC1 activation.
Orexin Activates mTORC1 via the Lysosome v-ATPase Pathway—Both amino acids and growth factors-induced mTORC1 activation require the lysosome v-ATPase-Ragulator-RAG pathway (18, 25, 26, 40). To examine whether this lysosome pathway is also required for the orexin-induced mTORC1 activation, we treated N41/OX1R and 293T/OX1R cells with two specific v-ATPase inhibitors, saliPhe (50) and bafilomycin A1 (51). In both cell lines, saliPhe inhibited the orexin-A-induced S6K and S6 phosphorylation in a dose-dependent manner (Fig. 5A). Likewise, bafilomycin A1 blocked both orexin-A and IGF-1-stimulated mTORC1 activation (Fig. 5B). A similar phenomenon was observed with orexin-B treatment of the N41/OX2R and 293T/OX2R cells (Fig. 5C). Furthermore, small hairpin RNA (shRNA)-mediated knockdown of RagC GTPase reduced the orexin-induced S6K and S6 phosphorylation in the 293T/OX1R cells (Fig. 5D). A similar phenomenon was observed with orexin-B treatment of the N41/OX2R and 293T/OX2R cells (Fig. 5C). In both cell lines, saliPhe inhibited the orexin-A-induced S6K and S6 phosphorylation in a dose-dependent manner (Fig. 5A). Likewise, bafilomycin A1 blocked both orexin-A and IGF-1-stimulated mTORC1 activation (Fig. 5B). A similar phenomenon was observed with orexin-B treatment of the N41/OX2R and 293T/OX2R cells (Fig. 5C). Furthermore, small hairpin RNA (shRNA)-mediated knockdown of RagC GTPase reduced the orexin-induced S6K and S6 phosphorylation in the 293T/OX1R cells (Fig. 5D). Because we could not achieve efficient shRNA-mediated knockdown in the N41/OX1R neurons, we constructed MEFs expressing OX1R as a substitute for this experiment. In the MEF/OX1R cells, orexin-A also activated the mTORC1-mediated phosphorylation of S6K and S6, which was diminished by the shRNA-mediated knockdown of RagC expression (Fig. 5C). These results demonstrate that orexin/GPCR signaling stimulates mTORC1 activation via the lysosome v-ATPase-Rag GTPase pathway.

Intracellular Calcium Surge Stimulates mTORC1 Activation in a v-ATPase-dependent Manner—Because Ca\(^{2+}\) influx was essential for the orexin-induced mTORC1 activation (Fig. 3), we wanted to ask whether an intracellular Ca\(^{2+}\) surge was sufficient to mimic orexin signaling to mTORC1. To address this question, we incubated N41/OX1R cells with ionomycin or thapsigargin. Whereas ionomycin triggers extracellular Ca\(^{2+}\) influx, thapsigargin causes the release of Ca\(^{2+}\) from intracellular stores (52, 53). Our studies showed that both ionomycin and thapsigargin efficiently induced the phosphorylation of S6K and S6, which were abolished by BAPTA/AM treatment (Fig. 6, A and B). Intracellular calcium-stimulated S6K and S6 phosphorylation was also abolished by rapamycin treatment (Fig. 6C), suggesting that cytoplasmic Ca\(^{2+}\) increase is sufficient to mimic orexin signaling to mTORC1 activation in the N41/OX1R cells.

In contrast, ionomycin, but not thapsigargin, resulted in the activation of Erk1/2 (Fig. 6, A and B). Moreover, orexin-induced phosphorylation of Erk1/2 was only inhibited by EGTA, an extracellular Ca\(^{2+}\) chelator, but not by BAPTA/AM, an extracellular Ca\(^{2+}\) chelator (Fig. 4, D and E). These results suggest that the event of Ca\(^{2+}\) influx through plasma membrane, but not the subsequent cytoplasmic Ca\(^{2+}\) surge, is responsible for orexin-stimulated activation of the MAPK/Erk pathway.
Together with our finding that orexin does not activate Akt, these results further support our conclusion that orexin induces mTORC1 activation independent of Erk and Akt.

Furthermore, this intracellular calcium-stimulated mTORC1 activation was diminished not only by pharmacological inhibition of v-ATPase activity by saliPhe or bafilomycin A1 (Fig. 6, D and E), but also by the shRNA-mediated knockdown of RagC GTPase (Fig. 6F). Taken together, our studies suggest that cytoplasmic calcium transient is necessary and sufficient for orexin/GPCR signaling to stimulate mTORC1 activation through the lysosome v-ATPase-Ragulator-RAG pathway.

**DISCUSSION**

Although orexin and its receptors were discovered 16 years ago, their downstream signaling pathways have not been fully characterized. Here, we have discovered that orexin/GPCR signaling results in rapid and robust activation of the mTORC1 pathway in human HEK-293T cells, MEFs, and mouse hypothalamic N41 neurons that express either OX1R or OX2R. Accordingly, overexpression of orexin caused hyperactivation of the mTORC1 pathway in the CAG/orexin mouse brain. In contrast to previous reports of Erk-dependent mTORC1 activation in response to GPCR signaling (24, 28), we showed that neither of the two well known upstream mTORC1 activators, Erk and Akt, played a critical role in orexin/GPCR signaling to the mTOR pathway. Rather, the orexin-induced mTORC1 activation is dependent on cytoplasmic calcium transient that activates the lysosomal v-ATPase pathway through an unknown mechanism. This study uncovers a novel regulatory link that the mTORC1 pathway functions as a key component of orexin/GPCR signaling network.

**Involvement of Calcium in mTORC1 Activation**—It is known that intracellular Ca$^{2+}$/H$^{+}$ transient is also necessary for the activation of mTORC1 in response to amino acids and growth factors (24, 52, 54, 55). However, the mechanism of calcium involvement in mTORC1 activation remains unclear. Previous studies proposed that mTORC1 formed a “signalosome” in the phosphatidylinositol 3-phosphate-rich endosome structure (56). Amino acids could increase intracellular Ca$^{2+}$/H$^{+}$ level to enhance Ca$^{2+}$/H$^{+}$/calmodulin interaction with hVps34, thus activating hVps34 kinase activity and elevating the phosphatidylinositol 3-phosphate level on the endosomes (54, 57). However, a recent study contradicted this idea by showing that hVps34 bound to calmodulin, but its activity was not suppressed by BAPTA, EGTA, or calmodulin-inhibitor W7 in vivo (58). Furthermore, the function of Vps34 in mTORC1 activation was not substantiated by genetic studies in *Drosophila*, although the
amino acid-induced TORC1 activation is conserved from yeast to mammals (59). In our study, neither PI3K inhibitors nor calmodulin-inhibitor W7 had any effect on the orexin-stimulated mTORC1 activation (data not shown). Thus, more detailed studies are required to elucidate the specific role of calcium in mTORC1 activation in the future.

How Does Calcium Signal to Lysosome v-ATPase?—We have shown that intracellular calcium surge is sufficient to mimic orexin/GPCR signaling to activate mTORC1 through the lysosome v-ATPase pathway. The v-ATPases are highly conserved proton pumps consisting of a peripheral membrane subcomplex called V₁, which contains the sites of ATP hydrolysis, and
an integral membrane subcomplex called V₀, which encompasses the proton pore and is attached to V₁ (60). The ATPase activity of the v-ATPase and the associated rotation of its V₀ section appear to be essential to relay the amino acids signal from the lysosome lumen to the Ragulator and Rag GTPase complex on the lysosome surface, but exactly how the v-ATPase functions to do so is unknown (26). A previous study reported that v-ATPases exhibited both Mg²⁺/H⁺- and Ca²⁺/H⁺-dependent ATPase activity (39, 61). Unlike the Mg²⁺/H⁺-dependent v-ATPase activity, the Ca²⁺/H⁺-dependent v-ATPase activity decays with time and is inhibited by ADP in vitro (61). Additionally, the proton pump activity is detected only in the presence of Mg²⁺, but not in the presence of Ca²⁺ (39). Thus, the differential regulation of v-ATPase by Mg²⁺ and Ca²⁺ may provide a potential mechanism for the cytoplasmic Ca²⁺ surge to directly regulate the functional state of v-ATPase and couple it to mTORC1 activation on the lysosome surface. There are more than 800 GPCRs encoded by the human genome, of which mainly the Gq-coupled GPCRs upon activation trigger intracellular calcium transient. It is plausible that activation of the mTORC1 pathway is a general cellular response to Gq-coupled GPCR signaling in a wide variety of physiological processes. Consistent with this hypothesis, activated mTORC1 regulates energy homeostasis through multiple mechanisms. For example, mTORC1 activates the transcription factor HIF1α to stimulate specific metabolic pathways, including glycolysis and the oxidative arm of the pentose phosphate pathway (65, 67–69). Therefore, the orexin-mTORC1 signaling axis may provide a plausible explanation for the metabolic phenotypes resulting from chronic gain or loss-of-function of orexin/GPCR signaling. Finally, a complete understanding of orexin/GPCR signaling network is essential to understanding the functions of orexin from cellular to organismal levels and for developing new therapeutic approaches to restore the balance of the orexin/GPCR signaling system disturbed in many disease states.

FIGURE 6. Calcium activates mTORC1 via the lysosome v-ATPase pathway. A and B, serum-starved N41/OX1R cells were treated with 50 μM orexin-A or 1 μM ionomycin (A) or 5 μM thapsigargin (TG; B) for 1 h in the absence or presence of 20 μM BAPTA/AM. C, serum-starved N41/OX1R cells were treated with thapsigargin or ionomycin in the absence or presence of 20 nm rapamycin. D, serum-starved N41/OX1R cells were treated with 1 μM ionomycin for 1 h in the absence or presence of 5 μM or 10 μM bafilomycin A1. E, serum-starved N41/OX1R cells were treated with 5 μM thapsigargin for 1 h in the absence or presence of 10 μM saliPhe or bafilomycin A1. F, 293T/OX1R cells were infected with control shGFP or shRagC lentivirus for 24 h. After hygromycin selection for 72 h, the infected cells were serum-starved for 24 h followed by ionomycin treatment for 1 h. The levels of S6K, RagC, and Raptor and phosphorylations of S6 and S6K were detected by Western blotting.

Orexin Activates mTOR
REFERENCES

1. Mignot, E. (1998) Genetic and familial aspects of narcolepsy. *Neurology* 50, S16–S22.

2. Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmqquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98, 437–451.

3. Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P. J., Nishino, S., and Mignot, E. (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98, 365–376.

4. Mignot, E., Lammers, G. J., Ripley, B., Okun, M., Nevsimalova, S., Overeem, S., Vankova, J., Black, J., Harsh, J., Bassetti, C., Schrader, H., and Nishino, S. (2002) The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. *Arch. Neurol.* 59, 1553–1562.

5. de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 322–327.

6. Sakurai, T., Amemiya, A., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585.

7. Carter, M. E., Schiafﬁ Borg, J., and de Lecea, L. (2009) The brain hypocretins and their receptors: mediators of atalostatic arousal. *Curr. Opin. Pharmacol.* 9, 39–45.

8. Sakurai, T., Mieda, M., and Tsujino, N. (2010) The orexin system: roles in sleep/wake regulation. *Ann. N.Y. Acad. Sci.* 1200, 149–161.

9. Wei, W., Mootoike, T., Krzeszinski, J. Y., Jin, Z., Xie, X. J., Dechow, P. C., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585.

10. Mignot, E., Lammers, G. J., Ripley, B., Okun, M., Nevsimalova, S., Overeem, S., Vankova, J., Black, J., Harsh, J., Bassetti, C., Schrader, H., and Nishino, S. (2002) The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. *Arch. Neurol.* 59, 1553–1562.

11. de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 322–327.

12. Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585.

13. Carter, M. E., Schiafﬁ Borg, J., and de Lecea, L. (2009) The brain hypocretins and their receptors: mediators of atalostatic arousal. *Curr. Opin. Pharmacol.* 9, 39–45.

14. Sakurai, T., Mieda, M., and Tsujino, N. (2010) The orexin system: roles in sleep/wake regulation. *Ann. N.Y. Acad. Sci.* 1200, 149–161.

15. Wei, W., Mootoike, T., Krzeszinski, J. Y., Jin, Z., Xie, X. J., Dechow, P. C., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585.

16. Mignot, E., Lammers, G. J., Ripley, B., Okun, M., Nevsimalova, S., Overeem, S., Vankova, J., Black, J., Harsh, J., Bassetti, C., Schrader, H., and Nishino, S. (2002) The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. *Arch. Neurol.* 59, 1553–1562.

17. de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 322–327.
promoting phorbol esters and activated Ras inactivate the tuberous sclero-
torsus tumor suppressor complex via p90 ribosomal S6 kinase. Proc. Natl.
Acad. Sci. U.S.A. 101, 13489–13494

36. Carrière, A., Cargnelu, M., Julien, L. A., Gao, H., Bonneil, E., Thibault, P.,
and Roux, P. P. (2008) Oncogenic MAPK signaling stimulates mTORC1 ac-
tivity by promoting RSK-mediated raptor phosphorylation. Curr. Biol. 18,
1269–1277

37. Carrière, A., Romeo, Y., Acosta-Jaquez, H. A., Moreau, J., Bonneil, E.,
Thibault, P., Fingar, D. C., and Roux, P. P. (2011) ERK1/2 phosphoryla-
tion of Raptor to promote Ras-dependent activation of mTORC1 (1
mTORC1). J. Biol. Chem. 286, 567–577

38. Forster, C., and Kane, P. M. (2000) Cytosolic Ca\(^{2+}\) homeostasis is a
constitutive function of the V-ATPase in Saccharomyces cerevisiae. J. Biol.
Chem. 275, 38245–38253

39. Crider, B. P., and Xie, X. S. (2003) Characterization of the functional cou-
pling of bovine brain vacuolar-type H\(^{-}\)-translocating ATPase. Effect of
divalent cations, phospholipids, and subunit H (SF6). J. Biol. Chem. 278,
44281–44288

40. Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., and Saba-
tini, D. M. (2010) Regulator-Rag complex targets mTORC1 to the lyso-
somal surface and is necessary for its activation by amino acids. Cell 141,
290–303

41. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005)
Phosphorylation and regulation of Akt/PKB by the rictor-mTOR com-
plex. Science 307, 1098–1101

42. Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C.,
Bar-Peled, L., and Sabatini, D. M. (2008) The Rag GTPases bind raptor and
mediate amino acid signaling to mTORC1. Science 320, 1496–1501

43. Marcus, J. N., Aschenkasi, C. J., Lee, C. E., Chemelli, R. M., Saper, C. B.,
Yanagisawa, M., and Elmqquist, J. K. (2001) Differential expression of
orexin receptors 1 and 2 in the rat brain. J. Comp. Neurol. 435, 6–25

44. Belsham, D. D., Cai, F., Cui, H., Smukler, S. R., Salapatek, A. M., and
Shkreta, L. (2004) Generation of a phenotypic array of hypothalamic neu-
ronal cell models to study complex neuroendocrine disorders. Endocrinol-
ogy 145, 393–400

45. Mieda, M., Willie, J. T., Hara, J., Sinton, C. M., Sakurai, T., and Yanagisawa,
M. (2004) Orexin peptides prevent cataplexy and improve wakefulness in
an orexin neuron-ablated model of narcolepsy in mice. Proc. Natl. Acad.
Sci. U.S.A. 101, 4649–4654

46. Funato, H., Tsai, A. L., Willie, J. T., Kisanuki, Y., Williams, S. C., Sakurai,
T., and Yanagisawa, M. (2009) Enhanced orexin receptor-2 signaling pre-
vents diet-induced obesity and improves leptin sensitivity. Cell Metab. 9,
64–76

47. Ammoun, S., Johansson, L., Ekholm, M. E., Holmqvist, T., and Seeley, R. J.
(2011) Activation of a metabolic gene regulatory network down-
stream of mTORC1. J. Biol. Chem. 286, 26409–26422

48. Graves, L. M., He, Y., Lambert, J., Hunter, D., Li, X., and Earp, H. S. (1997)
An intracellular calcium signal activates p70 but not p90 ribosomal S6
kinase in liver epithelial cells. J. Biol. Chem. 272, 1920–1928

49. Conus, N. M., Hemmings, B. A., and Pearson, R. B. (1998) Differential
regulation by calcium reveals distinct signaling requirements for the ac-
tivation of Akt and p70S6k. J. Biol. Chem. 273, 4776–4782

50. Gulati, P., Gaspers, L. D., Dann, S. G., Joaquín, M., Nobukuni, T., Natt, F.,
Kozma, S. C., Thomas, A. P., and Thomas, G. (2008) Amino acids activate
mTORC1 complex 1 via Ca\(^{2+}\)/CaM signaling to hVps34. Cell Metab. 7,
456–465

51. Ito, N., Ruegg, U. T., Kudo, A., Miyagoe-Suzuki, Y., and Takeda, S. (2013)
Activation of calcium signaling through Trpv1 by nNOS and peroxynitrite
as a key trigger of skeletal muscle hypertrophy. Nat. Med. 19, 101–106

52. Hannan, K. M., Thomas, G., and Pearson, R. B. (2003) Activation of SK6
(p70 ribosomal protein S6 kinase) 1 requires an initial calcium-dependent
priming event involving formation of a high-molecular-mass signaling
complex. Biochem. J. 370, 469–477

53. Nobukuni, T., Joaquín, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P.,
Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J., and
Thomas, G. (2005) Amino acids mediate mTOR/raptor signaling through
activation of class 3 phosphatidylinositol 3OH-kinase. Proc. Natl. Acad.
Sci. U.S.A. 102, 14238–14243

54. Yan, Y., Flinn, R. J., Wu, H., Schnur, R. S., and Backer, J. M. (2009) hVps15,
but not Ca\(^{2+}\)/CaM, is required for the activity and regulation of hVps34 in
mammalian cells. Biochem. J. 417, 747–755

55. Juhász, G., Hill, J. H., Yan, Y., Sass, M., Baehrecke, E. H., Backer, J. M., and
Neufeld, T. P. (2008) The class III PI(3)K Vps34 promotes autophagy and
derendocytosis but not TOR signaling in Drosophila. J. Cell Biol. 181,
655–666

56. Kane, P. M. (2012) Targeting reversible disassembly as a mechanism of
controlling V-ATPase activity. Curr. Protein Pept. Sci. 13, 117–123

57. Gräf, R., Harvey, W. R., and Wieczorek, H. (1996) Purification and prop-
erties of a cytosolic V1-ATPase. J. Biol. Chem. 271, 20908–20913

58. Sok, S. W., Overeem, S., Visscher, T. L., Lammers, G. J., Seidell, J. C., Pijl,
H., and Meinders, A. E. (2003) Hypocretin deficiency in narcoleptic hu-
mans is associated with abdominal obesity. Obes. Res. 11, 1147–1154

59. Cota, D., Proulx, K., Smith, K. A., Kozma, S. C., Thomas, G., Woods, S. C.,
and Seeley, R. J. (2006) Hypothalamic mTORC1 signaling regulates food
intake. Science 312, 927–930

60. Harlan, S. M., Guo, D. F., Morgan, D. A., Fernandes-Santos, C., and Rah-
mouni, K. (2013) Hypothalamic mTORC1 signaling controls sympathetic
nerve activity and arterial pressure and mediates leptin effects. Cell Metab.
17, 599–606

61. Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L.,
Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., Vander Heiden, M. G.,
MacKeigan, J. P., Finan, P. M., Clish, C. B., Murphy, L. O., and Manning,
B. D. (2010) Activation of a metabolic gene regulatory network down-
stream of mTORC1 complex 1. Mol. Cell 39, 171–183

62. Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Levers, S.,
Griffiths, J. R., Hong, Y. L., and Schulze, A. (2008) SREBP activity is regu-
lated by mTORC1 and contributes to Akt-dependent cell growth. Cell Metab. 8,
224–236

63. Feng, Y., Liu, T., Li, X., Lui, Y., Zhu, X. Y., Jankovic, J., Pan, T. H., and
Wu, Y. C. (2014) Neuroprotection by Orexin-A via HIF-1a induction in a
cellular model of Parkinson’s disease. Neurosci. Lett. 579, 35–40

64. Yuan, L. B., Dong, H. L., Zhang, H. P., Zhao, R. N., Gong, G., Chen, X. M.,
Zhang, L. N., and Xiong, J. (2011) Neuroprotective effect of orexin-A is
mediated by an increase of hypoxia-inducible factor-1 activity in rat. Ar-
esthesiology 114, 340–354

65. Sidker, D., and Kodadek, T. (2007) The neurohormone orexin stimulates
hypoxia-inducible factor-1 activity. Genes Dev. 21, 2995–3005