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mTORC1 pathway disruption abrogates the effects of the ciliary neurotrophic factor on energy balance and hypothalamic neuroinflammation

Caroline André [a,b,1], Caterina Catania [a,b,1], Julie Remus-Borel [c,d], Elodie Ladeveze [a,b], Thierry Leste-Lasserre [a,b], Wilfrid Mazier [a,b], Elke Binder [a,b], Delphine Gonzales [a,b], Samantha Clark [a,b], Omar Guzman-Quevedo [a,b], Djoher Nora Abrous [a,b], Sophie Layé [c,d], Daniela Cota [a,b,*]

[a] INSERM, Neurocentre Magendie, Physiopathologie de la Plasticité Neuronale, U1215, F-33000 Bordeaux, France
[b] University of Bordeaux, Neurocentre Magendie, Physiopathologie de la Plasticité Neuronale, U1215, F-33000 Bordeaux, France
[c] INRA, Nutrition et Neurobiologie Intégrée, UMR 1286, F-33076 Bordeaux, France
[d] University of Bordeaux, Nutrition et Neurobiologie Intégrée, UMR 1286, F-33076 Bordeaux, France

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ABSTRACT

Ciliary neurotrophic factor (CNTF) potently decreases food intake and body weight in diet-induced obese mice by acting through neuronal circuits and pathways located in the arcuate nucleus (ARC) of the hypothalamus. CNTF also exerts pro-inflammatory actions within the brain. Here we tested whether CNTF modifies energy balance by inducing inflammatory responses in the ARC and whether these effects depend upon the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which regulates both energy metabolism and inflammation. To this purpose, chow- and high fat diet (HFD)- fed mice lacking the S6 kinase 1 (*S6K1*$^{-/-}$), a downstream target of mTORC1, and their wild-type (WT) littermates received 12 days continuous intracerebroventricular (icv) infusion of the CNTF analogue axokine ($CNTF_{Ax15}$). Behavioral, metabolic and molecular effects were evaluated.

Central chronic administration of $CNTF_{Ax15}$ decreased body weight and feed efficiency in WT mice only, when fed HFD, but not chow. These metabolic effects correlated with increased number of iba-1 positive microglia specifically in the ARC and were accompanied by significant increases of IL-1β and TNF-α mRNA expression in the hypothalamus. Hypothalamic iNOS and SOCS3 mRNA, molecular markers of pro-inflammatory response, were also increased by $CNTF_{Ax15}$. All these changes were absent in *S6K1*$^{-/-}$ mice.

This study reveals that $CNTF_{Ax15}$ requires a functional S6K1 to modulate energy balance and hypothalamic inflammation in a diet-dependent fashion. Further investigations should determine whether S6K1 is a suitable target for the treatment of pathologies characterized by a high neuroinflammatory state.

1. Introduction

A complex combination of central and peripheral mechanisms regulates energy intake and consumption in order to maintain energy balance. Neurotrophic factors, such as the ciliary neurotrophic factor (CNTF), are known to regulate food intake and body weight and their actions are of interest so to better understand underlying molecular pathways involved (Pasquin et al., 2015; Xu and Xie, 2016).

CNTF is a cytokine expressed by both neuronal and glial cells belonging to the interleukin-6 (IL-6) family, which reduces body weight and ameliorates metabolic responses in obese rodents and humans by acting both centrally and peripherally (Gloaguen et al., 1997; Lambert et al., 2001; Ettinger et al., 2003; Sleeman et al., 2003; Bluher et al., 2004; Watt et al., 2006; Crowe et al., 2008). In particular, CNTF and it analogue axokine ($CNTF_{Ax15}$) reduce food intake and body weight by exerting their action within the arcuate nucleus (ARC) of the hypothalamus, where they modulate leptin-like pathways, such as the signal transducer and activator of transcription 3 (STAT3), and the activity of pro-opiomelanocortin (POMC) producing neurons (Lambert et al., 2001; Kelly et al., 2004; Kokoeva et al., 2005; Janoschek et al., 2006; Borg et al., 2016).

* Corresponding author at: Group "Energy Balance and Obesity", INSERM U1215, Neurocentre Magendie, 146 Rue Léo Saignat, 33077 Bordeaux, France.
E-mail address: daniela.cota@inserm.fr (D. Cota).
[1] These authors contributed equally to the work.

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However, CNTF has also endogenous pyrogenic and inflammatory activity *in vivo* (Shapiro et al., 1993; Kelly et al., 2004; Lin et al., 2009; Solymar et al., 2011), which might ultimately explain its action on energy balance. In fact, when centrally or peripherally infused, both CNTF and $CNTF_{Ax15}$ can cause in a dose-dependent manner fever, anorexia and reduced locomotor activity, suggesting the development of sickness behavior (Espat et al., 1996; Kelly et al., 2004; Solymar et al., 2011). Other studies have further shown that CNTF administration activates microglia *in vitro* and *in vivo* and increases the expression of glial fibrillary protein (GFAP) in astrocytes in otherwise healthy animals (Kahn et al., 1995; Clatterbuck et al., 1996; Lee et al., 2009; Lin et al., 2009). Thus, it remains to be clarified whether the central action of CNTF on the regulation of energy balance might depend upon its pro-inflammatory effect. Besides, underlying molecular pathways possibly linking the actions of CNTF on both energy balance and neuroinflammation are currently unknown.

The mammalian or mechanistic target of rapamycin (mTOR) is an evolutionary conserved serine/threonine kinase that acts in cells by forming two distinct complexes, respectively called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR and its related intracellular pathway controls cell proliferation and metabolism in response to the presence of nutrients, growth factors, mitogens, hormones and cellular stress (Haissaguerre et al., 2014; Saxton and Sabatini, 2017). mTORC1 signaling has also a critical role in the regulation of energy balance at the level of the hypothalamus (Cota et al., 2006; Blouet et al., 2008; Cota et al., 2008; Dagon et al., 2012), where this pathway is involved in mediating the acute appetite-suppressant effects of $CNTF_{Ax15}$ (Cota et al., 2008). Accordingly, mice lacking the 70-kDa ribosomal protein S6 kinase 1 (S6K1), a key downstream target of mTORC1 (Saxton and Sabatini, 2017), do not show acute $CNTF_{Ax15}$-induced hypophagia (Cota et al., 2008). Interestingly, mTORC1 signaling also regulates immune function and inflammatory responses (Cobbold, 2013; Perl, 2015; Linke et al., 2017). Indeed, recent studies suggest a link between mTORC1 activation in glial cells and production of pro-inflammatory cytokines (Dello Russo et al., 2009; Xie et al., 2014; Li et al., 2016). In particular, activation of mTORC1 signaling in microglia leads to a pro-inflammatory response (Li et al., 2016), while administration of the mTORC1 inhibitor rapamycin *in vitro* blunts lipopolysaccharide (LPS)-induced microglia activation, strongly inhibiting the expression of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Dello Russo et al., 2009; Lisi et al., 2011; Mengke et al., 2016; Liu et al., 2017; Yang et al., 2017). Whether the role exerted by the mTORC1 pathway in the modulation of neuroinflammation might be relevant for the regulation of energy balance is not known.

In the present study we aimed at investigating if the mTORC1 pathway determines the effects of $CNTF_{Ax15}$ on energy balance and neuroinflammation. To this purpose, we explored the behavioral, metabolic and molecular effects of 12 days continuous intracerebroventricular (icv) infusion of $CNTF_{Ax15}$ or its vehicle in chow- and high fat diet (HFD)- fed male mice lacking the *S6K1* gene ($S6K1^{-/-}$) and their wild-type (WT) littermates.

These studies reveal that $CNTF_{Ax15}$ requires a functional S6K1 in order to affect energy balance and that reduction of body weight is associated with induction of inflammatory responses within the hypothalamus in a diet-dependent fashion.

2. Material and methods

2.1. Animals

All experiments were performed in accordance with the European Union recommendations (2010/63/EU) and were approved by the local ethical committee of the University of Bordeaux (protocol N° 5012028A). Every effort was made to minimize suffering and the number of animal used.

Male $S6K1^{-/-}$ mice and their WT littermates were obtained and genotyped as described (Shima et al., 1998). Primers used for genotyping were S6K1_neo_Fwd: TGGCGGACCGCTATCAGGACA TAGCGTTGG, S6K1_Fwd: ACATACGCTGTGTCCCTTCCTCT, and S6K1_Rev: CTACTGGCTATTGGGGAAGGACAGTA. The S6K1 mouse strain was out-crossed at least twice to C57BL/6J mice upon arrival in the animal facility of the Neurocentre Magendie and was maintained on heterozygous breeding. Eight-weeks old animals were housed individually in standard plastic rodent cages and maintained on a 12h light/dark cycle (light off at 1pm) with free access to water and either standard rodent chow (Standard Rodent Diet A03, SAFE, France) or HFD (60% of fat, D12492, Research Diet, USA) for 8 weeks so to induce obesity, before undergoing stereotaxic surgery and treatment with $CNTF_{Ax15}$ or its vehicle. A total number of 29 chow-fed (20 WT and 9 $S6K1^{-/-}$) and 84 HFD-fed (38 WT and 46 $S6K1^{-/-}$) mice were used. At the end of the study, animals were sacrificed and tissues collected for further neuroanatomical and molecular analyses. Number of mice used for the different experiments is further detailed in the figure panels and in the figure legends.

2.2. Surgery and intracerebroventricular (icv) infusion of $CNTF_{Ax15}$

Sixteen week-old mice fed chow or HFD were anesthetized with a mix of ketamine and xylazine (100mg/kg and 10mg/kg respectively) then implanted with a cannula into the lateral ventricle (anteroposterior: −0.3mm from bregma, lateral: −1mm to bregma and dorsoventral: −2.5mm below skull, see Supplementary Fig. 1A) using a stereotaxic apparatus (David Kopf Instruments, USA). Correct cannula placement was verified at time of sacrifice and during neuroanatomical studies further described below by assessing the location of the track of the cannula. The cannula was connected to an Alzet osmotic minimpump (flow rate of 0.25 μl/h for 14d, Alzet model 1002 Alzet Charles River, France) via a 120 mm-long vinyl tubing (inner diameter 0.69 mm filled with artificial cerebrospinal fluid (aCSF, Tocris), which allowed delivery of aCSF in all animals during the first 2 days after the surgery. Delivery of the drug treatment started on day 3. The minipumps were filled with either aCSF alone as vehicle solution or aCSF with 63 ng/μl of a human recombinant variant of CNTF ($CNTF_{Ax15}$, Regeneron Pharmaceuticals USA) then primed overnight at 37°C in 0.9% saline.

2.3. Body weight, food intake, and feed efficiency

Body weight and food intake (expressed as g of food eaten daily) were measured daily for the length of the treatment with $CNTF_{Ax15}$ or its vehicle. Body weight change was expressed as percentage of the first day of $CNTF_{Ax15}$ treatment. Feed efficiency was calculated as body weight gain over caloric intake (g/cal × 100). For the body weight and food intake studies we used 20 WT and 9 $S6K1^{-/-}$ chow-fed mice and 10 WT and 13 $S6K1^{-/-}$ HFD-fed mice.

2.4. Body composition analysis

In order to assess degree of obesity in HFD-fed animals, 10 WT and 13 $S6K1^{-/-}$ mice underwent body composition analysis by EchoMRI (EchoMedical Systems, Houston, TX, USA) before the start of the pharmacological treatment, as in (Binder et al., 2013).

2.5. Western blot analysis

A different group of 14 WT and 12 $S6K1^{-/-}$ HFD-fed mice was used. After removal of the brains at sacrifice, a wedge of the

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