Brief Communication

MOLECULAR METABOLISM

Endocannabinoid-dependent disinhibition of orexinergic neurons: Electrophysiological evidence in leptin-knockout obese mice

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ABSTRACT

Objectives: In the *ob/ob* mouse model of obesity, chronic absence of leptin causes a significant increase of orexin (OX) production by hypothalamic neurons and excessive food intake. The altered OX level is linked to a dramatic increase of the inhibitory innervation of OX producing neurons (OX neurons) and the over expression of the endocannabinoid 2-arachidonoylglycerol (2-AG) by OX neurons of *ob/ob* mice. Little is known about the function of the excitatory synapses of OX neurons in *ob/ob* mice, and their modulation by 2-AG. In the present study, we fill this gap and provide the first evidence of the overall level of activation of OX neurons in the *ob/ob* mice.

Methods: We performed *in vitro* whole-cell patch-clamp recordings on OX neurons located in the perifornical area of the lateral hypothalamus in acute brain slices of wt and *ob/ob* mice. We identified OX neurons on the basis of their electrophysiological membrane properties, with 96% of concordance with immunohistochemisty.

Results: We found that OX neurons of *ob/ob* mice are innervated by less efficient and fewer excitatory synapses than wt mice. Consequently, *ob/ob* OX neurons show more negative resting membrane potential and lower action potential firing frequency than wt. The bath application of the cannabinoid type-1 receptor agonist WIN55,212-2, depresses both the excitatory and the inhibitory synapses in *ob/ob* animals, but only the excitatory synapses in wt animals. Finally, the physiologic release of 2-AG induces a prevalent depression of inhibition (disinhibition) of OX neurons in *ob/ob* animals but not in wt.

Conclusions: In *ob/ob* mice, chronic absence of leptin induces a 2-AG mediated functional disinhibition of OX neurons. This helps explain the increase of OX production and, consequently, the excessive food intake of *ob/ob* mice.

Keywords Obesity; Leptin; Orexin; Endocannabinoids

1. INTRODUCTION

Neurons releasing orexin (OX, a neuropeptide also known as hypocretin) are uniquely found in the perifornical area of the lateral hypothalamus (LH) [1–6]. OX is overexpressed in neurons of leptin-deficient obese mice (the *ob/ob* mouse strain) [7]. OX neurons have been implicated in a variety of functions, such as wakefulness, motivated behavior and reward, autonomic functions, nociception and, of relevance for the present study, energy balance and food seeking and intake (see for review: 8–10). In particular, the injection of orexin into the brain stimulates feeding behavior [6,11]. In the hypothalamus, the blood brain barrier is permeable to several hormones, including leptin. Leptin is released by adipocytes following food intake and exerts its anorexigenic effects on different types of neurons throughout the hypothalamus and other brain nuclei [12,13]. The effectiveness of excitatory synapses on OX neurons is depressed by leptin [14], and the firing activity of OX neurons is consequently reduced [14,15]. Conversely, the reduction of leptin level during fasting increases the activity of OX neurons [16,17].

Variations in the amount of circulating leptin, due to modifications of fasting/feeding states, cause substantial synaptic rewiring in hypothalamic nuclei involved in the control of food intake, such as the arcuate nucleus (ARC) and the LH. In the ARC, chronic lack of leptin, as in *ob/ob* mice, causes a functional and morphological synaptic plasticity that increases the excitation of neurons expressing orexigenic (neuropeptide Y and agouti-related peptide) and the inhibition of those expressing anorexigenic (pro-opiomelanocortin and cocaine-amphetamine regulated transcript) peptides [18]. Accordingly, leptin administration to *ob/ob* mice reduces food intake and body weight [19]. In the LH, overnight food deprivation is sufficient to enhance the excitatory drive to OX neurons of wt mice, an effect reversed by leptin administration [20]. We have recently demonstrated that chronic leptin deficiency or resistance in *ob/ob* and in high-fat diet (HFD) fed mice, respectively, causes a morphological switch from predominantly

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excitatory to predominantly inhibitory innervation of OX neurons [7]. The exceeding inhibitory innervation is functionally depressed by the activity of the endocannabinoid 2-arachidonoylglycerol (2-AG), produced by OX neurons and acting retrogradely at presynaptic cannabinoid type-1 (CB1) receptors. As a consequence, OX neurons of *ob/ob* and HFD animals are disinhibited and release more OX in target brain areas [7,21].

Our previous work provided functional evidence for the leptin-driven synaptic rewiring of OX neurons in *ob/ob* mice [7]. However, a comprehensive characterization of the retrograde effect of endocannabinoids on excitatory and inhibitory inputs to OX neurons of wt and *ob/ob* mice is still missing. This topic is of special interest also for human pathology since a 2-AG mediated modulation of OX neurons may be involved in human obesity, in which leptin resistance is frequently found [22]. Similarly, the psychoactive drug Δ^9-tetrahydrocannabinol present in marijuana may exert its appetite stimulating effect by acting on hypothalamic CB1 receptors [23].

2. METHODS

2.1. Ethics statement and animal maintenance

All experimental protocols have been approved by the Ministry of Health of Italy. Weaned (5–9 weeks of age) *ob/ob* mice [24] and their wt littermates were used. Mice were weaned at P23, bred in-house, kept in same-sex groups of maximal 6 mice under a light/dark cycle of 12 h (light on at 8 am). Mice had free access to water and were fed *ad libitum* with standard rodent lab chow. A total of 51 wt and 47 *ob/ob* mice have been utilized.

2.2. Slice preparation

Slices were cut as previously described [7]. In brief, mice were anesthetized by inhalation of ether or ketamine i.p. injection (100 mg/kg), transcardially perfused with ice-cold dissection solution, the brains quickly removed and hypothalamic 200–300 μm thick coronal slices were cut in ice-cold dissection solution (Vibratome Series 1000, St. Louis, MO, USA). Slices were then incubated in artificial cerebral spinal fluid (aCSF) at 32 °C for 30 min and subsequently kept at room temperature. Recordings were performed at room temperature in a submerged chamber superfused with aCSF (4 ml min^{-1}). Solutions were equilibrated with 95% O_2 and 5% CO_2; details in Cristino et al. [7].

2.3. Electrophysiology

Whole-cell patch-clamp recordings were performed during the animals' light phase with an Axopatch 200B amplifier (Axon Instruments, CA, U.S.A.) and 4–7 MΩ recording electrodes. Neurons were visualized with differential interference contrast optics and an infrared video camera (Hamamatsu, Japan). Recordings were filtered at 2–10 kHz and digitized at 20 kHz (Digidata 1322A, Axon Instr.).

For recording miniature excitatory postsynaptic currents (mEPSCs), evoked excitatory postsynaptic currents (eEPSCs), and evoked post synaptic potentials (ePSPs), the internal solution consisted of (in mM): $KMeSO_4$ 135, $MgCl_2$ 4, HEPES 10, Na-GTP 0.4, Na-ATP 4, phosphocreatine disodium salt 10, and EGTA 0.2–0.5, pH 7.3 with KOH, 320 mOsm/L. Liquid junction potential (LJP) 8.2 mV, corrected online. For recording resting membrane potential (RMP) and action potential (AP) firing activity, the internal solution consisted of (in mM): K-gluconate 125, KCl 5, $CaCl_2$ 0.5, HEPES 10, BAPTA 5, Tris-GTP 0.33, Mg-ATP 5, pH 7.3 with KOH, 280 mOsm/L. LJP 12.1 mV, corrected offline. For recording evoked inhibitory postsynaptic currents (eIPSC), the internal solution consisted of (in mM): KCl 145, HEPES 10, EGTA 0.2, Mg-ATP 2, Na-GTP 0.5, pH 7.3 with KOH, 290 mOsm/L.

According to several studies performed both in mice [7,25,26] and in rats [27,28], OX immunopositive neurons are concentrated in the perifornical area of the LH and are functionally characterized by the following voltage responses to injected currents: i) a tonic, non-adaptive repetitive spiking in response to a supra-threshold depolarizing pulse; ii) a time- and voltage-dependent rectification of membrane potential in response to a hyperpolarizing pulse; iii) abortive spikes evoked by a sub-threshold depolarizing pulse (Figure 1A). We accepted as orexinergic those

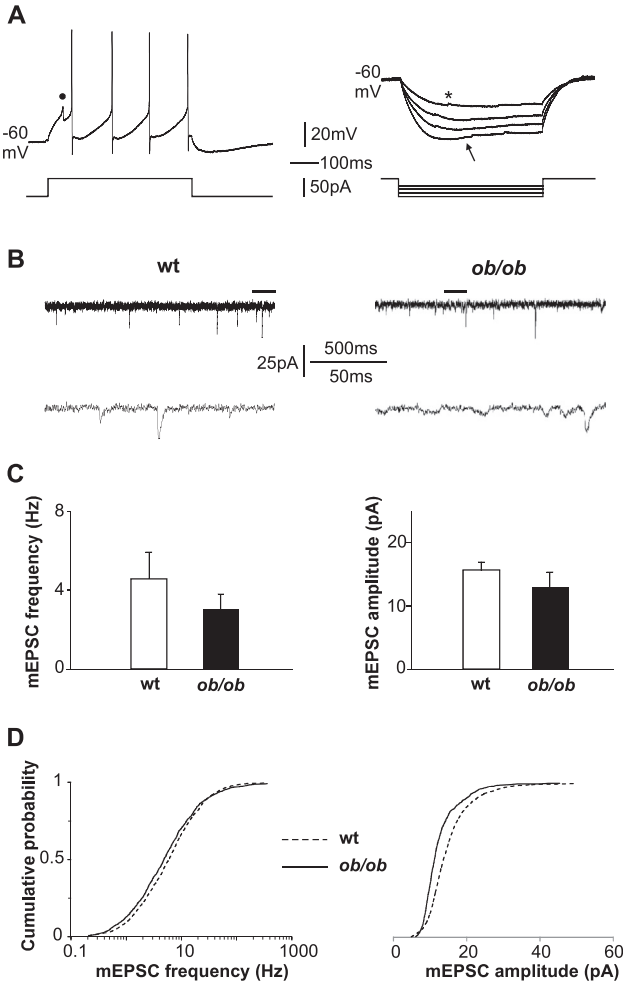

Figure 1: Adult *ob/ob* OX neurons have less functional excitatory synapses than wt neurons. (**A**) Electrophysiological criteria utilized to identify hypothalamic OX neurons. Left: representative voltage recording of tonic non-adapting AP firing (note the regular inter-spike time intervals) and of an abortive spike (black dot) in response to a depolarizing current pulse. Right: four superimposed representative voltage recordings characterized by a "sag" at negative potentials (arrow). The traces also show several spontaneous excitatory postsynaptic potentials (an example is indicated by *). (**B**) Representative recordings of mEPSCs from OX neurons voltage-clamped at −70 mV. Lower traces: expansions of the segments indicated with black lines in the upper traces. (**C**) Mean ± SEM frequency (left graph) and amplitude (right graph) of mEPSCs in wt and *ob/ob* OX neurons (frequency, wt *vs ob/ob*: 4.6 ± 1.34 Hz, n = 10/6 *vs* 3.0 ± 0.75 Hz, n = 7/5, U = 32.5, p = 0.8; amplitude, wt *vs ob/ob*: 15.8 ± 1.41 pA *vs* 13.1 ± 2.1 pA, U = 53.0, p = 0.08). (**D**) Cumulative probability distribution of mEPSCs frequency (left, semi-logarithmic) and amplitude (right), showing smaller values in *ob/ob* OX neurons (frequency: $p \leq 0.003$; amplitude: $p \leq 0.001$).

