Loss of Leptin Receptors on Hypothalamic POMC Neurons Alters Synaptic Inhibition

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Chun SK, Jo YH. Loss of leptin receptors on hypothalamic POMC neurons alters synaptic inhibition. J Neurophysiol 104: 2321–2328, 2010. First published September 15, 2010; doi:10.1152/jn.00371.2010. Adaptive changes in hypothalamic neural circuitry occur in response to alterations in nutritional status. This plasticity at hypothalamic synapses contributes to the control of food intake and body weight. Here we show that genetic ablation of leptin receptor gene expression in pro-opiomelanocortin (POMC) neurons (POMC: Lepr<sup>−/−</sup> GFP) induces alterations at synapses on POMC neurons in the arcuate nucleus of the hypothalamus. Our studies reveal that POMC: Lepr<sup>−/−</sup> GFP mice have decreased frequency of spontaneous GABAergic, but not glutamatergic, post synaptic currents (sIPSCs) onto POMC neurons in POMC: Lepr<sup>−/−</sup> GFP mice is significantly slower than that of sIPSCs in control animals. While analysis of individual miniature IPSCs shows lowered baseline activity, this tonic decrease is associated with an increased amplitude and slow decay of mini-IPSCs onto POMC neurons in POMC: Lepr<sup>−/−</sup> GFP mice. Moreover, POMC neurons receive greater total ionic flux per GABAergic event in the absence of leptin receptor signaling. In addition, treatment with the alpha 3 subunit-containing GABA<sub>A</sub> receptor modulator SB-205384 enhances GABAergic transmission only onto POMC neurons in POMC: Lepr<sup>−/−</sup> GFP mice. Single-cell RT-PCR analysis further supports the expression of the alpha 3 subunit of the GABA<sub>A</sub> receptor on POMC neurons in POMC: Lepr<sup>−/−</sup> GFP mice. Finally, the responses to the GABA<sub>A</sub> receptor agonist isoguvacine of POMC neurons are significantly smaller in POMC: Lepr<sup>−/−</sup> GFP than in control animals. Therefore our present work demonstrates that loss of leptin signaling in POMC neurons induces synaptic alterations at POMC synapses that may play an essential role in energy homeostasis.

INTRODUCTION

The hypothalamus plays a major role in regulating energy homeostasis by integrating hormonal and nutritional signals (Flier 2004; Morton et al. 2006; Schwartz and Porte 2005; Schwartz et al. 2000; Seeley and Woods 2003). Two sets of hypothalamic arcuate nucleus (ARC) neurons play key roles in the regulation of energy homeostasis: one set is the orexigenic pro-opiomelanocortin (POMC) and cocaine-aminophylline regulated transcript (CART) expressing neurons (Vrang et al. 1999) and the other set is the orexigenic neuropeptide Y (NPY) and the agouti gene related peptide (AgRP) expressing neurons (Hahn et al. 1998). Orexigenic NPY/AgRP neurons also release GABA onto POMC neurons (Cowley et al. 2001, 2003). POMC cells thus integrate diverse peripheral and central signals to match transmitter release to the body’s energy status.

Leptin, an adipocyte-derived hormone, modulates POMC neuron excitability via two mechanisms: decreased GABA release from NPY/AgRP neurons and direct activation of nonselective cation channels on POMC neurons (Cowley et al. 2001). Although it is well characterized that manipulations disrupting leptin signaling change the expression of feeding related neuropeptides in hypothalamic neurons (Bates et al. 2003; Cohen et al. 2001; McMinn et al. 2005), it also appears that GABA, a fast inhibitory neurotransmitter, plays a critical role in the control of ingestive behavior (Tong et al. 2008). Moreover, synaptic plasticity associated with dynamic structural changes at synapses in the hypothalamus appears to be involved in the control of food intake as well as the development of obesity (Gao et al. 2007; Pinto et al. 2004). Despite the important physiological impact of GABAergic tone in the ARC, little information is available on synaptic plasticity at GABAergic synapses that may contribute to the control of energy homeostasis. Pinto et al. demonstrated a marked net increase in inhibitory tone to POMC neurons in hypothalamic slices from ob/ob mice, consistent with previous findings indicating that loss of leptin signaling induces synaptic alterations at synapses on POMC neurons (Pinto et al. 2004). Interestingly, mice with a selective genetic deletion of leptin receptors in POMC neurons show only mild obesity and have increased leptin levels (Balthasar et al. 2004). This is consistent with the idea that the increased adiposity in mice lacking leptin receptors may activate compensatory mechanisms. In this study, we thus sought to determine whether loss of leptin receptors on POMC neurons alters synaptic activity at synapses on POMC neurons using POMC: Lepr<sup>−/−</sup> GFP mice (Balthasar et al. 2004).

METHODS

Animals

All mouse care and experimental procedures were approved by the Institutional Animal Care Research Advisory Committee of the Albert Einstein College of medicine. Mice used in these experiments include POMC-CRE, Z/EG (POMC: GFP), and POMC-CRE, Z/EG, Lepr<sup>flex</sup>P<sup>flex</sup> (POMC: Lepr<sup>−/−</sup> GFP) mice (Balthasar et al. 2004).
Slice preparation

Transverse brain slices were prepared from transgenic mice at postnatal age 21–28 days. Animals were anesthetized with a mixture of ketamine and xylazine. After decapitation, the brain was transferred into a sucrose-based solution bubbled with 95% O2-5% CO2 and maintained at ~3°C. This solution contained the following (in mM): 248 sucrose, 2 KCl, 1 MgCl2, 1.25 KH2PO4, 26 NaHCO3, 1 sodium pyruvate, and 10 glucose. Transverse coronal brain slices (200 μm) were prepared using a vibratome. Slices were equilibrated with an oxygenated artificial cerebrospinal fluid (aCSF) for >1 h at 32°C before transfer to the recording chamber. The slices were continuously superfused with ACSF at a rate of 1.5 ml/min containing the following (in mM): 113 NaCl, 3 KCl, 1 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, 1 MgCl2, and 5 glucose in 95% O2-5% CO2.

Electrophysiological recordings

Brain slices were placed on the stage of an upright, infrared-differential contrast microscope (Olympus BX50WI) mounted on a Gibraltar X-Y table (Burleigh) and visualized with a ×40 water-immersion objective by infrared microscopy (DAGE MTI). The POMC neurons were identified by the presence of enhanced green fluorescent protein (eGFP) resulting from the transgene. Whole cell patch clamp recordings and single-cell RT-PCR experiments were made from >300 POMC neurons in the arcuate nucleus from POMC: GFP and POMC: Lepr+/− GFP animals. The internal solution contained the following (in mM): 130 CsCl, 5 CaCl2, 10 EGTA, 10 HEPES, 2 MgATP, 0.5 Na2GTP, and 5 phosphocreatine. All recordings were made at 28°C. GABAergic currents were isolated with the addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-amino-phosphovaleric acid (D-AP-5, 50 μM). Membrane currents were recorded with a Multiclamp 700B in whole cell configuration. Electrophysiological signals were low-pass filtered at 2–5 kHz, stored on a PC, and analyzed off-line with pClamp 10 software (Molecular Devices) and mini analysis 6 software (Synaptosoft).

SB-205384 (Tocris Bioscience) was prepared as 10 mM stock solution in dimethyl sulfoxide and dissolved at a final concentration in aCSF just before the recording session. Picrotoxin (Sigma-Aldrich; 100 μM) was prepared as 100 mM stock solution in 100% EtOH. CNQX and D-AP-5 were purchased from Ascent Scientific and prepared as a 1,000× stock solution. To record GABA_A receptor-mediated currents, we used the GABA receptor agonist isoguvacine (Sigma-Aldrich). Isoguvacine was applied by pressure from a puff pipette positioned in the near proximity of the recorded cell (<20 μm).

Analysis of spontaneous mini IPSCs

Spontaneous miniature inhibitory postsynaptic currents (mini IPSCs) were recorded in the presence of tetrodotoxin (TTX; 1 μM; Sigma). Autodetected events with amplitudes of more than ~5 pA and rise time of <2 ms were also visually examined to correct for noise fluctuation (Mini analysis 6, Synaptosoft). Analysis of mini IPSC decay phase (Clampfit 10, Molecular Devices) was based on the following criteria: single events only (i.e., no multiple events), events having stable baselines 15 ms before the rise, and smooth transition from 0 current to peak amplitude [<20% deviation in d(PA)/dt during rise]. Aligned IPSCs were averaged and the decays were fit by a double exponential function, \( D(t) = A_{fast}e^{-t/\tau_{fast}} + A_{slow}e^{-t/\tau_{slow}} \), where \( D(t) \) is the decay of the IPSCs as a function of time (t), \( A_{fast} \) and \( A_{slow} \) are constants, and \( \tau_{fast} \) and \( \tau_{slow} \) are the fast and slow decay time constants, respectively. The weighted decay time constant was calculated \( \tau_w = (A_{fast} + A_{slow})/(A_{fast} + A_{slow})(t) \).

Single-cell RT-PCR

Single-cell samples were collected from brain slice preparations via aspiration into the patch pipette. The initial reverse transcription (RT) reaction was conducted after pressure ejection of the single-cell samples into freshly prepared RT mix A solution (RT Mix A: 20U of RNase OUT, 300 ng of random primers, 0.5% NP-40 and RNase free water). Samples were sonicated in a total volume of 10 μl at 4°C for 5 min and then incubated for 3 min at 65°C prior to addition of 10 μl RT mix B (RT mix B: 500 μM dNTP, 1X RT buffer, 5 mM MgCl2, 10 mM DTT and 200 U Superscript II). The tubes were incubated at 25°C for 5 min, at 42°C for 1 h, and at 65°C for 10 min.

Two rounds of amplification were performed for the detection of the GABA_A alpha-1–5 subunits transcript. In the first amplification (final volume 50 μl), the reaction mixture contained 10 μl of cDNA, 1× PCR buffer with Mg2+ (Roche), 0.2 mM dNTP, 0.1 μM of each primer, and 2 U of Taq polymerase (Roche). For the second amplification, the reaction mixtures contained 3 μl of the first round PCR product, 1× PCR buffer with Mg2+ (Roche), 0.5 mM dNTP, 1 μM of each primer, 1 M of Betaine and 2 U of Taq polymerase in a final volume of 30 μl. Following denaturation for 3 min at 94°C, the target cDNAs were amplified for 35 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s) followed by 10 min at 72°C. After amplification, the PCR products were analyzed on 2% agarose gels. The sequences of the primers used are the following: alpha 1 F1: 5’-CCATGAGGTTGACGTAGAC-3’; alpha 1 R1: 5’-TCAACCCCTGTTAAGTGCTTAGC-3’; alpha 1 F2: 5’-TGGAGACGTCCTATAGGATG-3’; alpha 1 R2: 5’-GTATGCTGTTGCTGTCTTAC-3’; alpha 2 F1: 5’-GGGACTTCAATAGCTTCTGAC-3’; alpha 2 R1: 5’-GGAAGCTTTCTGGCTTGCAGTG-3’; alpha 2 F2: 5’- GCTGCCTGCTGATGCCTACCG-3’; alpha 2 R2: 5’-GGTTCCTGTGTTGCTGTCTGAC-3’; alpha 3 F1: 5’-AGTACAGACAGATGCTTACG-3’; alpha 3 R1: 5’-GTTGTGTGTGAAGATGCTTACCG-3’; alpha 3 F2: 5’-GTTGTGTGTGAAGATGCTTACCG-3’; alpha 3 R2: 5’-GAGATGTGTGGAAGAAGCTGATAC-3’; alpha 4 F1: 5’- TGATGTGTTGAAATGGAATACCAAT-3’; alpha 4 R1: 5’-AGCAGCACAATCATAAAGGCGCGG-3’; alpha 4 R2: 5’-GTTCCTGTGAACTACAACTGAC-3’; alpha 5 F1: 5’-TGCGCACAACATGAGCAACC-3’; alpha 5 R1: 5’-GACTGCTGGGAAATATCAATGACGGCC-3’; alpha 5 R2: 5’-CTGACAGACAACTGACACCTCC-3’ (Sim et al. 2000); GAPDH (as a control) F1: 5’-TGAGGACAGTGTGCTCTTCT-3’; GAPDH (as a control) R1: 5’-GCCCTTGGTCTGATACCGT-3’; Nested GAPDH R2: 5’-GCTGCTGGGATGAAATTGGAG-3’ (Sim et al. 2000). All primers were purchased from Invitrogen (Carlsbad, CA) and amplified at equivalent efficiencies.

Statistics

Statistical analyses were performed on all POMC neurons examined using the Mann-Whitney U test. The mean values were reported from the entire population tested (Sigmaplot 12.0; Systat Software, San Jose, CA). chi-square tests were also used for statistical analysis of single-cell RT-PCR experiments (SPSS version 15.0; SPSS, Chicago, IL). The Kolomogorov-Smirnov test was used to compare the cumulative distribution of the data. Data were considered significantly different when the P value was <0.05. All statistical results are given as means ± SE.

RESULTS

Decreased GABAergic IPSCs onto POMC neurons in mice lacking leptin receptors in POMC neurons

We examined whether loss of leptin signaling in POMC neurons altered synaptic activity on ARC POMC neurons. POMC: Lepr+/− animals developed mild obesity associated with hyperleptinemia (Balthasar et al. 2004). This hyperleptinemia led to increased hyperphagia and body weight gain. The increase in body weight is likely due to the loss of leptin signaling in POMC neurons. This increase in body weight is likely due to the loss of leptin signaling in POMC neurons.
Deletion of leptin receptors alters GABAergic input onto POMC neurons. A: examples of spontaneous synaptic events onto POMC neurons from POMC: GFP and POMC: Lepr−/− GFP animals. Spontaneous events were recorded in the absence of any antagonists at a holding potential (HP) of −70 mV in whole cell voltage clamp mode. They are glutamatergic and GABAergic currents. There was a significant decrease in the frequency of spontaneous events in POMC neurons (sIPSCs; Fig. 1E) was significantly lower in POMC: Lepr−/− GFP than in POMC: GFP mice (C and D). There was no observable change in the mean amplitude of sIPSCs (Fig. 1D). Furthermore, analysis of individual sIPSCs revealed a significant difference in the decay phase between the two groups of neurons from POMC: GFP mice: 22.6 ± 0.7 ms; P < 0.01; n = 13 and 15 neurons, respectively). (POMC: GFP; ●; POMC: Lepr−/− GFP; ○).

FIG. 1. Decreased GABAergic inhibitory postsynaptic currents (IPSCs) onto pro-opiomelanocortin (POMC) neurons in mice lacking leptin receptors in POMC neurons. A: examples of spontaneous synaptic events onto POMC neurons from POMC: GFP and POMC: Lepr−/− GFP animals. Spontaneous events were recorded in the absence of any antagonists at a holding potential (HP) of −70 mV in whole cell voltage clamp mode. They are glutamatergic and GABAergic currents. There was a significant decrease in the frequency of spontaneous events in POMC: Lepr−/− GFP animals (top: POMC: GFP mouse (POMC). bottom: POMC: Lepr−/− GFP mice (POMC: Lepr−/−)). B: pooled data of frequency and amplitude of both GABAergic and glutamatergic events recorded in POMC neurons from the 2 strains [mean frequency (left): 4.5 ± 0.8 vs. 1.7 ± 0.2 Hz; P < 0.01; mean amplitude (middle): −78.1 ± 10.7 vs. −67.0 ± 7.3 pA; n = 15 and n = 27 neurons, respectively; P < 0.05]. Right: pooled data showing the frequency vs. the amplitude of sIPSCs (POMC: GFP; ●; POMC: Lepr−/− GFP; ○). C: recording samples showing spontaneous GABA_A receptor-mediated IPSCs. The frequency of these sIPSCs was significantly lower in POMC neurons from POMC: Lepr−/− GFP than in control mice (top: POMC: GFP mice. bottom: POMC: Lepr−/− GFP mice). HP = −70 mV. D: pooled data of frequency or amplitude of sIPSCs recorded from 13 and 15 different neurons, respectively. Deletion of leptin receptors on POMC neurons significantly decreased the mean frequency, but not the mean amplitude, of sIPSCs [mean frequency (left): 3.4 ± 0.5 vs. 1.9 ± 0.3 Hz; P < 0.05; mean amplitude (middle): −53.3 ± 4.2 vs. −58.3 ± 5.4 pA; P > 0.05]. Right: pooled data showing the frequency versus the amplitude of sIPSCs (POMC: GFP; ●; POMC: Lepr−/− GFP; ○). E: an example of recordings showing that the GABA_A receptor antagonist picrotoxin (100 μM) completely blocked sIPSCs. The sIPSCs were mediated by activation of GABA_A receptors. HP = −70 mV. F: an example of recordings shows sIPSCs recorded in POMC neurons. Left: superimposition of traces of sIPSCs. Right: normalized traces of average amplitude of sIPSCs to shows the difference in the decay time course of sIPSCs. G: pooled data of weighted decay time of sIPSCs. The weighted decay was significantly slower in POMC neurons from POMC: Lepr−/− GFP than in POMC: GFP animals (POMC: GFP mice: 17.0 ± 0.4 ms; POMC: Lepr−/− GFP mice: 22.6 ± 0.7 ms; P < 0.01; n = 13 and 15 neurons, respectively). H and I: pooled data showing the integrated area versus the frequency of sIPSCs. The mean integrated area that represents the total ion flux was slightly greater in POMC neurons from POMC: Lepr−/− GFP than POMC: GFP animals (H). (POMC: GFP; ●; POMC: Lepr−/− GFP; ○).
and POMC: Lepr<sup>−/−</sup> GFP animals (Fig. 1F). The decay phase of sIPSCs was best fitted with the sum of two exponential functions, thus the weighted decay time constant (τ<sub>d</sub>) was calculated. As shown in Fig. 1G, the calculated weighted decay in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals was significantly slower than that in POMC neurons from control mice. The mean integrated area of sIPSCs was 1204.3 ± 109.2 pA · ms in POMC neurons from POMC: GFP mice and 1493.8 ± 137.2 pA · ms in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice (Fig. 1, H and I; P > 0.05; n = 13 and 15 neurons, respectively). In contrast to lower inhibitory tone onto POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals, there was no significant difference in glutamatergic transmission at synapses on POMC neurons (mean frequency: POMC: GFP mice: 1.1 ± 0.3 Hz, POMC: Lepr<sup>−/−</sup> GFP mice: 1.0 ± 0.2 Hz; P > 0.05; mean amplitude: −13 ± 0.6 vs. −14 ± 1 pA; P > 0.05; decay: 4.3 ± 0.6 vs. 4.5 ± 0.7 ms; P > 0.05; n = 10 and n = 10 neurons, respectively).

As only GABAergic sIPSCs were depressed in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals, we next examined GABAergic IPSCs in the presence of tetrodotoxin (TTX; mini IPSCs). As shown in Fig. 2A, POMC: Lepr<sup>−/−</sup> GFP mice exhibited a marked decrease in inhibitory synaptic events at synapses on POMC neurons (Fig. 2A, 1–3). While the frequency of miniIPSCs was significantly lower, the amplitude of miniIPSCs was greater in POMC neurons from leptin-deficient than control animals (Fig. 2, B and C). We found that there was no difference in the mean rise time between the two groups of neurons from the 2 strains (mean frequency: 2.0 ± 0.2 vs. 0.8 ± 0.1 Hz; mean amplitude: −54.5 ± 1.7 vs. −69.5 ± 3.2 pA; n = 55 and n = 39 neurons; P < 0.001).

**FIG. 2.** Properties of mini IPSCs in POMC neurons from the 2 strains. A: representative recording samples show mini IPSCs recorded in POMC neurons from the 2 strains (A1, top: POMC: GFP animal; bottom: POMC: Lepr<sup>−/−</sup> GFP animal). HP = −70 mV. There was a marked decrease in the frequency of mini IPSCs in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals. (A2 and A3) Cumulative probability of mini IPSCs inter-event interval (left) and amplitude (right). The cumulative probability distributions for inter-event interval and amplitudes significantly differed between the 2 strains (P < 0.01 and P < 0.01, respectively). B: pooled data showing the frequency versus the amplitude of mini IPSCs (POMC: GFP: ●; POMC: Lepr<sup>−/−</sup> GFP: ○; n = 55 and n = 39 neurons, respectively). C: pooled data of frequency and amplitude of mini IPSCs. The mean frequency as well as the mean amplitude was significantly different between the 2 groups of neurons from the 2 strains (mean frequency: 2.0 ± 0.2 vs. 0.8 ± 0.1 Hz; mean amplitude: −54.5 ± 1.7 vs. −69.5 ± 3.2 pA; n = 55 and n = 39 neurons; P < 0.01). D: pooled data showing the mean rise time of mini IPSCs. The mean rise time was not significantly different between the 2 groups of POMC neurons from the 2 strains (mean rise time: 1.64 ± 0.07 vs. 1.72 ± 0.13 ms; n = 55 and n = 39 neurons; P > 0.05). E: superimposition of traces of average amplitude of mini IPSCs (left). Right: normalized traces of average amplitude of mini IPSCs. F: summary plot of weighted decay data from 55 (POMC: GFP) and 39 (POMC: Lepr<sup>−/−</sup> GFP) neurons. The mean decay time was greater in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP than POMC: GFP animals. G and H: pooled data showing the integrated area vs. the frequency of mini IPSCs. Although the mean frequency of mini IPSCs was lower, the mean integrated area that represents the total ion flux was greater in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP than POMC: GFP animals (H). (POMC: GFP: ●; POMC: Lepr<sup>−/−</sup> GFP: ○).
neurons (Fig. 2D, mean rise time: 1.64 ± 0.07 vs. 1.74 ± 0.13 ms; n = 55 and n = 39 neurons, P > 0.05). Of particular interest is that the decay time of individual mini IPSCs in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice were significantly prolonged [Fig. 2, E and F; fast (τ<sub>fast</sub>): POMC: GFP 6.5 ± 0.6 ms, POMC: Lepr<sup>−/−</sup> GFP 5.9 ± 0.5 ms, n = 55 neurons, P > 0.05; Slow (τ<sub>slow</sub>): POMC: GFP 14.8 ± 2.5 ms, POMC: Lepr<sup>−/−</sup> GFP 23.6 ± 1.8 ms, n = 39 neurons, P < 0.01; weighted decay (τ<sub>W</sub>): 17.2 ± 0.7 ms versus 22.5 ± 0.5 ms; n = 55 neurons and 39 neurons, respectively, P < 0.01].

As the amplitude and the decay time was greater in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice, we compared the total charge transfer measured as integrated individual mini IPSCs (Fig. 2G). The mean integrated area was 1103.0 ± 46.3 pA · ms in POMC neurons from POMC: GFP and 1537.0 ± 88.2 pA · ms in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice (Fig. 2H; n = 55; n = 39 neurons, respectively; P < 0.01). While the frequency of mini GABAergic IPSCs was lower in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice, it appears that POMC neurons receive a greater total ionic flux per GABAergic event in the absence of leptin receptor signaling.

Expression of distinct GABA<sub>α</sub> receptors in ARC POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice

The composition of GABA<sub>α</sub> alpha subunits determines the decay time phase (Gingrich et al. 1995; Kokksma et al. 2005; Ortinski et al. 2004). In particular, it has been demonstrated that the decay time courses of currents mediated by receptors containing α3 were slower than those containing α1 or, both α1 and α3 (Verdoorn 1994). Hence we examined whether the composition of GABA<sub>α</sub> receptor subunits in POMC neurons from two strains were molecularly distinct. We first tested the effect of the alpha 3 subunit-containing GABA<sub>α</sub> receptor modulator SB-205384 on mini GABAergic transmission (Meadows et al. 1997). Treatment with SB-205384 (5 μM) in the presence of TTX enhanced mini GABAergic transmission only onto POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice. As shown in Fig. 3B, the frequency of GABAergic mini IPSCs was increased from 0.8 ± 0.1 to 1.3 ± 0.2 Hz following application of SB-205384 (5 μM; Fig. 3C; mean increase: 167.3 ± 9.1% of control; n = 6; P < 0.01). This enhancement was associated with an increased amplitude of mini IPSCs in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice (Fig. 3D). However, there was no significant difference in the decay time (Fig. 3D). In contrast to the robust enhancement of GABAergic transmission onto POMC neurons from POMC: Lepr<sup>−/−</sup> GFP mice, there were no differences in mean frequency and amplitude in POMC neurons from control animals following treatment with SB-205384 (Fig. 3, A, C and D; mean frequency: before: 1.4 ± 0.1 Hz, after: 1.4 ± 0.2 Hz, n = 8; P > 0.05). To elucidate the molecular basis for the differences observed, we performed single-cell RT-PCR to examine GABA<sub>α</sub> alpha subunit expression in ARC POMC neurons. Single-cell RT-PCR analysis revealed the expression of distinct GABA<sub>α</sub> receptors in the ARC POMC neurons of POMC: Lepr<sup>−/−</sup> GFP mice compared with POMC: GFP mice (Fig. 4A and Table 1).

Expression of α1–α5 subunits in ARC POMC neurons from the two strains. Expression of α3 subunits was markedly different between two strains. Most POMC neurons harvested from POMC: Lepr<sup>−/−</sup> GFP mice expressed the α3 subunit. Figure 4A provides an example of α subunit signals detected in four neurons from each strain. Consideration of the kinetic analysis of IPSCs, together with subunit expression patterns suggests that the significant differences in decay time constants in POMC neurons from the two strains may correlate with differences in the expression of α subunits. Finally, we examined the responses to the GABA<sub>α</sub> receptor agonist isoguvacine (IGV). We found that the mean amplitude of IGV-induced maximum currents was approximately three times larger in POMC: Lepr<sup>−/−</sup> GFP mice.
Our results demonstrate that inhibitory synaptic signals onto POMC neurons differ considerably in POMC: Lepr<sup>−/−</sup> GFP mice compared with POMC: GFP mice. This could be due to differences in GABA<sub>A</sub> receptor subunit composition. Mice selectively lacking leptin receptor expression in POMC neurons show decreased GABA inhibitory tone onto ARC POMC neurons. This appears to be neuron-specific as loss of 

TABLE 1. Single cell RT-PCR analysis of GABA<sub>A</sub> receptor alpha subunits (from 1 to 5) in POMC: GFP and POMC: Lepr<sup>−/−</sup> GFP animals

<table>
<thead>
<tr>
<th>Subunit</th>
<th>POMC</th>
<th>POMC: Lepr&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Total Cells</td>
<td>Positive Cells</td>
</tr>
<tr>
<td>α1</td>
<td>17</td>
<td>12 (70.6)</td>
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<tr>
<td>α2</td>
<td>17</td>
<td>12 (70.6)</td>
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<td>α3</td>
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<td>α5</td>
<td>17</td>
<td>11 (64.7)</td>
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Parentheses enclose percentages. POMC, pro-opiomelanocortin.
thereby increasing the excitability of POMC neurons. If this is the case, this increase in POMC neuron excitability would attenuate the effect of loss of leptin signaling in POMC neurons.

Alternatively, changes in feeding and anorexigenic/orexigenic hormone signals via direct and/or indirect mechanisms could modify the synapse number and neurotransmitter release onto POMC neurons (Pinto et al. 2004). Pinto et al. (2004) showed that there was a net increase in GABAergic transmission but no change in excitatory tone onto POMC neurons in ob/ob mice. In contrast to their electrophysiological observations, the electron microscopic analysis showed a remarkable decrease in excitatory synapse number but no change in inhibitory synapse number on POMC neurons in ob/ob animals. Thus disruption of leptin signaling may alter not only the synapse number but also the probability of neurotransmitter release.

In line with this idea, we demonstrated a decreased GABAergic, but not glutamatergic, synaptic transmission at synapses on POMC neurons in POMC: Lepr<sup>−/−</sup> GFP mice. This could be due to lower probability of GABA release from presynaptic inputs, lower number of synaptic boutons, or lower number of GABA<sub>A</sub> receptors on the postsynaptic membrane in POMC: Lepr<sup>−/−</sup> GFP than in POMC: GFP mice. For example, it has been shown that GABAergic synaptic transmission onto POMC neurons was modulated by hormones and neuropeptides that regulate energy homeostasis (Acuna-Goycolea and van den Pol 2005; Cowley et al. 2001, 2003; Smith et al. 2007). Hence changes in hormonal signals associated with mild obesity could affect GABAergic inhibitory tone onto POMC neurons. We think that these synaptic changes occurred in cell types that are unlikely to have been affected by the specific CRE-mediated deletion of the leptin receptor.

Alternatively, it has been shown that leptin deficiency induces abnormal development of POMC neuronal processes (i.e., dendrite and axon) (Bouret et al. 2004). Hence genetic deletion of leptin receptors on POMC neurons would decrease the number of synapses on POMC neurons. Additionally, because membrane expression of GABA<sub>A</sub> receptors is regulated by levels of neuronal activity (Holopainen and Lauren 2003; Penschuck et al. 1999) and leptin-activated JAK/STAT pathway (Lund et al. 2008), the loss of leptin signaling in POMC neurons could disrupt the GABA<sub>A</sub> receptor alpha subunit gene expression. Indeed we found that the kinetics of GABAergic IPSCs at synapses on POMC neurons was slower in POMC: Lepr<sup>−/−</sup> GFP animals and that there was a distinct pharmacological difference in GABA<sub>A</sub> receptors in POMC neurons from the two strains. These results suggest that there are distinct differences in the composition of GABA<sub>A</sub> receptor alpha subunits in ARC POMC neurons from POMC: Lepr<sup>−/−</sup> GFP and POMC: GFP mice. Indeed we found that the positive modulator of alpha3-subunit containing GABA<sub>A</sub> receptors SB-205384 (Meadows et al. 1997) increased mini-IPSC frequency and amplitude in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP. Interestingly, it has been reported that the greatest effect on decay time was observed only with the alpha3beta2gamma1 subunit combination with small effects seen with alpha3beta2gamma2 and alpha3beta1gamma1 (Meadows et al. 1998). Furthermore, the potentiation of the peak amplitude of the GABA-activated currents by SB-205384 was less specific for a particular subunit combination (Meadows et al. 1998). We thus speculate that ARC POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals preferentially express GABA<sub>A</sub> receptors composed of alpha3, beta2 (or beta1), and gamma1 subunits.

What is the physiological significance of the expression of alpha3-containing GABA<sub>A</sub> receptors in ARC POMC neurons from POMC: Lepr<sup>−/−</sup> GFP animals? We consistently found that peak mini IPSC amplitudes as well as decay time constants were greater in POMC: Lepr<sup>−/−</sup> GFP than in control mice. It thus appears that POMC neurons compensate for the loss of leptin signaling with molecularly distinct GABA<sub>A</sub> receptors as POMC neurons received less inhibitory synaptic currents in POMC: Lepr<sup>−/−</sup> GFP animals. In fact, we show that the total charge transfer measured as integrated individual IPSCs was greater in POMC neurons from POMC: Lepr<sup>−/−</sup> GFP than POMC: GFP animals. It seems likely that GABAergic synapses in POMC: Lepr<sup>−/−</sup> GFP animals are able to selectively express GABA<sub>A</sub> receptors having slow decay kinetics during development. As synaptic duration of individual events may determine synaptic efficacy or strength, we think that this functional preference probably represents the compensatory mechanisms that counteract decreased inhibitory tone onto ARC POMC neurons.

Accumulating evidence suggests that changes in GABA<sub>A</sub> receptor alpha subunit composition may be important in the molecular pathogenesis of several common CNS conditions including epilepsy, alcoholism, anxiety, and stress (Hsu et al. 2003). Thus it is interesting to examine whether alterations in GABA<sub>A</sub> receptor alpha subunit composition are involved in the development of mild or severe obesity in animal models. In fact, the observed increase in alpha3 expression is important precisely because it contributes to compensation in circuits that control energy homeostasis, resulting in a less severe extent of obesity than would be expected by loss of leptin signaling in these POMC cells. Understanding the cellular mechanisms underlying discrete synaptic adaptations at synapses on POMC neurons in response to physiological adaptations would be useful in providing targets for the development of improved therapies for the treatment of obesity and obesity-related diseases.

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