Leptin down-regulates KCC2 activity and controls chloride homeostasis in the neonatal rat hippocampus.

Camille Dumon[1#], Yasmine Belaidouni[1], Diabe Diabira[1], Suzanne M. Appleyard[2], Gary A. Wayman[2] and Jean-Luc Gaiarsa[1*].

[1]Aix-Marseille Univ UMR 1249, INSERM (Institut National de la Santé et de la Recherche Médicale) Unité 1249, INMED (Institut de Neurobiologie de la Méditerranée), Parc Scientifique de Luminy, Marseille, France.

[2]Program in Neuroscience, Department of Integrative Physiology and Neuroscience, Washington State University, Pullman, WA, USA.

[#]Present address: Neurochlore Parc Scientifique et Technologique de Luminy, Bâtiment Beret Delaage, Zone Luminy Entreprises Biotech, Marseille, France

[*]Correspondence to: jean-luc.gaiarsa@inserm.fr

ABSTRACT

The canonical physiological role of leptin is to regulate hunger and satiety acting on specific hypothalamic nuclei. Beyond this key metabolic function; leptin also regulates many aspects of development and functioning of neuronal hippocampal networks throughout life. Here we show that leptin controls the chloride homeostasis in the developing rat hippocampus *in vitro*. The effect of leptin relies on the donx-regulation of the activity of the potassium/chloride extruder KCC2 and is present during a restricted period of postnatal development. This study confirms and extends the role of leptin in the ontogenesis of functional GABAergic inhibition and helps understanding how abnormal levels of leptin may contribute to neurological disorders.

Key words: GABA, KCC2, chloride homeostasis, hippocampus, rat, leptin, maternal obesity.

INTRODUCTION

Leptin, the product of the obese (ob) gene, is a circulating hormone secreted mainly from the white adipocytes and transported across the blood brain barrier to the hypothalamus to suppress appetite and enhance metabolism in adult (1). The hypothalamus is not the only central nervous system target for leptin, as a high density of leptin receptors are expressed in other brain areas including the hippocampus where leptin receptors regulate many aspects of synaptic plasticity and cognitive function (2,3). A large body of evidence indicates that leptin also acts as an important neurodevelopmental factor during the perinatal period (4–6). Thus, while plasma leptin levels reflect adiposity in the adult rodents, leptin levels surge during the two first postnatal week of life regardless of the animal's weight or body fat mass (1). A similar restricted surge of plasma levels is observed during the last trimester of gestation in human (7). Along with the leptin surge, the leptin receptors are expressed and functional in several brain regions at embryonic and postnatal stages and promote neuronal networks development (8–14). Due to the many important physiological and developmental functions of leptin, dysregulation in its availability or signaling has been proposed as causal factors for the occurrence of neurological disorders (15–23).

Abnormalities in GABAergic synaptic transmission are strongly associated with neurological disorders (24,25). Therefore understanding whether and how leptin controls the development and efficacy of the GABAergic transmission is warranted. Leptin deficient (*ob/ob*) mice exhibit a lower number of GABAergic synapses impinging hypothalamic (26) and hippocampal (12) neurons highlighting the role of leptin in GABAergic synaptogenesis. Likewise, leptin modulates the GABAergic synaptic activity *in vitro* in the hypothalamus (27,28) and hippocampus (12,29). The homeostasis of intra-neuronal Cl^- concentration ($[Cl^-]_i$) is an essential determinant of GABA functioning and alterations in $[Cl^-]_i$ is implicated in the etiology of numerous neurological and

psychiatric disorders (30–32). In a previous study, we reported that the absence of leptin signaling accelerates the ontogenesis of functional GABAergic inhibition in the newborn mice hippocampus *in vivo* (13). In the present study we show that leptin acts directly on hippocampal neurons to control Cl^- homeostasis and the activity of the K^+-Cl^- co-transporter KCC2 in the rat hippocampus during a restricted developmental window.

MATERIALS and METHODS

All animal procedures were carried out in accordance with the European Union Directive of 22 September (2010/63/EU). The protocol was approved by the INSERM Local committee (Number 0287.01, delivered by the French Ministry of Education and Research). Experiments were performed on both male and female postnatal day (P) 1 to 10 Wistar rats. Animals were housed in a temperature-controlled environment with a 12 light/dark cycle and free access to food and water.

Hippocampal slice preparation

Brains were removed and immersed into ice-cold (2-4°C) artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 126 NaCl, 3.5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose, pH 7.4 equilibrated with 95% O_2 and 5% CO_2. Hippocampal slices (600 µm thick) were cut with a McIlwain tissue chopper (Campden Instruments Ltd.) and kept in ACSF at room temperature (25°C) for at least one hour before recording. Slices were then transferred to a submerged recording chamber perfused with oxygenated (95% O_2 and 5% CO_2) ACSF (3 ml/min) at 34°C.

Electrophysiological recordings

E_{GABA} measurement: Perforated patch-clamp recordings were made from CA3 pyramidal neurons using an axopatch 200B (Axon Instrument) or Multiclamp 700B (Molecular devices) amplifier. Glass recording electrodes had resistances of 4-7 MΩ when filled with KCl solution containing 150mM KCl and 10 mM HEPES, pH adjusted to 7.2 with Tris-OH. The pipettes were tip filled with a gramicidin-free KCl solution and then backfilled with the same solution containing

gramicidin A (50µg/ml, diluted from a 50mg/ml stock solution in DMSO). $GABA_A$ receptor-mediated postsynaptic currents ($eGABA_A$-PSCs) were evoked in the presence of glutamatergic receptor antagonists (NBQX 5µM and D-APV 40µM) at a frequency of 0.01Hz with a bipolar tungsten electrode placed in the CA3 *stratum radiatum*. After the access resistance had dropped (40 to 80 MΩ) and stabilized (15-30 min), a current-voltage relationship was constructed by measuring the peak amplitude of averaged $eGABA_A$-PSCs (3 single sweeps) at different holding potentials in 10 mV increment recorded in the presence of NBQX 5µM and D-APV 40µM. Measurements were not corrected for the liquid junction potentials. A linear regression was used to calculate the best-fit line of the voltage dependence of the synaptic currents. Spontaneous rupture into whole-cell was evidenced by large inward synaptic currents due to E_{Cl} of 0mV.

Isoguvacine effect on neuronal firing: Loose cell attached patch clamp recordings were performed from CA3 pyramidal neurons using an axopatch 200B (Axon Instrument) with glass electrodes (4-7 MΩ) filled with KCl solution containing 150mM KCl and 10 mM HEPES, pH adjusted to 7.2 with Tris-OH. After a baseline period of at least 10 min in the presence of NBQX (5µM) and D-APV (40µM), isoguvacine (10µM) was bath applied for 2 min. The effect of isoguvacine was quantified as the mean frequency of action potential following application of isoguvacine (4-8 min) versus baseline frequency (-10-0 min). Synaptic activity was recorded with Axoscope software version 8.1 (Axon Instruments) and analyzed offline with Mini Analysis Program version 6.0 (Synaptosoft).

Statistics

No statistical methods were used to predetermine sample sizes, but our sample sizes correspond to those reported in previous publications (12,13). To ensure the consistency and reproducibility

of our results, we conducted repeated trials in different acute hippocampal slices prepared from at least three different animals for each experimental condition. A Mann Whitney test was used to analyze difference between two individual groups. A two-tailed paired Student's *t*-test was used to analyze differences within one group across conditions. All data are expressed as Mean $\pm$ standard error to the mean (S.E.M.). Data are judged significantly different when $P<0.05$. In the figures, box plots represent the 1^{rst} and 3^{rd} quartiles; whiskers show data range; horizontal lines show the median.

RESULTS

Leptin controls chloride homeostasis *in vitro*.

Our first aim was to determine whether leptin directly acts on hippocampal cells to control Cl^- homeostasis in the neonatal rat. We used acute postnatal (P) day 5 rat hippocampal slices and stimulated presynaptic GABAergic neurons while gramicidin perforated patch-clamp recordings were made from CA3 pyramidal neurons in the presence of the glutamatergic receptor blockers NBQX (5µM) and D-APV (40µM). $GABA_A$ receptor-mediated postsynaptic currents ($eGABA_A$-PSCs) were evoked at different holding potentials, before and during the application of leptin, to determine the impact of the adipocyte hormone on their reversal potential (E_{GABA}). We found that leptin induced an average depolarizing shift of E_{GABA} (ΔE_{GABA}) of 5.4±1.7 mV (from -48.2±2.8 mV to -42.8±3.7 mV, n=10, p=0.01 two-tailed paired Student's *t*-test, Fig. 1A1 and B). In control experiments in which leptin was omitted E_{GABA} did not change over the same recording duration (ΔE_{GABA}= 1.3±0.5 mV, from -45.6±3.8 mV to -45.2±3.8 mV, n=8, p=0.7 two-tailed paired Student's *t*-test, Fig. 1A2 and B). Leptin applied at a concentration of 20 nM had no effect on E_{GABA} (ΔE_{GABA}= -0.5±1.6 mV, from -53.6±2.4 mV to -54.8±3.1 mV, n=6, p=0.4 two-tailed paired Student's *t*-test, Fig. 1B). We next determined whether the depolarizing shift of E_{GABA} induced by leptin was associated with increased neuronal excitation. To this end we recorded action potentials in loose patch mode in the presence of NBQX (5µM) and D-APV (40µM). After a baseline period (10 min), leptin (100 nM) was added to the perfusion medium for 20 minutes. We assessed the effect of leptin on action potential firing at the end of the leptin application (15-20 min) versus the baseline period (-10-0 min, Fig. 1C). Leptin led to a significant increase in the frequency of action potentials (from 0.46±0.14 Hz to 1.02±0.32 Hz, n=7, p=0.03 compared to baseline, two-tailed paired Student's *t*-test, Fig. 1C and D). In interleaved control experiments in

which leptin was omitted the spike firing remained constant (from 0.32±0.12 Hz to 0.43±0.16 Hz, n=7, p=0.2 compared to baseline, two-tailed paired Student's *t*-test, Fig. 1C and D). In agreement with the lack of effect of leptin at 20 nM on E_{GABA} (Fig. 1B), bath applied leptin at the same concentration (20 nM, 20 min) had no effect on the firing frequency of CA3 pyramidal neurons (from 0.47±0.14 Hz to 0.44±0.16 Hz, n=6, p=0.6 compared to baseline, two-tailed paired Student's *t*-test, Fig. 1B). Altogether these data show that bath applied leptin shifts E_{GABA} towards depolarizing values and increases the neuronal excitation of P5 CA3 pyramidal neurons on rat hippocampal slices.

Leptin controls KCC2 activity *in vitro*.

Chloride homeostasis and the strength of $GABA_A$-mediated synaptic inhibition are mainly controlled by the activity of two cation-chloride cotransporters: the Na^+-K^+-$2Cl^-$ (NKCC1) co-transporter that accumulates Cl^- intracellularly and the K^+-Cl^- (KCC2) co-transporter that lowers intracellular Cl^- concentration (33,34). We therefore asked whether leptin acts on KCC2 and/or NKCC1 activity. We found that the depolarizing shift of E_{GABA} induced by leptin was prevented by the diuretic bumetanide at a concentration of 100µM, to block both NKCC1 and KCC2 (ΔE_{GABA}= -0.1±2 mV, from -54±4 mV to -54±6 mV, n=10, *P*=0.9, two-tailed paired Student's *t*-test, Fig. 2A). However, bumetanide at 20µM to block NKCC1 shifted E_{GABA} toward hyperpolarizing values (from -51±3mV (n=20) to -75±5 mV (n=10), p=0.08, Mann Whitney test, Fig. 2B) but failed to prevent the effect of leptin on E_{GABA} (ΔE_{GABA}= 8±3 mV, from -75±5 mV to -63±6 mV, n=7, *P*=0.04, two-tailed, paired Student's *t*-test, Fig. 2A). These results suggest that leptin down-regulates KCC2 activity. Accordingly, the selective KCC2 blocker VU0463271 (20

μM) led to a depolarizing shift of E_{GABA} (from -56±13mV to -39±7 mV, n=10, p=0.005 two-tailed paired Student's *t*-test, Fig. 2B) and prevented the effect of leptin (ΔE_{GABA}= -0.8±1.2 mV from -43±8 mV to -44±7 mV, n=7, *P*=0.5, two-tailed paired Student's *t*-test, Fig. 2A).

To determine whether the increase in spike firing induced by bath applied leptin (Fig. 1 C and D) also resulted from a down regulation of KCC2 activity and modification of GABAergic strength, the same experiment was repeated in the continuous presence of the selective $GABA_A$ receptor antagonist Gabazine (5μM) or in the presence of the selective KCC2 blocker VU0463271. We found that Gabazine (5μM) completely abolished the leptin-induced increase in firing. The frequency of action potential was respectively 0.81±0.22 Hz and 0.85±0.28 Hz before and during the application of leptin (n=8, p=0.57 compared to baseline, two-tailed paired Student's *t*-test, Fig. 2C). Likewise, the selective KCC2 blocker VU0463271 (20μM) also prevented the effect of leptin (from 0.15±0.01 to 0.17±0.02 Hz before and during the application of leptin, n=4, p=0.15 compared to baseline, two-tailed paired Student's *t*-test, Fig. 2C). Altogether, these data show that leptin down-regulates KCC2 activity shifting E_{GABA} towards depolarizing values in P5 rat hippocampal slices.

The action of leptin *in vitro* on chloride homeostasis is developmentally regulated

Previous studies reported that the responsiveness of leptin is regulated during development (29,35–37). We therefore asked whether the leptin-induced depolarizing shift of E_{GABA} is developmentally regulated. We found a non-linear U-shaped relationship between the age of the rats and the responsiveness of leptin. Thus, while bath applied leptin (100nM, 20 min) led to a significant depolarizing shift of E_{GABA} at P5 (Fig. 1B), the same application had no effect on the

reversal potential of $GABA_A$ receptor-mediated postsynaptic currents evoked on hippocampal slices at P2 (ΔE_{GABA}=-0.75±2.1 mV, from -45.6±7 mV to -47.6±6 mV, n=5, p=0.37 two-tailed paired Student's *t*-test, Fig. 3A) and P10 (ΔE_{GABA}=0.5±1.6 mV, from -70.8±2.1mV to -70.3±2.7 mV, n=6, p=0.7 two-tailed paired Student's *t*-test, Fig. 3A). Of note, the effect of leptin on E_{GABA} was not correlated to the initial polarity of the GABAergic responses (Fig. 3B). Likewise, leptin failed to increase the firing frequency of CA3 pyramidal neurons when applied at P10 (from 0.55±0.13 to 0.64±0.13 Hz before and during the application of leptin, n=11, p=0.13 compared to baseline, two-tailed paired Student's *t*-test, Fig. 3C). We were unable to test the effect of leptin at P2 because of a sparse action potentials and low frequency discharge. Altogether, these data show that the effects of leptin on chloride homeostasis *in vitro* are restricted to a narrowed developmental window.

DISCUSSION

Besides its key role in regulating energy balance, leptin exerts many other important developmental and physiological functions throughout life (1,2,4,7,38). In the present study, we show that leptin acts directly on newborn rat hippocampal neurons to control the chloride homeostasis and the strength of GABAergic inhibition *in vitro*. We further show that the effects of leptin rely on the control of the activity of the K/Cl cotransporter KCC2 and are present during a restricted developmental window. The present study complements previous reports of leptin modulating GABAergic synaptic transmission in the developing rat hippocampus *in vitro* (12,29) and extends our previous report of leptin controlling the ontogenesis of functional GABAergic inhibition in the developing mice hippocampus *in vivo* (13).

Our data demonstrate that bath applied leptin regulates the activity of KCC2 in the developing rat hippocampus. We have shown that leptin treatment induces a depolarizing shift of E_{GABA} and increases the firing frequency of CA3 pyramidal neurons. Both effects were prevented by the selective KCC2 blocker VU0463271. How leptin controls KCC2 activity is presently unknown. The ion transport activity of KCC2 depends on transcriptional factors (i.e the protein abundance) as well as post-translational regulations by (de)phosphorylation of the protein (33,34). We previously showed that newborn leptin receptor deficient (*db/db*) mice showed an increased expression of KCC2 compared to their wild type littermates (13). We also showed that chronic (24h) treatment of hippocampal neuronal cultures with leptin decerased the amount of KCC2 and increased the phosphorylation of the threonine 906 and 1007 residues (Thr906/Thr1007) of KCC2 (13), known to decrease the membrane expression and activity of the transporter (39,40). In the presence study, the acute (20 min) application of leptin was unlikely to

induce transcriptional modifications, and a post-translational regulation is the most expected mechanism to account for the reduced activity of KCC2.

Developmental changes in leptin's actions and downstream signaling pathways have been reported in the hippocampus (29,35,37) and hypothalamic (36) neurons. We found that the effects of leptin on E_{GABA} and firing of CA3 pyramidal neurons are also developmentally regulated. Different mechanisms, including a developmentally regulated expression of the leptin receptors as well as downstream signaling pathways and/or effectors could account for this observation. The former hypothesis is unlikely since both molecular (8,10) and functional (11,12) studies revealed the presence of functional leptin receptors in the newborn rodent hippocampus. The later hypothesis could be considered even if the downstream pathway linking leptin and the activity of KCC2 remains to be elucidated. The With No lysine family of serine/threonine kinase (WNK)-dependent phosphorylation of the Thr906/Thr1007 residues of KCC2 is a key player in the regulation of chloride homeostasis during development (39,40). We previously obtained evidence that leptin promotes the phosphorylation of the Thr906/Thr1007 residues of KCC2 via a WNK-dependent pathway on hippocampal neuronal cultures (13). Developmental changes in WNK signaling and WNK-dependent control of chloride homeostasis have been observed both *in vitro* and *in vivo* in cortical and hippocampal neurons (39,40). Moreover, Thr906/Thr1007 residues becomes progressively dephosphorylated during neuronal development (40,41). Thus the high level of endogenous Thr906/Thr1007 phosphorylated KCC2 at birth and the absence of KCC2-dependent control of chloride homeostasis by endogenous WNK in mature neurons are possible explanations for the restricted effects of leptin.

We have shown that an elevated concentration (i.e. 100 nM, but not 20 nM) of leptin affects the chloride homeostasis in the developing rat hippocampus. Similarly, in a previous study

we reported that hyperleptinemia induced *in vivo* by daily sub-cutaneous injections of leptin delayed the emergence of functional GABAergic inhibition in the newborn mice hippocampus (13). Elevated circulating leptin levels have been observed in patient with neurodevelopmental disorders such as Autistic spectrum disorder (ASD) and Rett syndrome (15,16,18,42,43) and in animal models of the diseases (44,45). Moreover, accumulating evidence indicate that impaired chloride homeostasis is a common feature of numerous neurological disorders associated with impairments in hippocampal-dependent cognitive processes (30–32). Targeting the leptin signaling pathway may therefore have therapeutic potential in neurological and neuropsychiatric disorders.

LIST of ABBREVIATION

APV, 5-amino-phosphono-valeric acid; ASD, Autistic spectrum disorder; *db/db* mice, Leptin-receptor deficient mice; $eGABA_A$-PSCs, evoked $GABA_A$ receptor-mediated postsynaptic currents; GABA, γ-aminobutyric acid; KCC2, K^+-Cl^- cotransporter; NBQX, 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; NKCC1, Na^+-K^+-$2Cl^-$ co-transporter; *ob/ob* mice, Leptin deficient mice; Thr906/Thr1007, threonine 906 and 1007 residues; WNK, With No lysine family of serine/threonine kinase.

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AUTHOR CONTRIBUTIONS

JLG, GW, SA and CD conceived and designed the experiments. CD, YB and DD performed the experiments and analyzed the data. JLG drafted the manuscript and all authors participated in critical revision of the manuscript. All authors approved the final version of the manuscript.

ETHICS APPROVAL and CONSENT TO PARTICIPATE

All animal procedures were carried out in accordance with the European Union Directive of 22 September (2010/63/EU). The protocol was approved by the INSERM Local committee (Number 0287.01, delivered by the French Ministry of Education and Research).

DATA AVAILABILITY STATEMENT

The data sets generated for this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

CONSENT for PUBLICATION

Not applicable

FIGURES LEGEND

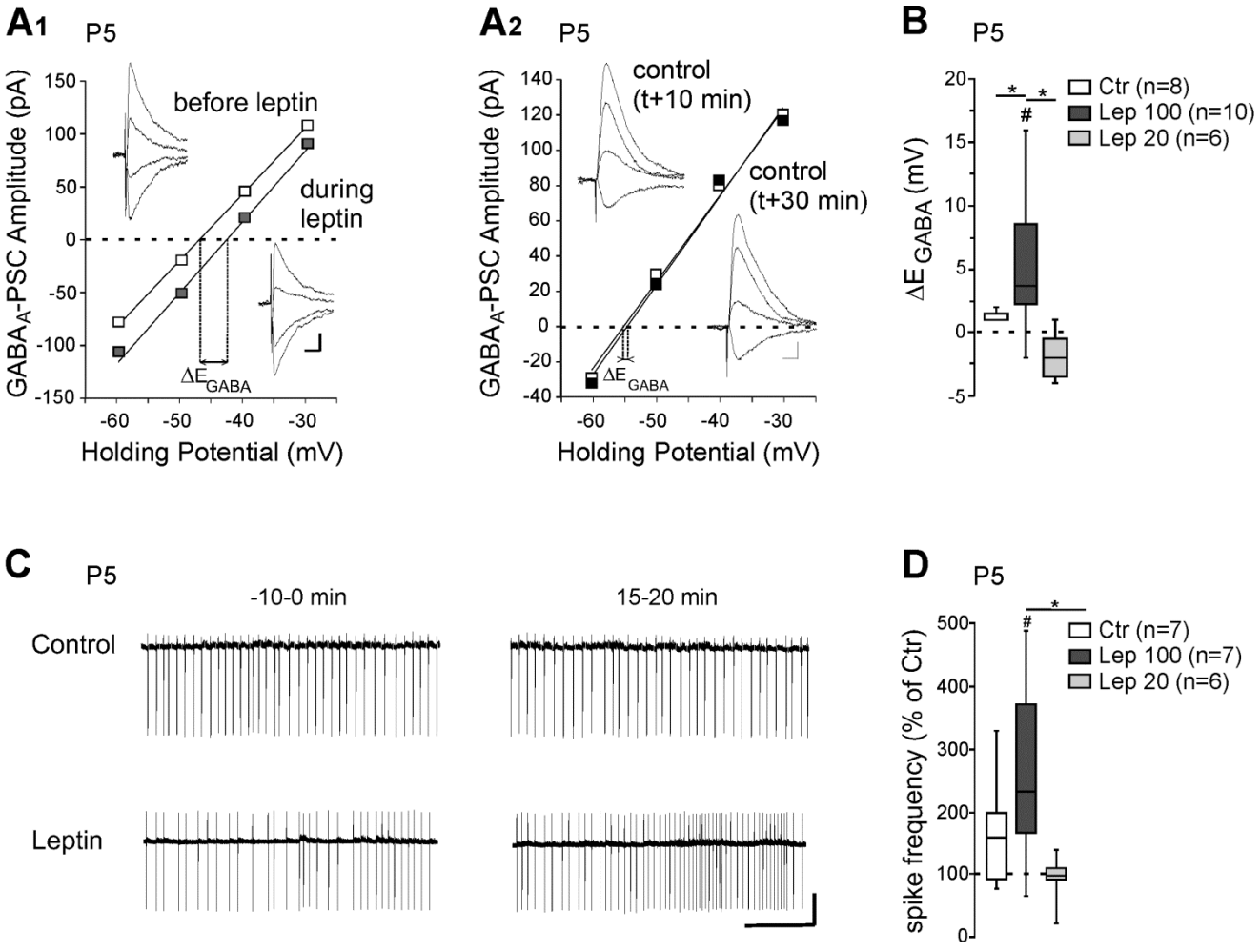

Figure 1: Leptin controls chloride homeostasis in rat hippocampal slices.

(**A**) Current-voltage (I-V) relationships for evoked GABAergic synaptic currents before and during leptin application (100nM, 20 min) (**A1**) and in control experiments (**A2**) during which neurons were recorded following the same protocol but leptin was omitted. The intercepts of the linear regression of the I-V curves was used to calculate E_{GABA} changes induced by leptin (ΔE_{GABA}). Insets depict the GABAergic synaptic currents. Scale bars, 10 ms, 20 pA. (**B**) Box plots of ΔE_{GABA} induced by leptin 100 and 20 nM. In control experiments (Ctr), neurons were recorded following the same protocol in the absence of leptin. **(C)** Loose patch recordings of

CA3 pyramidal neurons on acute hippocampal slices before (-10-0 mins) and during (15-20 mins) the application of leptin (100nM, 20min) and in control experiment, during which neurons recorded following the same protocol in the absence of leptin. Scale bar, 2 min, 50pA. **(D)** Box plots of leptin action on spike activity in the indicated conditions. *P<0.05 when compared to leptin experiments, Mann Whitney test. $^{\#}$P<0.05 when compared to pre-leptin values, two-tailed paired Student *t*-test.

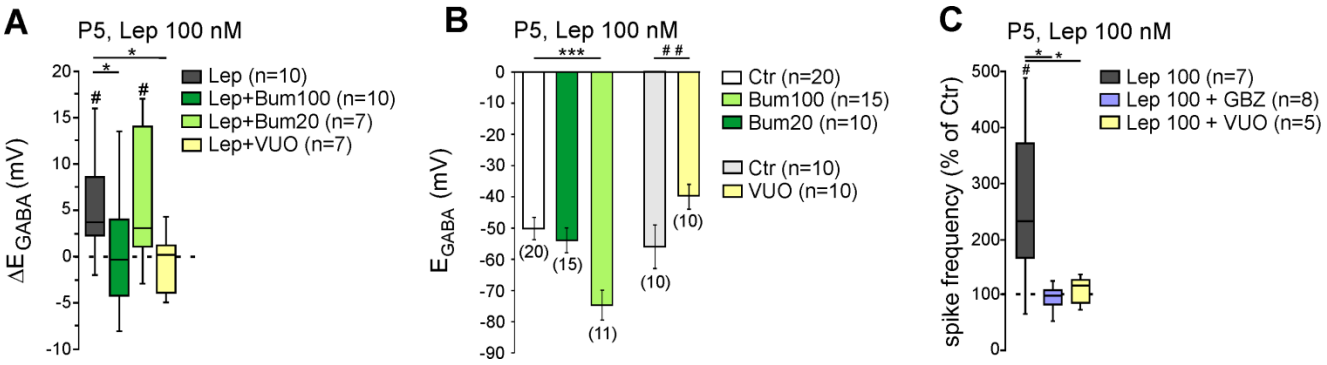

Figure 2: Leptin controls KCC2 activity in rat hippocampal slices.

(**A**) Box plots of ΔE_{GABA} induced by leptin 100 nM in control condition (Lep), in the presence of bumetanide 100 and 20 µM, or in the presence of VU0423271 (VU0, 10µM). (**B**) Bar plots of the mean and standard error to the mean of the reversal potential of $GABA_A$ receptor-mediated postsynaptic currents (E_{GABA}) in the indicated conditions. **(C)** Box plots of leptin action on spike activity in the presence of Gabazine (GBZ, 5µM), or in the presence of VU0423271 (VU0, 10µM). *P<0.05 and ***P<0.001 when compared to leptin experiments, Mann Whitney test. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 when compared to pre-leptin values, two-tailed paired Student *t*-test.

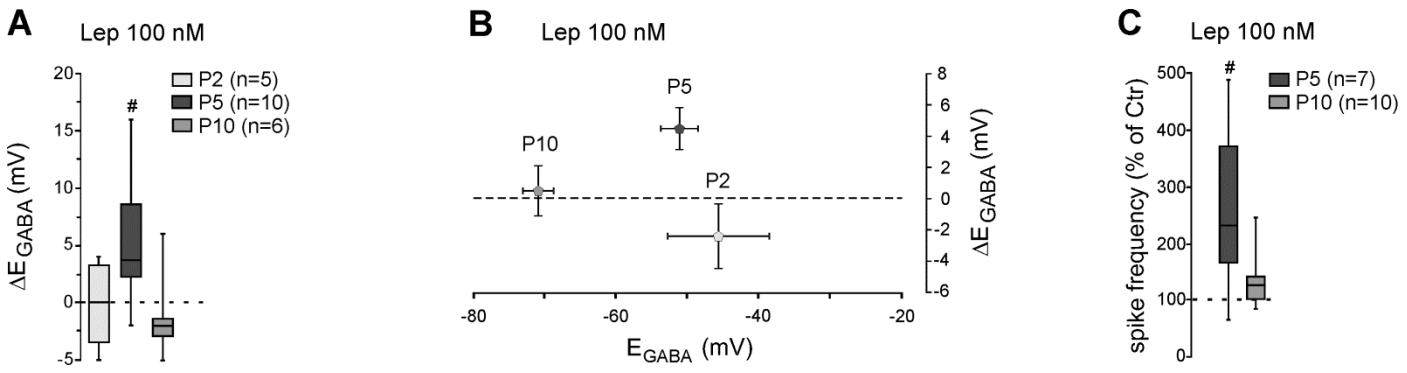

Figure 3: The action of leptin of chloride homeostasis is developmentally regulated.

(**A**) Box plots of ΔE_{GABA} induced by leptin 100nM at postnatal (P) day 2, 5 and 10. (**B**) Plots of mean and standard error to the mean of the ΔE_{GABA} induced by leptin 100nM versus the reversal potential of $GABA_A$ receptor-mediated postsynaptic currents (E_{GABA}) at P2, P5 and P10. (**C**) Box plots of leptin action on spike activity at P5 and P10. $^{\#}P<0.05$ when compared to pre-leptin values, two-tailed paired Student *t*-test.

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