Behavioral/Systems/Cognitive

Enhanced Excitatory Input to Melanin Concentrating Hormone Neurons during Developmental Period of High Food Intake Is Mediated by GABA

Ying Li and Anthony N. van den Pol
Department of Neurosurgery, Yale University School of Medicine, New Haven, Connecticut 06520

In contrast to the local axons of GABA neurons of the cortex and hippocampus, lateral hypothalamic neurons containing melanin concentrating hormone (MCH) and GABA send long axons throughout the brain and play key roles in energy homeostasis and mental status. In adults, MCH neurons maintain a hyperpolarized membrane potential and most of the synaptic input is inhibitory. In contrast, we found that developing MCH neurons received substantially more excitatory synaptic input. Based on gramicidin-perforated patch recordings in hypothalamic slices from MCH-green fluorescent protein transgenic mice, we found that GABA was the primary excitatory synaptic transmitter in embryonic and neonatal ages up to postnatal day 10. Surprisingly, glutamate assumed only a minor excitatory role, if any. GABA plays a complex role in developing MCH neurons, with its actions conditionally dependent on a number of factors. GABA depolarization could lead to an increase in spikes either independently or in summation with other depolarizing stimuli, or alternately, depending on the relative timing of other depolarizing events, could lead to shunting inhibition. The developmental shift from depolarizing to hyperpolarizing occurred later in the dendrites than in the cell body. Early GABA depolarization was based on a Cl^--dependent inward current. An interesting secondary depolarization in mature neurons that followed an initial hyperpolarization was based on a bicarbonate mechanism. Thus during the early developmental period when food consumption is high, MCH neurons are more depolarized than in the adult, and an increased level of excitatory synaptic input to these orexigenic cells is mediated by GABA.

Introduction

Melanin concentrating hormone (MCH) neurons play an important role in the regulation of energy homeostasis. Intracerebral injection of MCH increased food intake in rodents (Qu et al., 1996; Rossi et al., 1997). MCH-deficient mice have reduced food intake and a lean phenotype (Shimada et al., 1998). MCH receptor-deficient mice also have a reduced body weight (Marsh et al., 2002). Fasting increases MCH mRNA expression (Qu et al., 1996). During early development, animals and humans tend to eat frequently to provide the nutrition necessary for growth and development (Hall and Rosenblatt, 1978; Blass and Teicher, 1980; Matheny et al., 1990); in part this may be due to an increased activity of orexigenic neurons. MCH is a cyclic 19-amino-acid peptide (Saito et al., 1999) synthesized in the hypothalamus, primarily in the lateral region. MCH neurons send long axonal projections throughout the CNS (Bittencourt et al., 1992) and the MCH receptor has a wide distribution throughout the brain (Lembo et al., 1999) indicating that MCH neurons may modulate many different CNS functions. MCH receptor antagonists have antidepressant effects (Borowsky et al., 2002).

Received Aug. 25, 2009; revised Oct. 7, 2009; accepted Oct. 20, 2009.

This work was supported by National Institutes of Health–National Institute of Neurological Disorders and Stroke Grants NS34887, NS41454, and NS48476. We thank Y. Yang, V. Rogulin, and J. N. Davis for excellent technical facilitation and L. Y. Fu for suggestions.

Correspondence should be addressed to Anthony N. van den Pol, Department of Neurosurgery, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520. E-mail: anthony.vandenpol@yale.edu.

DOI:10.1523/JNEUROSCI.4203-09.2009

 0270-6474/09/2915195-10$15.00/0

GABA is the dominant inhibitory neurotransmitter in the adult hypothalamus (Tappaz et al., 1982; van den Pol and Tsujimoto, 1985; van den Pol, 1986; Decavel and van den Pol, 1990). In mature neurons, GABA hyperpolarizes the membrane potential and reduces input resistance (Kim and Dudek, 1992; Tasker and Dudek, 1993). MCH neurons colocalize the amino acid transmitter GABA and its synthesizing enzyme glutamate decarboxylase (Elias et al., 2001, 2008; Dallvechia-Adams et al., 2002). The primary synaptic input to adult MCH neurons is GABA mediated (van den Pol et al., 2004), underlining the importance of GABA regulation of the activity of MCH cells. In mature identified MCH neurons, GABA is inhibitory (Gao et al., 2003; van den Pol et al., 2004).

GABA excitation during development plays a role in neurogenesis, cell migration, differentiation and synapse formation (Barbin et al., 1993; LoTurco et al., 1995; Heck et al., 2007; Wang and Kriegstein, 2008). Early excitatory actions of GABA have been found throughout the brain (Connor et al., 1987; Yamashita and Fukuda, 1993; Obrietan and van den Pol, 1995), but have been studied in depth primarily in cortical structures such as the hippocampus and cortex (Ben-Ari et al., 1989; Cherubini et al., 1991; Yuste and Katz, 1991; Ben-Ari, 2002; Owens and Kriegstein, 2002). In the hippocampus, a primary role of depolarizing GABA actions is to remove the voltage-dependent Mg^{2+} block of the NMDA receptor, facilitating a glutamate-mediated giant depolarizing potential (Ben-Ari et al., 1989). Increasingly sophisticated models of how GABA and glutamate interact during development are based in large part on studies of excitatory py-

ramidal neurons with long projection axons. The GABA neurons in cortex and hippocampus are primarily involved in local inhibition. In striking contrast to cortical regions, many inhibitory neurons of the hypothalamus send long axons outside the hypothalamus; long efferent inhibitory axons arise not only from the MCH neurons which send extensive long axons throughout the CNS, but also from the GABA neurons of the suprachiasmatic and arcuate nuclei (Elias et al., 1998). Mature synaptic input to the MCH neurons shows a preponderance of GABA, with substantially less excitatory input than found in cortical regions. These findings suggest that GABA excitation in the developing hypothalamus may not fully follow models developed in cortex or hippocampus.

To study the actions of GABA on a developing GABAergic neuron that projects widely throughout the brain, we used gramicidin-perforated patch and whole-cell recording in hypothalamic slices from transgenic mice that express GFP selectively in MCH neurons.

Materials and Methods

Hypothalamic slices. We used transgenic mice that express enhanced green fluorescent protein (GFP) selectively in MCH neurons (van den Pol et al., 2004). One experiment includes a comparison with hypocretin/orexin neurons, detected in a different transgenic mouse (from Dr T.Sakurai) expressing GFP selectively in hypocretin neurons (Li et al., 2002). Briefly, embryonic to 56-d-old adult MCH-GFP transgenic mice were anesthetized with hypothermia (embryonic and early neonatal) or Nembutal (100 mg/kg) and decapitated. The brains were quickly removed and immersed in an ice-cold and oxygenated (95% O_2, 5% CO_2) high-sucrose solution containing the following (in mM): 220 sucrose, 2.5 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 1.23 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose, adjusted to pH 7.4 with NaOH. Coronal hypothalamic slices (250–400 μm thick) containing MCH neurons were cut on a vibratome and then incubated for at least 2 h at room temperature in an oxygenated (95% O_2, 5% CO_2) artificial CSF (ACSF) solution containing the following (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.23 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose, adjusted to pH 7.4 with NaOH. After the incubation period, slices were transferred to a recording chamber (0.5–1.0 ml volume) and perfused continuously with oxygenated (95% O_2, 5% CO_2) ACSF preheated to 32°C. The recording chamber was mounted on the stage of an Olympus BX51WI upright microscope equipped with a 40× water-immersion objective (Olympus). Experiments were approved by the Yale University Committee on Animal Care and Use.

Patch-clamp recordings. Whole-cell patch recordings were collected from GFP-expressing MCH neurons. Individual MCH neurons were visualized using a differential interference contrast (DIC) optical system combined with an infrared (IR) filter, a GFP fluorescent filter, a monochrome CCD camera and a monitor. Electrodes used for whole-cell recordings were pulled from thin-walled borosilicate glass capillary tubes (World Precision Instruments) and had resistances between 4 and 6 MΩ when filled with recording solution containing the following (in mM): 145 $KMeSO_4$, 1 $MgCl_2$, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, and 0.5 Na_2-GTP, adjusted to pH 7.3 with KOH. HEPES buffer was used in some experiments (in mM: NaCl 150, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, glucose 10, pH was adjusted to 7.4 with NaOH). The pipette resistance was 2–3 MΩ for pipettes used for perforated whole-cell patch-clamp recording. Neurons in which the series resistance was >20 MΩ and changed by >15% during the experiment were judged unstable, and were excluded from the statistics.

Most of the experiments are based on recordings with gramicidin-perforated patches. Gramicidin-perforations are advantageous in that they allow recording without disturbing the intracellular level of Cl^- (Ebihara et al., 1995; Chen et al., 1996; Fu and van den Pol, 2007). Recordings were made using an EPC-9 amplifier with Pulse 8.5 software (HEKA). A Grass SD9 stimulator was used for electric stimulation of slices (Grass Medical Instruments). Data were analyzed using Pulsefit 8.5 and Axograph 4.8 and exported to IgorPro Carbon 4.07 software (WaveMetrics) to make plots and perform statistical analysis.

PSCs were recorded at a holding potential of −60 mV. Only events with amplitudes >5 pA were counted. This procedure has been described in detail previously (Gao and van den Pol, 1999). Kolmogorov–Smirnov statistical tests were used to measure the cumulative probability of synaptic events. The statistical test used, except where indicated, was a one-way ANOVA. In the text and figures, values are presented as a mean ± SE and $p < 0.05$ was considered statistically significant.

Pressure application (3–5 psi, 4–6 ms) with a Picospritzer II (Parker-Hannefin, Fairfield, NJ) was used to deliver muscimol locally to dendrites or soma. The tip of the application pipette (1 μm diameter tip) was positioned 4–10 μm away from dendrites or soma. Bath applications of drug were applied at the concentrations indicated. Stock solutions for all drugs were prepared and kept frozen until immediately before flow pipette (400 μm diameter tip) delivery or bath application. TTX was obtained from Alomone Labs. GABA, muscimol bromide, bicuculline methiodide (BIC), DL-2-amino-5-phosphonovaleric acid (AP-5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Sigma-Aldrich.

Results

The MCH promoter is active early in hypothalamic development

We first examined the expression of GFP under control of the MCH promoter during early brain development. Substantial GFP could be detected in sections from embryonic hypothalamus. GFP expression is clearly seen at embryonic day 18 (E18) (Fig. 1*A–C*) and postnatal day 1 (P1) (Fig. 1*D*). The early expression of GFP allowed us to record selectively from these cells during embryonic and neonatal stages, and suggests that these cells synthesize MCH early in development, consistent with data from other species (Bresson et al., 1987; Steininger et al., 2004; Mancera and Fernández-Llebrez, 1995).

Negative shift in Cl^- reversal membrane potential during MCH neuron development

Both the Cl^- reversal potential and the resting membrane potential (RMP) can influence the polarity of GABA responses in neurons. To investigate developmental shifts in MCH neurons, we used MCH-GFP transgenic mice ranging in age from E20 to adult P56. The perforated whole-cell patch recording technique was used so that the intracellular Cl^- concentration was not artifactually changed by perfusion with the pipette solution (Ebihara et al., 1995). The holding potential of MCH neurons was systematically varied between −20 mV, −40 mV, −60 mV and −80 mV. Application of the $GABA_A$ agonist muscimol (50 μM) to MCH neurons produced either an inward or outward current depending on the holding potential (Fig. 2*A–C*). GABA reversal membrane potentials and resting membrane potentials (RMP) were determined for each neuron and plotted as a function of age (Fig. 2*D*). The reversal potentials of the muscimol-evoked currents ranged from −32 mV to −60 mV in E20–P9 MCH neurons, and from −54 mV to −72 mV in P10–P56 neurons ($n = 53$). The RMPs of E20–P10 MCH neurons were between −40 mV and −58 mV, and between −48 mV and −64 mV for P10–P56. Both Cl^- reversal potentials and recorded RMPs became more negative with cellular maturity; in immature cells the E_{Cl^-} was positive to the RMP up to P10, and negative to the RMP in cells older than P10 (Fig. 2*D*). Muscimol depolarized the membrane potential during early periods of MCH neuron development from E20–P12, but hyperpolarized the membrane potential in P10– adult neurons.

Between P8 and P12, muscimol (50 μM) depolarized some MCH neurons and hyperpolarized others in the same slice, sug-

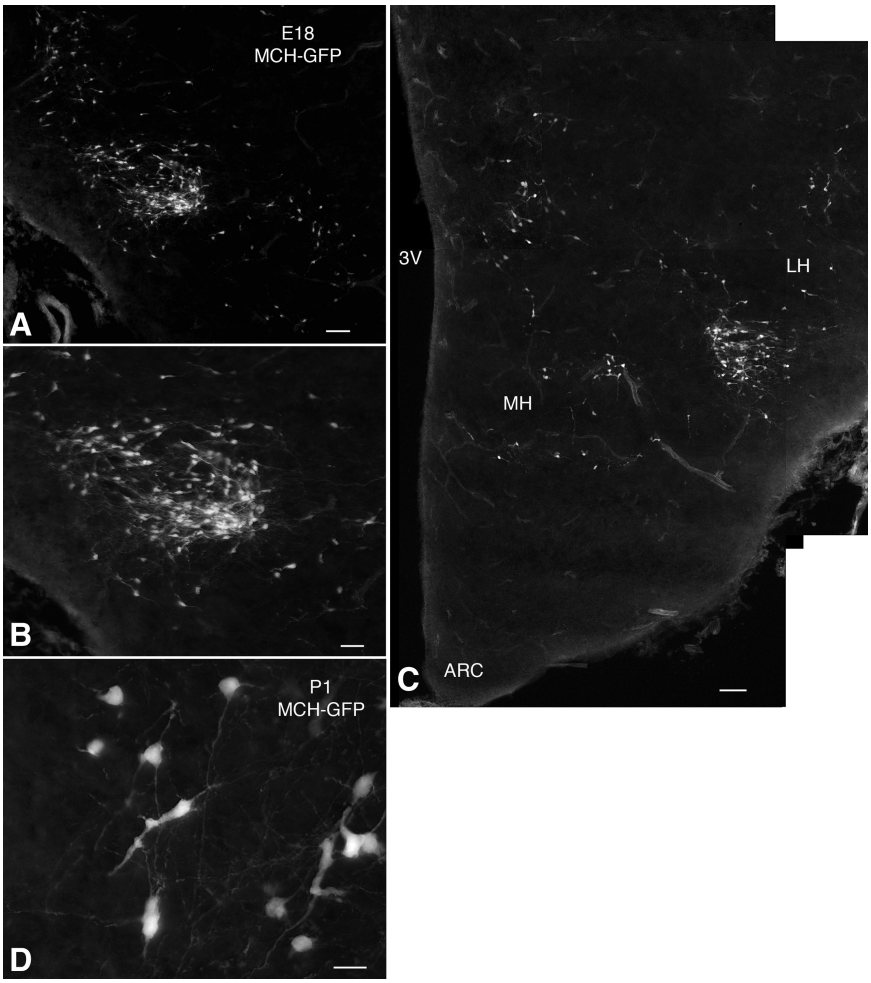

Figure 1. MCH-GFP is expressed early in development. ***A***, ***B***, GFP driven by the MCH promoter is strong even at E18 (***A***), seen in higher magnification in ***B***. ***C***, A photomicrograph of the medial and lateral hypothalamus at E18 shows MCH cells in both regions, similar to the adult. ***D***, At P1, MCH neurons extend multiple processes. Scale bars: ***A***, 55 μm; ***B***, 25 μm; ***C***, 60 μm; ***D***, 15 μm.

gesting that MCH neurons can respond to GABA with either an excitatory or inhibitory action during this period. Thus during development, both the resting membrane potential and the GABA reversal potential move in a negative direction. The GABA reversal potential passes the resting membrane potential by about P10, and stabilizes ~10 mV negative to resting potential in the mature MCH cell. A number of reports have suggested that the RMP of developing neurons becomes more negative with maturity (Spigelman et al., 1992; Zhou and Hablitz, 1996). One factor that may influence this apparent trend is the combination of leak currents during recording coupled with the reduced input resistance in mature neurons (Tyzio et al., 2008). To account for this, the apparent RMP was subtracted from the E_{GABA} to generate the curve in Figure 2*E*, giving a trend for E_{GABA} relative to the RMP.

Early depolarizing action of GABA dependent on Cl^-, secondary depolarization of mature neurons dependent on bicarbonate

GABA-mediated depolarization could be due to Cl^- efflux, or to a bicarbonate flux (Kaila et al., 1993). When cells were recorded in a HEPES buffer, the GABA depolarization mediated by bicarbonate is blocked (Staley et al., 1995). To test which mechanism was operating in the developing MCH cell, we compared the effect of the GABA agonist on neurons in our normal bicarbonate-based ACSF and HEPES buffers using gramicidin-perforation recording. In developing neurons <10-d-old, muscimol evoked similar amplitude depolarizing actions in both buffers (Fig. 3*A*, 11 ± 2.2 mV in bicarbonate-buffered solution; 10.5 ± 1.4 mV in HEPES-buffered solution, $n = 6$), suggesting that the mechanism underlying GABA-mediated depolarization was based primarily on Cl^- efflux from the recorded cell.

In mature MCH neurons (P30 or older), a brief application (~2 s) of muscimol (20 μM) evoked only a hyperpolarization in bicarbonate-buffered solution (7 ± 2.6 mV, $n = 12$) or HEPES solution (8 ± 2.4 mV, $n = 6$) with no subsequent depolarization (Fig. 3*B*). In contrast, extended application of muscimol (20 μM) induced an initial hyperpolarization (−5 ± 1 mV) followed by a late slow depolarization (11 ± 2.2 mV, $n = 6$) in bicarbonate-buffered ACSF (Fig. 3*C*,*D*). When MCH cells were perfused with HEPES solution, extended application of muscimol then evoked only a hyperpolarization (Fig. 3*C*, 10 ± 2.4 mV; Fig. 3*D*) without a secondary depolarization. This suggests that in older MCH neurons, muscimol has an initial hyperpolarizing action mediated by an inward Cl^- movement, followed by a secondary depolarization mediated by bicarbonate.

Delayed maturation of dendrites relative to soma with respect to GABA response

The density and composition of Cl^- transporters may vary in the cell body and dendrites, and this may result in differing Cl^- levels in different regions of the somatodendritic complex. To determine whether Cl^- concentrations in MCH neuronal dendrites and soma are the same, different stages of development were tested, including three groups consisting of P5–P7, P10–P12 and P18–P24. MCH neurons were recorded with a patch pipette on the soma using perforated whole-cell recording and muscimol (50 μM) was microapplied to either the dendrites (100 μm-250 μm from the parent cell body) or the soma with pressure application (4–6 psi, 5–8 ms) from a micropipette with a diameter of 1 μm that allowed very focal application of the GABA agonist (Fig. 4*E*). When muscimol was puffed to either the dendrite or soma, P5–P7 neurons displayed a depolarization response (Fig. 4*A*). Dendritic application of muscimol depolarized the membrane potential by 7.9 ± 0.5 mV (P5–P7, $n = 6$, $p < 0.05$, ANOVA) and application to the soma depolarized the membrane potential by 6.3 ± 0.3 mV (P5–P7, $n = 6$, $p < 0.05$, ANOVA) (Fig. 4*D*).

P10–P12 MCH neurons depolarized following application of muscimol (50 μM) to the dendrite. In contrast, these same neurons displayed a hyperpolarizing response when muscimol was applied to the soma (Fig. 4*B*). Dendritic application of muscimol induced a 1.9 ± 1.8 mV depolarization in membrane potential (P10–P12, $n = 6$, $p > 0.05$, ANOVA), but application to the soma hyperpolarized the membrane potential by −3.4 ± 1.7 mV (P10–P12, $n = 6$, $p > 0.05$, ANOVA) (Fig. 4*D*). P18–P24 MCH

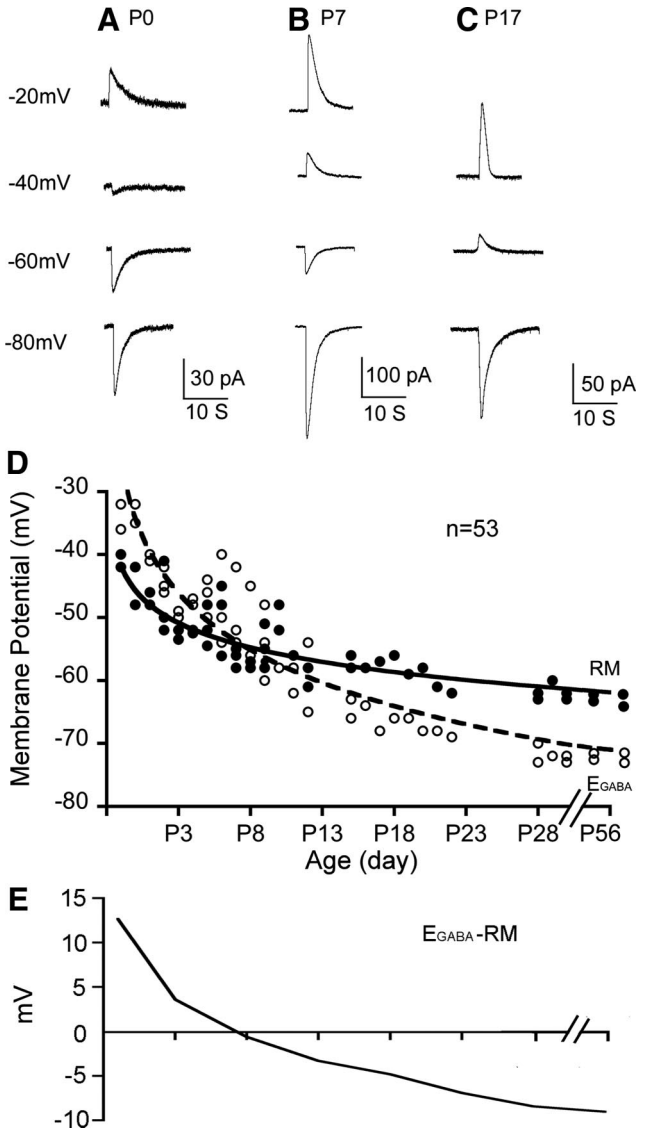

Figure 2. The GABA-A reversal potential becomes negative relative to the resting membrane potential as MCH neurons develop. ***A–C***, Muscimol (50 μM) induced inward and outward currents in MCH neurons recorded with gramicidin perforated patch at different holding potentials and different ages (***A***, P0; ***B***, P7; ***C***, P17) under voltage clamp. ***D***, A plot of the GABA reversal potential and resting membrane potential (RMP) in 53 MCH neurons as a function of animal age. RM, Resting membrane potential. ***E***, RMP (RM) values were subtracted from E_{GABA} data to show polarity of GABA response relative to RMP. E_{GABA}, GABA reversal potential. ***A–E***, All recordings were done with gramicidin-perforated patch.

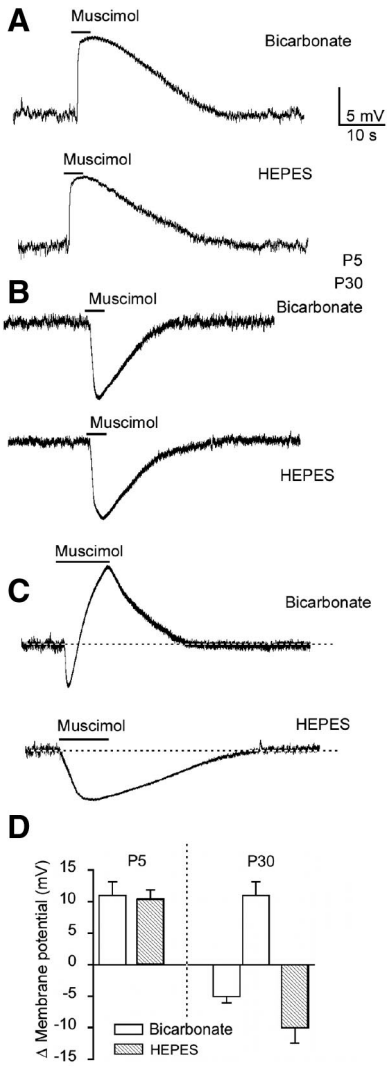

Figure 3. $GABA_A$-induced depolarization is Cl-dependent. ***A***, Top: Muscimol (20 μM) depolarized the membrane potential of P5 MCH neurons in bicarbonate buffer. Resting membrane potential (RMP) −48 mV. Bottom: Muscimol (20 μM) depolarized membrane potential of P5 MCH neurons in HEPES buffer. ***B***, In P30 MCH neurons, brief application of muscimol hyperpolarizes neurons in both bicarbonate and HEPES buffer. RMP, −62 mV. ***C***, Top: Extended muscimol (20 μM) application evoked an initial hyperpolarization, followed by depolarization of the membrane potential of P30 MCH neurons in bicarbonate buffer. Bottom: Extended muscimol (20 μM) application hyperpolarized membrane potential of P30 MCH neurons in HEPES. ***D***, Bar graph showing muscimol-induced changes in membrane potential on P5 and P30 MCH neurons. To the right of the vertical dotted line, the first bar represents the peak hyperpolarization, and the second bar represents the subsequent peak depolarization. Error bars are SEM.

neurons were hyperpolarized by muscimol (50 μM) application to either the dendrites or soma (Fig. 4*C*). Dendritic application of muscimol to these neurons hyperpolarized the membrane potential by -7.1 ± 0.4 mV (P18–P24, $n = 6$, $p < 0.05$, ANOVA) and application to the soma hyperpolarized the membrane potential by -10 ± 0.6 mV (P18–P24, $n = 6$, $p < 0.05$, ANOVA) (Fig. 4*D*). These results suggest that, during the P10–P12 developmental period, the reversal membrane potential of Cl^- in the dendrite is positive relative to the resting membrane potential, whereas in the same neuron, the Cl^- reversal membrane potential of the cell body is negative relative to the resting membrane potential.

GABA initiates spikes in silent developing MCH neurons

GABA depolarization could be excitatory and lead to spike initiation, or could lead to shunting inhibition. If GABA is excitatory to developing MCH neurons, then it would be expected to initiate spike activity in these cells. When muscimol (10 μM) was puffed onto the soma of P5 MCH neurons using a brief 4–6 ms duration setting, the cell generated one spike following each application of muscimol (Fig. 5*A1*). Figure 5*A2* is a higher resolution trace of one of the spikes shown in Figure 5*A1*. When a positive current was injected (100 pA, 2 ms) into developing P5 MCH neurons, a spike was evoked after each current injection. Whereas the application of muscimol depolarized the cell, it blocked the current injection-induced spike (Fig. 5*B*). To investigate further the mechanisms that underlie depolarization leading to excitation versus inhibition, the experiments below were undertaken.

We investigated how input resistance changed during the responses to muscimol. A negative current (30 pA, 500 ms) was

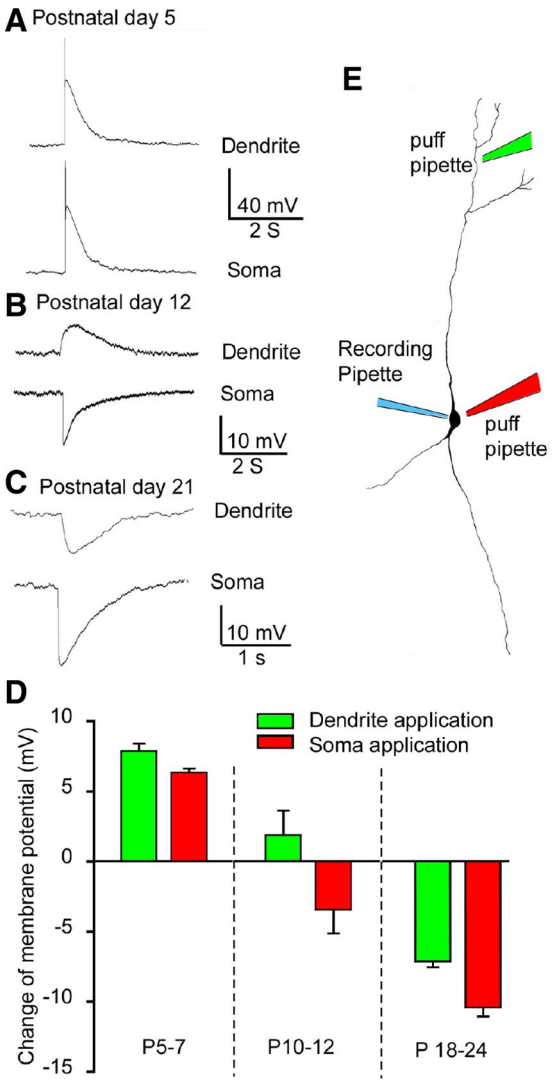

Figure 4. E_{GABA} is more positive in dendrites compared with soma. ***A***, Typical recording showing that muscimol (50 μM) induced a spike when applied to the soma or dendrite under current clamp at P5. RMP, −51 mV. ***B***, Representative neuron showing that muscimol (50 μM) hyperpolarized the membrane potential when applied to the soma, but depolarized the membrane potential when applied to the dendrite during P10–P12. RMP, −54 mV. ***C***, Traces showing hyperpolarization induced by application of muscimol to soma or dendrite at P21. RMP, −60 mV. ***D***, Bar graph shows change in membrane potential after application of muscimol to dendrite or soma. ***E***, Schematic illustration of the recording site and puff site of the recorded neuron.

injected into P5 MCH neurons repeatedly, as shown in Figure 5*C*; muscimol depolarized the cell and reduced the input resistance, with the lowest input resistance found at the peak of the depolarization. The input resistance began to recover after the peak depolarization, and showed full recovery by the time the membrane potential returned to control levels. We next injected positive current (15 pA, 20 ms) at various intervals to depolarize the membrane potential (Fig. 5*D2*) and emulate responses due to excitatory transmitters such as glutamate. Muscimol induced an inward current (Fig. 5*D3*) and depolarization (Fig. 5*D1*). Positive current injection at the peak depolarization and peak current induced a further small depolarization, but no spike. The muscimol-induced current showed a faster recovery than did the membrane potential. With the recovery of the current, due to the closing of Cl^- channels, the input resistance also recovered, and thus the second and third current injections initiated spikes at a time during which the resting membrane potential showed only partial recovery. This suggests that GABA can initiate spikes or induce shunting inhibition in the same developing cell, depending on timing relative to ion channel closure while the membrane potential is still depolarized.

GABA can enhance or reduce spike probability depending on timing

To determine the importance of timing of GABA actions relative to other excitatory stimuli, muscimol application was coupled with direct current injection of the recorded cell, emulating excitatory synaptic input. To evoke a depolarization, subspike threshold inward current (20–30 pA) was injected into the recorded cell in P5 MCH neurons. Brief puff application of muscimol (10 μM, arrow) was also used to induce depolarization. When muscimol was puffed independent of current injection, both stimuli evoked a depolarization, but no spike was generated (Fig. 5*E*,*F*). Similarly, when muscimol was puffed a few milliseconds before the current injection, no spike was generated (Fig. 5*G*). However, puff application of muscimol consistently evoked a spike when it was applied a couple of ms after the initiation of the current injection (Fig. 5*H*,*I*). With this short interval, and only within this interval, current injection acted synergistically with GABA application to evoke an action potential. If a suprathreshold current injection was used, then application of GABA before the current injection reduced the spike probability (data not shown). In older P15 MCH neurons, injection of inward current (20–30 pA) induced depolarization, and puff application of muscimol consistently induced hyperpolarization; at this age, spikes were not evoked regardless of the temporal relationship between the two stimuli (Fig. 5*J*).

GABAergic postsynaptic activity is predominant in developing MCH neurons, but not in nearby developing hypocretin neurons

MCH neurons (P7–P8) were set at a −60 mV holding potential using perforated whole-cell recording. Under these conditions, the frequency of postsynaptic currents (PSCs) was 4.4 ± 1.5 Hz ($n = 6$). BIC (30 μM) substantially reduced the frequency of PSCs to 8 ± 3.3% of control (P7–P8, $n = 6$, $p < 0.05$, ANOVA) (Fig. 6*A*,*B*). After washout, the frequency of PSCs returned to 92 ± 6.6% of the control. In contrast, AP-5 (50 μM) and CNQX (10 μM) had no significant effect on the frequency of PSCs (16% reduction, $n = 6$, $p > 0.05$, ANOVA) (Fig. 6*A*,*B*). After washout, the frequency of PSC was 91 ± 6% of the control. These data suggest that most synaptic events in these developing MCH cells are due to GABA release.

Hypocretin/orexin neurons are interspersed with MCH neurons in the lateral hypothalamus, and project to many of the same regions of the brain (Bittencourt et al., 1992; Broberger et al., 1998; Peyron et al., 1998). The hypocretin neuropeptide is expressed at birth, and physiological responses to hypocretin are found at birth, indicating early maturation of the hypocretin system (van den Pol et al., 2001). To determine whether the GABAergic responses of MCH cells are unique or shared with other neurons in the same anatomical region, hypocretin neurons (P7–P8) were tested, and, unlike MCH neurons, showed primarily glutamatergic postsynaptic activity. Application of BIC (30 μM) to hypocretin neurons yielded no significant effect on the frequency of PSCs (92 ± 5.3% of the control, ANOVA, $p > 0.05$, Fig. 6*C*,*D*). After BIC was washed out, the frequency was 95 ± 6.6% of the control. AP-5 (50 μM) and CNQX (10 μM) significantly decreased the frequency of PSCs to 14.5 ± 8.8% of the

control ($n = 6$, $p < 0.05$, ANOVA, Fig. 6*C*,*D*). After washout of AP-5 and CNQX, the frequency of PSC returned to 96 ± 6% of the control. Together, these results suggest that developing MCH neurons at P7/P8 receive predominantly GABAergic input, whereas developing hypocretin neurons receive a greater amount of glutamatergic rather than GABAergic input.

The study of spontaneous synaptic activity above may not reveal the actions of silent synapses, possibly glutamatergic, on MCH neurons. To study active + silent synapses, in parallel experiments, we enhanced synaptic activity by electrically evoking postsynaptic currents from developing MCH neurons <P10 to test further what type of input MCH neurons receive. A bipolar stimulation electrode was placed at least 300 μm away (medial or lateral) from the recorded neuron. Electrical stimulation (50–100 μA, 0.2–0.5 ms, 0.1–0.2 Hz) evoked an inward postsynaptic current (Amplitude: 15 ± 3 pA, Fig. 6*E*, left). Application of BIC (30 μM) completely blocked this evoked postsynaptic current (Fig. 6*E*, middle), indicating that the current was mediated by GABA. In contrast, the glutamate receptor antagonists AP-5 (50 μM) and CNQX (10 μM) showed relatively little attenuation of the evoked inward current (95 ± 2.5% of the control, Fig. 6*E*, right). Thus at this stage of development, GABA accounts for a greater excitatory synaptic current than does glutamate.

Excitatory synaptic input is primarily GABAergic in early development

In the paragraph above, we examined the contribution of GABA to synaptic events. To test whether GABA is the predominant excitatory neurotransmitter driving action potentials in developing MCH neurons, we used theta glass pipettes as bipolar electrodes to evoke synaptic responses in MCH neurons. The tungsten electrode was placed 300 μm away (medial or lateral) from the recorded neuron. The experiment was performed using P4–P9 MCH neurons. Electrical stimulation (30–70 V, 1 ms, 0.25 Hz) evoked spikes on P4 MCH neurons (Fig. 7*A*,*B*). When BIC (30 μM) was perfused in the bath, the electrical stimulation failed to initiate spikes, suggesting a dependence on synaptically activated GABA-A receptors. The evoked spikes returned after washout of BIC. In contrast, when AP-5 (50 μM) and CNQX (10 μM) were perfused in the bath, electrical stimulation still initiated spikes. This finding suggests that developing MCH neurons are excited to generate action potentials primarily by GABA input, and glutamate plays an inconsequential role in spike initiation.

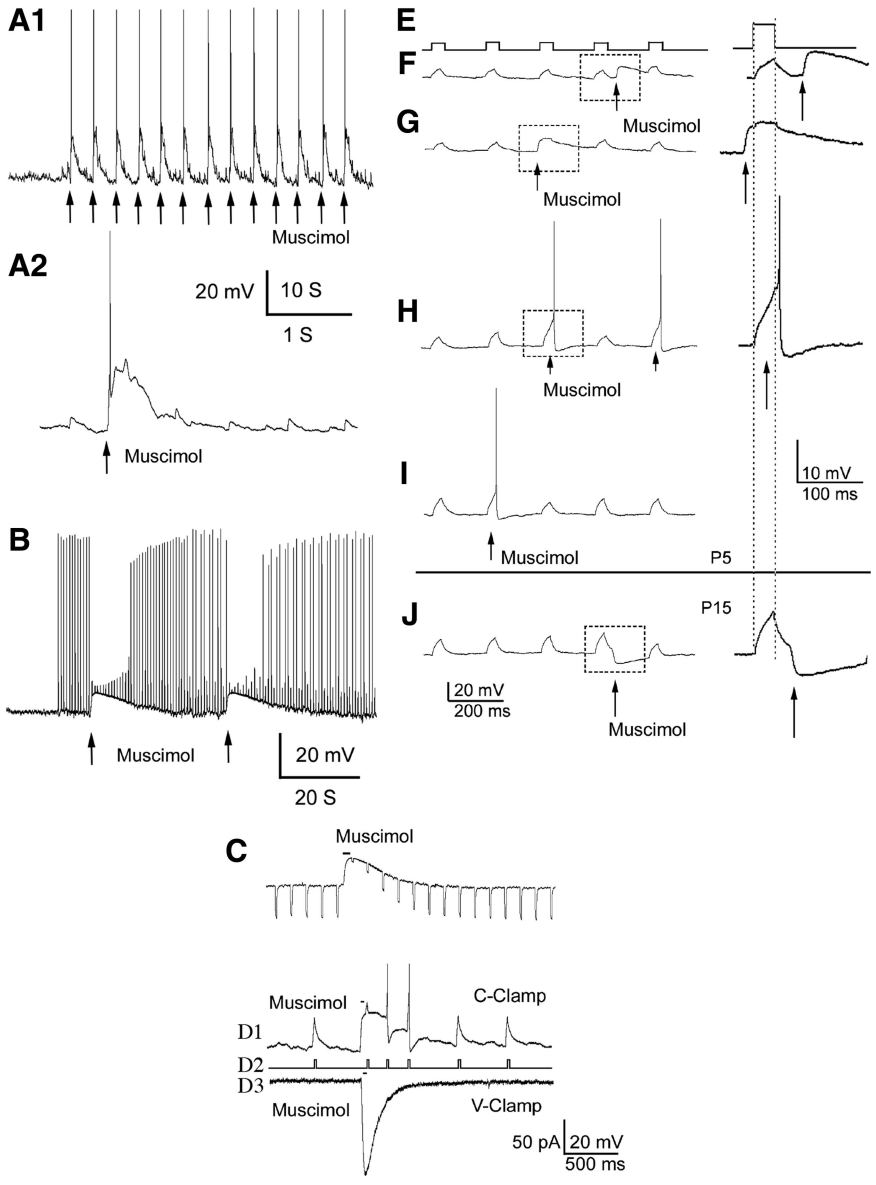

Figure 5. Muscimol-induced depolarization of MCH neurons could initiate spikes or shunt other stimulation-induced excitation during early development. ***A1***, Trace from a typical cell showing that muscimol (vertical arrows, 10 μM) induces spikes (RMP, −45 mV). ***A2***, Faster time scale of a spike shown in ***A1***. ***B***, A P2 MCH neuron was injected with a positive current every 1 s to initiate spikes (RMP, −42 mV). Muscimol (25 μM) depolarized the neuron, transiently reducing spikes. Arrows in panels indicate muscimol puff application. ***C***, Muscimol (30 μM) depolarized the developing MCH neuron and reduced input resistance at peak depolarization. ***D***, Electrical stimulation initiated action potentials only after muscimol-induced current returned to control level but before membrane potential returned to control level. A single MCH neuron was used in ***D1–D3***. ***D1***, Current clamp, electrical stimulation alone depolarized the membrane potential, the first electric stimulation right after the application of muscimol induced further depolarization of the membrane potential but no spike, the second and third electric stimulations initiated spikes (RMP, −48 mV). ***D2***, Electric stimulation pattern. ***D3***, Voltage clamp in same neuron showing muscimol induced a large transient inward current. ***E–J***, GABA action and electrical stimulation effect can be synergistic depending on timing. ***E***, Recorded cells received a subthreshold inward current of similar amplitude to depolarize at regular intervals. ***F***, Subthreshold concentrations of muscimol also induced depolarization when administered between two electrical stimulation-induced depolarizations (RMP, −45 mV). The trace on the right side is the enlarged image of part of the trace to the left. ***G***, Muscimol-induced depolarization when applied a few milliseconds before electrical stimulation. ***H***, ***I***, Muscimol initiated spikes only when applied immediately after the beginning of the inward current injection (top and bottom are different cells). ***J***, At P15 and older, muscimol consistently induced hyperpolarization regardless of its timing relative to inward current injection-mediated depolarization, and did not lead to spikes at any relative time point (RMP, −58 mV).

When an older P9 slice was stimulated electrically, BIC (30 μM) blocked synaptically evoked spikes. AP-5 (50 μM) and

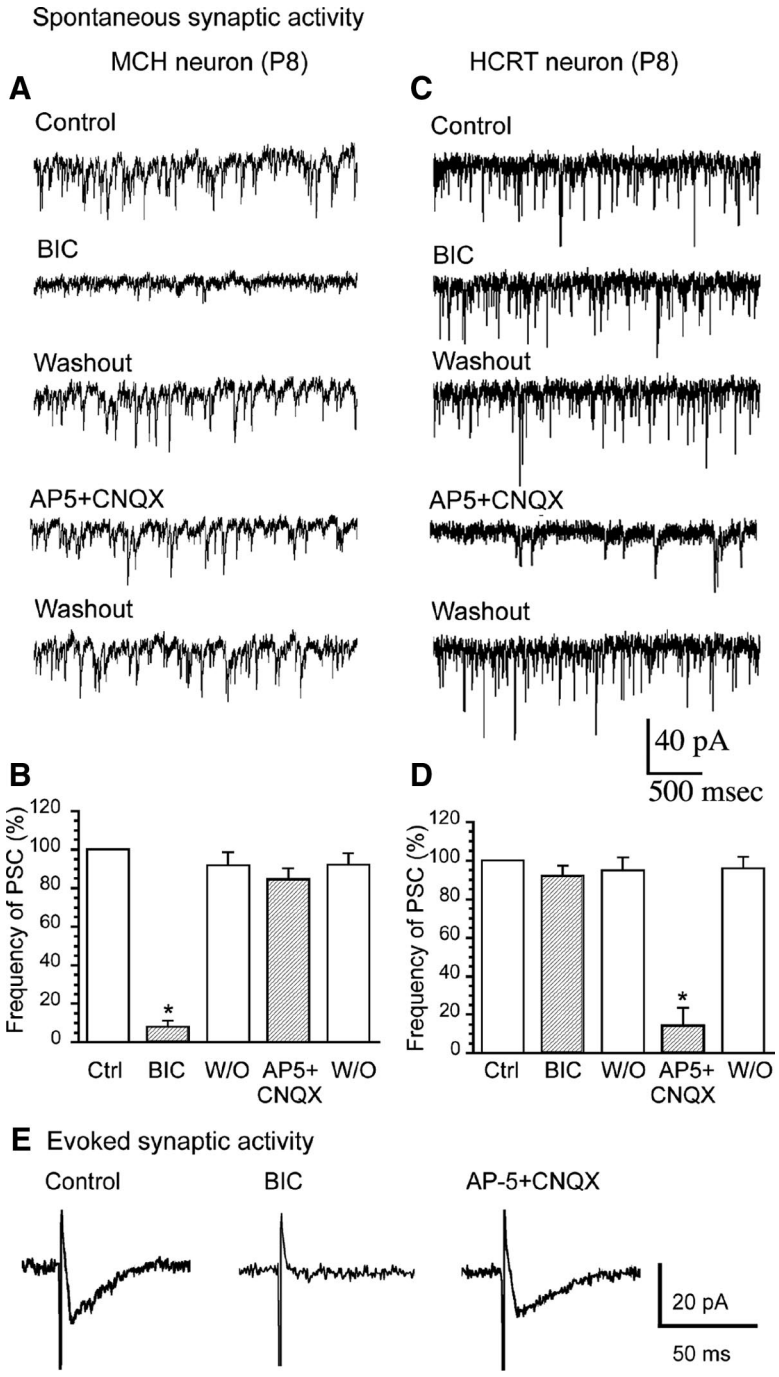

Figure 6. Synaptic activity in MCH neurons is driven primarily by GABA, whereas in hypocretin neurons it is driven by glutamate. ***A***, Sample traces showing that MCH neurons receive GABA inputs. ***B***, Bar graph showing that postsynaptic currents of MCH neurons are primarily GABA mediated, and blocked by BIC, but not by AP-5/CNQX. ***C***, In contrast, in HCRT neurons a large part of the synaptic activity is glutamatergic, and blocked by glutamate receptor antagonists AP-5/CNQX but not by BIC. ***D***, Bar graph showing mean synaptic activity in the presence of control solution (Ctrl), BIC, washout (W/O), or AP-5/CNQX. ***E***, Evoked inward postsynaptic current in MCH neurons is blocked in large part by BIC.

CNQX (10 μM) reduced the evoked spike frequency by 30 $\pm$ 5.2% (P7–P9, Fig. 7*C*, $n = 6$, ANOVA, $p < 0.05$). This suggests that at this stage of development, GABA is still the dominant excitatory transmitter, but glutamate also plays a minor excitatory role. When slices from older mice (P21) were tested, BIC did not block synaptically evoked spikes.

Figure 7*D* shows subthreshold evoked EPSP before, during and after BIC and AP-5 + CNQX respectively in P9 MCH neurons. BIC blocked the evoked EPSP. AP-5 and CNQX reduced the amplitude of the evoked postsynaptic potential by 28 $\pm$ 5.8% (P7–P9, Fig. 7*E*, $n = 7$, ANOVA, $p < 0.05$), thus supporting the view that GABA plays a greater depolarizing role than glutamate at this stage of development.

To evaluate the NMDA and AMPA contribution to the evoked EPSPs, AP-5 (50 μM) and CNQX (10 μM) were applied sequentially to the slice while the slice was stimulated electrically. The time-voltage integral (area of EPSP) of EPSPs was 28 $\pm$ 3 mV · s. BIC reduced the integral of evoked EPSPs to 32.3 $\pm$ 4.4% of control (P7–P9, $n = 12$, ANOVA, $p < 0.05$, Fig. 7*F*). AP-5 reduced the integral of evoked EPSPs to 72 $\pm$ 5.0% of control, CNQX reduced the area of evoked EPSPs to 77 $\pm$ 5.2% of control; the attenuation mediated by the AMPA receptor antagonist CNQX was not significantly different from the one induced by the NMDA receptor antagonist, AP-5. These results suggest that GABA plays a greater depolarizing role than glutamate acting at either NMDA or AMPA receptors during neonatal development in terms of generation of action potentials, and that GABA's depolarizing action is relatively independent of the NMDA receptor.

Discussion

Glutamate is the dominant excitatory transmitter in the mature hypothalamus (van den Pol et al., 1990). In mature MCH neurons, most synaptic input is inhibitory (van den Pol et al., 2004). In contrast, when the synaptic input to developing MCH neurons was examined, GABA accounted for the majority of excitatory synaptic activity during development, with glutamate playing only a minor role. This was not a general feature of hypothalamic development, but was a particular feature of inhibitory MCH neurons, because the nearby excitatory hypocretin cells showed substantially more glutamatergic synaptic activity at the same stages of brain development.

GABA is primary excitatory transmitter in developing MCH neurons, and is independent of the NMDA receptor

GABA plays a key role in developing hippocampal pyramidal cells where it reduces the voltage-dependent Mg^{2+} block of the NMDA receptor by initiating a depolarization, allowing a strong glutamate-mediated giant depolarization (Ben-Ari et al., 1989; Ben-Ari, 2002). In contrast, synaptic glutamate activity played a relatively minor role in depolarizing developing MCH neurons. Both spontaneous and evoked synaptic activity was primarily GABA-mediated. Action potentials could be generated by muscimol application, or by evoking synaptic release of GABA, and the synaptically evoked

spikes could be blocked with the GABA-A receptor antagonist BIC, but not by glutamate receptor antagonists AP-5/CNQX. Where BIC reduced the giant depolarizing potential in hippocampal pyramidal cells, it blocked synaptic depolarization in developing MCH neurons. The glutamate receptor antagonists AP-5 and CNQX failed to block synaptic depolarization in the young MCH cells. Together, these data suggest that, unlike the hippocampus where GABA plays a supportive role for activation of the NMDA receptor (Ben-Ari et al., 1989; Leinekugel et al., 1997), in the MCH neurons, GABA synaptic activity plays a direct excitatory role, independent of glutamate-mediated excitation. The independence of GABA actions from the NMDA receptor may generalize to other hypothalamic GABA systems including the suprachiasmatic or arcuate neurons with long projection axons and strong GABA synaptic input.

The primary mechanism for the early depolarization and inward current evoked by GABA was due to Cl^- movement out of the cell. However, in more mature MCH neurons, after an initial hyperpolarization, cells sometimes showed a secondary depolarization in response to GABA agonists. In contrast to the Cl^--dependent mechanism in developing MCH neurons, the mechanism for the secondary depolarization in more mature MCH cells appeared to be based on bicarbonate. Previous studies in the hippocampus showed that a bicarbonate-free HEPES buffer would block the depolarizing action of bicarbonate ions (Grover et al., 1993; Staley et al., 1995), and we found that the GABA-mediated depolarization in older neurons, but not immature neurons, was blocked by an extracellular HEPES buffer. These data further suggest that even in mature MCH neurons, GABA can still depolarize, but by a mechanism different from the one found in developing MCH neurons. Whether this depolarization in adult MCH cells leads to spike generation would depend on the ongoing activity of the cell and other synaptic input.

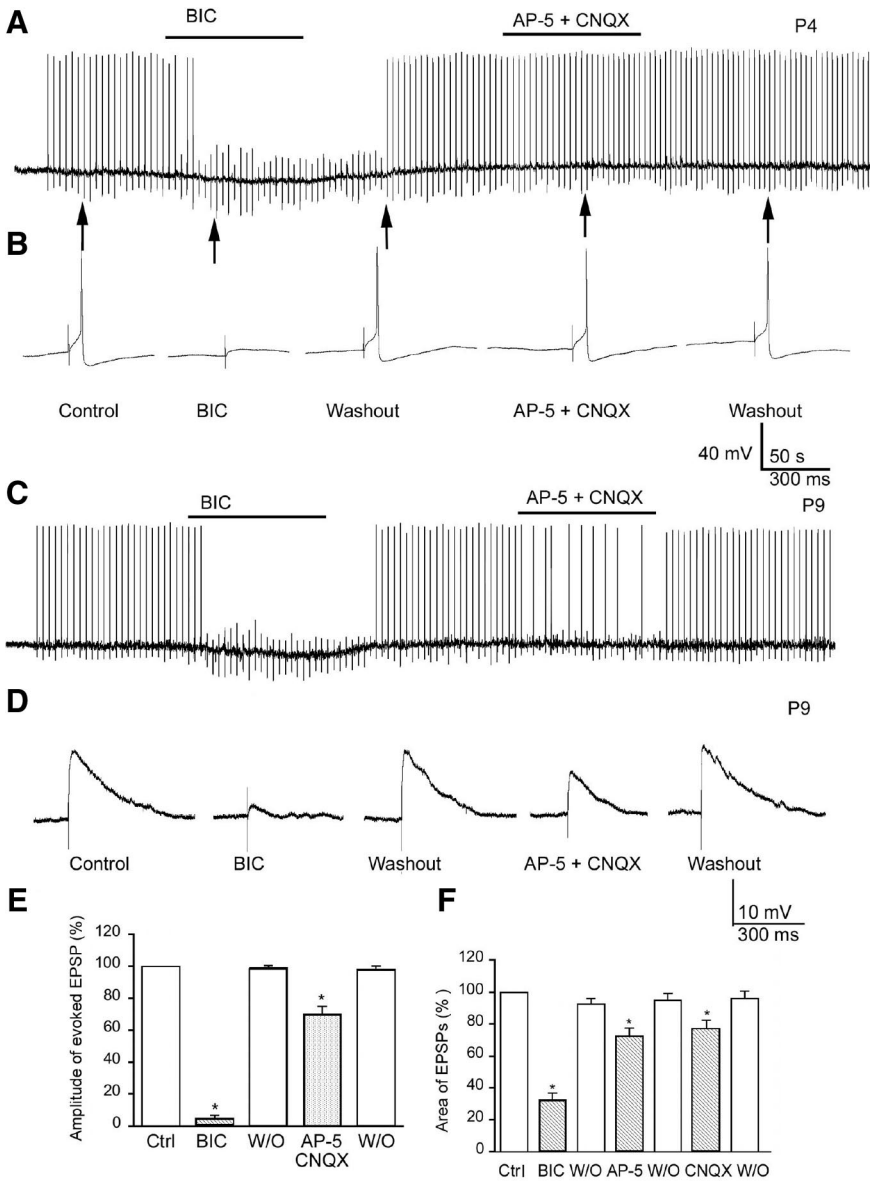

Figure 7. Bicuculline blocks evoked synaptic release-mediated spikes. Synaptic release was evoked by electrical stimulation of the slice 300 μm away from the recorded cell. ***A***, BIC (30 μM) blocked synaptically evoked spikes but AP-5 (50 μM) and CNQX (10 μM) did not block evoked spikes in P4 neurons (RMP, −47 mV). ***B***, Five individual traces taken from A show control, BIC, washout, AP-5 + CNQX, and washout. ***C***, In P9 MCH neurons, BIC (30 μM) blocked evoked spikes and AP-5 (50 μM) plus CNQX (10 μM) reduced the evoked spike frequency (RMP, −52 mV). ***D***, Five single traces showing BIC (30 μM) blocked evoked postsynaptic potential and AP-5 (50 μM) plus CNQX (10 μM) reduced the amplitude of the evoked postsynaptic potential. ***E***, Bar graph showing mean data from P9, related to ***D***. ***F***, Bar graph showing mean voltage-time integral (area) of evoked EPSPs before, during, and after application of BIC, AP-5 and CNQX, respectively.

Developmental difference in GABA response from dendrite and soma

In early development, microapplication of GABA agonist to dendrite or soma gave the same depolarizing response. However, between P10 and P12, the response to GABA was hyperpolarizing in the cell body, but depolarizing in the dendrites of the same cell, suggesting that during this period, the Cl^- concentration in dendrites was greater than in the soma. One hypothetical mechanism for this may be that an inward Cl^- transporter such as NKCC1 may be expressed in greater density in dendrites, leading to a higher local concentration of Cl^-, or that with similar densities of transporter, the smaller volume of the dendrite may reveal active Cl^- transport more readily than would be found in the soma where the volume to surface area ratio is greater. In mature MCH neurons, dendrites and perikaryon responded to GABA similarly, with hyperpolarization. This contrasts with the cortex, where dendrites of mature neurons can show a depolarizing response to GABA whereas the cell body shows a hyperpolarizing response (Gulledge and Stuart, 2003).

Timing is critical for GABA excitation

Early actions of GABA can generate a depolarizing potential leading to action potential generation, or can produce shunting inhibition in developing MCH neurons. In contrast, in mature neurons, GABA evokes an initial hyperpolarizing response with a

subsequent depolarizing action. A key mechanistic factor that determines whether GABA depolarization leads to spike induction or inhibition is the timing of the GABA-mediated event relative to other events. We show that GABA-mediated depolarization can summate with another depolarizing event to produce spikes, but primarily during the period when the GABA-induced decrease in membrane input resistance has returned toward its elevated control level, but while the membrane potential is still depolarized. Because many of the GABA-opened Cl^- channels rapidly close, the input resistance recovers while the cell is still depolarized, enhancing the summation of GABA with other depolarizing activity leading to spike generation.

The depolarizing response of immature MCH neurons to GABA raises the question of whether GABA is released during this period. GABA is secreted early in neuronal development; the growth cones of developing hypothalamic axons release GABA even in the absence of a postsynaptic partner (Gao and van den Pol, 2000), and growth cones of unidentified hypothalamic neurons respond to GABA application (Obrietan and van den Pol, 1996).

Relevance to development and energy homeostasis

Here we found that the MCH promoter was active in embryonic development (E18 or earlier), leading to selective expression in cells and axons in the hypothalamus. During early development, feeding is generally upregulated and food intake is relatively continuous (Hall and Rosenblatt, 1978; Blass and Teicher, 1980; Matheny et al., 1990). As MCH neurons increase food intake, it is interesting to note that GABA generates an excitatory tone to the MCH neurons that is lost as animals develop and feeding is reduced. The membrane potential of developing MCH neurons is substantively depolarized compared with the mature neuron. That MCH cells may play an early functional role is further suggested by the appearance of MCH in neurons in the brains of embryonic fish at 36 h post-fertilization (Mancera and Fernández-Llebrez, 1995). In humans, MCH cells are identifiable as early as in the 7 week fetus (Bresson et al., 1987); in rat MCH cells are detectable by E16 (Steininger et al., 2004). Neuropeptide modulation of feeding has been described early in development, even at birth (Capuano et al., 1993). An interesting model of developmental changes in circuitry regulating food intake, not mutually exclusive from our work on MCH neurons, found that neuropeptide Y (NPY)-mediated inhibition of GABA input to the paraventricular nucleus (PVN) was reduced in the mature brain compared with P7–P9, leading to the suggestion that there was a reduced NPY attenuation of inhibitory circuits with maturity (Melnick et al., 2007). Although we have not tested PVN neurons, our results suggest an additional interpretation, that if the P7–P9 rat PVN is similar to the developmental pattern found here in MCH cells, then the strong NPY presynaptic inhibition at this early stage may actually have reduced excitatory (GABA), not inhibitory, tone in the P7–P9 PVN.

In summary, our results show an increased level of excitatory synaptic input to developing MCH neurons that is primarily GABA-mediated. We speculate that this may contribute to MCH release during early brain development.

References

Barbin G, Pollard H, Gaïarsa JL, Ben-Ari Y (1993) Involvement of GABA-A receptors in the outgrowth of cultured hippocampal neurons. Neurosci Lett 152:150–154.

Ben-Ari Y (2002) Excitatory actions of GABA during development: the nature of the nurture. Nat Rev Neurosci 3:728–739.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303–325.

Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W, Sawchenko PE (1992) The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 319:218–245.

Blass EM, Teicher MH (1980) Suckling. Science 210:15–22.

Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, DeLeon J, Lagu B, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, Branchek TA, Gerald C, Vaysse PJ, Forray C (2002) Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. Nat Med 8:825–830.

Bresson JL, Clavequin MC, Fellmann D, Bugnon C (1987) [Ontogenetic data on the peptidergic interneuronal population in immunoreactivity of the GRF 37 type serum of the human posterolateral hypothalamus. Immunocytochemical studies using anti-GRF 37 and anti-MCH (melanin-concentrating hormone) immune sera.] C R Séances Soc Biol Fil 181:376–382.

Broberger C, De Lecea L, Sutcliffe JG, Hökfelt T (1998) Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J Comp Neurol 402:460–474.

Capuano CA, Leibowitz SF, Barr GA (1993) Effects of paraventricular injection of neuropeptide Y on milk and water intake of pre-weanling rats. Neuropeptides 24:177–182.

Chen G, Trombley PQ, van den Pol AN (1996) Excitatory actions of GABA in developing rat hypothalamic neurones. J Physiol 494:451–464.

Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. 14:515–519.

Connor JA, Tseng HY, Hockberger PE (1987) GABA induced excitement in other brain area: depolarization- and transmitter-induced changes in intracellular Ca^{2+} of rat cerebellar granule cells in explant cultures. J Neurosci 7:1384–1400.

Dallvechia-Adams S, Kuhar MJ, Smith Y (2002) Cocaine- and amphetamine-regulated transcript peptide projections in the ventral midbrain: colocalization with gamma-aminobutyric acid, melanin-concentrating hormone, dynorphin, and synaptic interactions with dopamine neurons. J Comp Neurol 448:360–372.

Decavel C, van den Pol AN (1990) GABA: a dominant neurotransmitter in the hypothalamus. J Comp Neurol 302:1019–1037.

Ebihara S, Shirato K, Harata N, Akaike N (1995) Gramicidin-perforated patch recording: GABA response in mammalian neurones with intact intracellular chloride. J Physiol 484:77–86.

Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK (1998) Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21:1375–1385.

Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, Elmquist JK (2001) Characterization of CART neurons in the rat and human hypothalamus. J Comp Neurol 432:1–19.

Elias CF, Sita LV, Zambon BK, Oliveira ER, Vasconcelos LA, Bittencourt JC (2008) Melanin-concentrating hormone projections to areas involved in somatomotor responses. J Chem Neuroanat 35:188–201.

Fu LY, van den Pol AN (2007) GABA excitation in mouse hilar neuropeptide Y neurons. J Physiol 579:445–464.

Gao XB, van den Pol AN (1999) NT-3 potentiates excitatory GABAergic synaptic transmission in cultured developing hypothalamic neurones of the rat. J Physiol 518:81–95.

Gao XB, van den Pol AN (2000) GABA release from mouse axonal growth cones. J Physiol 523:629–637.

Gao XB, Ghosh PK, van den Pol AN (2003) Neurons synthesizing melanin-concentrating hormone identified by selective reporter gene expression after transfection in vitro: transmitter responses. J Neurophysiol 90:3978–3985.

Grover LM, Lambert NA, Schwartzkroin PA, Teyler TJ (1993) Role of HCO3- ions in depolarizing GABAA receptor-mediated responses in pyramidal cells of rat hippocampus. J Neurophysiol 69:1541–1555.

Gulledge AT, Stuart GJ (2003) Excitatory actions of GABA in the cortex. Neuron 37:299–309.

Hall WG, Rosenblatt JS (1978) Development of nutritional control of food intake in suckling rat pups. Behav Biol 24:413–427.

Heck N, Kilb W, Reiprich P, Kubota H, Furukawa T, Fukuda A, Luhmann HJ (2007) GABA-A receptors regulate neocortical neuronal migration in vitro and in vivo. Cereb Cortex 17:138–148.

Kaila K, Voipio J, Paalasmaa P, Pasternack M, Deisz RA (1993) The role of bicarbonate in GABAA receptor-mediated IPSPs of rat neocortical neurones. J Physiol 464:273–289.

Kim YI, Dudek FE (1992) Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: inhibitory synaptic mechanisms. J Physiol 458:247–260.

Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R (1997) Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron 18:243–255.

Lembo PM, Grazzini E, Cao J, Hubatsch DA, Pelletier M, Hoffert C, St-Onge S, Pou C, Labrecque J, Groblewski T, O'Donnell D, Payza K, Ahmad S, Walker P (1999) The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. Nat Cell Biol 1:267–271.

Li Y, Gao XB, Sakurai T, van den Pol AN (2002) Hypocretin/orexin excites hypocretin neurons via a local glutamate neuron—a potential mechanism for orchestrating the hypothalamic arousal system. Neuron 36:1169–1181.

LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR (1995) GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15:1287–1298.

Mancera JM and Fernández-Llebrez P (1995) Development of melanin-concentrating hormone-immunoreactive elements in the brain of gilthead seabream (*Sparus auratus*). Cell Tissue Res 282:523–526.

Marsh DJ, Weingarth DT, Novi DE, Chen HY, Trumbauer ME, Chen AS, Guan XM, Jiang MM, Feng Y, Camacho RE, Shen Z, Frazier EG, Yu H, Metzger JM, Kuca SJ, Shearman LP, Gopal-Truter S, MacNeil DJ, Strack AM, MacIntyre DE, et al. (2002) Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. Proc Natl Acad Sci U S A 99:3240–3245.

Matheny RJ, Birch LL, Picciano MF (1990) Control of intake by human-milk-fed infants: relationships between feeding size and interval. Dev Psychobiol 23:511–518.

Melnick I, Pronchuk N, Cowley MA, Grove KL, Colmers WF (2007) Developmental switch in neuropeptide Y and melanocortin effects in the paraventricular nucleus of the hypothalamus. Neuron 56:1103–1115.

Obrietan K, van den Pol AN (1995) GABA neurotransmission in the hypothalamus: developmental reversal from Ca^{2+} elevating to depressing. J Neurosci 15:5065–5077.

Obrietan K, van den Pol AN (1996) Growth cone calcium elevation by GABA. J Comp Neurol 372:167–175.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715–727.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015.

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 6571:243–247.

Rossi M, Choi SJ, O'Shea D, Miyoshi T, Ghatei MA, Bloom SR (1997) Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. Endocrinology 138:351–355.

Saito Y, Nothacker HP, Wang Z, Lin SH, Leslie F, Civelli O (1999) Molecular characterization of the melanin-concentrating-hormone receptor. Nature 6741:265–269.

Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. Nature 6712:670–674.

Spigelman I, Zhang L, Carlen PL (1992) Patch-clamp study of postnatal development of CA1 neurons in rat hippocampal slices: membrane excitability and K+ currents. J Neurophysiol 68:55–69.

Staley KJ, Soldo BL, Proctor WR (1995) Ionic mechanisms of neuronal excitation by inhibitory GABAA receptors. Science 269:977–981.

Steininger TL, Kilduff TS, Behan M, Benca RM, Landry CF (2004) Comparison of hypocretin/orexin and melanin-concentrating hormone neurons and axonal projections in the embryonic and postnatal rat brain. J Chem Neuroanat 27:165–181.

Tappaz ML, Oertel WH, Wassef M, Mugnaini E (1982) Central GABAergic neuroendocrine regulations: pharmacological and morphological evidence. Prog Brain Res 55:77–96.

Tasker JG, Dudek FE (1993) Local inhibitory synaptic inputs to neurones of the paraventricular nucleus in slices of rat hypothalamus. J Physiol 469:179–192.

Tyzio R, Minlebaev M, Rheims S, Ivanov A, Jorquera I, Holmes GL, Zilberter Y, Ben-Ari Y, Khazipov R (2008) Postnatal changes in somatic c-aminobutyric acid signaling in the rat hippocampus. Eur J Neurosci 27:2515–2528.

van den Pol AN (1986) Gamma-aminobutyrate, gastrin releasing peptide, serotonin, somatostatin, and vasopressin: ultrastructural immunocytochemical localization in presynaptic axons in the suprachiasmatic nucleus. Neuroscience 17:643–659.

van den Pol AN, Tsujimoto KL (1985) Neurotransmitters of the hypothalamic suprachiasmatic nucleus: immunocytochemical analysis of 25 neuronal antigens. Neuroscience 15:1049–1086.

van den Pol AN, Wuarin JP, Dudek FE (1990) Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 250:1276–1278.

van den Pol AN, Patrylo PR, Ghosh PK, Gao XB (2001) Lateral hypothalamus Early developmental expression and response to hypocretin (orexin). J Comp Neurol 433:349–363.

van den Pol AN, Acuna-Goycolea C, Clark KR, Ghosh PK (2004) Physiological properties of hypothalamic MCH neurons identified with selective expression of reporter gene after recombinant virus infection. Neuron 42:635–652.

Wang DD, Kriegstein AR (2008) GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 28:5547–5558.

Yamashita M, Fukuda Y (1993) Calcium channels and GABA receptors in the early embryonic chick retina. J Neurobiol 24:1600–1614.

Yuste R, Katz LC (1991) Control of postsynaptic Ca2+ influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron 6:333–344.

Zhou FM, Hablitz JJ (1996) Postnatal development of membrane properties of layer I neurons in rat neocortex. J Neurosci 16:1131–1139.