Supporting Online Material for

Maternal Oxytocin Triggers a Transient Inhibitory Switch in GABA Signaling in the Fetal Brain During Delivery
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Materials and methods

Slice preparation. Experiments were performed on fetuses and neonatal Wistar rat pups from the embryonic day [E]18 to the postnatal day [P]5 (term is E21). E21 fetuses were recovered from pregnant rats during the early phase of delivery characterized by the appearance of vaginal bleeding. Neonatal (P0) rats were divided in two groups: (i) “early P0” rats were obtained from 5 to 15 minutes after delivery and (ii) “late P0” rats were obtained from 6 to 12 hours after delivery. All of the research was performed in compliance with the national guidelines on humane care and use of laboratory animals and approved by INSERM. Hippocampal slices (300-500 µm thick) were prepared using a Microm tissue slicer (International GmbH, Germany) as described previously (1). Slices were kept in oxygenated (95% O2 / 5% CO2) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 126, KCl 3.5, CaCl2 2.0, MgCl2 1.3, NaHCO3 25, NaH2PO4 1.2 and glucose 11 (pH 7.4) at room temperature (20-22 °C) at least 1 hour before use.

Electrophysiology. For recordings, slices were placed into a conventional fully submerged chamber superfused with ACSF at a rate of 2-3 ml/min at room temperature. Patch clamp recordings from visually identified CA3 pyramidal cells in cell-attached configuration were performed using EPC-10 amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany). Patch pipette solution contained (in mM): (1) for recordings of single GABA(A) channels: NaCl 120, KCl 5, TEA-Cl 20, 4-aminopyridine 5, CaCl2 0.1, MgCl2 10, glucose 10, Hepes-NaOH 10 buffered to pH 7.2-7.3. (2) for recordings of single NMDA channels: nominally Mg2+ free ACSF with NMDA (10 µM) and glycine (1 µM). Analysis of currents trough single channels and current-voltage relationships were performed using Clampfit 9.2 (Axon Instruments, Union City, CA) as described previously (2,3). A picospritzer (General Valve Corporation, Fairfield, NJ) was used to puff-apply isoguvacine (10-100 µM in ACSF) from a glass pipette in stratum radiatum at a distance of about 100 µm from soma in cell-attached recordings of action potentials. The pressure was from 10 to 20 kPa, and the duration of the puff varied from 50-100 ms. Anoxic-aglycemic episodes were induced in the intact hippocampi in vitro (4) by superfusion with ACSF in which N2 was replaced for O2 and sucrose was substituted for glucose at 32-34°C. Anoxic depolarization was detected using extracellular field potentials recordings from a pyramidal cell layer or stratum radiatum of CA3 subfield with WPI amplifier (bandpass 0-4 kHz; x1000) (5). Recordings were digitised (10 kHz) online with Digidata-1200 interface card (Axon Instruments, Union City, CA) and analysed offline with Axon package, MiniAnalysis (Synaptsoft, Decatur, GA), and Origin (Microcal Software, Northampton, MA).

Calcium and chloride imaging. For the dye loading, slices were incubated in ACSF containing 10 µM Fura2-AM or 6 mM MQAE [N-(methoxyquinolyl) acetoethyl ether] for 20-30 minutes in the dark at 35–37°C for calcium and chloride imaging, respectively. Slices were transferred to recording chamber and perfused with oxygenated ACSF at 30-32°C. Imaging was performed with a multi-beam two-photon LASER scanning system (Trimscope-LaVision Biotec, Germany) coupled to an Olympus Microscope. This system is based on a beamsplitter that splits up the incoming femtosecond LASER beam (provided by a Ti:Sapphire LASER source, Chameleon, Coherent, USA), into 64 beamlets, which are scanned simultaneously (scan rate 2KHz) in slice. Images were acquired through a CCD Camera (La Vision Imager 3QE), with a time resolution of 100 ms for full frames (90 to 150ms). Slices were imaged using a low-magnification, high numerical aperture objective (20X, NA-0.95, Olympus). The size of
the imaged field was typically \(430 \times 380 \ \mu \text{m}^2\) (2X2 binning, pixel size: 600nm). Calcium and chloride fluorescence signals were obtained by the excitation wavelength of the LASER source at 780 nm and 740 nm, respectively. Analysis was performed with custom-made software in Matlab (MathWorks, Natick, MA) using an advanced version of the previously designed software for cortical slices analysis (6). Image processing was performed on time averages of recorded movies. The calcium signal of each cell was the average fluorescence within the contour of that cell, measured as a function of time. Signal processing algorithms of MiniAnalysis software (Synaptosoft, Decatur, GA) were used to detect the onsets and offsets (time of half amplitude decay) of calcium signals within the traces of individual cells.

**Histology.** Brains from embryos and postnatal rats were fixed with paraformaldehyde (4% in PBS). Brain sections (50 \(\mu \text{m}\)) were obtained with a vibratome (Leica, Germany) and permeabilised for 10 min at room temperature (RT) in PBS–Triton X-100 (0.1%)– goat serum (5%). After permeabilisation, slices were incubated overnight at RT with rabbit anti-oxytocin receptor antibodies (abcam, Cambridge, UK; ab13076; 1: 200). The slices were rinsed three times in PBS and incubated for 2 h at RT in Cy-3 conjugated goat anti-rabbit antibodies diluted 1: 200 (Jackson ImmunoResearch). After three final washes in PBS, slices were mounted on glass slides and cover-slipped in Gel Mount (Biomedia). Experiments were repeated three times with sections from 4 different brains per developmental stage. Double labelling experiments were performed with Neuro Trace green fluorescent (Molecular probes, Oregon, USA) and mouse anti GAD-67 antibodies (1: 200; Chemicon Int.) revealed with goat anti rabbit FITC-conjugated antibodies (1: 200; Jackson ImmunoResearch). Sections were examined under a Zeiss LSM510 confocal microscope using 10X, 20X and 65X objectives; images were digitized using the built-in software and exported in tiff format for quantitative analysis with the analysis software ImageJ 1.33d (Wayne Rasband, NIH). Optical fluorescence density was measured in the hippocampus and neocortex, and fluorescence values from the corpus callosum were used as a reference value for background.

**Statistics.** Group measures are expressed as means ± SEM; error bars also indicate SEM. The statistical significance of differences was assessed with the Students \(t\) - test. The level of significance was set at \(P<0.05\).

**Pharmacology.** All drugs except isoguvacine (Cambridge Research Biochemicals) were purchased from Sigma. Oxytocin receptor antagonist SSR126768A was a gift from Sanofi-Synthelabo. SSR126768A (1 mg/kg bodyweight) was administered to pregnant rats per orally in 50 ml of water solution starting from E20. Fura2-AM and MQAE [N-(methoxyquinolyl) acetoethyl ether] were purchased from Molecular Probes.
Supplementary Figure 1. Developmental changes of oxytocin-receptor immunoreactivity in the rat hippocampus and neocortex. (A) Hippocampal sections from an E15 embryo. Oxytocin-receptor immunostaining was particularly high in the choroids plexus (cp). Inset illustrates a higher magnification of the hippocampal plate where all neurons display a dense puncta-staining around the cell bodies. (B) Hippocampal sections from an E18 embryo. Immunoreactivity is particularly important within the hippocampal plate and absent from the ventricular zone (vz). Also note a dense immunostaining of blood vessels in the hippocampal fissure (hf). Inset: the hippocampal plate at higher magnification; note a dendritic-like extension decorated with puncta-staining. (C) Oxytocin-receptor immunostaining of neocortical sections from E15, E18, P0 and P15 rats. Meninges are highly immunoreactive and a few blood vessels also immunopositive. Note the absence of staining of the ventricular zone (vz). (D) Hippocampal sections from a newborn (P0) rat. Inset illustrates at higher magnification the presence of puncta-staining around the cell bodies of pyramidal cells. (E) Quantitative analysis of receptor immunoreactivity in the hippocampus and neocortex of maturing rats. Receptor immunoreactivity declined in P20 rats. Bars: 200 µm (A-B, D) 100 µm (C) and 20 µm (insets).
Supplementary Figure 2

Supplementary Figure 2. Oxytocin and oxytocin receptor immunostaining of hippocampal CA3 pyramidal field from newborn rats (A) Immunostaining of CA3 pyramidal cell layer with Cy3 conjugated anti rabbit antibodies after incubation with rabbit antibodies to Oxytocin receptors (OT-R, abcam; 1: 200), normal rabbit serum (RS, Sigma; 1: 100) or rabbit antibodies to oxytocin (OT, abcam; 1: 2000). Note that hippocampal neurons express oxytocin receptors but do not produce oxytocin. Scale bar: 10 µm. (B) Pairs of confocal images representative of the immunostaining of CA3 pyramidal cells from a P0 rat (optical 2 µm thick sections). Sections were immunostained with Oxytocin receptor antibodies (in red) and Nissl counterstained (in green). Note that red spots are observed within the cytosol and by the periphery of the cell (arrows). The immunostaining obtained is compatible with the presence of oxytocin receptors at the neuronal membrane. Scale bar: 15 µm.
Supplementary Figure 3

Supplementary Figure 3. Washing out and washing in oxytocin via intracardial perfusion efficiently modifies $DF_{GABA}$. Summary histogram showing GABA(A) driving force ($DF_{GABA}$) deduced from cell-attached single GABA(A) channels recordings in CA3 pyramidal cells of E21 fetal rat hippocampal slices. Note that $DF_{GABA}$ is negative in control (non-perfused) animal. Prolonged (30 min) intracardial perfusion of fetuses with ACSF at term produces strong depolarizing shift of $DF_{GABA}$. Addition of 1 µM-oxytocin at the end of perfusion restored hyperpolarizing values of $DF_{GABA}$. Bath application of atosiban (5 µM) to the slices obtained from the fetuses that have been initially intracardially perfused with ACSF and then with Oxytocin (1 µM, at the end of perfusion), induces depolarizing shift of $DF_{GABA}$. Pooled data from 45 cells. These results are compatible with the hypothesis that residual maternal oxytocin remaining in fetal blood vessels provides tonic activation of oxytocin receptors in a slice.
Supplementary Figure 4. Oxytocin reverses the GABA(A)-mediated calcium changes. (A) In a P4 rat hippocampal slice loaded with Fura2-AM and imaged with two-photon excitation (time resolution: 145 ms/frame), application of isoguvacine (Isog, 10µM) produces a significant increase in [Ca^{2+}], associated to an increase in the frequency of spontaneous calcium fluorescence events as shown by the histogram representing the percentage of imaged cells that are detected as being active at each movie frame. Fluorescence traces illustrate the [Ca^{2+}] changes produced by isoguvacine application in the same movie. In this example, a majority of imaged neurons are excited by isoguvacine application as shown in the contour plot of the imaged slice (filled red contours are excited by isoguvacine while open contours are not affected by the drug). (B) In a slice previously incubated in oxytocin (1µM), isoguvacine produced opposite effects inducing an increase in [Ca^{2+}], only in a minority of neurons (red fluorescence trace, red filled contours in the contour map). Most cells showed a decrease in the frequency of spontaneous calcium events in response to isoguvacine (blue fluorescence traces, and blue filled contours in the contour map).
Supplementary Figure 5. Action of oxytocin on GABA signaling is mediated via down-regulation of the bumetanide-sensitive chloride transport. Histograms of $DF_{GABA}$ measured in CA3 pyramidal cells using cell-attached recordings of single GABA(A) channels in E18 (A) and E21(B) rat hippocampal slices. (A) At E18, bumetanide (Bm, 10 µM) mimics and occludes the depolarizing to hyperpolarizing switch in the GABA signaling mediated by exogenous oxytocin (OT, 1 µM). (B) At E21, bumetanide does not affect $DF_{GABA}$ in control conditions when GABA is already hyperpolarizing. In the presence of atosiban (AT, 5 µM) to block the effects of endogenous oxytocin, bumetanide switches $DF_{GABA}$ from depolarizing to hyperpolarizing. Pooled data from 45 cells (E18) and 50 cells (E21).
Supplementary Figure 6. Effect of blockade of oxytocin receptors on the gene expression in newborn rats. Transcript abundance assayed using quantitative real-time PCR normalized to Hypoxanthine- guanine phosphoribosyltransferase (HPRT) did not reveal any significant changes between groups (shown as x-fold change, mean ± SEM) mRNAs levels were similar in control and oxytocin antagonist treated pups, suggesting that exposure to maternal oxytocin during delivery did not significantly modify the expression levels of the mRNAs encoding for different determinants of intracellular chloride concentration as cation chloride cotransporters (NKCC1, KCC1, KCC2, KCC3, KCC4), anion exchangers (AE3, NDBCE), the chloride channel ClC-2 or the kinase WNK3 known to regulate cation chloride cotransporter activity. Methods: Oxytocin antagonist SSR126768A (1 mg/kg bodyweight) was administered to pregnant rats per orally starting from E18. Immediately after delivery total hippocampi were dissected from 6 newborn rats, which had been exposed to the antagonist, and 6 control animals, which had not been exposed to the antagonist. Total RNA was prepared using the High Pure RNA purification kit (Qiagen). First strand cDNA was generated using the SuperScript II cDNA kit (Invitrogen) with random hexamers (Invitrogen). Real-time PCR was performed for NKCC1 (NM_031798), KCC1 (NM_019229), KCC2 (NM_134363), KCC3 (ENSRNOT00000007144), KCC4 (XM_001071999), AE3 (NM_017049), ClC-2 (NM_017137), NDBCE (NM_199497), and WNK3 (XR_008488) using the Rotor-Gene real time cycler (Corbett Research, Australia) with the SYBR green PCR master mix (Applied Biosystems) and normalized to HPRT cDNA (primer sequences available on request). The cycling protocol was: 95°C for 5 min, 95°C for 10 sec, 60°C for 15 sec, and 72°C for 20 sec, with steps 2 to 4 repeated 40 times. Each sample was amplified in duplicate and gave consistent results, with amplification efficiency normalized to that of HPRT.
References


