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A STUDY OF THE ROLE OF NEURO-GLIAL REMODELING IN THE OXYTOCIN SYSTEM AT LACTATION

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Abstract—Under conditions of strong secretion of neurohypophysial hormone, such as during parturition, lactation and dehydration, the hypothalamic oxytocin-system displays a remarkable morphological plasticity such that astrocytic coverage of its neurones diminishes, their surfaces become directly juxtaposed and contacted by an increased number of synapses. A growing body of evidence indicates that these anatomical changes have an impact on glutamatergic neurotransmission in the supraoptic nucleus, and may be therefore of physiological consequence. We here evaluated the consequences of the inhibition of such plasticity on the overall activity of the oxytocin system during lactation. Remodeling was prevented by performing hypothalamic microinjections in gestating rats of endoneuraminidase, an enzyme that removes polysialic acid from the neural cell adhesion molecule. Our earlier studies established that the presence of polysialic acid is a prerequisite for remodeling of the oxytocin system in the supraoptic and paraventricular nuclei. In dams in which polysialic acid was absent in all magnocellular nuclei after bilateral endoneuraminidase injections, parturition was normal and neither the frequency nor the amplitude of suckling-induced reflex milk ejections was different from vehicle-treated dams. The weight gain of pups was also normal as was water intake by the dams. We then assessed the electrical activity of antidromically identified magnocellular neurones in the polysialic acid-free supraoptic nucleus of isoflurane-anesthetized lactating rats. Basal and bursting activity characteristic of oxytocin neurones before each reflex milk ejection was not significantly different from that recorded in the supraoptic nucleus of rats with normal levels of polysialic acid. Our results indicate that neuro-glial remodeling, despite its role on fine modulation of oxytocin neuronal activity, is not essential to parturition and lactation. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: PSA-NCAM, morphological plasticity, neuro-glial interactions, endosialidase.

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Abbreviations: aCSF, artificial cerebrospinal fluid; AVP, vasopressin; endoN, endoneuraminidase; HNS, hypothalamo-neurohypophysial system; NCAM, neural cell adhesion molecule; OT, oxytocin; PSA, polysialic acid; PVN, paraventricular nucleus; SON, supraoptic nucleus.

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It is now evident that glial cells take an active part in synaptic function, including the regulation of synaptogenesis during development (see Ullian et al., 2004) and neurotransmission (see Auld et al., 2003). The hypothalamo-neurohypophysial system (HNS), and in particular its oxytocin (OT) component, displays a significant reduction in glial coverage of neuronal surfaces, which are left directly juxtaposed and contacted by an increased number of synapses under certain conditions of stimulation, like parturition, lactation and dehydration (for a review see Theodosis, 2002). Recent work has revealed that such a reduction has direct effects on the fine-tuning of synaptic transmission in the hypothalamic magnocellular nuclei *in vitro* (Oliet et al., 2001; Piet et al., 2004). However, it is not known whether remodeling is of consequence to the overall physiological activity of the OT system, notably at parturition and lactation, when it exhibits a characteristic electrical activity, leading to the release of OT into the bloodstream, and then to contraction of the uterus or to milk ejection from the mammary gland (Lincoln and Wakerley, 1974). In the adult, the OT system continues to express molecular features characteristic of developing neural systems undergoing neuro-glial and synaptic transformations (reviewed in Theodosis et al., 2004a). Of these, expression of polysialic acid (PSA)-enriched neural cell adhesion molecule (NCAM), is particularly striking (Theodosis et al., 1991, 1999; Bonfanti et al., 1992; Kiss et al., 1993). Because of its large hydrated volume and negative charge, PSA on cell surfaces is thought to greatly reduce adhesion and to be essential for dynamic cell interactions and plasticity (see Rutishauser and Landmesser, 1996). In an earlier study (Theodosis et al., 1999), we removed PSA from all cell surfaces in one supraoptic nucleus (SON) by making unilateral microinjections in the vicinity of the nucleus of a phage enzyme, endoneuraminidase (endoN) that specifically removes PSA, leaving NCAM undisturbed (Rutishauser et al., 1985). The effect of the enzyme was long lasting and when microinjections were made at the end of gestation, the morphological remodeling associated with parturition and lactation did not take place (Theodosis et al., 1999). This offers a useful tool to study the physiological consequences of morphological plasticity in the OT system. In the present study, therefore, we evaluated in detail the physiological consequences of PSA removal from magnocellular nuclei, and the subsequent lack of neuro-glial or synaptic remodeling in the OT system. We thus performed microinjections of endoN close to the SON and paraventricular nucleus (PVN) in late gestating rats and then assessed physiological parameters linked to OT secretion during parturition and lactation as well as

the electrophysiological activity of antidromically identified magnocellular neurones *in vivo* in the SON during suckling.

EXPERIMENTAL PROCEDURES

Animals

Female Wistar rats, at least 3 months of age, and raised under controlled temperature and light conditions (12-h light/dark; water and food *ad libitum*) were used ($n=55$). They were mated and pregnancy was confirmed by the presence of a vaginal plug on the next day (gestation day 0); they were then housed in individual cages. All experiments were carried out according to international guidelines on the ethical use of animals. Moreover, the protocol elaborated here was such as to limit the number of animals used and minimize their suffering.

In vivo endoN application

Rats on days 15–18 of gestation were anesthetized with isoflurane (2.5%) through an adapted facemask allowing placement in a Narishige stereotaxic frame (TEM, Bordeaux, France). A hole was drilled in the skull and a cannula (OD 0.25 mm) was lowered in the hypothalamus. The enzyme (gift of G. Rougon) was used at a dilution of 1:200 in artificial cerebrospinal fluid (aCSF) from a stock solution containing 1 mg/ml protein. The activity of the enzyme was titrated to be 3100 U/mg. EndoN degrades linear polymers of sialic acid with α-2.8-linkage with a minimum length of seven to nine residues associated with NCAM (Rutishauser et al., 1985). The aCSF solution was composed of 124 mM NaCl, 5 mM KCL, 25 mM $NaHCO_3$, 5 mM D-glucose, 2 mM $CaCl_2$; pH adjusted to 7.4.

Bilateral injections. Three injections of 1 μl of endoN ($n=12$) or vehicle (aCSF; $n=11$) were made at 10 min intervals, on each side of the hypothalamus, between the SON and PVN (AP: 8; 7.8; 7.6 L: 1.6; 1.8; 1.2 H: 8.5; 8.5; 8.0, according to the atlas of Paxinos and Watson, 1986).

Unilateral injections. In another set of experiments, three injections of endoN ($n=24$) or aCSF ($n=8$) were made, as described above, on a single side of the hypothalamus, between the SON and PVN.

Animals were allowed to recover and placed in their cages for observation and further experimentation.

Physiological observations

Once the rats gave birth, the daily weight gain of each litter (reduced to eight pups) was noted each morning. For the group of rats that had received bilateral injections of endoN or aCSF, at 8–11 days of lactation, we assessed the frequency of spontaneous milk ejections that occurred during 2 h by monitoring the characteristic stretch reactions of the pups, which took place whenever they received milk. Dams were then separated from all their pups except one. The following morning, they were anesthetized in a Plexiglas chamber containing 5% isoflurane; they were then placed under 2.5% isoflurane through a facemask for subsequent surgical manipulations; this was further reduced to 1% to maintain anesthesia for the duration of the experiments. A fine polyurethane tubing (OD 0.61 mm) was placed in the teat duct of a thoracic mammary gland, connected to a pressure transducer (AI 415, Axons Instruments Inc., Foster City, CA, USA). The catheter allowed the recording of intramammary pressure variations to assess the frequency and amplitude of reflex milk ejections. Rectal temperature was maintained at 37.5 °C by a thermostatically controlled electric blanket (Animal Blanket Control Unit, Ealing, UK). In these bilaterally treated groups, we also measured water consumption each morning at 10 a.m., beginning on the day after microinjections until the end of the experiments. We monitored the daily water intake as a general indicator of the normal functioning of the hypothalamic system after our treatment.

Extracellular recordings

On days 8–11 of lactation, rats given unilateral injections of endoN or aCSF were separated from all but one of their pups. The following morning, they were anesthetized as described above. A cannula was inserted into the trachea and a fine polyurethane tubing (OD 0.61 mm) was placed in the right jugular vein; the animal was then paralyzed by continuous i.v. injection of bromure pancuronium (Faulding Pharmaceuticals Plc., Warwickshire, UK) while being artificially ventilated with a veterinary anesthesia workstation (Hallowell EMC Model AWS, Pittsfield, MA, USA). The level of expiratory CO_2 as well as the level of O_2 and isoflurane, was continuously monitored, with a Capnomac II (DATEX Instruments, Helsinki, Finland) during the whole experiment. Variations of intramammary pressure were recorded as described in the previous paragraph using a pressure transducer, and rectal temperature was maintained at 37.5 °C. With the animal placed in a stereotaxic frame, a bipolar stimulating electrode (SNEX 100, Rhodes Medical Instrument, Tujunga, CA, USA) was lowered into the pituitary stalk to enable antidromic activation of magnocellular neurones while a glass micropipette, filled with a solution of NaCl (0.8 M) and Pontamine Sky Blue (2%) was positioned into the SON using Paxinos and Watson (1986) coordinates. Anesthesia was then reduced to 1% isoflurane and one hour later eight pups were positioned on the nipples of the dam.

Extracellular neuronal activity was recorded through an ultra low-noise differential amplifier (AI 402x50, Axons instruments Inc.) connected to a CyberAmp series signal conditioner (CyberAmp 320, Axons Instrument, Inc.). Signals were visualized on an oscilloscope (Hameg, Mainhausen, Germany, F HM 407-2.01), recorded on magnetic tapes and sent to a discriminator (N750 spike analyzer, Mentor, Minneapolis, MN, USA). Final analysis of data was carried out using the Spike 2 software for the interface CED 1401 (Cambridge Electronic Design, Cambridge, UK).

At the end of the recording, the position of the electrode was ascertained by electrophoresis of Pontamine Sky Blue using a current of 5 μA for 10 min followed by a further 20 μA for 10 min.

Immunohistochemistry for PSA

At the end of each experiment, all animals were deeply anesthetized with 55 mg/kg i.p. pentobarbital (Sanofi, Libourne, France), injected intracardially with heparin and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. After post-fixation of the brain in 4% of paraformaldehyde overnight at 4 °C, brains were cut on a vibratome to obtain frontal slices (50 μm) of the whole hypothalamus between and including the magnocellular nuclei; sections underwent immunoperoxidase labeling for PSA as described previously (Theodosis et al., 1999). Briefly, all sections were incubated in a monoclonal mouse IgM antibody (1:8000) that specifically recognizes PSA on NCAM, for at least 48 h at 4 °C (Rougon et al., 1986). Affinity-purified anti-mouse IgM immunoglobulins conjugated to horseradish peroxidase (HRP, 1:100, Sigma, Les Ullis, France) were used as immunolabels. The reaction product was revealed with the glucose oxydase–nickel–DAB method (Shu et al., 1988). Sections from animals that had obtained aCSF microinjections were treated concomitantly. All sections were examined with a Leica DMR microscope with bright field optics.

Statistical analyses

An unpaired *t*-test was used to compare data from two groups; when more than two groups were compared, a one-way analysis