

Video Article

Assessing Cellular Stress and Inflammation in Discrete Oxytocin-secreting Brain Nuclei in the Neonatal Rat Before and After First Colostrum Feeding

Benjamin Y. Klein¹, Hadassah Tamir^{1,2,3}, Muhammad Anwar¹, Robert J. Ludwig¹, Jasmine H. Kaidbey¹, Sara B. Glickstein⁴, Martha G. Welch^{1,2}

¹Department of Psychiatry, Columbia University

²Department of Pathology & Cell Biology, Columbia University Medical Center

³Department of Psychiatry, Division of Molecular Imaging and Neuropathology, New York State Psychiatric Institute

⁴EB Sciences

Correspondence to: Benjamin Y. Klein at benjamin.klein@gmail.com, Martha G. Welch at mgw13@cumc.columbia.edu

URL: <https://www.jove.com/video/58341>

DOI: [doi:10.3791/58341](https://doi.org/10.3791/58341)

Keywords: Immunology and Infection, Issue 141, Nutrient insufficiency, nucleus tractus solitarius (NTS), stress response, general control nonderepressible 2 (GCN2), nuclear factor- κ B (NF- κ B), eukaryotic translation initiation factor 2A (eIF2a)

Date Published: 11/14/2018

Citation: Klein, B.Y., Tamir, H., Anwar, M., Ludwig, R.J., Kaidbey, J.H., Glickstein, S.B., Welch, M.G. Assessing Cellular Stress and Inflammation in Discrete Oxytocin-secreting Brain Nuclei in the Neonatal Rat Before and After First Colostrum Feeding. *J. Vis. Exp.* (141), e58341, [doi:10.3791/58341](https://doi.org/10.3791/58341) (2018).

Abstract

The goal of this protocol is to isolate oxytocin-receptor rich brain nuclei in the neonatal brain before and after first colostrum feeding. The expression of proteins known to respond to metabolic stress was measured in brain-nuclei isolates using Western blotting. This was done to assess whether metabolic stress-induced nutrient insufficiency in the body triggered neuronal stress. We have previously demonstrated that nutrient insufficiency in neonates elicits metabolic stress in the gut. Furthermore, colostrum oxytocin modulates cellular stress response, inflammation, and autophagy markers in newborn rat gut villi prior to and after first feed. Signaling protein markers associated with the endoplasmic reticulum stress [ER chaperone binding immunoglobulin protein (BiP), eukaryotic translation initiation factor 2A (eIF2a), and eIF2a kinase protein kinase R (p-PKR)], as well as two inflammation-signaling proteins [nuclear factor- κ B (NF- κ B) and inhibitor κ B (I κ B)], were measured in newborn brain nuclei [nucleus of the solitary tract (NTS), paraventricular nucleus (PVN), supra-optic nucleus (SON), cortex (CX), striatum nuclei (STR), and medial preoptic nucleus (MPO)] before the first feed (unprimed by colostrum) and after the start of nursing (primed by colostrum). Expression of BiP/GRP78 and p-eIF2a were upregulated in unprimed and downregulated in primed NTS tissue. NF- κ B was retained (high) in the CX, STR, and MPO cytoplasm, whereas NF- κ B was lower and unchanged in NTS, PVN, and SON in both conditions. The collective BiP and p-eIF2 findings are consistent with a stress response. eIF2a was phosphorylated by dsRNA dependent kinase (p-PKR) in the SON, CX, STR, and MPO. However, in the NTS (and to a lesser extent in PVN), eIF2a was phosphorylated by another kinase, general control nonderepressible-2 kinase (GCN2). The stress-modulating mechanisms previously observed in newborn gut enterocytes appear to be mirrored in some OTR-rich brain regions. The NTS and PVN may utilize a different phosphorylation mechanism (under nutrient deficiency) from other regions and be refractory to the impact of nutrient insufficiency. Collectively, this data suggests that brain responses to nutrient insufficiency stress are offset by signaling from colostrum-primed enterocytes.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58341/>

Introduction

In contrast to our understanding of early brain development occurring over the course of days-to-weeks postpartum, relatively little is known about the myriad of dynamic changes occurring in the first hours of life in rats. A key challenge has been the small size of the neonatal rat brain and a requirement for high-tech tools to isolate discrete brain regions or single cells. Studies often assess gene transcription and not translation^{1,2}, which does not give a firm understanding of functional levels of activated signaling molecules. Others examine expression using immunohistochemistry to reference brain regions, which does not allow for the quantification of expression levels³. No study to date has examined the activation of signaling pathways associated with rats' first colostrum feed in discrete brain regions, which requires rapid isolation and sacrifice and measurement of protein expression and protein phosphorylation using Western blotting. While brain microdissection is performed on older and larger brains, we have not identified a reference performing a non-single-cell brain punch in a P0 brain. This paper presents a protocol for isolating restricted regions of the neonatal brain using a relatively low-tech punch technique and a Western blotting procedure to measure protein expression in relatively small samples. This protocol may be suitable for research questions that require the assessment of protein expression and post-translational modifications (e.g., phosphorylation) in relatively restricted regions of small brains of any species, provided that the user can visually identify the brain region of interest with an atlas and identifiable landmarks.

This technique was developed to understand changes occurring in the brain as a result of the neonatal rats' first colostrum feed, which is rich in oxytocin (OT). OT has been long known for its ability to stimulate milk let-down and uterine contraction. However, OT is now known to play a wide range of roles in the regulation of many bodily functions and behaviors⁴. For example, OT opposes stress and inflammation in conjunction

with adaptive affiliative behaviors⁵, delays gastric emptying, and slows intestinal transit. OT receptors (OTR) have been identified in enteric neurons and intestinal epithelium^{6,7,8}. The gastrointestinal effects of OT are particularly important to the infant during the early postnatal period. For instance, breastfeeding is associated with the delivery of significant quantities of OT to the neonatal gut^{9,10}, and data show that the OTR is heavily overexpressed in duodenal villi during the milk suckling period⁸.

In vitro experiments using a gut cell line have demonstrated at the cellular level that oxytocin modulates important molecules in the stress signaling pathway^{11,12} and plays a regulatory role in translation of proteins¹². These studies suggest that components of milk, including exogenous oxytocin from the mother, are important in the unfolded protein response in neonates to reduce cellular stress¹³.

In vivo and *ex vivo* studies have shown that colostrum OT modulates the cellular stress response, inflammation, and autophagy markers in newborn rat gut villi. Newborn enterocytes suffer substantial cellular stress on their luminal side when the gut is simultaneously exposed to microbiota from the mother in colostrum^{14,15} and numerous proteins, including hormones such as OT^{9,10,16}.

The effects of OT on the brain have been studied¹⁷. However, the OT signaling mechanisms demonstrated in the gut during the early postnatal period have not been studied in the brain. In this paper, a method for isolating discrete brain nuclei in the neonatal rat brainstem and hypothalamus using electrophoresis is used to profile isolated brain regions. The overall goal of this method is to capture the state of cell signaling in brain areas as close as possible to birth, before and after the first milk suckling, in brain tissue with the lowest glial/neuronal index. The rationale for the development of this technique is that it allows for the rapid isolation of restricted, microscopic brain regions in neonatal pups with a more homogenous collection of neurons for *ex vivo* studies using an automated Western blotting methodology, offering highly consistent results on relatively small dissected samples. A shortcoming of prior work includes more gross dissection (brain slices or whole brain) and older animals^{18,19}. The brains of young pups are incredibly dynamic, featuring waves of glial differentiation after birth. In order to study brain changes influenced by the pups' first feeding, studying restricted neuronal nuclei with reproducible dissection is necessary.

Milk feed is usually analyzed for its immunological and nutritional impact on health or gene expression (for example, in enterocytes^{20,21}), whereas its effect on brain areas during brain development is rarely studied. The effect of milk transit in the gut on brain function was analyzed in reference to gut cholecystokinin receptors vagal relay to brain stem nuclei, but not to intracellular signaling pathways²². There is a vast literature on vulnerability of the developing neonate brain to malnutrition of mothers during pregnancy²³, but the stress and inflammation signals are not addressed. Importantly, the current method takes advantage of a phenomenon in day-zero rat newborns that isolates the blood-born colostrum stimuli from vagal relay of visceral stimuli. This is the so-called stress hypo-responsiveness period characterized by immature nucleus tractus solitarius (NTS)-hypothalamic circuit immediately after birth^{24,25} that restricts NTS, paraventricular nucleus (PVN), and supraoptic nucleus (SON) signals to blood-born stimuli.

This method is useful for analysis of multiple signaling pathways and relatively restricted to neuronal cells, provided that brain tissue is harvested at postnatal day-0 in rats, in addition to whether mothers have been challenged or not by any kind of treatment during pregnancy. Litters can be analyzed for the effects of colostrum feed versus pre-feeding signaling. When comparing signals between brain areas with poor versus rich protein yield, this method enables in-capillary determination of total protein of the polypeptide bands in capillaries run parallel to immun-quantitation of protein antigens. This method enables the quantitative comparison, using arbitrary units, of results obtained by the same antibody without standard quantitative curves and by reference to total protein per capillary. Comparing results obtained by different antibodies is possible only using quantitative standard curves.

This method allowed for the assessment of bidirectional signaling occurring between the gut and the brain and that can impact function in both organs²⁶. The association between oxytocin and food intake, which has been extensively studied in recent years²⁷, supports a link between increased oxytocin signaling and nutrient availability. These studies also support the converse concept that energy deficits are coupled with reductions in hypothalamic oxytocin signaling.

Earlier studies of the effect of OT on brain activity demonstrated that induced gut inflammation elicited cFos transcription in hypothalamic PVN, amygdala, and piriform cortex which was refractory to vagotomy²⁸. However, systemic infusion of OT with secretin decreased the brain cFos response to the provoked inflammatory reaction in the gut²⁸. This suggested that the effect of exogenous OT was carried out by routes other than vagal relays, possibly via blood-borne signaling molecules carried through the area postrema^{6,29}.

In this study, the cellular stress signaling pathways that have previously observed in the gut were assessed in the brain. The hypothesis was that milk components may protect or defer the effect of inflammation on gut permeability to microbial and other metabolites, and in turn, the effects on brain function. The clear antagonistic differences in IκB versus BiP signaling found in villi, before and after priming by colostrum¹³, suggested that the brains of neonates, still in the process of developing, may sense these colostrum-induced gut signals.

Signaling protein markers used in previous gut experiments that are associated with endoplasmic reticulum stress were measured. They include the ER chaperone BiP, translation initiation factor eIF2α (which serves as a stress response integrator³⁰), eIF2α kinase p-PKR, and two inflammation-signaling proteins (NF-κB and its inhibitor, IκB).

Six brain regions based on their ability in adults to secrete or respond to OT were chosen. The NTS, located at the upper medulla, is the first relay of the visceral input and receives direct signaling from vagal sensory neurons in the gut³¹ and possibly blood-born cytokines, toxins, and hormones via the adjacent area-postrema³². The PVN, supraoptic nucleus (SON), striatum nuclei (STR), cerebral cortex (CX), and medial preoptic nucleus (MPO) receive signaling from the gut via the NTS.

Results showed that the cellular stress response during the immediate postnatal period prior to colostrum priming and immediately after first feeding is different in NTS compared to PVN and SON. Signaling in CX, STR, and MPO differed from that of PVN and SON, as well. The distinct protective functions of OT shown previously to modulate cell stress and inflammation in the gut are likely sensed by some areas of the brain. Collectively, the data indicate that at the cellular level, during the first hours after birth, the brain responds to the metabolic stress associated with nutrient insufficiency. The data also show that the extent and direction of the modulating effects of the colostrum feed are region-dependent and that in some regions, they mirror OT effects shown previously in the gut.

Protocol

This study was approved by the Institutional Animal Care and Use Committees at Columbia University and the New York State Psychiatric Institute.

1. Tissue Preparation

1. Order timed pregnant rats from vendor.
2. Follow timed pregnant rats by observing their growing abdomens in the weeks after their arrival and subsequently looking for pups on the expected delivery date by inspecting the cage every 2 h until delivery begins.
3. Remove pups with a gloved hand by their tail before their first feed for unprimed pups (no white milk belly is apparent when viewing abdomen) or after the first feed for primed pups (at which point a white stomach will be visible on their abdomen) as described in the timeline (**Figure S1**).
Note: The first colostrum feed is termed as priming the pup; thus, a pup is unprimed until the first feed, after which they are colostrum-primed.
4. Quickly decapitate the unanesthetized pup using sharp, clean surgical scissors.
5. Remove the brain by cutting the skin down the midline and top surface of the skull to the nose. Then, using forceps, gently pry away the bone to expose the brain (**Figure 1A**) and localize the bregma, marking it with a pen as the bone plates are removed (**Figure 1B**).
6. Rapidly place the whole brain in a polymethyl methacrylate brain mold at room temperature for coronal slicing at room temperature (**Figure 1C**).
7. Without delay, make 500 μm -thick slices using a fresh razor blade. Lay the slices rostral to caudal in a Petri dish to maintain orientation of sections (**Figure 2**).
8. Quickly add artificial cerebrospinal fluid (ACSF; 1.0 mM KH_2PO_4 , 26 mM NaHCO_3 , 118.6 mM NaCl , 3.0 mM KCl , 203.3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) without glucose and incubate the slices for 60 min at 28–30 °C, constantly stirring on an orbital shaker to metabolically and differentially challenge the unprimed versus colostrum-primed tissues.
9. Identify the brain nuclei that are required to punch using a brain atlas³³ and anatomic landmarks on the tissue section. Place this slice with the nuclei of interest in a Petri dish and move it to the dissecting microscope.
10. Once visualized, quickly punch out 4 of 6 different nuclei using a coring tool, selecting the size to best punch the nucleus in question and consistently between samples (**Figure 2**).
Note: The remaining brain slice will now have a hole where brain tissue was removed. In this study, we excised the following nuclei using the below coordinates. All anterior/posterior (A/P) coordinates are from Bregma (except NTS, which is with reference to the Calamus Scriptorius). All dorsal/ventral (D/V) coordinates are from the surface of the cortex (except NTS, which is from the surface of the medulla). The following coordinates include A/P, medial/lateral (M/L), and D/V in mm: 1) solitary tract nucleus (NTS, A/P, 0.4 to 0.8; L, ± 0.2 ; D/V, 0.3 (from the surface of the medulla), 2) paraventricular nucleus (PVN, -0.8; ± 0.2 ; 0), 3) supra-optic nucleus (SON, -1.1; ± 1.4 ; 4.3), 4) cortex (CX, partial cortex area 1, -2.8; ± 1.5 ; 0.6), 5) striatum nuclei (STR, -0.0; ± 1.6 ; 1.8), and 6) medial preoptic nucleus (MPO, -0.6; ± 0.2 ; 4.2).
11. Rapidly immerse the punched nuclei in 0.06 mL of ice-cold, protein extraction buffer containing protease inhibitors and phosphatase inhibitors for 60 minutes (see step 2.3).

2. Protein Extraction

1. Prepare the protein extraction solution using the protein lysis kit (**Table of Materials**) on the day before expected pup delivery.
2. Thaw (on ice) the frozen (-20 °C) aqueous solution of the protease and phosphatase inhibitors of the lysis buffer kit and place the lysis buffer and DMSO solution of the proteases/phosphatases inhibitors on ice.
3. Add 1.85 mL of lysis buffer into a clean, ice-cold 15 mL tube. Then, add 0.1 mL of aqueous solution of inhibitors and 0.05 mL of DMSO-dissolved inhibitors. Finally, cap and briefly vortex the tube and keep it at -20 °C until use.
4. Label 24 clean microcentrifuge tubes (0.5 mL each) for the lysis procedure. Designate 12 tubes per brain for the colostrum-unprimed group (U) [6 left (L) and 6 right (R) brain nuclei], and label according to nuclei acronym, side, and condition (e.g., NTS-L-U, NTS-R-U, etc.). Label the second group of 12 tubes for the colostrum-primed samples.
5. Label two additional sets of tubes as done in step 2.4 for the stock protein extracts (using 1.5 mL Eppendorf-style tubes) and for the first set of sample preparation (using 0.5 mL tubes). Keep these tubes in two separate, labeled freezing boxes (each designed for 100 tubes). One box will be used for the unprimed samples and the other for the primed samples.
6. Thaw the lysis solution on ice on the day that the tubes are delivered, and while incubating brain slices in ACSF, aliquot 0.06 mL lysis solution into the lysis procedure tubes (from step 2.4) and add nuclei punches and incubate in ice for 60 min.
7. Centrifuge the incubated lysed nuclei for 30 min in a cooled mini-centrifuge at 14000 rpm (10,000 $\times g$) and carefully aspirate 0.055 mL of supernatant with a properly set pipette. Transfer the supernatant into the pre-cooled 1.5 mL stock tubes (from step 2.5) and put them on ice. Before freezing (at -20 °C) the protein stock tubes, transfer 0.012 mL of supernatant into the 0.5 mL pre-cooled tubes for the first sample preparation (from step 2.5) and leave them on ice.

3. Sample Preparation for In-capillary Protein Measurement

1. Use a kit and prepare the reagents for separation according to manufacturer's directions. Add 0.003 mL of master-mix reagent to each of the 12 samples in the 0.5 mL labeled tubes (from step 2.5) on ice that contain 0.012 mL of the protein extracts.
2. Turn on the heating block to 95 °C and add 0.004 mL of the reagent prepared in step 3.1 to a biotinylated molecular-weight (MW) ladder in a tube with 0.016 mL of deionized water. To denature the ladder and samples, place the ladder tube and the 12 samples of unprimed protein extracts in the heat block at 95 °C for 5 min and store them at 4 °C until use.
3. Repeat steps 2.0 to 3.2, from protein extraction to sample preparation, for the nuclei punched from colostrum-primed rats.

4. Electrophoresis Preparation

1. Thaw the biotin labeling reagent (stored in the deep freezer at -80°C) on the bench and prepare the protein detection kit on ice at 4°C following the manufacturer's directions.
2. Mix 0.15 mL of luminol with 0.15 mL of peroxide and load 0.01 mL into 25 wells (row E, wells 1-25) of the plate for the automated Western machine. Load 0.008 mL of Streptavidin-HRP from the kit into wells D1 to D25.
3. Load 0.01 mL of the antibody diluent solution into wells C1 to C25 and B1.
4. Spin the 24 protein samples and ladder tubes briefly (2-3 seconds) in a minicentrifuge to pool down evaporated water from the tube caps.
5. Load 0.003 mL of 12 unprimed samples into wells A2 to A13, 0.003 mL of 12 colostrum-primed samples into wells A14 to A25, and 0.005 mL of the biotinylated ladder into well A1.
6. Leave row F empty and load 0.45 mL of wash buffer into each of the 5 compartments in each of the 3 rows below row F. Cover the plate with its plastic lid to avoid evaporation during the remaining procedures.
7. Briefly vortex the thawed biotin labeling reagent and add 0.15 mL of the reagent to its designated tube; then add to it 0.15 mL of the total protein reconstitution agent and mix them to homogeneity.
8. Remove the cover from the plate and load 0.01 mL of agents 1 and 2 into wells B2 to B25. Cover the plate and centrifuge it for 10 min at $1,000 \times g$ to remove any air bubbles from the various solutions. Use an empty plate in the centrifuge for balance.

5. Electrophoresis

1. While the plate is spinning, open a run file in the automated Western machine-attached computer by indicating in the dropdown page from "file" to run a total protein assay and clicking the respective spot.
2. Annotate the samples by well in the computer. Then, remove the plate from the centrifuge remove the cover and carefully peel off the aluminum cover from the separation solution compartments.
3. Place the plate in the automated Western instrument, peel off the cover from the capillary cartridge box, insert the cartridge in its designated place, and close the door.
4. Click the "RUN" button, and when prompted with the type of the assay ("total protein"), type in the name of the samples (for example, "unprimed 2-13 and colostrum-primed 14-25"). Click "OK", and when prompted by the activated run date and ID number of the run file, make a note of the time when the run ends.
5. At the end of the run (170 min after the start), open the instrument door, remove the capillary cartridge, and discard it into the sharps disposal. Discard the plate in the biological matter disposal.
6. Click the separation curves icon in the analysis page of the run file, and check that all the samples have run properly and are showing multiple protein curves in all capillaries.

6. Analysis of Signaling Proteins

1. In a labeled 1.5 mL tube, add 0.003 mL of rabbit anti phospho-eIF2a (p-eIF2a) antibody and suspend it in 0.3 mL (1:100 dilution) in antibody diluent from the suitable detection kit. Then, keep it on ice.
2. Label a luminol tube and add 0.15 mL of luminol and 0.15 mL of peroxide from this detection module kit and dispense 0.01 mL into wells E1 to E25 of a fresh, automated Western plate.
3. Dispense 0.01 mL of the secondary anti rabbit antibody into wells D2 to D25, and add 0.01 mL of streptavidin-HRP from the kit to well D1.
4. Dispense 0.01 mL of the primary antibody (from step 6.1) into wells C2 to C25, and add 0.01 mL of antibody diluent 2 solution to wells C1 and B1 to B25.
5. Leave the row F wells empty and fill 0.45 mL of wash buffer into the 5 compartments of 3 rows below the F row.
6. Briefly spin the refrigerated samples for 3 s, and add 0.003 mL of each sample to row A in the same order they were added for the total protein assay, starting from A2 to A25. Add 0.005 mL of the biotinylated MW ladder to well A1 and cover the plate.
7. Centrifuge the plate as done in step 4.8. While the plate is spinning, open (in the automated Western-associated software and computer) a new run file and indicate in the dropdown page from "File" to run a molecular size assay by clicking the respective spot.
8. In the assay page, type the sample names in each capillary, then type the name of the primary antibody in the allocated spot and the secondary anti-rabbit antibody below it.
9. At the end of centrifugation, repeat steps 5.3–5.5, except the name given to the run file this time should be "p-eIF2a on unprimed 2–13 and colostrum-primed 14–25".
10. After the electrophoresis separation, check the run file for immune-reactivity peaks of antigens at sizes of 40–43 kDa. Where MW of peaks this size are missing, right-click below the curve and indicate inside the dropdown list to add MW to the peak, which ensures that the size and arbitrary quantity below the curve are recorded.

7. Processing the Results

1. Open a spreadsheet file for total protein run in the automated Western run file and provide an ID number.
2. Open the run file of the total protein assay at the analysis page at the curve mode and mark all the peaks in individual capillaries. Then, copy and paste them into the spreadsheet and sum the areas under the curve of all peaks recorded in the entire capillary.
3. In a separate column, arrange the total amount of protein for each column with capillary numbers, names of the respective brain nuclei, and the ID numbers of the run files.
4. Open a spreadsheet for the p-eIF2a antigen, and in a single column, record the area under the curve from each capillary side-by-side with its respective capillary number, name of brain nucleus, and ID number of the run file.
5. Copy the total protein column (parallel to the p-eIF2a curve quantities) into a third spreadsheet and compute p-eIF2a:total protein ratios in a third column.

6. Collect results from steps 4 to 6 for each brain nucleus, arrange them in groups of nuclei, and generate a bar graph.

Representative Results

The representative bands of immunoreactivity relative to total protein show that there are brain nuclei with very low harvested protein. This requires the use of the automated Western blot technique, which is highly sensitive compared to the canonical Western blot. This approach can be run with fortyfold less protein per capillary compared to the per-lane in Western blots.

Differential effects of colostrum priming on BiP levels in brain nuclei

Coronal sections of rat brains were harvested before the first colostrum feeding (unprimed) and after the first feeding (primed). Samples were fractionated by capillary electrophoresis (on WES), and proteins were quantified using the kit for in-capillary total protein staining (see Protocol). Additional immune capillary fractionation on the automated Western provided identification of the respective protein antigens. In **Figure 3A**, representative BiP protein expression is shown in the upper panel, with the corresponding total protein shown below. BiP is an ER chaperone protein. In **Figure 3B**, quantitated immunoreactivities are presented by bar graphs relative to the corresponding total protein.

Within the unprimed tissue samples, BiP levels in NTS were significantly higher compared to all other regions ($p < 0.05$, $n = 4$ nuclei). Priming of gut tissue with colostrum had an opposite effect in NTS, compared with increased BiP in other regions, relative to unprimed tissue (**Figure 3B**). Priming decreased BiP in NTS (**Figure 3B**) and had no effect on PVN and SOPT. However, priming increased BiP in CX, STR, and MPO relative to unprimed tissue. This data implies that BiP levels in the various tested brain nuclei respond differently to gut priming and that there not yet any demonstrated cross-talk between the various nuclei tested.

Differential effects of colostrum priming on eIF2a and p-eIF2a levels in brain nuclei

Brain tissue samples were prepared, analyzed, and quantified as shown in **Figure 3A** and **3B**. As with BiP levels (**Figure 3B**), eIF2a and p-eIF2a levels in NTS were elevated in the unprimed condition relative to other nuclei (**Figure 4B** and **4C**). As demonstrated with BiP levels, the response in NTS to priming of gut tissue with colostrum was opposite to that in other nuclei. Levels of both eIF2a and p-eIF2a in NTS were reduced relative to unprimed tissue. In all other tested nuclei, priming increased levels in eIF2a and p-eIF2a relative to unprimed tissue (**Figure 4B** and **4C**).

Phosphorylation of eIF2a by kinase GCN2 in NTS and PVN after colostrum priming

Brain tissue samples were prepared, analyzed, and quantified as shown in **Figure 3**. Priming decreased p-PKR in PVN ($\chi^2 p = 0.025$), unlike in all other regions where it was increased. Contrary to p-eIF2a findings (**Figure 4C**), levels of p-PKR were low in unprimed samples relative to primed samples in NTS (**Figure 5A**). Phosphorylation of eIF2a is usually catalyzed by its kinase PKR in response to viruses³⁴ and by OT in the gut, as previously demonstrated¹¹. However, the fact that p-PKR levels were very low in unprimed versus primed NTS (relative to other nuclei, **Figure 5A**) strongly suggests that another kinase must be involved in phosphorylating eIF2a (**Figure 4C**). Accordingly, GCN2 levels were tested in NTS and PVN because it is also a known kinase of eIF2a³⁵. Levels of p-GCN2 (active form) were higher in unprimed samples compared to primed samples in NTS and PVN (**Figure 5B**), and GCN2 (inactive form), compared to p-GCN2, was inversely expressed in PVN (**Figure 5C**). pGCN2 in NTS (**Figure 5C**) was expressed inversely to pPKR (**Figure 5A**) in both unprimed and primed NTS. In primed tissue, p-eIF2a was considerably lower in NTS than in all other brain regions [PVN ($p < 0.01$), SON ($p < 0.01$), CX ($p < 0.01$), and MPO ($p = 0.02$)]. This indicates that pGCN2, rather than pPKR, was responsible for eIF2 inhibition in NTS.

Colostrum priming inhibits NF- κ B in CX, STR, and MPO

Figure 6A shows that I κ B levels were significantly higher in SON unprimed samples vs primed samples. I κ B levels in SON, CX, STR, and MPO trended higher in the same direction. In **Figure 6C**, NF- κ B levels in CX, STR, and MPO were significantly higher in primed versus unprimed tissue. However, NF- κ B levels were significantly lower in primed NTS tissue, which suggests that colostrum had a distinct anti-inflammatory effect on this brain region. Cytosolic NF- κ B was higher (retained) in unprimed NTS samples, whereas its inhibitor I κ B was low and unchanged in primed samples. This suggests that a distinct anti-inflammatory mechanism is regulating NF- κ B in NTS. This nutrient insufficiency stress effect is consistent with current findings that stress markers BiP and p-eIF2a are both regulated by the presence of colostrum priming (**Figures 3** and **4**, respectively), also shown in previous BiP and p-eIF2a findings¹⁰. The reason for the distinct response of I κ B and NF- κ B in NTS compared with CX, STR, and MPO is not yet fully understood. However, it is consistent with the observation that the NTS, which relays signals from the gut to the brain, may play a crucial role in responding uniquely and oppositely versus other brain regions compared to peripheral inflammation³⁶. Levels of I κ B in CX, STR, and MPO were low (**Figure 6A**), compared to high levels of NF- κ B in the same areas (**Figure 6C**). While this finding apparently contradicts the premise that I κ B is binding and retaining NF κ B in the cytosol, different calculation of the data which combines and compares all primed samples in CX, STR, and MPO using regression analysis shows that NF- κ B highly correlates with I κ B ($p < 0.0001$, $n = 24$).

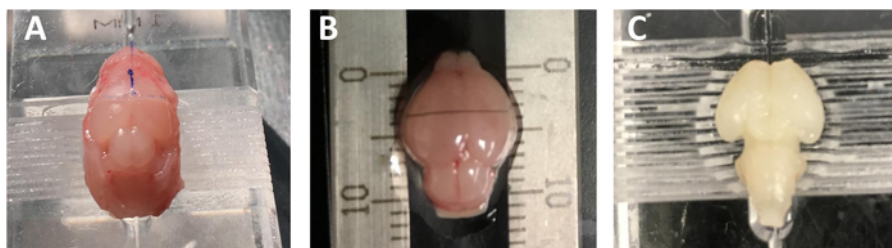


Figure 1: Dissected neonatal rat brain. (A) The dissected brain is shown with bones removed rostral to bregma. (B) The brain is shown after a line has been drawn at bregma, after which the remaining bone plates were removed. (C) The brain is shown in a polymethyl methacrylate brain mold just before being sliced with a razor blade. [Please click here to view a larger version of this figure.](#)

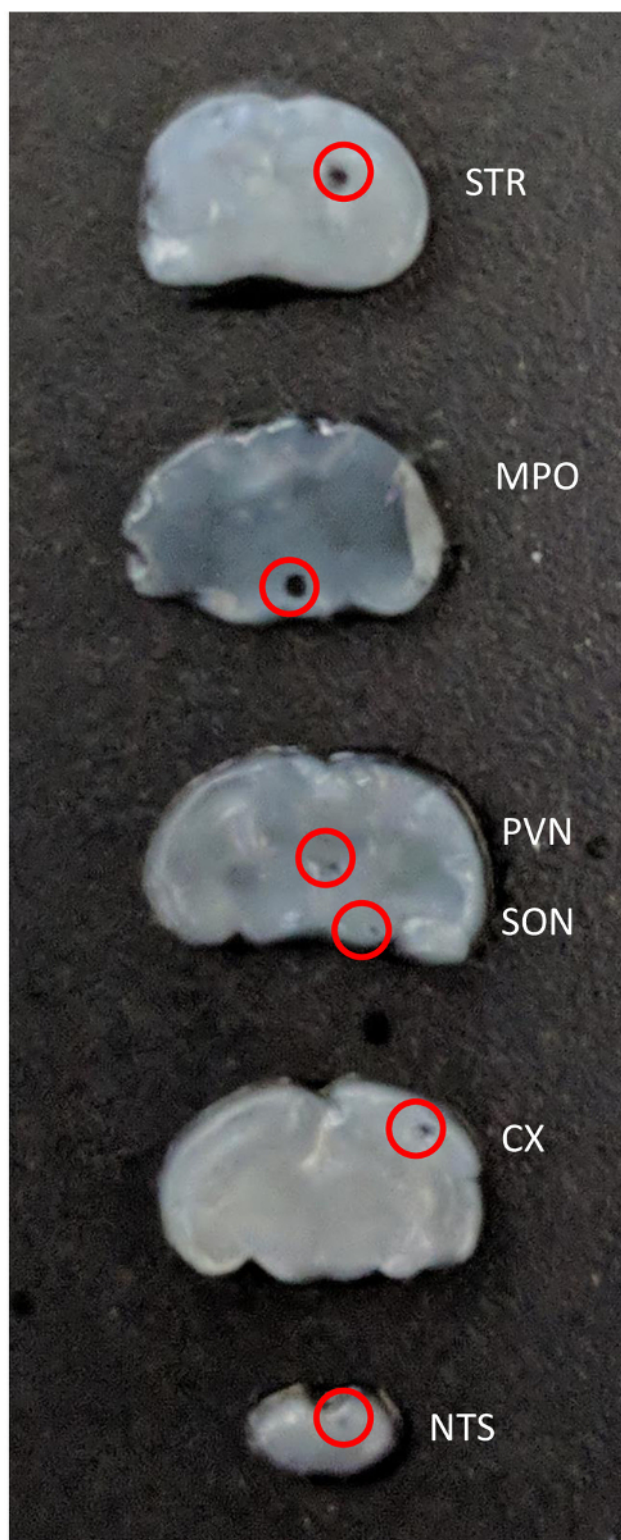


Figure 2: Brain slices arranged from rostral to caudal with punches (red circles). Abbreviations: CX: cortex, parietal lobe; MPO: medial preoptic area; NTS: nucleus tractus solitarius; PVN: paraventricular nucleus; SON: supraoptic nucleus; and STR: striatum. [Please click here to view a larger version of this figure.](#)

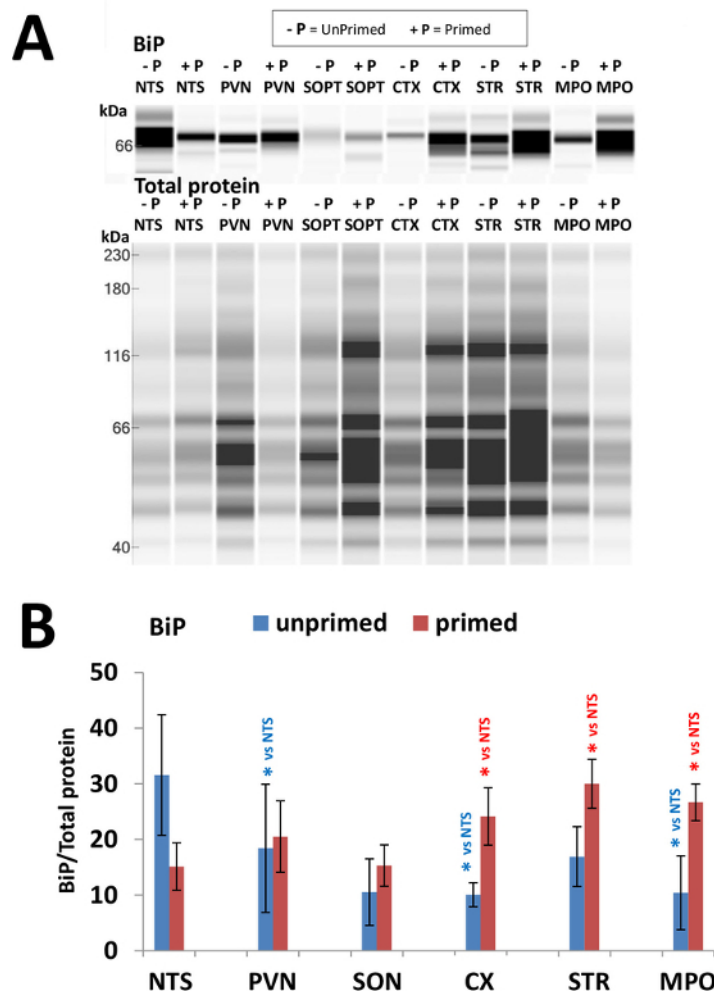


Figure 3: BiP/GRP78 response in NTS is inverted to that of other nuclei. (A) Representative BiP protein expression in the upper panel relative to the total protein presented in the panel below. Note that the total protein density in the various capillaries are heterogeneous, and these are used to equalize the respective BiP expression per protein in panel B. (B) Shown is the mean BiP relative to total protein in brain nuclei of unprimed samples (blue) versus colostrum-primed samples (brown). Asterisks of respective colors designate significant differences ($p < 0.05$, $n = 4$ nuclei) between BiP in NTS versus BiP in the other nuclei. BiP expression in unprimed versus primed samples in NTS are significantly inverted to those in STR (χ^2 $p = 0.028$, $n = 4$). [Please click here to view a larger version of this figure.](#)

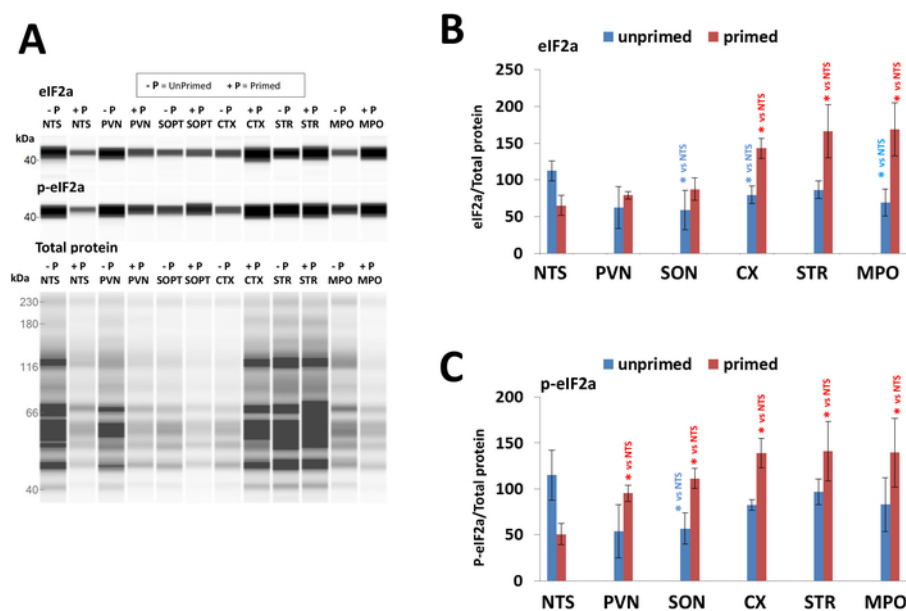


Figure 4: Active and inactive eIF2a (p-eIF2a) response to colostrum priming versus unprimed samples in NTS inverted to that of other nuclei. (A) Representative eIF2a and p-eIF2a protein expressions are shown in the upper panels. The total protein lanes are presented at the lower panel. (B) Shown is the mean eIF2a of 4 samples expressed relative to total protein (from lower panel A) in brain nuclei of unprimed samples (blue) versus colostrum-primed samples (brown), and (C) the inactive form of eIF2a (p-eIF2a). Asterisks of respective colors designate significant differences ($p < 0.05$, $n = 4$ nuclei) between both eIF2a forms in NTS versus respective levels in the other nuclei. Active eIF2a expression in unprimed versus primed samples in NTS are significantly inverted to those in CX, STR, and MPO ($\chi^2 p < 0.05$ versus all three nuclei, $n = 4$). Error bars represent standard error. [Please click here to view a larger version of this figure.](#)

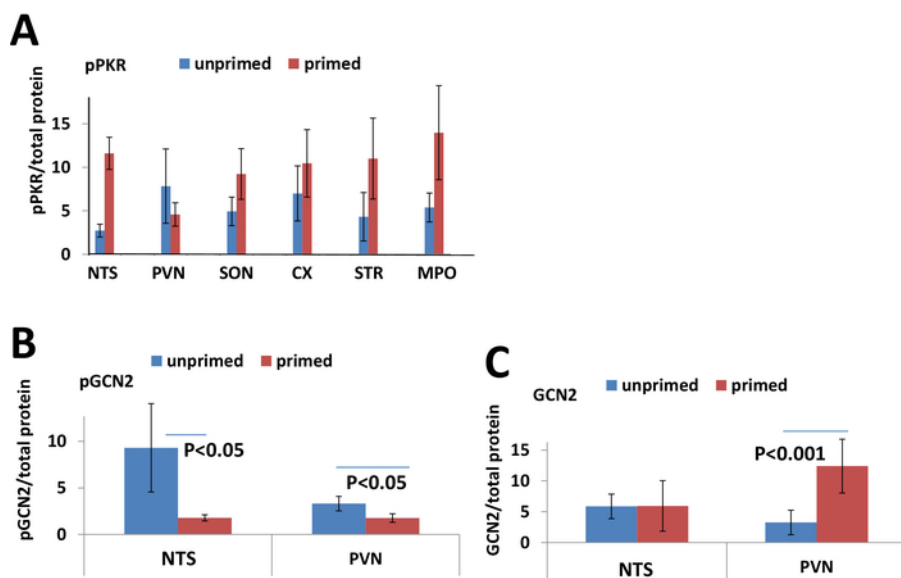


Figure 5: NTS expresses eIF2a kinase p-GCN2 in accord with p-eIF2 level and inversely to p-PKR level. (A) The level of p-PKR (activated dsRNA kinase of eIF2a) is expressed in unprimed versus colostrum-primed NTS inversely to 1) the level of p-PKR in PVN ($\chi^2 p = 0.025$) and 2) its expected targeted substrate eIF2a after its phosphorylation (to p-eIF2a, **Figure 2C**). Note that the pattern of p-PKR in SON, CX, STR, and MPO of unprimed versus colostrum-primed samples fits the respective patterns of p-eIF2a expression from **Figure 2C**. (B) Activated eIF2a kinase (p-GCN2) in unprimed versus primed NTS samples follows a similar pattern of p-eIF2a expression from **Figure 2C** and an inverse pattern of pPKR expression from **Figure 3A** ($\chi^2 p = 0.028$). C = mean levels of inactive GCN2. Error bars represent standard error. [Please click here to view a larger version of this figure.](#)

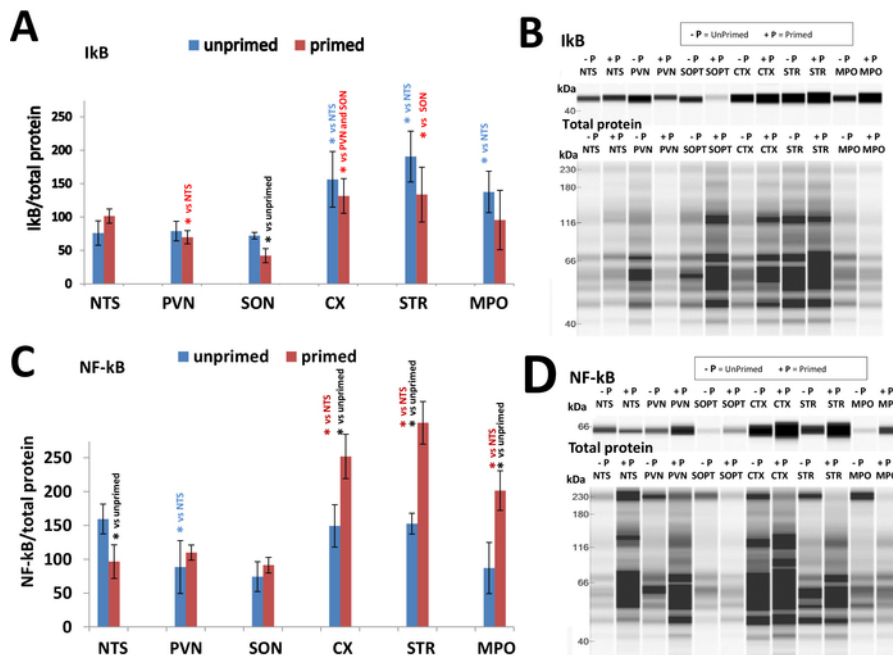


Figure 6: Cytoplasmic expression of NF-κB and IκB in brain nuclei from unprimed and colostrum-primed gut samples. (A) Mean cytoplasmic expression of IκB relative to total protein (from panel C) at indicated brain nuclei. (B) Representative cytoplasmic bands of IκB protein are presented in the upper panel and total protein density in respective lanes in the lower panel. (C) Mean cytoplasmic expression of NF-κB relative to total protein (from panel D) at indicated brain nuclei. (D) Representative cytoplasmic bands of NF-κB bands are presented in the upper panel and total cytoplasmic protein density in respective lanes in the lower panel. Colored asterisks designate significant differences between indicated samples ($p < 0.05$, $n = 4$). Note that cytoplasmic NF-κB levels are higher in colostrum-primed CX, STR, and MPO than in hypothalamic nuclei NTS, PVN, and SON in disagreement with the levels of NF-κB inhibitor (IκB). [Please click here to view a larger version of this figure.](#)

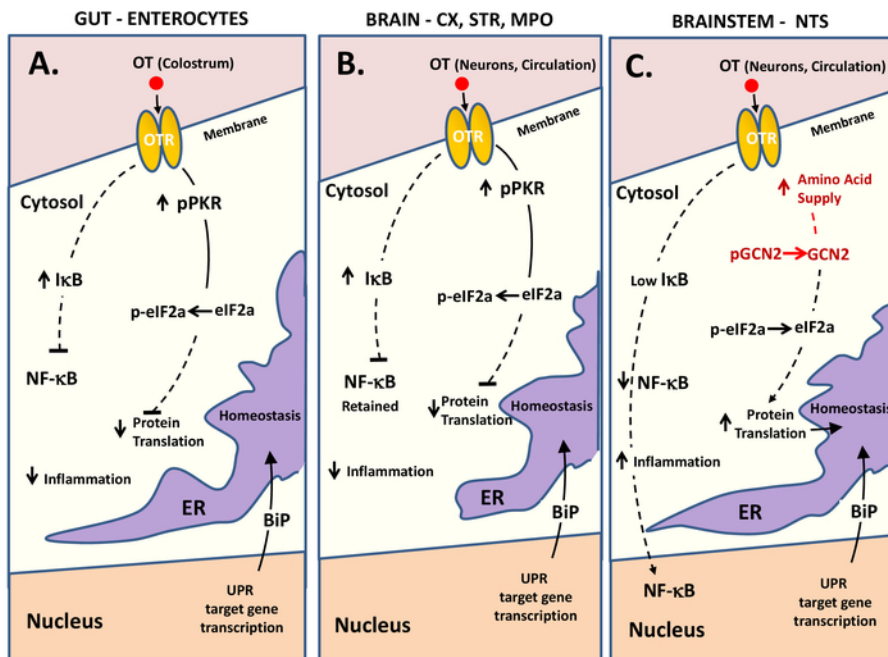
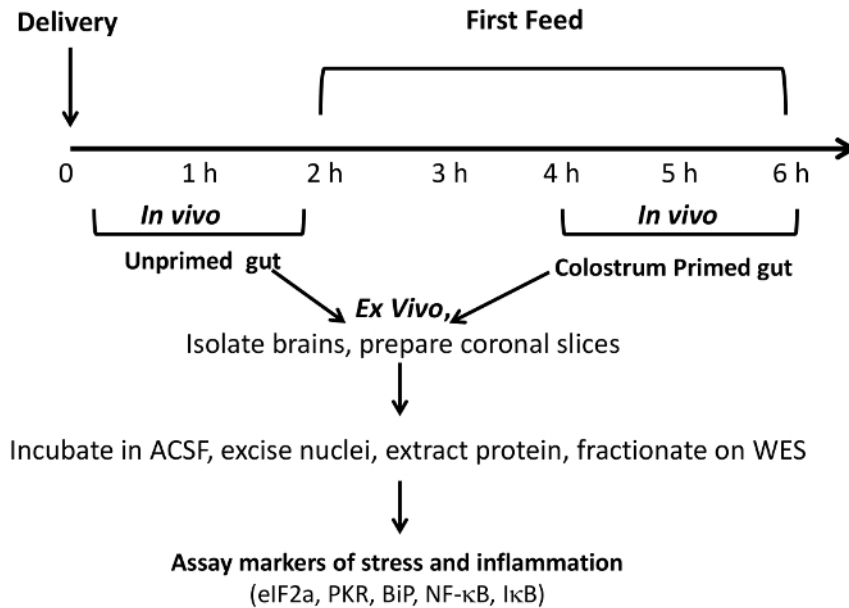


Figure 7: Schematic comparing hypothesized OT signaling in colostrum-primed gut and brain regions. (A) Colostrum OT in enterocytes activates its receptor via direct interaction with the OTR, reducing inflammation via increased IκB downstream signaling. Protein translation is inhibited by increasing p-PKR, which decreases eIF2a activity through phosphorylation. Homeostasis is restored via increased BiP gene expression^{13,37}. (B) Neuronal or circulatory OT activates its receptor in the CS, STR, and MPO regions of the brain, downregulating inflammation and protein translation via the same molecules as the gut (see panel A). (C) The hypothesized effects of colostrum priming in the NTS region of the brain are distinct and opposite from those in panels A and B. GCN2, which is sensitive to amino acid supply, increases both eIF2a and protein translation. At the same time, low levels of IκB allow NF-κB to enter the nucleus and induce inflammation. Note that BiP acts similarly to restore homeostasis in the gut and in NTS in the brain. [Please click here to view a larger version of this figure.](#)



Supplementary Figure 1: Schematic showing the protocol timeline. [Please click here to view a larger version of this figure.](#)

Discussion

A technique for microdissection of discrete, OTR-rich brain nuclei in the neonatal rat brain is presented in this paper. It is well recognized that neurons are highly specialized, even within well-characterized nuclei in the brain. This highly reproducible approach to isolate specific OTR-rich nuclei enables robust hypothesis testing. Using automated Western blotting, the consistency and reproducibility of the results were further improved. While a limitation of this technique remains modest brain punch variability; this technique represents an advance over single-cell, whole brain or brain slice approaches. Single-cell approaches are complicated by providing very limited snap shots. Using the whole brain, any findings may be diluted if the effect is restricted to a brain region or neuronal subtype. Brain slice approaches without microdissection can introduce profound variability in results within mere microns in a slice, which is especially problematic for the hypothalamus in this study, due to tight clustering of distinct nuclei. Using a standardized punch and the assistance of a microscope, the variability in protein measurements was reduced.

There are several critical steps within this protocol. These include adherence to the timing of tissue harvest with respect to the first feed, using a minimal volume of protein extraction buffer, incubation of brain slices in ACSF so that molecular inhibitors or stimulants may be used, and using a sensitive immune-electrophoretic device. Possible modifications include the choice of reagents for incubation and the duration of incubation and extending the time of unprimed starvation to compare brains at equivalent times. Important limitations include the presence of some non-neuronal cells (even during this very early neonatal period) and a restricted number of samples that can be prepared from one litter of newborns in one day. Furthermore, this approach is completely dependent on when the pups are born. With respect to existing methods, gene expression in the brain at this age is typically and more conveniently assessed at the transcriptional level. However, for cell signaling analysis, the proteomic approach executed by core facility equipment is more expensive than the desktop automated instruments. The conventional Western blot method requires much more protein, takes several days to complete, and involves several manipulation steps by the technical manpower. This automated approach requires 0.8 µg protein per capillary, once loaded into the instrument all the steps are performed without human hands interference and takes 2 h and 50 min to complete the run. This technique can be used for studying the immediate postnatal brain development in rats and wide range of genetically manipulated mouse models.

Using this technique, the effects of OT at the cellular level, specifically in the very critical period between birth and feeding, were investigated when the OTR is maximally expressed in the epithelium⁸. The modulating effects of OT on cell signaling molecules in gut cells, both in a cell line³⁷ and in vivo¹³, were previously demonstrated. In the present study, the effects observed in gut cells were assessed in brain regions rich in OTR. The expression of BiP/GRP78 and p-eIF2α was upregulated in unprimed (consistent with an expected response to stress) and downregulated in primed NTS tissue (consistent with an attenuated response to stress), whereas NF-κB was high and stable in both conditions³⁸. Expressions of BiP and NF-κB were the same in the other tested regions in both unprimed and primed conditions. eIF2α was phosphorylated by dsRNA dependent kinase (pPKR) in the SON, CX, STR, and MPO. However, in the NTS, and to a lesser extent in PVN, eIF2α was phosphorylated by another kinase, general control nonderepressible-2 kinase (GCN2). A schematic depicting hypothesized differences in signaling in the brain and gut elicited by colostrum exposure is presented in **Figure 7**.

This microdissection technique makes it possible to test hypotheses related to the impact of the first colostrum feed on stress-related signaling in discrete OTR-rich nuclei in the neonatal brain. This data suggest that the stress modulating mechanisms previously observed in new born gut enterocytes are mirrored in specific brain regions rich in OTR, as shown by increased cytosolic retention of NFKB (PVN, SON CX, STR, and MPO). Results also indicate that NTS and PVN utilize a different phosphorylation mechanism from the other regions that may be refractory to the impact of nutrient insufficiency. Collectively, the data indicate that cell signaling in the brain associated with nutrient and hormonal insufficiency during the first hours following birth is similar to cell signaling in the gut under the same conditions. This data supports the importance of breast

milk at the time of birth for stress modulation in both gut and brain. These results underscore the need for further exploration of the impact of colostrum on brain function.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank Manon Ranger and Alexandra Schulz for their assistance in preparing this protocol.

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