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Colostrum in the newborn gut modulates cellular stress and inflammation in oxytocin-secreting nuclei in the brain
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1 **TITLE:**
2 Assessing Cellular Stress and Inflammation in Discrete Oxytocin-Secreting Brain Nuclei in the
3 Neonatal Rat Before and After First Colostrum Feeding
4
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27 **KEYWORDS:**
28 Nutrient insufficiency, nucleus tractus solitarius (NTS), stress response, general control
29 nonderepressible 2 (GCN2), nuclear factor-kB (NF-kB), eukaryotic translation initiation factor 2A
30 (eIF2a)
31
32 **SUMMARY:**
33 Here, we present a protocol to isolate brain nuclei in the neonatal rat brain in conjunction with
34 first colostrum feeding. This technique allows the study of nutrient insufficiency stress in the
35 brain as modulated by enterocyte signaling.
36
37 **ABSTRACT:**
38 The goal of this protocol is to isolate oxytocin-receptor rich brain nuclei in the neonatal brain
39 before and after first colostrum feeding. The expression of proteins known to respond to
40 metabolic stress was measured in brain-nuclei isolates using Western blotting. This was done to
41 assess whether metabolic stress-induced nutrient insufficiency in the body triggered neuronal
42 stress. We have previously demonstrated that nutrient insufficiency in neonates elicits metabolic
43 stress in the gut. Furthermore, colostrum oxytocin modulates cellular stress response,
44 inflammation, and autophagy markers in newborn rat gut villi prior to and after first feed.

45   Signaling protein markers associated with the endoplasmic reticulum stress [ER chaperone
46   binding immunoglobulin protein (BiP), eukaryotic translation initiation factor 2A (eIF2a), and
47   eIF2a kinase protein kinase R (p-PKR)], as well as two inflammation-signaling proteins [nuclear
48   factor-κB (NF-kB) and inhibitor κB (IkB)], were measured in newborn brain nuclei [nucleus of the
49   solitary tract (NTS), paraventricular nucleus (PVN), supra-optic nucleus (SON), cortex (CX),
50   striatum nuclei (STR), and medial preoptic nucleus (MPO)] before the first feed (unprimed by
51   colostrum) and after the start of nursing (primed by colostrum). Expression of BiP/GRP78 and p-
52   eIF2a were upregulated in unprimed and downregulated in primed NTS tissue. NF-kB was
53   retained (high) in the CX, STR, and MPO cytoplasm, whereas NF-kB was lower and unchanged in
54   NTS, PVN, and SON in both conditions. The collective BiP and p-eIF2 findings are consistent with
55   a stress response. eIf2a was phosphorylated by dsRNA dependent kinase (p-PKR) in the SON, CX,
56   STR, and MPO. However, in the NTS (and to a lesser extent in PVN), eIf2a was phosphorylated by
57   another kinase, general control nonsuppressed-2 kinase (GCN2). The stress-modulating
58   mechanisms previously observed in newborn gut enterocytes appear to be mirrored in some
59   OTR-rich brain regions. The STN and PVN may utilize a different phosphorylation mechanism
60   (under nutrient deficiency) from other regions and be refractory to the impact of nutrient
61   insufficiency. Collectively, this data suggests that brain responses to nutrient insufficiency stress
62   are offset by signaling from colostrum-primed enterocytes.
63
64 **INTRODUCTION:**
65   In contrast to our understanding of early brain development occurring over the course of days-
66   to-weeks postpartum, relatively little is known about the myriad of dynamic changes occurring
67   in the first hours of life in rats. A key challenge has been the small size of the neonatal rat brain
68   and a requirement for high-tech tools to isolate discrete brain regions or single cells. Studies
69   often assess gene transcription and not translation[1,2], which does not give a firm understanding
70   of functional levels of activated signaling molecules. Others examine expression using
71   immunohistochemistry to reference brain regions, which does not allow for the quantification of
72   expression levels[3]. No study to date has examined the activation of signaling pathways associated
73   with rats' first colostrum feed in discrete brain regions, which requires rapid isolation and
74   sacrifice and measurement of protein expression and protein phosphorylation using Western
75   blotting. While brain microdissection is performed on older and larger brains, we have not
76   identified a reference performing a non-single-cell brain punch in a P0 brain. This paper presents
77   a protocol for isolating restricted regions of the neonatal brain using a relatively low-tech punch
78   technique and a Western blotting procedure to measure protein expression in relatively small
79   samples. This protocol may be suitable for research questions that require the assessment of
80   protein expression and post-translational modifications (*e.g.*, phosphorylation) in relatively
81   restricted regions of small brains of any species, provided that the user can visually identify the
82   brain region of interest with an atlas and identifiable landmarks.
83
84   This technique was developed to understand changes occurring in the brain as a result of the
85   neonatal rats' first colostrum feed, which is rich in oxytocin (OT). OT has been long known for its
86   ability to stimulate milk let-down and uterine contraction. However, OT is now known to play a
87   wide range of roles in the regulation of many bodily functions and behaviors[4]. For example, OT
88   opposes stress and inflammation in conjunction with adaptive affiliative behaviors[5], delays

89   gastric emptying, and slows intestinal transit. OT receptors (OTR) have been identified in enteric
90   neurons and intestinal epithelium[6-8]. The gastrointestinal effects of OT are particularly important
91   to the infant during the early postnatal period. For instance, breastfeeding is associated with the
92   delivery of significant quantities of OT to the neonatal gut[9,10], and data show that the OTR is
93   heavily overexpressed in duodenal villi during the milk suckling period[8].

94

95   *In vitro* experiments using a gut cell line have demonstrated at the cellular level that oxytocin
96   modulates important molecules in the stress signaling pathway[11,12] and plays a regulatory role in
97   translation of proteins[12]. These studies suggest that components of milk, including exogenous
98   oxytocin from the mother, are important in the unfolded protein response in neonates to reduce
99   cellular stress[13].

100

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

106

107   The effects of OT on the brain have been studied[17]. However, the OT signaling mechanisms
108   demonstrated in the gut during the early postnatal period have not been studied in the brain. In
109   this paper, a method for isolating discrete brain nuclei in the neonatal rat brainstem and
110   hypothalamus using electrophoresis is used to profile isolated brain regions. The overall goal of
111   this method is to capture the state of cell signaling in brain areas as close as possible to birth,
112   before and after the first milk suckling, in brain tissue with the lowest glial/neuronal index. The
113   rationale for the development of this technique is that it allows for the rapid isolation of
114   restricted, microscopic brain regions in neonatal pups with a more homogenous collection of
115   neurons for *ex vivo* studies using an automated Western blotting methodology, offering highly
116   consistent results on relatively small dissected samples. A shortcoming of prior work includes
117   more gross dissection (brain slices or whole brain) and older animals[18,19]. The brains of young
118   pups are incredibly dynamic, featuring waves of glial differentiation after birth. In order to study
119   brain changes influenced by the pups' first feeding, studying restricted neuronal nuclei with
120   reproducible dissection is necessary.

121

122   Milk feed is usually analyzed for its immunological and nutritional impact on health or gene
123   expression (for example, in enterocytes[20,21]), whereas its effect on brain areas during brain
124   development is rarely studied. The effect of milk transit in the gut on brain function was analyzed
125   in reference to gut cholecystokinin receptors vagal relay to brain stem nuclei, but not to
126   intracellular signaling pathways[22]. There is a vast literature on vulnerability of the developing
127   neonate brain to malnutrition of mothers during pregnancy[23], but the stress and inflammation
128   signals are not addressed. Importantly, the current method takes advantage of a phenomenon
129   in day-zero rat newborns that isolates the blood-born colostrum stimuli from vagal relay of
130   visceral stimuli. This is the so-called stress hypo-responsiveness period characterized by
131   immature nucleus tractus solitarius (NTS)-hypothalamic circuit immediately after birth[24,25] that
132   restricts NTS, paraventricular nucleus (PVN), and supraoptic nucleus (SON) signals to blood-born

133   stimuli.

134

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

145

146   This method allowed for the assessment of bidirectional signaling occurring between the gut and
147   the brain and that can impact function in both organs[26]. The association between oxytocin and
148   food intake, which has been extensively studied in recent years[27], supports a link between
149   increased oxytocin signaling and nutrient availability. These studies also support the converse
150   concept that energy deficits are coupled with reductions in hypothalamic oxytocin signaling.

151

152   Earlier studies of the effect of OT on brain activity demonstrated that induced gut inflammation
153   elicited cFos transcription in hypothalamic PVN, amygdala, and piriform cortex which was
154   refractory to vagotomy[28]. However, systemic infusion of OT with secretin decreased the brain
155   cFos response to the provoked inflammatory reaction in the gut[28]. This suggested that the effect
156   of exogenous OT was carried out by routes other than vagal relays, possibly via blood-borne
157   signaling molecules carried through the area postrema[6,29].

158

159   In this study, the cellular stress signaling pathways that have previously observed in the gut were
160   assessed in the brain. The hypothesis was that milk components may protect or defer the effect
161   of inflammation on gut permeability to microbial and other metabolites, and in turn, the effects
162   on brain function. The clear antagonistic differences in IkB versus BiP signaling found in villi,
163   before and after priming by colostrum[13], suggested that the brains of neonates, still in the
164   process of developing, may sense these colostrum-induced gut signals.

165

166   Signaling protein markers used in previous gut experiments that are associated with endoplasmic
167   reticulum stress were measured. They include the ER chaperone BiP, translation initiation factor
168   eIF2a (which serves as a stress response integrator[30]), eIF2a kinase p-PKR, and two inflammation-
169   signaling proteins (NF-kB and its inhibitor, IkB).

170

171   Six brain regions based on their ability in adults to secrete or respond to OT were chosen. The
172   NTS, located at the upper medulla, is the first relay of the visceral input and receives direct
173   signaling from vagal sensory neurons in the gut[31] and possibly blood-born cytokines, toxins, and
174   hormones via the adjacent area-postrema[32]. The PVN, supraoptic nucleus (SON), striatum nuclei
175   (STR), cerebral cortex (CX), and medial preoptic nucleus (MPO) receive signaling from the gut via
176   the NTS.

177

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

187

188 **PROTOCOL:**

189

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

192

193 **1. Tissue Preparation**

194

195 1.1. Order timed pregnant rats from vendor.

196

197 1.2. Follow timed pregnant rats by observing their growing abdomens in the weeks after their
198 arrival and subsequently looking for pups on the expected delivery date by inspecting the cage
199 every 2 h until delivery begins.

200

201 1.3. Remove pups with a gloved hand by their tail before their first feed for unprimed pups (no
202 white milk belly is apparent when viewing abdomen) or after the first feed for primed pups (at
203 which point a white stomach will be visible on their abdomen) as described in the timeline (**Figure
204 S1**).

205

206 Note: The first colostrum feed is termed as priming the pup; thus, a pup is unprimed until the
207 first feed, after which they are colostrum-primed.

208

209 1.4. Quickly decapitate the unanesthetized pup using sharp, clean surgical scissors.

210

211 1.5 Remove the brain by cutting the skin down the midline and top surface of the skull to the
212 nose. Then, using forceps, gently pry away the bone to expose the brain (**Figure 1A**) and localize
213 the bregma, marking it with a pen as the bone plates are removed (**Figure 1B**).

214

215 1.6. Rapidly place the whole brain in a polymethyl methacrylate brain mold at room temperature
216 for coronal slicing at room temperature (**Figure 1C**).

217

218 1.7. Without delay, make 500 μm-thick slices using a fresh razor blade. Lay the slices rostral to
219 caudal in a Petri dish to maintain orientation of sections (**Figure 2**).

220

221 1.8. Quickly add artificial cerebrospinal fluid (ACSF; 1.0 mM KH_2PO_4, 26 mM $NaHCO_3$, 118.6 mM
222 NaCl, 3.0 mM KCl, 203.3 mM $MgCl_2$-6H2O) without glucose and incubate the slices for 60 min at
223 28-30 °C, constantly stirring on an orbital shaker to metabolically and differentially challenge the
224 unprimed versus colostrum-primed tissues.

225

226 1.9. Identify the brain nuclei that are required to punch using a brain atlas[33] and anatomic
227 landmarks on the tissue section. Place this slice with the nuclei of interest in a Petri dish and
228 move it to the dissecting microscope.

229

230 1.10. Once visualized, quickly punch out 4 of 6 different nuclei using a coring tool, selecting the
231 size to best punch the nucleus in question and consistently between samples (**Figure 2**).

232

233 Note: The remaining brain slice will now have a hole where brain tissue was removed. In this
234 study, we excised the following nuclei using the below coordinates. All anterior/posterior (A/P)
235 coordinates are from Bregma (except NTS, which is with reference to the Calamus Scriptorius).
236 All dorsal/ventral (D/V) coordinates are from the surface of the cortex (except NTS, which is from
237 the surface of the medulla). The following coordinates include A/P, medial/lateral (M/L), and D/V
238 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
239 medulla), 2) paraventricular nucleus (PVN, -0.8; ± 0.2; 0), 3) supra-optic nucleus (SON, -1.1; ± 1.4;
240 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),
241 and 6) medial preoptic nucleus (MPO, -0.6; ± 0.2; 4.2).

242

243 1.11. Rapidly immerse the punched nuclei in 0.06 mL of ice-cold, protein extraction buffer
244 containing protease inhibitors and phosphatase inhibitors for 60 minutes (see step 2.3).

245

246 **2. Protein Extraction**

247

248 2.1 Prepare the protein extraction solution using the protein lysis kit (**Table of Materials**) on the
249 day before expected pup delivery.

250

251 2.2 Thaw (on ice) the frozen (-20 °C) aqueous solution of the protease and phosphatase inhibitors
252 of the lysis buffer kit and place the lysis buffer and DMSO solution of the proteases/phosphatases
253 inhibitors on ice.

254

255 2.3 Add 1.85 mL of lysis buffer into a clean, ice-cold 15 mL tube. Then, add 0.1 mL of aqueous
256 solution of inhibitors and 0.05 mL of DMSO-dissolved inhibitors. Finally, cap and briefly vortex the
257 tube and keep it at -20 °C until use.

258

259 2.4 Label 24 clean microcentrifuge tubes (0.5 mL each) for the lysis procedure. Designate 12 tubes
260 per brain for the colostrum-unprimed group (U) [6 left (L) and 6 right (R) brain nuclei], and label
261 according to nuclei acronym, side, and condition (*e.g.*, NTS-L-U, NTS-R-U, *etc.*). Label the second
262 group of 12 tubes for the colostrum-primed samples.

263

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2.5 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.

2.6 Thaw the lysis solution on ice on the day that the pups 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.

2.7 Centrifuge the incubated lysed nuclei for 30 min in a cooled mini-centrifuge at 14000 rpm (10000 x 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

3.1 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.

3.2 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.

3.3 Repeat steps 2.0 to 3.2, from protein extraction to sample preparation, for the nuclei punched from colostrum-primed rats.

4. Electrophoresis Preparation

4.1 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.

4.2 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

4.3 Load 0.01 mL of the antibody diluent solution into wells C1 to C25 and B1.

4.4 Spin the 24 protein samples and ladder tubes briefly (2-3 seconds) in a minicentrifuge to pool down evaporated water from the tube caps.

307 4.5 Load 0.0003 mL of 12 unprimed samples into wells A2 to A13, 0.003 mL of 12 colostrum-
308 primed samples into wells A14 to A25, and 0.005 mL of the biotinylated ladder into well A1.
309
310 4.6 Leave row F empty and load 0.45 mL of wash buffer into each of the 5 compartments in each
311 of the 3 rows below row F. Cover the plate with its plastic lid to avoid evaporation during the
312 remaining procedures.
313
314 4.7 Briefly vortex the thawed biotin labeling reagent and add 0.15 mL of the reagent to its
315 designated tube; then add to it 0.15 mL of the total protein reconstitution agent and mix them to
316 homogeneity.
317
318 4.8 Remove the cover from the plate and load 0.01 mL of agents 1 and 2 into wells B2 to B25.
319 Cover the plate and centrifuge it for 10 min at 1000 x g to remove any air bubbles from the various
320 solutions. Use an empty plate in the centrifuge for balance.
321
322 **5. Electrophoresis**
323
324 5.1 While the plate is spinning, open a run file in the automated Western machine-attached
325 computer by indicating in the dropdown page from "file" to run a total protein assay and clicking
326 the respective spot.
327
328 5.2 Annotate the samples by well in the computer. Then, remove the plate from the centrifuge
329 remove the cover and carefully peel off the aluminum cover from the separation solution
330 compartments.
331
332 5.3 Place the plate in the automated Western instrument, peel off the cover from the capillary
333 cartridge box, insert the cartridge in its designated place, and close the door.
334
335 5.4 Click the "RUN" button, and when prompted with the type of the assay ("total protein"), type
336 in the name of the samples (for example, "unprimed 2-13 and colostrum-primed 14-25"). Click
337 "OK", and when prompted by the activated run date and ID number of the run file, make a note
338 of the time when the run ends.
339
340 5.5 At the end of the run (170 min after the start), open the instrument door, remove the capillary
341 cartridge, and discard it into the sharps disposal. Discard the plate in the biological matter
342 disposal.
343
344 5.6 Click the separation curves icon in the analysis page of the run file, and check that all the
345 samples have run properly and are showing multiple protein curves in all capillaries.
346
347 **6. Analysis of Signaling Proteins**
348

349 6.1 In a labeled 1.5 mL tube, add 0.003 mL of rabbit anti phospho-eIF2a (p-eIF2a) antibody and
350 suspend it in 0.3 mL (1:100 dilution) in antibody diluent from the suitable detection kit. Then,
351 keep it on ice.
352
353 6.2 Label a luminol tube and add 0.15 mL of luminol and 0.15 mL of peroxide from this detection
354 module kit and dispense 0.01 mL into wells E1 to E25 of a fresh, automated Western plate.
355
356 6.3 Dispense 0.01 mL of the secondary anti rabbit antibody into wells D2 to D25, and add 0.01 mL
357 of streptavidin-HRP from the kit to well D1.
358
359 6.4 Dispense 0.01 mL of the primary antibody (from step 6.1) into wells C2 to C25, and add 0.01
360 mL of antibody diluent 2 solution to wells C1 and B1 to B25.
361
362 6.5 Leave the row F wells empty and fill 0.45 mL of wash buffer into the 5 compartments of 3
363 rows below the F row.
364
365 6.6 Briefly spin the refrigerated samples for 3 seconds, and add 0.003 mL of each sample to row
366 A in the same order they were added for the total protein assay, starting from A2 to A25. Add
367 0.005 mL of the biotinylated MW ladder to well A1 and cover the plate.
368
369 6.7 Centrifuge the plate as done in step 4.8. While the plate is spinning, open (in the automated
370 Western-associated software and computer) a new run file and indicate in the dropdown page
371 from "File" to run a molecular size assay by clicking the respective spot.
372
373 6.8 In the assay page, type the sample names in each capillary, then type the name of the primary
374 antibody in the allocated spot and the secondary anti-rabbit antibody below it.
375
376 6.9 At the end of centrifugation, repeat steps 5.3-5.5, except the name given to the run file this
377 time should be "p-eIF2a on unprimed 2-13 and colostrum-primed 14-25".
378
379 6.9 After the electrophoresis separation, check the run file for immune-reactivity peaks of
380 antigens at sizes of 40-43 kDa. Where MW of peaks this size are missing, right-click below the
381 curve and indicate inside the dropdown list to add MW to the peak, which ensures that the size
382 and arbitrary quantity below the curve are recorded.
383
384 **7. Processing the Results**
385
386 7.1 Open a spreadsheet file for total protein run in the automated Western run file and provide
387 an ID number.
388
389 7.2 Open the run file of the total protein assay at the analysis page at the curve mode and mark
390 all the peaks in individual capillaries. Then, copy and paste them into the spreadsheet and sum
391 the areas under the curve of all peaks recorded in the entire capillary.
392

393 7.3 In a separate column, arrange the total amount of protein for each column with capillary
394 numbers, names of the respective brain nuclei, and the ID numbers of the run files.
395
396 7.4 Open a spreadsheet for the p-eIF2a antigen, and in a single column, record the area under
397 the curve from each capillary side-by-side with its respective capillary number, name of brain
398 nucleus, and ID number of the run file.
399
400 7.5 Copy the total protein column (parallel to the p-eIF2a curve quantities) into a third
401 spreadsheet and compute p-eIF2a:total protein ratios in a third column.
402
403 7.6 Collect results from steps 4 to 6 for each brain nucleus, arrange them in groups of nuclei, and
404 generate a bar graph.
405
406 **REPRESENTATIVE RESULTS:**
407 The representative bands of immunoreactivity relative to total protein show that there are brain
408 nuclei with very low harvested protein. This requires the use of the automated Western blot
409 technique, which is highly sensitive compared to the canonical Western blot. This approach can
410 be run with fortyfold less protein per capillary compared to the per-lane in Western blots.
411
412 *Differential effects of colostrum priming on BiP levels in brain nuclei*
413 Coronal sections of rat brains were harvested before the first colostrum feeding (unprimed) and
414 after the first feeding (primed). Samples were fractionated by capillary electrophoresis (on WES),
415 and proteins were quantified using the kit for in-capillary total protein staining (see **Protocol**).
416 Additional immune capillary fractionation on the automated Western provided identification of
417 the respective protein antigens. In **Figure 3A,** representative BiP protein expression is shown in
418 the upper panel, with the corresponding total protein shown below. BiP is an ER chaperone
419 protein. In **Figure 3B**, quantitated immunoreactivities are presented by bar graphs relative to the
420 corresponding total protein.
421
422 Within the unprimed tissue samples, BiP levels in NTS were significantly higher compared to all
423 other regions ($p < 0.05$, n = 4 nuclei). Priming of gut tissue with colostrum had an opposite effect
424 in NTS, compared with increased BiP in other regions, relative to unprimed tissue (**Figure 3B**).
425 Priming decreased BiP in NTS (**Figure 3B**) and had no effect on PVN and SOPT. However, priming
426 increased BiP in CX, STR, and MPO relative to unprimed tissue. This data implies that BiP levels in
427 the various tested brain nuclei respond differently to gut priming and that there not yet any
428 demonstrated cross-talk between the various nuclei tested.
429
430 *Differential effects of colostrum priming on ElF2a and p-elF2a levels in brain nuclei*
431 Brain tissue samples were prepared, analyzed, and quantified as shown in **Figures 3A** and **3B**. As
432 with BiP levels (**Figure 3B**), eIF2a and p-eIF2a levels in NTS were elevated in the unprimed
433 condition relative to other nuclei (**Figures 4B** and **4C**). As demonstrated with BiP levels, the
434 response in NTS to priming of gut tissue with colostrum was opposite to that in other nuclei.
435 Levels of both eIF2a and p-eIF2a in NTS were reduced relative to unprimed tissue. In all other
436 tested nuclei, priming increased levels in eIF2a and p-eIF2a relative to unprimed tissue (**Figures**

437   **4B** and **4C**).

438

439   *Phosphorylation of eIF2a by kinase GCN2 in NTS and PVN after colostrum priming*
440   Brain tissue samples were prepared, analyzed, and quantified as shown in **Figure 3**. Priming
441   deceased p-PKR in PVN (χ^2 p = 0.025), unlike in all other regions where it was increased. Contrary
442   to p-eIF2a findings (**Figure 4C**), levels of p-PKR were low in unprimed samples relative to primed
443   samples in NTS (**Figure 5A**). Phosphorylation of eIF2a is usually catalyzed by its kinase PKR in
444   response to viruses[34] and by OT in the gut, as previously demonstrated[11]. However, the fact that
445   p-PKR levels were very low in unprimed versus primed NTS (relative to other nuclei, **Figure 5A**)
446   strongly suggests that another kinase must be involved in phosphorylating eIF2a (**Figure 4C**).
447   Accordingly, GCN2 levels were tested in NTS and PVN because it is also a known kinase of eIF2a[35].
448   Levels of p-GCN2 (active form) were higher in unprimed samples compared to primed samples in
449   NTS and PVN (**Figure 5B**), and GCN2 (inactive form), compared to p-GCN2, was inversely
450   expressed in PVN (**Figure 5C**). pGCN2 in NTS (**Figure 5C**) was expressed inversely to pPKR (**Figure
451   5A**) in both unprimed and primed NTS. In primed tissue, p-eIF2a was considerably lower in NTS
452   than in all other brain regions [PVN (p < 0.01), SON (p < 0.01), CX (p < 0.01), and MPO (p = 0.02)].
453   This indicates that pGCN2, rather than pPKR, was responsible for eIF2 inhibition in NTS.

454

455   *Colostrum priming inhibits NF-kB in CX, STR, and MPO*
456   **Figure 6A** shows that IkB levels were significantly higher in SON unprimed samples vs primed
457   samples. IkB levels in SON, CX, STR, and MPO trended higher in the same direction. In **Figure 6C**,
458   NF-kB levels in CX, STR, and MPO were significantly higher in primed versus unprimed tissue.
459   However, NF-kB levels were significantly lower in primed NTS tissue, which suggests that
460   colostrum had a distinct anti-inflammatory effect on this brain region. Cytosolic NF-kB was higher
461   (retained) in unprimed NTS samples, whereas its inhibitor IkB was low and unchanged in primed
462   samples. This suggests that a distinct anti-inflammatory mechanism is regulating NF-kB in NTS.
463   This nutrient insufficiency stress effect is consistent with current findings that stress markers BiP
464   and p-eIF2a are both regulated by the presence of colostrum priming (**Figures 3** and **4**,
465   respectively), also shown in previous BiP and p-eIF2a findings[10]. The reason for the distinct
466   response of IkB and NF-kB in NTS compared with CX, STR, and MPO is not yet fully understood.
467   However, it is consistent with the observation that the NTS, which relays signals from the gut to
468   the brain, may play a crucial role in responding uniquely and oppositely versus other brain regions
469   compared to peripheral inflammation[36]. Levels of IkB in CX, STR, and MPO were low (**Figure 6A**),
470   compared to high levels of NF-kB in the same areas (**Figure 6C**). While this finding apparently
471   contradicts the premise that IkB is binding and retaining NFkB in the cytosol, different calculation
472   of the data which combines and compares all primed samples in CX, STR, and MPO using
473   regression analysis shows that NF-kB highly correlates with IkB (p < 0.0001, n = 24)

474

475   **FIGURE AND TABLE LEGENDS:**
476   **Figure 1: Dissected neonatal rat brain.** (A) The dissected brain is shown with bones removed
477   rostral to bregma. (B) The brain is shown after a line has been drawn at bregma, after which the
478   remaining bone plates were removed. (C) The brain is shown in a polymethyl methacrylate brain
479   mold just before being sliced with a razor blade.

480

481 **Figure 2: Brain slices arranged from rostral to caudal with punches (red circles).** Abbreviations:
482 CX = cortex, parietal lobe; MPO = medial preoptic area; NTS = nucleus tractus solitaries; PVN =
483 paraventricular nucleus; SON = supraoptic nucleus; and STR = striatum.

485 **Figure 3: BiP/GRP78 response in NTS is inverted to that of other nuclei**. (A) Representative BiP
486 protein expression in the upper panel relative to the total protein presented in the panel below.
487 Note that the total protein density in the various capillaries are heterogeneous, and these are
488 used to equalize the respective BiP expression per protein in panel B. (B) Shown is the mean BiP
489 relative to total protein in brain nuclei of unprimed samples (blue) versus colostrum-primed
490 samples (brown). Asterisks of respective colors designate significant differences (p < 0.05, n = 4
491 nuclei) between BiP in NTS versus BiP in the other nuclei. BiP expression in unprimed versus
492 primed samples in NTS are significantly inverted to those in STR (χ^2 p = 0.028, n = 4).

494 **Figure 4: Active and inactive eIF2a (p-eIF2a) response to colostrum priming versus unprimed**
495 **samples in NTS inverted to that of other nuclei**. (A) Representative eIF2a and p-eIF2a protein
496 expressions are shown in the upper panels. The total protein lanes are presented at the lower
497 panel. (B) Shown is the mean eIF2a of 4 samples expressed relative to total protein (from lower
498 panel A) in brain nuclei of unprimed samples (blue) versus colostrum-primed samples (brown),
499 and (C) the inactive form of eIF2a (p-eIF2a). Asterisks of respective colors designate significant
500 differences (p < 0.05, n = 4 nuclei) between both eIF2a forms in NTS versus respective levels in
501 the other nuclei. Active eIF2a expression in unprimed versus primed samples in NTS are
502 significantly inverted to those in CX, STR, and MPO (χ^2 p < 0.05 versus all three nuclei, n = 4). Error
503 bars represent standard error.

505 **Figure 5: NTS expresses eIF2a kinase p-GCN2 in accord with p-eIF2 level and inversely to p-PKR**
506 **level**. (A) The level of p-PKR (activated dsRNA kinase of eIF2a) is expressed in unprimed versus
507 colostrum-primed NTS inversely to 1) the level of p-PKR in PVN (χ^2 p = 0.025) and 2) its expected
508 targeted substrate eIF2a after its phosphorylation (to p-eIF2a, **Figure 2C**). Note that the pattern
509 of p-PKR in SOPT, CX, STR, and MPO of unprimed versus colostrum-primed samples fits the
510 respective patterns of p-eIF2a from **Figure 2C**. (B) Activated eIF2a kinase (p-GCN2) in unprimed
511 versus primed NTS samples follows a similar pattern of p-eIF2a expression from **Figure 2C** and an
512 inverse pattern of pPRK expression from **Figure 3A** (χ^2 p = 0.028). C = mean levels of inactive
513 GCN2. Error bars represent standard error.

515 **Figure 6: Cytoplasmic expression of NF-kB and IkB in brain nuclei from unprimed and**
516 **colostrum-primed gut samples**. (A) Mean cytoplasmic expression of IkB relative to total protein
517 (from panel C) at indicated brain nuclei. (B) Representative cytoplasmic bands of IkB protein are
518 presented in the upper panel and total protein density in respective lanes in the lower panel. (C)
519 Mean cytoplasmic expression of NF-kB relative to total protein (from panel D) at indicated brain
520 nuclei. (D) Representative cytoplasmic bands of NF-kB bands are presented in the upper panel
521 and total cytoplasmic protein density in respective lanes in the lower panel. Colored asterisks
522 designate significant differences between indicated samples (p < 0.05, n = 4). Note that
523 cytoplasmic NF-kB levels are higher in colostrum-primed CX, STR, and MPO than in hypothalamic
524 nuclei NTS, PVN, and SON in disagreement with the levels of NF-kB inhibitor (IkB).

525

526 **Figure 7: Schematic comparing hypothesized OT signaling in colostrum-primed gut and brain**
527 **regions.** (A) Colostrum OT in enterocytes activates its receptor via direct interaction with the
528 OTR, reducing inflammation via increased IkB downstream signaling. Protein translation is
529 inhibited by increasing p-PKR, which decreases eIF2a activity through phosphorylation.
530 Homeostasis is restored via increased BiP gene expression[13,37]. (B) Neuronal or circulatory OT
531 activates its receptor in the CS, STR, and MPO regions of the brain, downregulating inflammation
532 and protein translation via the same molecules as the gut (see panel A). (C) The hypothesized
533 effects of colostrum priming in the NTS region of the brain are distinct and opposite from those
534 in panels A and B. GCN2, which is sensitive to amino acid supply, increases both eIF2a and protein
535 translation. At the same time, low levels of IkB allow NF-kB to enter the nucleus and induce
536 inflammation. Note that BiP acts similarly to restore homeostasis in the gut and in NTS in the
537 brain.

538

539 **Supplementary Figure 1: Schematic showing the protocol timeline.**

540

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

554

555 There are several critical steps within this protocol. These include adherence to the timing of
556 tissue harvest with respect to the first feed, using a minimal volume of protein extraction buffer,
557 incubation of brain slices in ACSF so that molecular inhibitors or stimulants may be used, and
558 using a sensitive immune-electrophoretic device. Possible modifications include the choice of
559 reagents for incubation and the duration of incubation and extending the time of unprimed
560 starvation to compare brains at equivalent times. Important limitations include the presence of
561 some non-neuronal cells (even during this very early neonatal period) and a restricted number
562 of samples that can be prepared from one litter of newborns in one day. Furthermore, this
563 approach is completely dependent on when the pups are born. With respect to existing methods,
564 gene expression in the brain at this age is typically and more conveniently assessed at the
565 transcriptional level. However, for cell signaling analysis, the proteomic approach executed by
566 core facility equipment is more expensive than the desktop automated instruments. The
567 conventional Western blot method requires much more protein, takes several days to complete,
568 and involves several manipulation steps by the technical manpower. This automated approach

569   requires 0.8 μg protein per capillary, once loaded into the instrument all the steps are performed
570   without human hands interference and takes 2 h and 50 min to complete the run. This technique
571   can be used for studying the immediate postnatal brain development in rats and wide range of
572   genetically manipulated mouse models.
573
574   Using this technique, the effects of OT at the cellular level, specifically in the very critical period
575   between birth and feeding, were investigated when the OTR is maximally expressed in the
576   epithelium[8]. The modulating effects of OT on cell signaling molecules in gut cells, both in a cell
577   line[37] and in vivo[13], were previously demonstrated. In the present study, the effects observed in
578   gut cells were assessed in brain regions rich in OTR. The expression of BiP/GRP78 and p-eIF2a
579   was upregulated in unprimed (consistent with an expected response to stress) and
580   downregulated in primed NTS tissue (consistent with an attenuated response to stress), whereas
581   NF-kB was high and stable in both conditions[38]. Expressions of BiP and NF-kB were the same in
582   the other tested regions in both unprimed and primed conditions. eIf2a was phosphorylated by
583   dsRNA dependent kinase (pPKR) in the SON, CX, STR, and MPO. However, in the NTS, and to a
584   lesser extent in PVN, eIf2a was phosphorylated by another kinase, general control
585   nonsuppressed2 kinase (GCN2). A schematic depicting hypothesized differences in signaling in
586   the brain and gut elicited by colostrum exposure is presented in **Figure 7**.
587
588   This microdissection technique makes it possible to test hypotheses related to the impact of the
589   first colostrum feed on stress-related signaling in discrete OTR-rich nuclei in the neonatal brain.
590   This data suggest that the stress modulating mechanisms previously observed in new born gut
591   enterocytes are mirrored in specific brain regions rich in OTR, as shown by increased cytosolic
592   retention of NFKB (PVN, SON CX, STR, and MPO). Results also indicate that NTS and PVN utilize
593   a different phosphorylation mechanism from the other regions that may be refractory to the
594   impact of nutrient insufficiency. Collectively, the data indicate that cell signaling in the brain
595   associated with nutrient and hormonal insufficiency during the first hours following birth is
596   similar to cell signaling in the gut under the same conditions. This data supports the importance
597   of breast milk at the time of birth for stress modulation in both gut and brain. These results
598   underscore the need for further exploration of the impact of colostrum on brain function.
599

606

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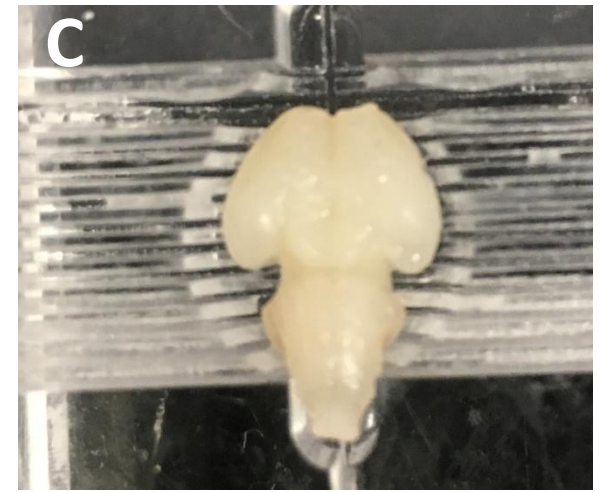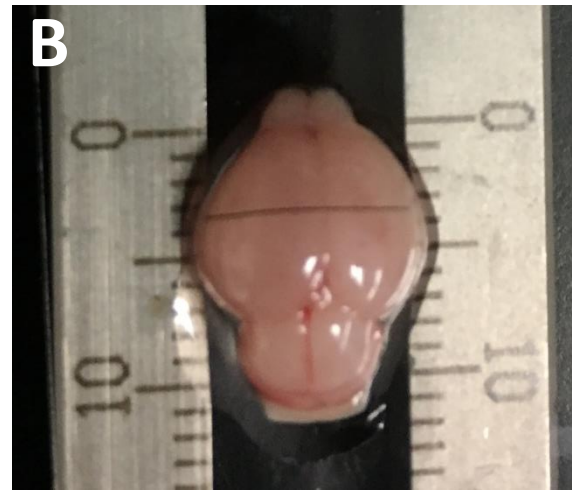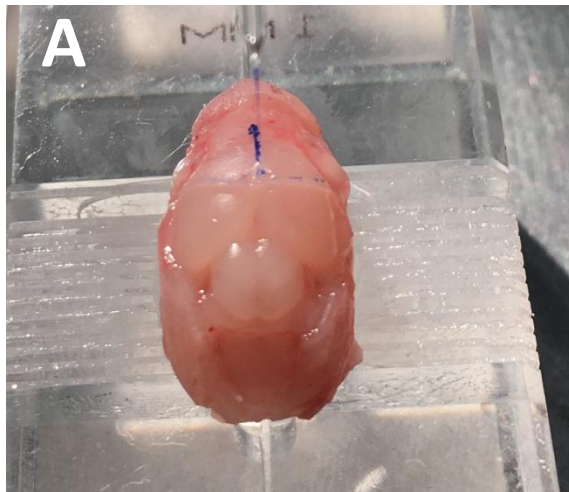689   rat. *Journal of Comparative Neurology.* **234** (3), 344-364 (1985).
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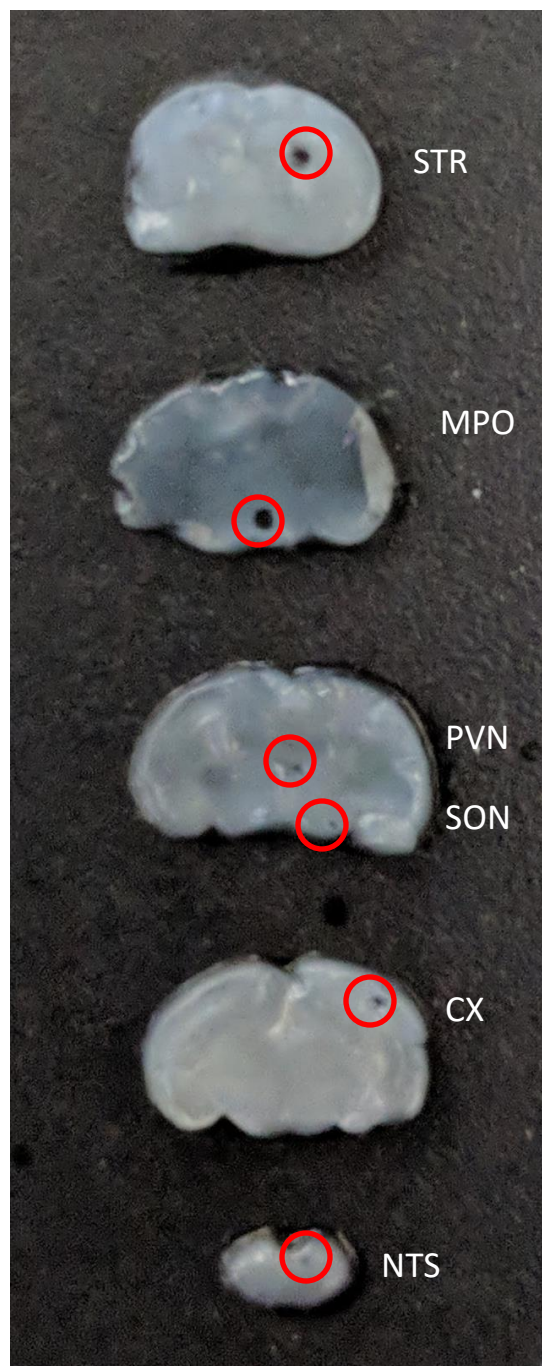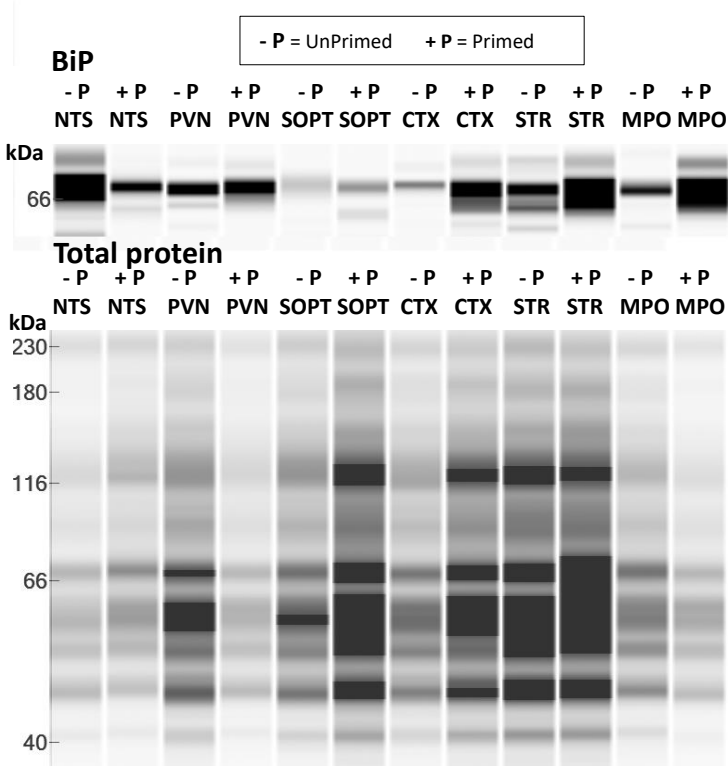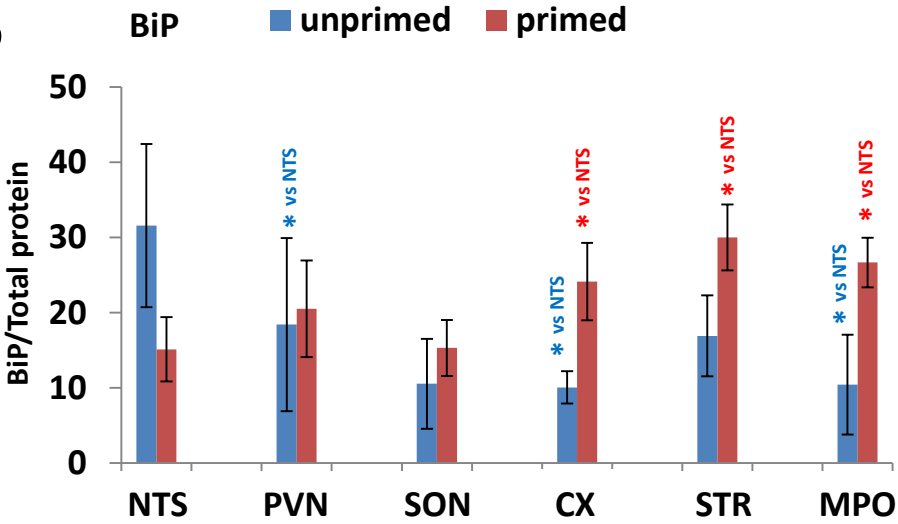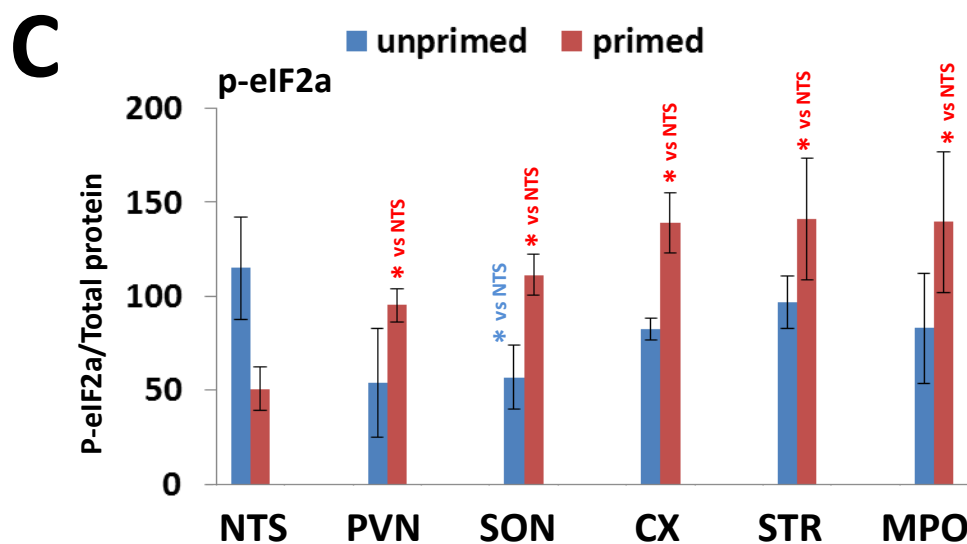702   *Perspectives in Biology.* **1** (3), a001271 (2009).
703

Figure

Figure 1

Figure 2

Figure 3

A

B

C

Figure 5

A. GUT - ENTEROCYTES

B. BRAIN - CX, STR, MPO

C. BRAINSTEM - NTS
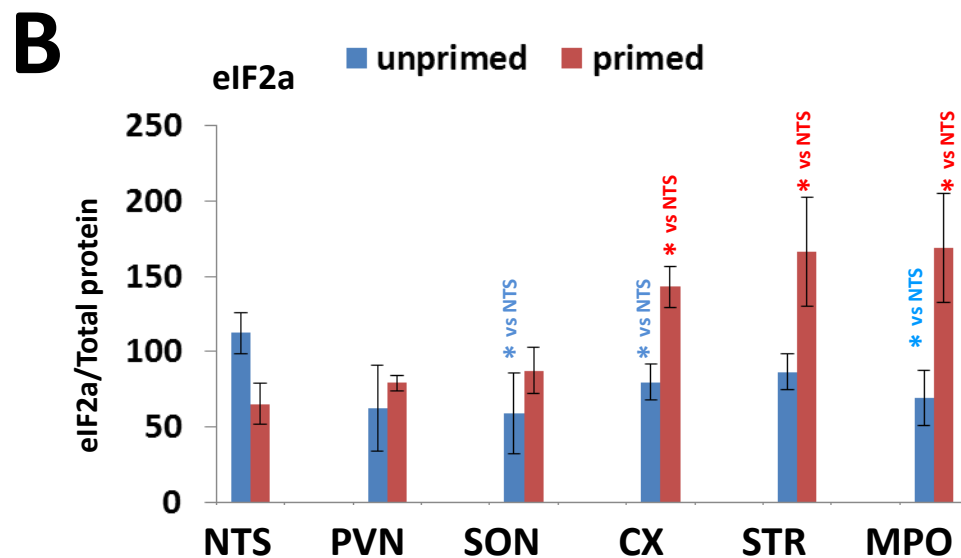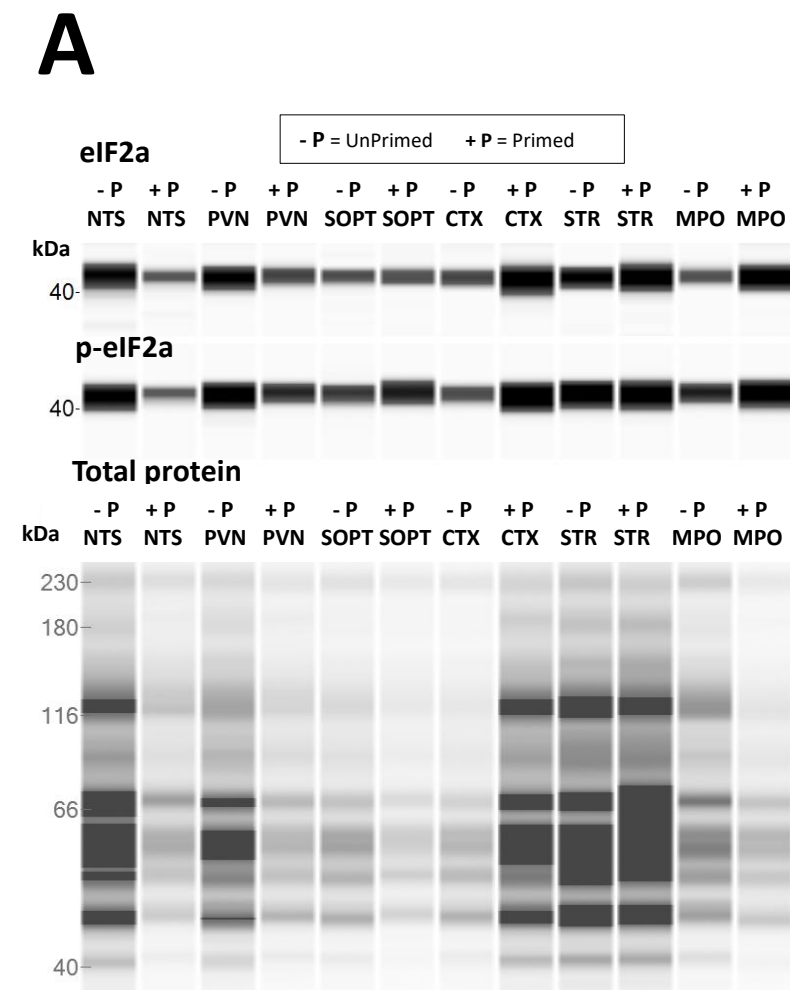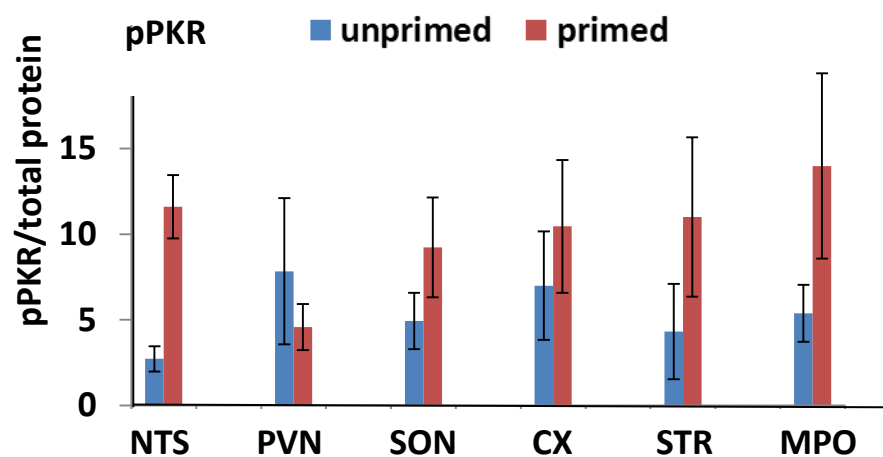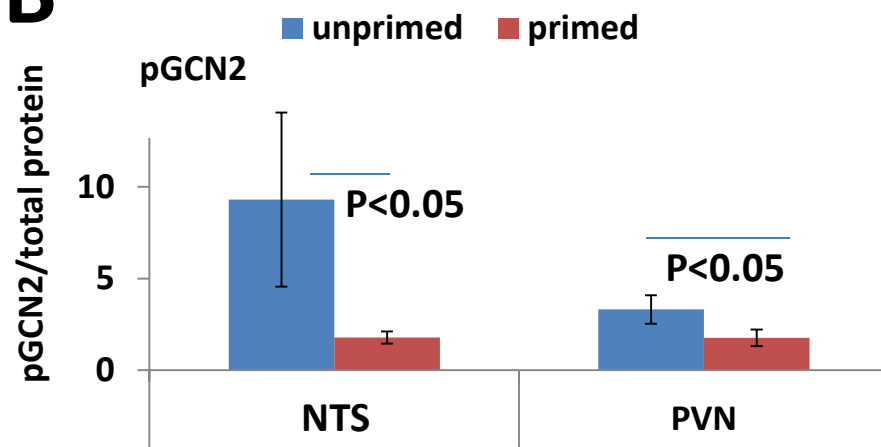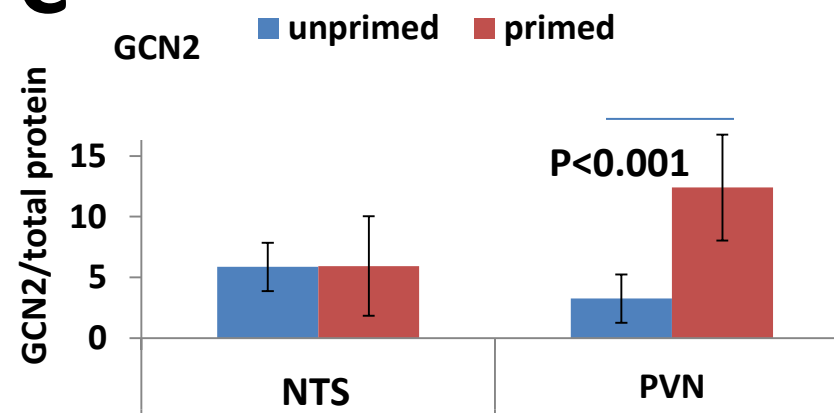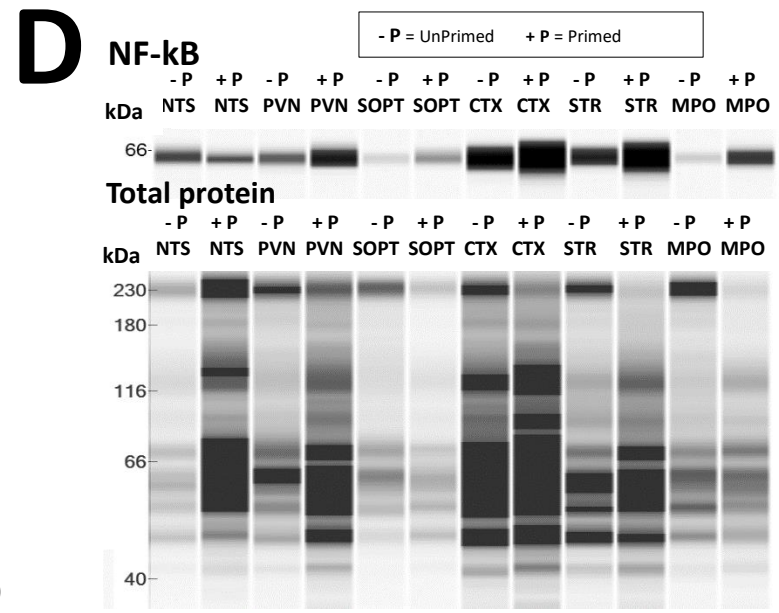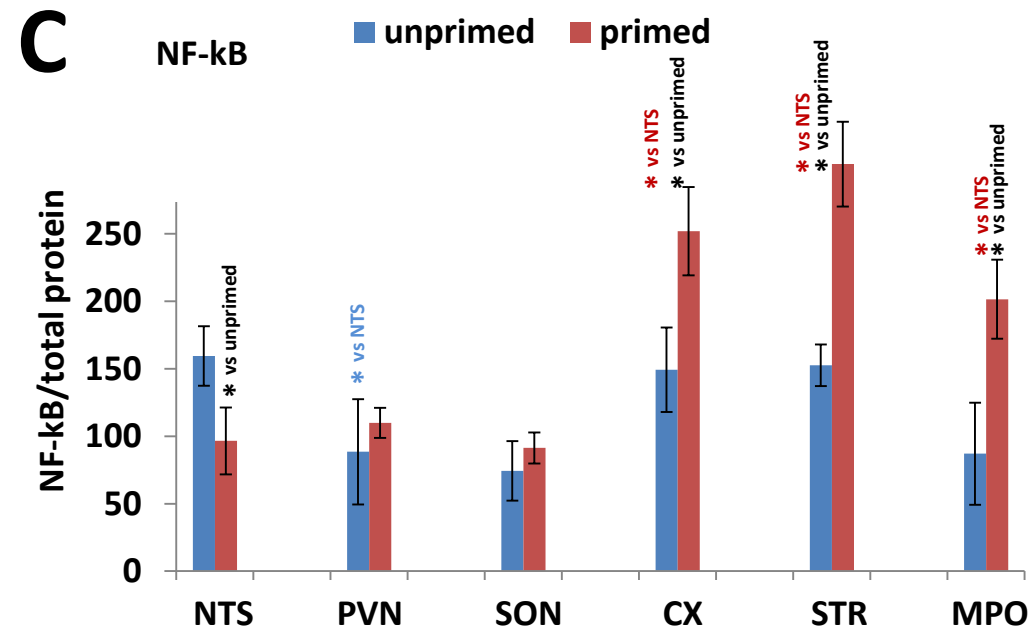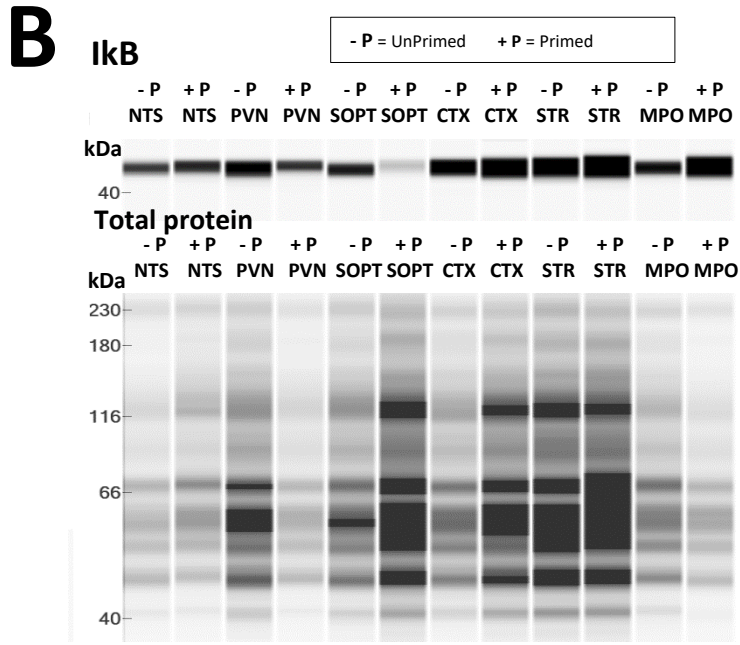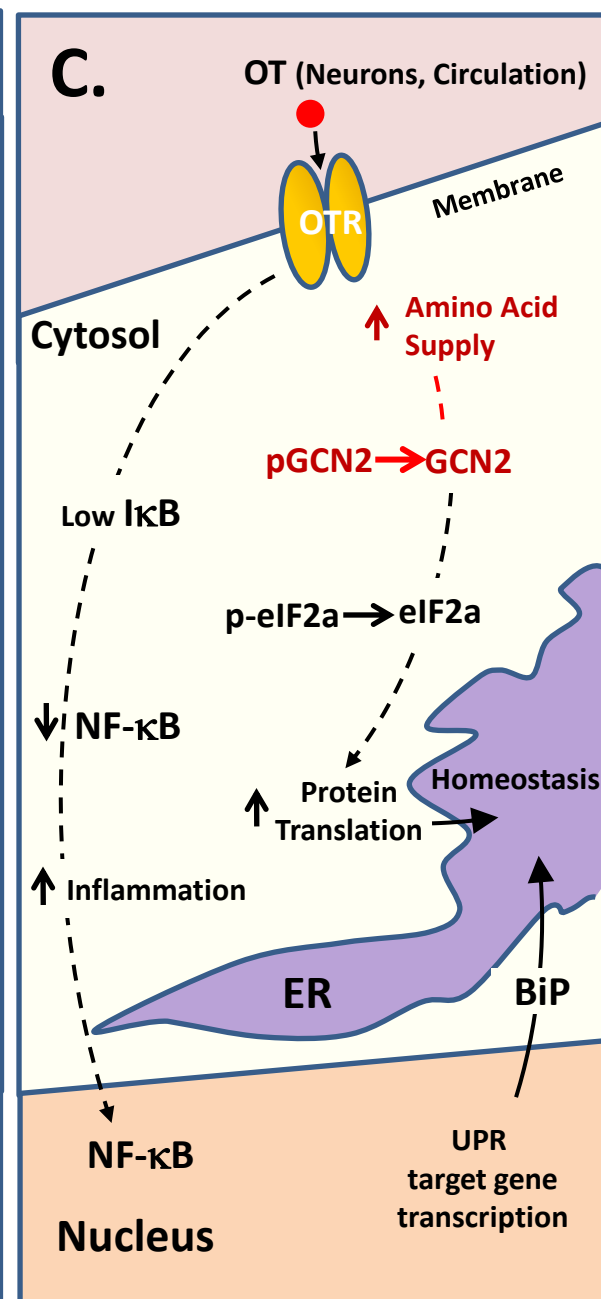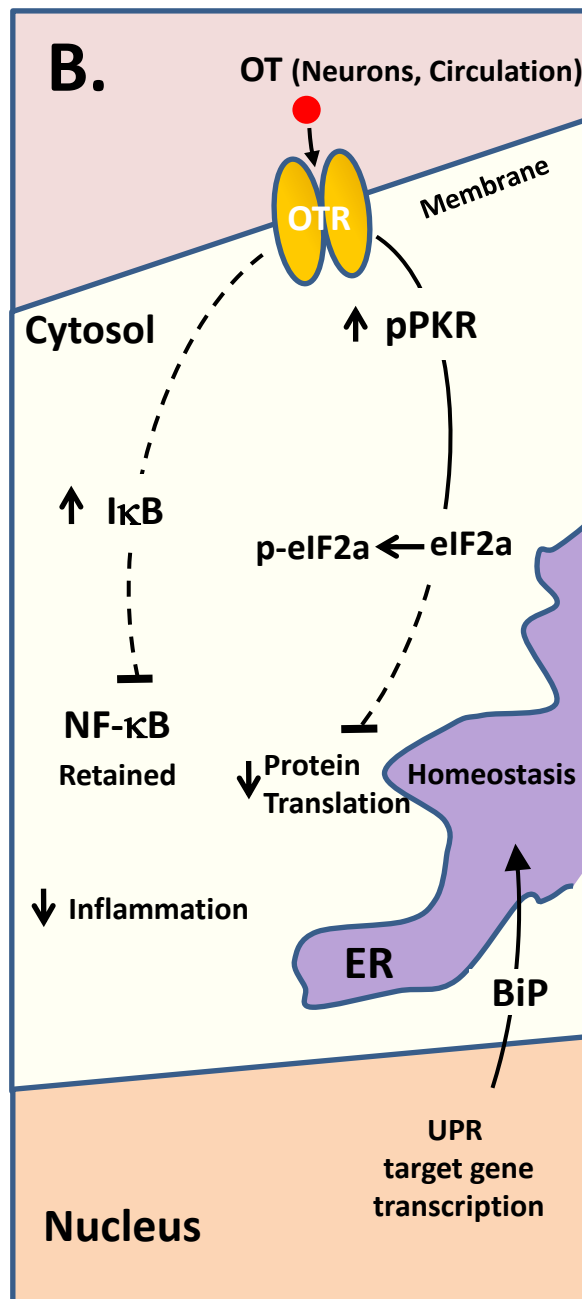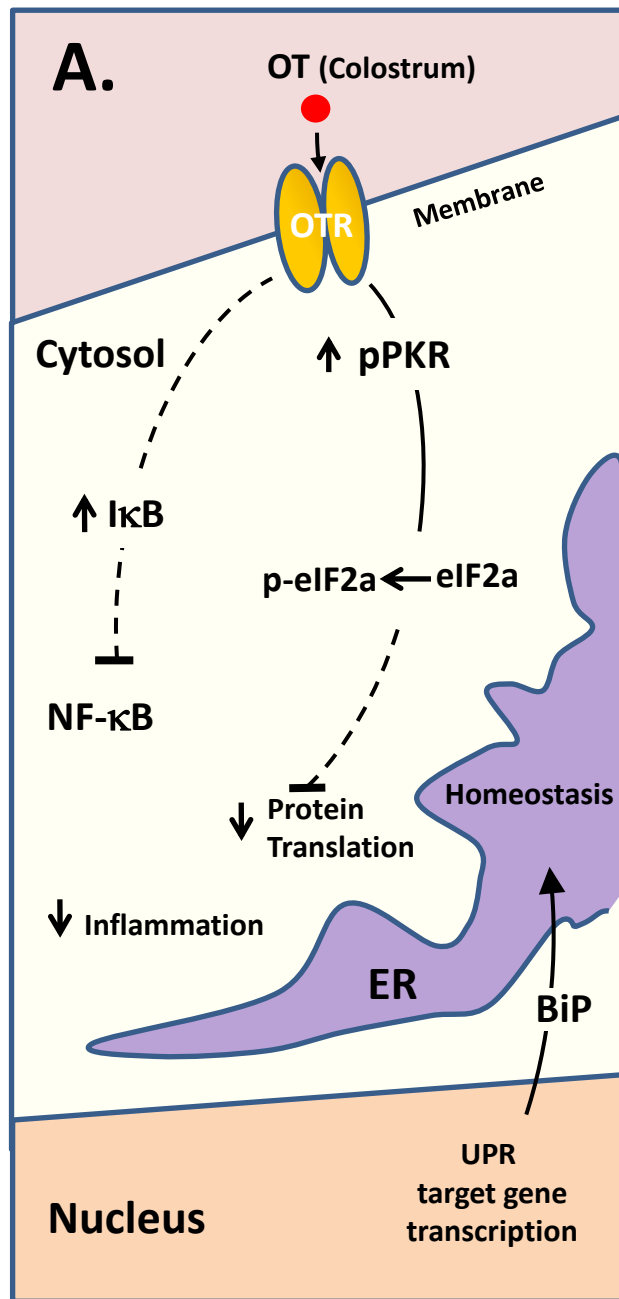
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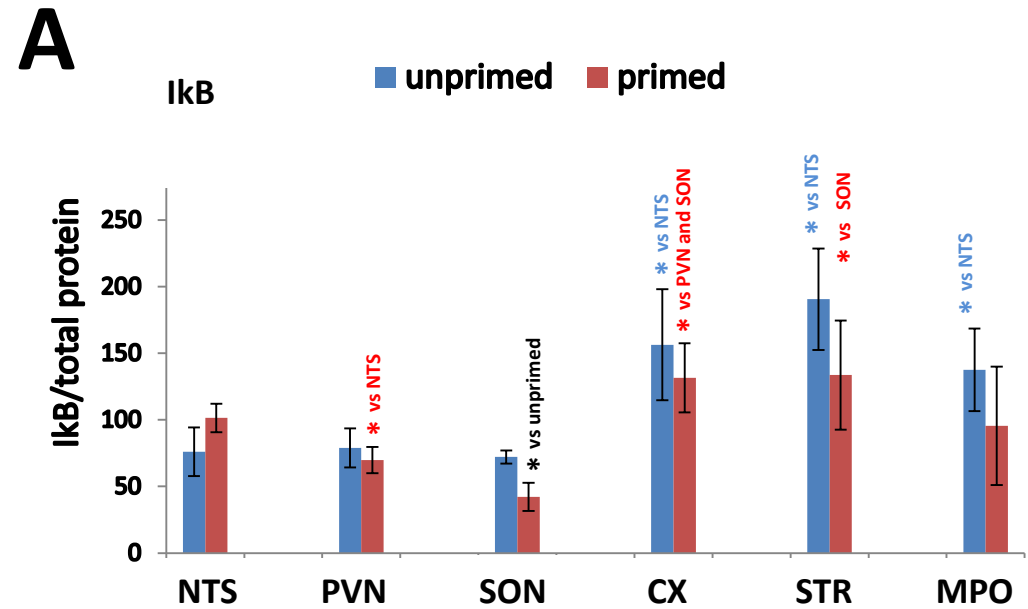
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Bradford solution	Bio Rad		
Protein lysis kit	Protein simple	CBS403	Bicine/CHAPS
WES kits	Protein simple	WES-Mouse 12-230 master kit (PS-MK15), WES-Rabbit 12-230 master kit (PS-MK14), WES 12-230 kDa total Protein master kit (PS-TP07)	
anti-mouse IgG HRP conjugate	Protein simple		
Rabbit anti-phospho-eIF2a	Cell Signaling technology	SER51, 9721	
mouse mAb anti-PKR	Cell Signaling technology	2103	
Rabbit anti-phospho-PKR	Millipore	Thr451, 07-886	
Rabbit mAb anti-PKR	Cell Signaling technology	12297	
rabbit mAb anti-GAPDH	Cell Signaling technology	2118	

mouse mAb anti-phospho-IKB	Cell Signaling technology	9246
mouse mAb anti-IKB	Cell Signaling technology	4814
rabbit anti-BiP	Cell Signaling technology	3183
Rabbit anti GCN2	Cell Signaling technology	3302
Rabbit mAb anti-phospho-GCN2	BIORBYT	T899
pregnant Sprague-Dawley rats	Charles River Laboratories	
Punch device	WellTech Rapid Core or Harris Uni-Core	0.35, 0.50, 0.75, 1.0, 1.20, 1.50

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Author(s): *Klein, BV, Tamir, H, Ludwig, R, Glickstein SB, Welch, MW*

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CORRESPONDING AUTHOR:

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Article Title: *Colostrum in the newborn gut modulates cellular stress and inflammation in certain sacred nuclei in the brain*

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Friday, July 6, 2018

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Re: Assessing Cellular Stress and Inflammation in Discrete Oxytocin-Secreting Nuclei in the Neonatal Rat Before and After First Colostrum Feeding

Dear Vineeta Bajaj,

Thanks to you and the reviewer for the careful review of our manuscript. Below we detail our responses to reviewer feedback. We have made the suggested revisions and believe this paper has benefited greatly from the review process.

Thank you for your consideration of our paper.

Respectfully,

Benjamin Y. Klein, MD and Martha G. Welch, MD
Bk2348@cumc.columbia.edu and mgw13@columbia.edu
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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please revise lines 93-95, 221-227, 237-239, 400-401, and 410-412 to avoid previously published text.

We have revised the text for lines 93-95 , 400-401, and 410-412. The text for lines 221-227 and 237-239 is methods text and remains as is.

3. Figures: Please list the panels in order from left to right and then top to bottom. Please be consistent throughout.

We have made this edit, which includes an updated Figure 4.

4. Figure 2: Please line up panels B and C. Please define error bars in the figure legend.

This figure has been updated and the legend updated to define error bars.

The figure legend has been updated.

We have made this edit.

We have added a statement (Lines 90-92).

We have added this (Lines 92-96).

We have added this text (Lines 96-100)

We have added this (Lines 100 – 112).

We have added this (Lines 114-123)

Done

Done.

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Bio Rad, ProteinSimple, WES-Mouse 12-230 master kit, Cell Signaling Technology (CST), Inc., Millipore, BIORBYT, Charles River Laboratories, etc.

Done

12. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Done

13. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Done

14. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added additional detail.

15. Lines 135 and 151: Please move material information to the Table of Equipment and Materials.

Moved.

16. 1.1.2/1.1.3/1.3.2: Please break up into sub-steps and add more details to each sub-step.

We have added additional substeps.

17. 1.2.2: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Done

18. 1.3.1: Please remove the weblink which contains commercial language. Please add more details to this step.

Done

19. Lines 229-249: Unclear what we can show here. Please describe the actions.

We have added additional detail.

20. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

Done (Lines 295-298)

21. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Done

22. JoVE article does not have a Conclusion section. Please move information in the Conclusion section or Results or Discussion section.

done

23. References: Please do not abbreviate journal titles.

done

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
To study the effect of colostrum oxytocin in gut on the activity of oxytocin receptor-rich brain regions, authors used a novel method for punching neonatal brain nuclei and then assessed BiP, eIF2a, NF-kB and IkB expressions using Western blotting before and after first colostrum feeding. The results showed that the stress modulating mechanisms previously observed in newborn gut enterocytes appear to be mirrored in some OTR-rich brain regions. Under nutrient deficiency the STN and PVN may utilize a different phosphorylation mechanism from other regions and be refractory to the impact of nutrient insufficiency. The authors concluded that brain responses to nutrient insufficiency stress are offset by signaling from colostrum-primed enterocytes. This work does show some innovation in the methods of sampling and in-capillary protein assay and is useful for establishing a theory that nutrient-insufficiency-related metabolic stress is a body-wide stressor. However, serious concerns about the methods and analyses should be addressed.

Major Concerns:
1. Feeding itself, even without milk or oxytocin, can strongly influence vagal input and brain activity (see Line 92). Thus a milk control containing no oxytocin or liquid without nutrients should be given.

We are grateful to the author for raising this important point. We did not pursue a "sham-feed" for two reasons. Frist, at Day 0 – 1 the vagal inputs is not yet developed fully and, second, the handling required for a sham-feed and equivalent handling in the primed group would also impact our measures and data interpretation.

2. Line 164, the sampling time between the unprimed and primed pups differed by 3-5 hours, which casts the

doubt that the priming effect could reflect the natural developing process or adaptation process to the external environment. Thus, it is necessary to set an unprimed control group wherein sampling time is the same as the primed group. Alternatively, the authors may consider to provide the priming at the same time of the unprimed group.

We agree with the authors that the timing between primed and unprimed groups requires further evaluation. We chose to sacrifice unprimed pups prior to any stress response to eliminate the impact of stress related to the pup's request for the first feed on gene expression. We will explore this feature in a subsequent paper.

3. Line 172: why was "artificial cerebrospinal fluid with 203.3mM MgCl 2 -6H2O used? No dehydration occurred?

Incubation in ACSF is necessary as a baseline for future options to examine the signaling response to selected inhibitors or stimulants for any signaling pathway.

4. In identification of said brain regions, histological presentation is necessary since the ordination or the surface marker of the skull was not showed.

We have indicated the distance from bregma, which allows for the identification.

5. Line 192, the meaning of "this extraction cocktail should contain protease inhibitors and phosphatase inhibitors" is not clear. Did it "contained" or did not contain" the inhibitors?

We have corrected, it contained inhibitors.

6. Line 109-201: "Protein Simple kit for Biotin-labeling of the total proteins concomitantly during electrophoresis to measure protein concentration, as opposed to adjusting the protein concentration to equal volumes in advance" raises the question if the quantification is valid.
As shown in Figure 2 that changes in the areas of eIF2a and p-eIF2a are almost the same, and so does the total protein (SOPT and MPO are exceptional). Without a visual hint of clear changes, how could the authors get the values shown in the bar graphs? Are the exemplary bands representative? If the WES detected the density correctly, a loading control with adjusting the protein solution to equal concentration/amount should be presented, at least.

The tissue yield of some brain nuclei is very small to normalize against protein.

7. Discussion should address the comments listed above.

We have had to substantially modify the discussion to meet journal style and discuss primarily the technique. We have added some of this detail to the introduction.

Minor Concerns:
Line 177: the name of brain atlas referred should be given.

We have added.

Reviewer #2:

Manuscript Summary:
This is modification of current techniques used to determine protein levels in brain tissue.

Major Concerns:
While present in the introduction, there is no premise/rationale presented in the abstract. There is nothing to pull the reader in to the topic of the research.

We have made these additions. Thank you for bringing this to our attention.

Minor Concerns:
1. The grammar is generally good, but could use an overall revision for a few minor issues.

We have given the manuscript a proofread.

2. Line 169, is this with a standard razor blade?

Yes, we have corrected.

3. Lines 203-206. This sentence is difficult to comprehend.
This sentence has been removed in accordance with journal requirements to remove any commercial information.

4. Line 208, should this be -80C?

Yes, thank you for noticing, this has been corrected.

5. The graphs with asterisks are visually challenging. I'm not sure if the use of letters would be preferable by all, but alternative designs should be explored.

We have improved.

Reviewer #3:
Manuscript Summary:
Traditionally Oxytocin was known only to involve in milk production and delivery. But the ever increasing roles of Oxytocin is further supported by the present study.
Although there are other techniques such as single cell or brain slice approaches currently popular, but the authors put forward a detailed description of an efficient method to specifically isolate different OTR-rich regions within the rat-brain. Expression of different signaling proteins were tested in different brain regions and were compared to their already published data from gut. Based on their results, authors concluded that cell signaling in the brain associated with nutrient and hormonal insufficiency during the first hours following birth is similar to the gut under similar conditions.
The method is well described and is probably reproducible, as authors claim. This method can probably be used to develop new protocols in different tissues too.

All the best

Thanks

Major Concerns:

NONE

Minor Concerns:
NONE

1   Hansson, J. *et al.* Time-resolved quantitative proteome analysis of in vivo intestinal
        development. *Mol Cell Proteomics.* **10** (3), M110 005231, doi:10.1074/mcp.M110.005231,
        (2011).
2   Mochizuki, K., Yorita, S. & Goda, T. Gene expression changes in the jejunum of rats during the
        transient suckling-weaning period. *J Nutr Sci Vitaminol (Tokyo).* **55** (2), 139-148 (2009).
3   Rinaman, L., Banihashemi, L. & Koehnle, T. J. Early life experience shapes the functional
        organization of stress-responsive visceral circuits. *Physiol Behav.* **104** (4), 632-640,
        doi:10.1016/j.physbeh.2011.04.008, (2011).
4   Dobbing, J. Boyd Orr memorial lecture. Early nutrition and later achievement. *Proc Nutr Soc.* **49**
        (2), 103-118 (1990).
5   Rinaman, L. Hindbrain noradrenergic A2 neurons: diverse roles in autonomic, endocrine,
        cognitive, and behavioral functions. *Am J Physiol Regul Integr Comp Physiol.* **300** (2), R222-235,
        doi:10.1152/ajpregu.00556.2010, (2011).
6   Walker, C. D., Toufexis, D. J. & Burlet, A. Hypothalamic and limbic expression of CRF and
        vasopressin during lactation: implications for the control of ACTH secretion and stress
        hyporesponsiveness. *Prog Brain Res.* **133** 99-110 (2001).

Figure S1

Delivery

First Feed

0 1 h 2 h 3 h 4 h 5 h 6 h

In vivo

In vivo

Unprimed gut

Colostrum Primed gut

Ex Vivo,
Isolate brains, prepare coronal slices

Incubate in ACSF, excise nuclei, extract protein, fractionate on WES

Assay markers of stress and inflammation
(eIF2a, PKR, BiP, NF-κB, IκB)