SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

SUBJECTS

This study evaluated 10 litters from control and PTU-treated parents. Different sample sizes are used for some outcome measures because some tissue samples were not adequate for assays or there was inadvertent trauma to a brain area during dissection.

MICRODISSECTION

For dissection, the brain was first placed in a beaker containing cold buffer for ~2 min. The buffer simulated cerebrospinal fluid (in mM: 126 NaCl, 5 KCl, 2.0 $CaCl_2$, 2.0 $MgSO_4$, 26.0 $NaHCO_3$, 1.25 NaH_2PO_4, 10 D-glucose; pH 7.4). Different regions of the brain were microdissected by the same experimenter to ensure similarity of dissection across animals and similar delays from euthanasia to freezing of harvested tissues (range: 3-5 min; see Supp. Fig. 1).

BDNF ELISA

Sample preparation

Frozen (-80^{o}C) tissue samples were weighed and a 10% homogenate was made with lysis buffer (20.0 mM Tris-HCl, pH 8.0, containing 1.0 mM EDTA, 137.0 mM sodium chloride, 1.0 mM phenyl methyl sulfonyl fluoride, 10.0 ug/uL aprotonin, 1.0 ug/uL leupeptin, 0.5 mM sodium ortho-vanadate, 1.0% NP40 and 10% glycerol). The tissue homogenates were centrifuged at 14,000 x g for 30 min at 4^{o}C in a microfuge Eppendorf, Hamburg, Germany). Supernatants were stored at -80^{o}C.

Protein Estimation

Aliquots of each sample lysate (supernatant) were analyzed in triplicate for total protein by the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL).

ELISA procedure

BDNF levels were determined with an Enzyme Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions (R & D Systems, Minneapolis, MN) with minor modifications, described below.

Microplates (96 wells) were treated with a monoclonal human anti-BDNF antibody (Capture antibody) in 0.1M Dulbecco's phosphate buffer saline (Invitrogen, Carlsbad, CA). The plate was covered with adhesive aluminum foil and incubated for 16-20 hrs at 4^{o}C with mild shaking (500 RPM). The next day, all the antibodies were aspirated, plates were washed 3 times with 400 µl "Wash Buffer" and blocked with 300 µlL "Reagent Diluent" for 2 hrs at room temperature with slow shaking (100 RPM). This washing step was repeated to ensure that all blocking reagent was removed. One hundred µl of standards (Recombinant human BDNF, 23.86 pg/ml – 1719 pg/ml), and samples (25 µg/25 µl in Reagent Diluent) were added to different wells in a total volume of 100 µl. The plate was then incubated while shaking (500 RPM) for 2 hrs at room temperature and washed 3 times with Wash Buffer. A biotinylated human anti-BDNF antibody (Detection antibody diluted in Reagent Diluent) was then added to the wells followed by shaking for 2 hrs at room temperature. After 3 washes with Wash buffer, Streptavidin-HRP conjugate (diluted in Reagent Diluent) was added to the wells and was shaken for 20 min at room temperature. After 3 more washes with Wash buffer, a peroxidase substrate (1:1 Reagent A:Reagent B) was added to the wells, and the plates were shaken for 30 min at room temperature in the dark. The reaction was stopped with 2.0 M sulfuric acid, and

absorbance was read in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) at 450, 540 and 570 nm (to read plate background) using Softmax Pro software (Molecular Devices).

The amount of BDNF protein in each sample was determined in triplicate in each experimental set of plates, and each run was repeated three times. Samples were counterbalanced so that all treatments, ages, and genders were represented on each plate. Preliminary testing established the appropriate lysate dilutions to employ so that sample values fell within the range of the standard curves for total protein and BDNF protein.

Supplemental Table 1. Comparison of control and PTU-treated litters.

A. Age at time of mating (days)	**Dams**		**Studs**	
	Control	**PTU**	**Control**	**PTU**
Mean	79.0	99.4	99.4	101.2
Sem	5.8	5.6	14.2	10.1
N	10	10	10	10

B. Time (mating to birth, days)	**Control**	**PTU**
Mean	23.9	24.3
SEM	0.4	0.5
N	10	10
p	0.5172	

C. Litter size (# of pups)	**Control**	**PTU**
Mean	13.3	13.3
Sem	0.7	0.5
N	10	10
p	0.7022	

D. Food intake (g/day)	**Control**	**PTU**
Mean	27.22	25.14
Sem	2.18	1.26
N	3	3
p	0.4558	

E. Water intake (ml/day)	**Control**	**PTU**
Mean	38.76	32.10
Sem	2.58	0.74
N	3	3
p	0.06242	

F. Body weight gain (g/day)	**Dams**		**Studs**	
	Control	**PTU**	**Control**	**PTU**
Mean	134.7	134.7	58.0	74.0
Sem	30.1	7.5	8.5	9.3
N	3	3	3	3

Legend:

Data are shown for parents of control and PTU-treated offspring.

A. Ages of dams and studs that were used for control and PTU-treatment are shown. Two-way ANOVA showed that there was no effect of PTU treatment ($F= 0.1323$; df 1,39; $p=0.7182$), sex ($F=3.7773$; df 1,39; $p=0.0598$), or sex x treatment interaction ($F= 0.03122$; df 1,39; $p=0.8608$).

B. Comparisons were made to determine if breeding pairs treated with PTU had a different time between mating and gestation than controls. For mating, animals were placed in the same cage and the day was arbitrarily defined as 0; the day of birth was the last day. A Student's t-test (two-tailed) was used for B-E.

C. Litter sizes of the control and PTU-treated litters were not statistically different.

D. Food intake was measured from the day of mating and for the next 21 days. Measurements were made Monday, Wednesday, and Friday, and the daily food intake was calculated as the mean daily intake (in g). There were no detectable effects of PTU on food intake. There was no significant correlation between food intake of parents and TSH levels in the offspring ($p=0.9826$). Significance of the correlation coefficient was determined using OriginPro (v.8; Originlabs, Northampton, MA).

E. Water intake was measured similar to the method used for to food intake. There were no detectable differences between control and PTU-treated breeding pairs. There was no significant correlation between water intake of parents and TSH levels in the offspring ($p=0.9981$).

F. Two-way ANOVA results showed that there was no effect of PTU treatment on body weight gain ($F=1.2310$; df 1,11; $p=0.2994$) but there was a significant effect of sex ($F=37.9298$; df 1,11; $p= 0.0002714$) and no sex x treatment interaction ($F= 0.9414$; df 1,11; $p=0.3603$). Body weight gain of the 3 dams weighed throughout gestation and treated with PTU was not significantly correlated with TSH levels in offspring ($p=0.2771$).

Supplemental Table 2. Statistical comparisons of thyroid hormone levels.

A. Parents

1. Free T_3

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	0.077	0.077	0.768	0.3911	0.768	0.128
Sex	1	0.223	0.223	2.243	0.1499	2.243	0.283
Trt x Sex	1	0.046	0.046	0.457	0.5066	0.457	0.096
Residual	20	1.991	0.100				

2. Free T_4

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	106.471	106.471	14.423	0.0011	14.423	0.964
Sex	1	743.150	743.150	100.671	<0.0001	100.671	1.000
Trt x Sex	1	0.650	0.650	0.088	0.7697	0.088	0.059
Residual	20	147.639	7.382				

3. Total T_4

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	478.827	478.827	10.144	0.0047	10.144	0.872
Sex	1	3308.802	3308.802	70.098	<0.0001	70.098	1.000
Trt x Sex	1	33.607	33.607	0.712	0.4088	0.712	0.122
Residual	20	944.045	47.202				

4. TSH

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	0.150	0.150	3.228	0.0875	3.228	0.387
Sex	1	0.309	0.309	6.661	0.0178	6.661	0.693
Trt x Sex	1	0.116	0.116	2.487	0.1305	2.487	0.309
Residual	20	0.929	0.046				

B. Offspring

1. Free T_3

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	0.0001763	0.0001763	0.327	0.5770	0.327	0.082
Sex	1	0.0001763	0.0001763	0.327	0.5770	0.327	0.082
Trt x Sex	1	0.001	0.001	0.994	0.3370	0.994	0.146
Residual	13	0.007	0.001				

2. Free T_4

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	0.621	0.621	9.663	0.0090	9.663	0.825
Sex	1	0.002	0.002	0.027	0.8719	0.027	0.053
Trt x Sex	1	0.002	0.002	0.027	0.8719	0.027	0.053
Residual	12	0.771	0.064				

3. Total T_4

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	0.295	0.295	61.959	<0.0001	61.959	1.000
Sex	1	0.197	0.197	41.443	<0.0001	41.443	1.000
Trt x Sex	1	0.197	0.197	41.443	<0.0001	41.443	1.000
Residual	14	0.067	0.005				

4. TSH

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	13.260	13.260	48.817	<0.0001	48.817	1.000
Sex	1	0.192	0.192	0.706	0.4149	0.706	0.119
Trt x Sex	1	0.002	0.002	0.009	0.9255	0.009	0.051
Residual	14	3.803	0.272				

Legend:

A. Parents.

1. Analysis of Free T_3 demonstrated no significant effects of PTU treatment or sex.

2. Free T_4 analysis showed that there were significant effects of both PTU treatment and sex because of markedly higher T_4 levels in males.

3. Total T_4 analysis demonstrated significant effects of both PTU treatment and sex because of markedly higher T_4 levels in males.

4. Analysis of TSH showed that there were significantly higher TSH levels in males, but no significant sex difference. Abbreviations: Trt, PTU or control treatment.

B. Offspring.

1. Free T_3. There was no significant effect of postnatal age or PTU treatment.

2. Free T_4. There was a significant effect of PTU treatment but not postnatal age.

3. Total T_4. There were significant effects of PTU treatment and postnatal age. The significant interaction effect reflects the marked increase in the total T_4 levels in controls at P7, which was suppressed by PTU.

4. TSH. There was a significant effect of PTU treatment, but not postnatal age.

Supplemental Table 3. Statistical comparisons of BDNF protein levels.

A. Parents

A. Hippocampus

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	124.437	124.437	0.021	0.8870	0.021	0.562
Sex	1	73574.603	73574.603	12.447	0.0042	12.447	0.912
Trt x Sex	1	10547.107	10547.107	1.784	0.2064	1.784	0.223
Residual	12	70932.635	5911.053				

B. Cerebellum

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt x Sex	1	547.107	547.107	0.018	0.8964	0.018	0.052
Sex	1	28025.240	28025.240	0.906	0.3599	0.906	0.136
Trt x Sex	1	245866.012	245866.012	7.951	0.0155	7.951	0.742
Residual	12	371084.980	30923.7				

B. Offspring

1 .Hippocampus

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	52510.660	52510.660	27.828	<0.0001	27.828	1.000
Sex	1	3915.570	3915.570	2.075	0.1539	2.075	0.280
Age	1	253340.298	253340.298	134.259	<0.0001	134.259	1.000
Trt x Sex	1	351.267	351.267	0.186	0.6674	0.186	0.070
Trt x Age	1	5.587	5.587	0.003	0.9568	0.003	0.050
Sex x Age	1	0.318	0.318	0.0001688	0.9897	0.0001688	0.050
Trt x Sex x Age	1	5654.321	5654.321	2.997	0.0876	2.997	0.384
Residual	74	139634.461	139634.461				

2. Cerebellum

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	218557.714	218557.714	3.469	0.0669	3.469	0.435
Sex	1	25314.479	25314.479	0.402	0.5283	0.402	0.093
Age	1	66284.224	66284.224	1.052	0.3087	1.052	0.164
Trt x Sex	1	4267.218	4267.218	0.0680	0.7955	0.0680	0.057
Trt x Age	1	21361.878	21361.878	0.339	0.5623	0.339	0.087
Sex x Age	1	15167.415	15167.415	0.241	0.6253	0.241	0.076
Trt x Sex x Age	1	87357.185	87357.185	1.387	0.2431	1.387	0.201
Residual	67	4220631.176	62994.495				

3. Brainstem

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	1534.164	1534.164	0.691	0.4107	0.691	0.123
Sex	1	400.622	400.622	0.181	0.6732	0.181	0.069
Age	1	782701.452	782701.452	352.65	<0.0001	352.65	1.000
Trt x Sex	1	248.810	248.810	0.112	0.7395	0.112	0.062
Trt x Age	1	813.571	813.571	0.367	0.5483	0.367	0.089
Sex x Age	1	586.008	586.008	0.264	0.6102	0.264	0.078
Trt x Sex x Age	1	5685.435	5685.435	2.5620	0.1174	2.5620	0.329
Residual	40	68779.388	68779.388				

Legend:

A. Parents.

1. Hippocampus. There was no significant effect of PTU on hippocampal BDNF of parents but there was a significant sex effect, reflecting the higher mean BDNF concentrations in females, which is consistent with previous studies (Scharfman et al., 2003). However, neither the treatment nor the sex x treatment interaction effects were statistically significant.

2. Cerebellum. Neither sex nor PTU treatment affected overall BDNF levels. The significant interaction effect reflects a slight increase in mean BDNF levels in males and a slight decrease in females, after PTU. Abbreviations: Trt, PTU or control treatment.

Reference: Scharfman HE, Mercurio TC, Goodman JH, Wilson MA, MacLusky NJ.(2003) Hippocampal excitability increases during the estrous cycle in the rat: a potential role for brain-derived neurotrophic factor. 23:11641-11652.

B. Offspring.

1. Hippocampus. There were significant effects of both PTU treatment and postnatal age on hippocampal BDNF protein levels, reflecting lower BDNF concentrations after PTU treatment and lower values in all treatment groups at P3 compared to P7.

2. Cerebellum. BDNF levels tended to be slightly higher after prenatal PTU, but this was not statistically significant ($p=0.0669$). None of the other main factors or interaction effects were significant.

3. Brainstem. Brainstem levels of BDNF were not influenced by sex or PTU treatment, but there was a significant increase overall between P3 and P7.

Supplemental Table 4. Statistical comparisons of BDNF levels in frontal regions, pituitary and serum from control and PTU-treated parents.

A. Dorsal and ventral frontal regions

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	4773.459	4774.459	0.013	0.9113	0.013	0.051
Sex	1	14203.147	14203.147	0.038	0.8477	0.038	0.054
Region	1	7045.834	7045.834	0.019	0.8024	0.019	0.052
Trt x Sex	1	110156.445	110156.445	0.292	0.5937	0.292	0.080
Trt x Region	1	6849.376	6849.376	0.018	0.8939	0.018	0.052
Sex x Region	1	31197.938	31197.938	0.083	0.7760	0.083	0.059
Trt x Sex x Region	1	12860.070	12860.070	0.034	0.8550	0.034	0.054
Residual	24	9043088.869	376795.370				

B. Pituitary

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	76.592	76.592	0.007	0.9348	0.007	0.051
Sex	1	25750.034	25750.034	2.319	0.1473	2.319	0.286
Trt x Sex	1	4935.846	4935.846	0.445	0.5145	0.445	0.094
Residual	16	177665.053	11104.066				

C. Serum

	DF	Sum of Squares	Mean Squares	F value	P value	Lambda	Power
Trt	1	111.143	111.143	0.080	0.7842	0.080	0.057
Sex	1	8654.292	8654.292	6.244	0.0370	6.244	0.591
Trt x Sex	1	260.028	260.028	0.188	0.6764	0.188	0.067
Residual	8	11088.527	1386.066				

Legend:

A. Frontal areas. Frontal brain areas were separated into a dorsal and ventral region (Supp. Fig. 1). There was no significant effect of PTU treatment or sex on BDNF levels in the frontal cortex, and no influence of region (dorsal or ventral).

B. Pituitary. There was no significant effect of PTU treatment on BDNF levels in the pituitary, or effect of sex.

C. Serum. There was no significant effect of PTU treatment on BDNF levels in serum, but there was an effect of sex, with higher levels of BDNF in male serum compared to females. However, there was no sex x treatment interaction.

Supplemental Figure 1. Methods used for dissection.

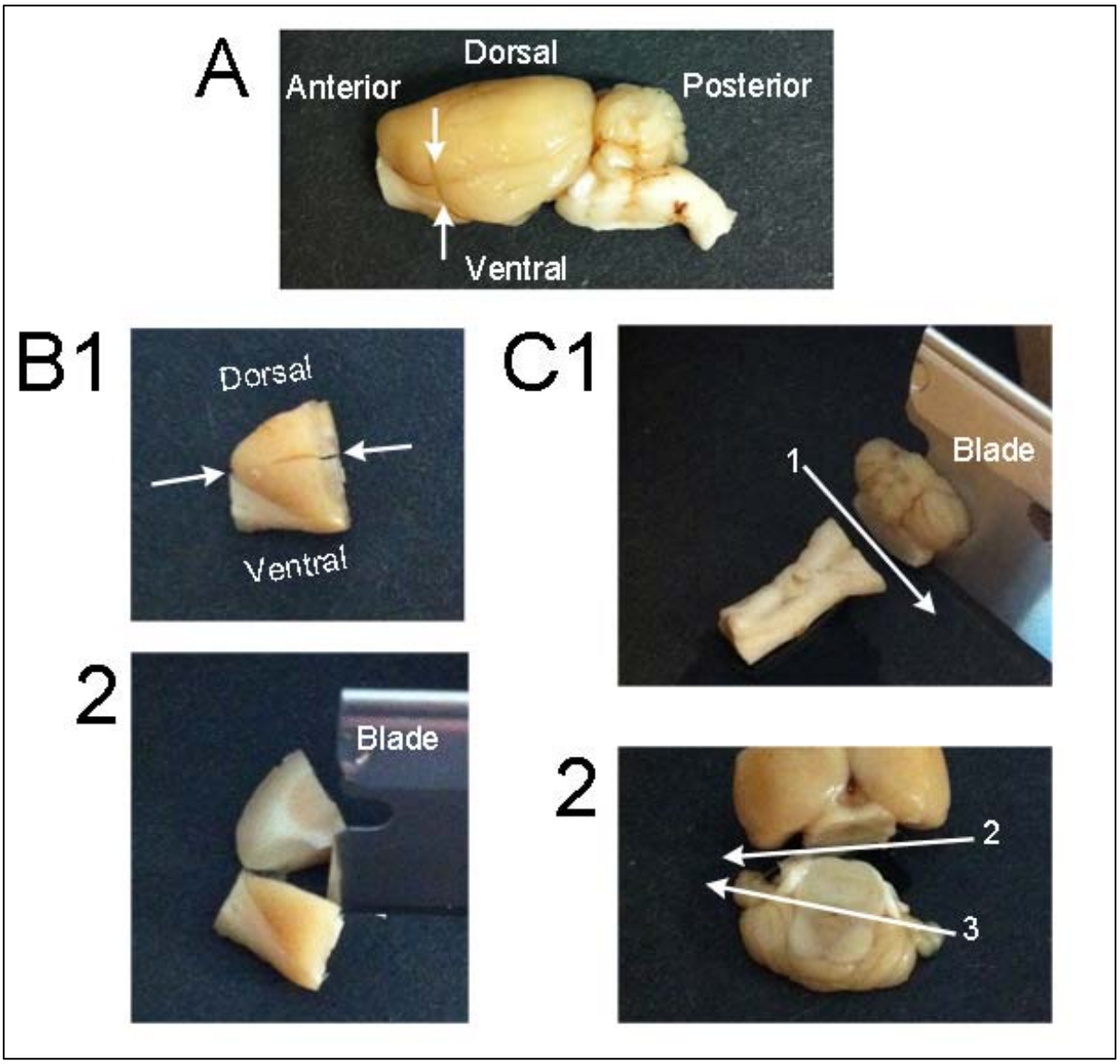

Figure Legend:

A. A side view of the rat brain is shown. A razor blade was used to make a coronal cut at the level of the middle cerebral artery (arrow) to separate frontal areas from more caudal regions.

B. The separation of the frontal region into a dorsal and ventral area is shown. The razor blade was used to separate the two areas (B1; arrows) using a cut in the horizontal plane across the

longest part of the frontal region. The result of the cut was a separation of the dorsal and ventral areas (B2).

C. The dissection of the cerebellum and brainstem is shown. A coronal cut with a razor blade was made immediately caudal to the cerebellum to separate the brainstem from the cerebellum (C1; arrow 1). Another cut was made in the coronal plane to separate the anterior portion of the cerebellum from forebrain (C2; arrow 2). After positioning the dissected region of brainstem and cerebellum on the anterior coronal surface of the cerebellum, a horizontal cut was made to separate the regions ventral to the cerebellum, referred to as brainstem, from the cerebellum itself (C2; arrow 3). The dissection of hippocampus used a curved spatula, inserted into the crevice between the ventricular wall and alveus. Gentle pressure was used to “roll out” the hippocampus from the ventricular surface. Areas adjacent to hippocampus were removed by trimming close to the edge of the hippocampus with the razor blade.