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Journal of the Autonomic Nervous System 70 (1998) 115–122

Journal of the Autonomic Nervous System

GDNF is abundant in the adult rat gut

Robert J. Peters [a], Mark A. Osinski [b], Jo-Anne Hongo [c], Gregory L. Bennett [c], Angela J. Okragly [d], Mary Haak-Frendscho [d], Miles L. Epstein [a,*]

[a] *Department of Anatomy and Neuroscience Training Program, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA*
[b] *Department of Gastroenterology, University of Wisconsin, 1300 University Avenue, Madison, WI 50376, USA*
[c] *Department of Antibody Technologies and BioAnalytical Technology, Genentech, 1 DNA Way, South San Francisco, San Francisco, CA 94080, USA*
[d] *Promega, 2800 Woods Hollow Road, Madison, WI 53711-5399, USA*

Received 29 September 1997; revised 9 February 1998; accepted 9 February 1998

Abstract

Glial derived neurotrophic factor (GDNF) is essential for the development of the enteric nervous system (ENS). Although previous work has measured GDNF mRNA levels, little is known about the concentration of GDNF protein produced in developing or adult tissues. The aim of this study was to quantitate the concentration of GDNF protein in various tissues of the developing and adult rat and in adult human gut. A two site antibody immunoassay was used to quantitate GDNF using recombinant rat GDNF as a standard. In the adult rat gastrointestinal tract the intestine contained the highest concentration of GDNF while the stomach and esophagus have the lowest concentrations. The isolated muscular wall of the intestine has approximately four times the GDNF concentration of the intact intestine. Other tissues with smooth muscle such as the aorta and urinary bladder contain moderate GDNF concentrations. In contrast, GDNF is barely detectable in the adult kidney and liver. High concentrations of GDNF were also detected in human colon and jejunum. As development proceeds in the rat, there is a tendency for the concentration of GDNF to increase in the intestine but decrease in other tissues. Treatment of the jejunum with the cationic surfactant benzyldimethyltetradecylammonium chloride (BAC) results in an increase in the number of smooth muscle cells, a decrease in myenteric neurons, and an increase in the concentration of GDNF in homogenates of intestine. The observations that GDNF concentrations are high in the adult intestine suggest that this growth factor may be important for the maintenance of the adult ENS.

Keywords: GDNF; Growth factor; Gastrointestinal tract; Smooth muscle; ELISA; Enteric nervous system; Myenteric neuron ablation; Rat; Human

1. Introduction

Glial derived neurotrophic factor (GDNF), a member of the TGF-β superfamily, was isolated and cloned based on its ability to support the survival of midbrain dopaminergic neurons in culture (Lin et al., 1993). Subsequent investigations showed that injection of GDNF protected dopamine neurons in the substantia nigra from damage in models of Parkinson's disease (Beck et al., 1995; Tomac et al., 1995). Recently, a novel method for exogenous expression, injection of adenovirus producing GDNF, was found to protect rat nigral neurons after injection of the neurotoxin 6-hydroxydopamine (Choi-Lundberg et al., 1997).

The expression of GDNF mRNA in the adult rat brain was localized to a number of target sites for nigral neurons including the striatum and nucleus accumbens (Trupp et al., 1997). These observations showed a moderate labeling over some neurons and suggest that neurons are producing this growth factor. Although GDNF mRNA is found in the brain, the role of GDNF in the brain is not clear.

In contrast to the adult, GDNF has a critical role in embryogenesis and is essential for the development of the enteric nervous system and kidneys as shown by targeted mutations of the murine GDNF gene (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Mutant mice died shortly after birth and lacked the enteric nervous system (ENS) and kidneys. They also showed a 20–50% reduction in the number of spinal motor neurons, and sensory neurons in the nodose-petrosal and dorsal root ganglia, but surprisingly not a reduction in the number of nigral neurons (Granholm et al., 1997).

Studies of expression of GDNF have measured or localized mRNA, mostly in developing rodents (Poulsen et al.,

* Corresponding author. Tel.: +1 608 2635074; fax: +1 608 2627306; e-mail: mepstein@facstaff.wisc.edu

0165-1838/98/$19.00

1994; Hellmich et al., 1996). RNAase protection assays show substantial levels in the developing gut (Trupp et al., 1995) and in situ hybridization experiments show substantial label in the gut wall (Nostrat et al., 1996). However, little is known about the levels of GDNF protein in either developing or adult tissues, particularly the gut. We measured GDNF protein with an ELISA and report that the adult rat intestine contains substantial concentrations relative to other tissues. To gain insight into the cellular source of GDNF we have treated jejunum with a surfactant that destroys most myenteric neurons and increases the number of smooth muscle cells. This treatment resulted in an increase in GDNF and suggests that GDNF might be produced by smooth muscle cells.

2. Methods

2.1. Animals

All procedures involving animal use were approved by the Animal Care Committee of the University of Wisconsin, Madison. Adult Sprague–Dawley (Madison, WI) rats (all male except one) were euthanized with carbon dioxide gas. Tissues were removed and placed in cold phosphate-buffered saline (PBS), pH 7.4. After the small intestine was isolated, several pieces from throughout its length were removed to serve as the intact intestine sample. From the remainder of the intestine, the mucosa was stripped with a scalpel, collected, and concentrated by centrifugation prior to weighing. The intestinal wall stripped of mucosa became the intestine stripped sample. Observations indicate that stripped muscle samples consisted of muscularis externa and not submucosa. The stomach was bisected along its longitudinal axis, half served as the intact sample and the other half as the stripped sample. Rats were also killed at postnatal day (P) 2 and 21. A pregnant female rat was killed, the fetuses (E17.5) were removed, staged, and dissected in cold PBS. Tissues from E17.5 and P2 animals were pooled when tissue from a single animal was insufficient to generate enough homogenate for the ELISA.

2.2. Antibodies

Mice were immunized with recombinant rat GDNF in Ribi adjuvant (RIBI Immunochem Research, Hamilton, MO) using a protocol analogous to one previously described (Hongo et al., 1995). The mAb 1693 used in this study binds rat GDNF and human GDNF (with $\sim$50% reduction in signal) and does not cross-react with recombinant human TGF-β1, NT3, NT4/5 and NGF. A polyclonal antiserum was generated by injecting rabbits with recombinant rat GDNF. The GDNF polyclonal antibody was tested for specificity in an ELISA format, in which the antibody was Protein A purified, biotinylated, and then used for capture and detection. Human and murine neurturin, and human TGF-β1 were all $<0.06\%$ cross-reactive when compared with rat GDNF.

2.3. Preparation of extract

We used the protocol of Okragly and Haak-Frendscho (11) for preparation of extracts. Tissue was weighed and homogenized 1:1 (weight:volume) in cold extraction buffer {Tris-buffered saline (TBS; 137 mM NaCl, 20 mM Tris), pH 8.0, with 1% NP-40, 10% glycerol, 0.5 mM sodium vanadate, and the protease inhibitors 1 mM PMSF (Sigma, St. Louis, MO), 10 μg/ml aprotinin (Sigma), and 1 μg/ml leupeptin (Sigma)} with a high speed mechanical homogenizer (Tissuemizer, Tekmar, Cincinnati, OH). The homogenate was frozen until assayed. At the time of assay 90 μl of homogenate was mixed with 360 μl of TBS (pH 7.6) acidified to pH 3 with $\sim$14 μl of 1.0 N HCl, incubated at room temperature for 15 min, neutralized with $\sim$15 μl of 1.0 N NaOH to $\sim$pH 7.5, and microfuged at 15,000 g at 4°C for 10 min. The resulting supernatant was removed and assayed. The pH of the homogenate was determined by measuring a drop of homogenate on pH paper.

2.4. Preparation of ELISA plates

We developed a two-antibody sandwich assay using 96-well plates from Nunc (Maxisorp F96). Each plate was coated with monoclonal mouse-anti-GDNF (1 μg/ml, Genentech mAb 1693) in carbonate buffer (25 mM $NaHCO_3$, 25 mM Na_2CO_3, pH 8.2) overnight at 4°C, washed once in wash buffer (411 mM NaCl, 20 mM Tris, pH 7.6, and 0.05% Tween-20), and blocked in blocking buffer (TBS, pH 7.6, containing 1% BSA, 10% goat serum, and 0.05% Tween-20) for 1 h at room temperature. Blocking buffer was removed and 100 μl aliquots of tissue extracts and GDNF standards were loaded in triplicate wells. For each assay, a fresh solution of 500 pg/ml GDNF standard (recombinant rat GDNF, Genentech) was prepared in a GDNF standard dilution buffer (TBS, pH 7.6, with 10% extraction buffer and 1% BSA). Serial twofold dilutions in dilution buffer gave a GDNF standard series of 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, and 7.8 pg/ml, as well as a 0 pg/ml blank of standard dilution buffer. The plate was incubated with the tissue extracts and GDNF standards for 6 h at room temperature, with shaking. After five washes with wash buffer, 100 μl of rabbit-anti-GDNF (1:3000 dilution in blocking buffer, Genentech) was added to each well and the plate was incubated at 4°C overnight. The plate was washed five times with wash buffer, and 100 μl of goat-anti-rabbit IgG conjugated to HRP (1:60 000 dilution in blocking buffer, Jackson, West Grove, PA) was added to each well, followed by incubation with shaking at

room temperature for 2.5 h. After five washes with wash buffer, 100 μl of TMB peroxidase substrate (Kirkegaard and Perry, Gaithersburg, MD) was added to each well, and the plate incubated for ~10 min at room temperature. Color development was stopped by addition of 100 μl of 1.0 M phosphoric acid to each well, and absorbances at 450 nm were measured with a plate reader.

2.5. Total protein determination

A portion of the diluted tissue extract was further diluted 1/20 in TBS. Serial dilutions of BSA in TBS with 0.5% extraction buffer gave a standard series with 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.063 mg/ml, and 0.031 mg/ml, as well as a blank of TBS/0.5% extraction buffer. Triplicate 10 μl samples of each tissue extract and BSA standard were placed in individual wells of a 96 well plate and incubated with 200 μl of BCA reagent (Pierce, Rockford, IL) for 2 h at room temperature. A plate reader was used to measure absorbances at 590 nm.

2.6. Data analysis

Microsoft Excel was used to perform all data manipulations. For both ELISA and BCA assays, the average absorbance value of the blank was subtracted from the average absorbance of each sample to give a corrected average absorbance for each sample.

A linear regression coefficient based on the standard series was used to estimate the GDNF or total protein concentration of the samples from ELISA and BCA assays, respectively. Concentrations were adjusted for dilution. GDNF concentrations reported in this study are given as the ratio of the GDNF (pg/ml) to the total protein (mg/ml) to give a value of pg GDNF per mg total protein. For each tissue, the GDNF concentration and standard error were calculated using the values for this ratio obtained from different animals.

2.7. Benzyldimethyltetradecylammonium chloride (BAC) treatment

Male albino rats (Sprague–Dawley), weighing 200–225 g, were anesthetized with an intraperitoneal injection of a 2:1 mixture of ketamine and xylazine. The procedure for exposing the jejunum and application of the BAC has been described (Fox et al., 1983). Briefly, after exposure of a 5 cm portion of the jejunum delineated by serosal suture tags, a solution of 2 mM BAC is applied to the serosa every 5 min for 30 min. The jejunum of control rats was treated with 0.9% saline. The rats were housed singly in cages after surgery and killed 12 days later. To assess the effectiveness of the BAC treatment, pieces of the delineated jejunum from five treated and three control animals were fixed and longitudinal sections were cut on a cryostat (Fox et al., 1983). Sections were immunostained with PGP9.5 and the number of neurons per millimeter of section length was counted in 5–10 sections from each animal as described previously (Fox et al., 1983). Sections were also stained with antibodies to S-100 (Dako, Westbury, NY) and glial fibrillary acidic protein (GFAP, Sigma). The thickness of the smooth muscle was measured in 5–10 toludine-blue stained frozen sections from each of the five treated and three control animals.

2.8. Human tissue

Human tissue procurement was approved by the Human Subjects Committee of the University of Wisconsin, Madison. Samples of full thickness colon (two adults) and jejunum (one adult, one juvenile) were obtained from surgical patients at University of Wisconsin Hospital and University of Iowa Hospital, respectively.

2.9. Statistical analysis

Comparisons of GDNF levels among adult rat tissues was made using Fisher's Protected Least Significant Difference method. This method first requires overall statistical significance from an analysis of variance (ANOVA) model. Post hoc comparisons are then made using *t*-tests.

Table 1
Effect of incubation time, pH during acidification, and freeze–thaw cycles on apparent GDNF concentration

Condition	% of value under standard conditions
Acidified to pH:	
pH 5	81.6 ± 1.7
pH 4	103.01 ± 3.0
pH 3	100.1 ± 1.0
pH 2	89.3 ± 3.8
pH 1	81.3 ± 2.5
Time at pH 3:	
0 min	42.5 ± 6.5
5 min	98.1 ± 0.7
15 min	100.0 ± 1.4
30 min	89.9 ± 1.8
60 min	72.1 ± 3.5
Neutralized to pH:	
pH 6	12.4 ± 0.8
pH 7	100.0 ± 1.4
pH 8	103.0 ± 2.5
pH 9	92.3 ± 4.7
Microfuge prior to acidification	94.6 ± 4.4
Freeze / thaw	
1 cycle	91.6 ± 1.9
2 cycles	81.1 ± 3.3
4 cycles	95.8 ± 2.9

When each factor was varied, the others were held constant.
Values are expressed as % ± SD of value obtained under standard conditions (pH 3, 15 min incubation, then pH 7).

Comparisons of GDNF levels between the same tissues from adult and postnatal or embryonic animals were made using two-sample *t*-tests.

3. Results

We wished to determine whether acidification was necessary for measurement of GDNF and if so, to optimize acidification conditions. Intestine from 12 postnatal-day 6 (P6) rats was homogenized and extracted as described above. Standard conditions were acidification to pH 3, incubation at room temperature for 15 min, and neutralization to pH 7. From these standard conditions, we varied the following parameters independently: the pH of acidification, the duration of incubation, and the pH after neutralization. The results are shown in Table 1.

The effect of varying the pH of acidification did not result in large changes in the GDNF values. Maximum values were obtained with pH 3–4 but little reduction was found at pH 1 or 5.

When the incubation time in acid was varied, the maximum value was obtained with a 15 min incubation. If the time was extended to 60 min, the GDNF level dropped to 72% of the maximum. If the extract was not acidified, the level was 42% of the maximal value. When the extract was microfuged before acidification, the level of GDNF measured was not altered, suggesting that most of the GDNF is soluble at the time of extraction.

Varying the pH after neutralization produced substantial changes on the levels of GDNF observed in our assay. The levels varied little between pH 7–9 but dropped to 12% of maximum when the extract was neutralized to only pH 6. Freeze and thaw of the samples four times did not reduce the levels of GDNF.

We have also tested the specificity of our ELISA for members of the TGF-β family. Even when TGF-β1 and TGF-β3 were applied in nanogram amounts, we found no detectable binding. We also added exogenous GDNF to determine the percentage of recovery. When 7.5 pg/ml was added, we obtained 75% recovery in adult intestine, 40% recovery in adult liver, and 101% in tubes without tissue.

We could distinguish concentrations as low as 1 pg/ml with a $P < 0.05$, and 3.9 pg/ml with $P < 0.001$. The sensitivity of the BCA protein assay was 0.031 mg/ml with $P < 0.005$. The coefficient of variation among triplicate wells was $< 7\%$ in 90% of cases for ELISA, and $< 14\%$ in 90% of cases for BCA.

We have measured GDNF in different tissues from adult rats (Table 2). The highest concentrations were found in the intestine, aorta, trigeminal nerve, and sciatic nerve; intermediate concentrations in the bladder and spinal cord; and low concentrations in the testis, whole brain, stomach, skeletal muscles, kidney, heart, spleen, and liver.

Table 2
GDNF concentrations in adult and developing rat tissues

Tissue		Adult rat		P21 rat		P2 rat		E17.5 rat	
		GDNF		GDNF		GDNF		GDNF	
		n	(pg/mg protein)	*n*	(pg/mg protein)	*n*	(pg/mg protein)	*n*	(pg/mg protein)
Intestine, str.[a]	A	9	2.818 ± 0.403	7	2.123 ± 0.414				
Mucosa	B	8	0.781 ± 0.205	4	0.208 ± 0.067				
Intestine	B	9	0.756 ± 0.106	4	0.435 ± 0.098	6	0.459 ± 0.084	1	0.294
Aorta		3	0.507 ± 0.222						
Trigeminal	C	6	0.399 ± 0.097	1	0.191				
Sciatic nerve	C,D	8	0.260 ± 0.084	2	0.452 ± 0.094				
Stomach, str.[a]	C,D	9	0.257 ± 0.073	5	0.305 ± 0.076	3	0.387 ± 0.122		
Bladder		3	0.186 ± 0.200			1	−0.245		
Ovary		1	0.133						
Spinal cord	C,D	9	0.113 ± 0.017	5	0.144 ± 0.027	1	0.276		
Blood		2	0.095 ± 0.116						
Testis	C,D	8	0.066 ± 0.013			1	1.896		
Brain	D	9	0.045 ± 0.013	7	0.038 ± 0.01	6	−0.011 ± 0.034	3	−0.023 ± 0.007
Stomach	D	12	0.043 ± 0.020	2	0.062 ± 0.033	3	0.302 ± 0.135	1	0.390
Muscle, skeletal	D	8	0.025 ± 0.005	4	0.095 ± 0.046				
Kidney	D	9	0.023 ± 0.008	6	0.068 ± 0.013	6	1.001 ± 0.153	1	4.646
Heart	D	9	0.023 ± 0.011	4	0.038 ± 0.008			1	−0.223
Spleen	D	9	0.011 ± 0.011	6	−0.002 ± 0.009	1	0.049		
Liver	D	12	0.009 ± 0.009	7	0.015 ± 0.011	3	−0.003 ± 0.006	3	−0.074 ± 0.018

Tissues are ranked, highest to lowest, according to GDNF level in the adult.
Values are expressed as mean ± SE. Adult values with the same letters are not significantly different while those with different letters are significantly different at $P < 0.05$. For instance, trigeminal nerve is different from brain while sciatic nerve is not different from brain. Samples with fewer than three observations were not included in the statistical analysis. All values obtained from 1 animal are preliminary.
[a] Stripped of mucosa.

After finding high concentrations of GDNF in homogenate of the entire small intestine, we attempted to further localize the source of GDNF. When the mucosa was removed, the levels in the muscular wall were increased four-fold over the levels in the whole intestine. The isolated mucosa also contained high levels of GDNF. Because GDNF is enriched in the smooth muscle portion of the intestine, we assayed other tissues containing smooth muscle such as aorta and bladder to determine if they also contained GDNF. Both the aorta and bladder contained large concentrations of GDNF. In contrast to other tissues with extensive smooth muscle, the stomach contained very low levels of GDNF (almost 20-fold lower than intestine). When stripped of mucosa, the stomach contained more GDNF, but was still 10-fold less than stripped intestine.

In another experiment to further localize the GDNF, we assayed different portions of the gastrointestinal tract. These tissues included the distal 2/3 of the esophagus, 1/2 of the stomach bisected along its longitudinal axis, the duodenum, a 15 cm length of jejunum 15 cm distal from the beginning of the jejunum (Ligament of Trietz), the distal 15 cm of ileum, and all of the colon. As shown in Table 3, the largest values are found in the colon and small intestine while low levels are found in the esophagus and stomach.

Human gut tissues were assayed and the GDNF concentrations were similar to those of rat intestine (Table 4). The highest concentrations were found in the muscular wall and the lowest values in the isolated mucosa.

GDNF found in the wall of the gut could be produced either in the muscle or the neuronal plexus. To distinguish these possibilities we investigated whether increases in gastrointestinal smooth muscle and loss of myenteric neurons would result in changes in GDNF levels. In previous experiments, serosal application of the surfactant BAC to the rat jejunum resulted in the destruction of longitudinal and much of the circular smooth muscle layer. The smooth muscle regenerated and after 10–14 days the number of cells in the longitudinal and circular muscle layer doubled (Fox et al., 1983; See et al., 1988). Most of the myenteric neurons were eliminated.

Animals were killed 12 days after BAC application. Samples of tissue were removed and fixed from BAC-treated and saline control animals. Examination of frozen sections showed a two-fold increase in the thickness of the muscular wall and an 82% reduction in the number of myenteric neurons in BAC-treated jejunum compared to saline-treated jejunum. The values for the changes in smooth muscle thickness and neuron number are similar to those previously reported (Fox et al., 1983; See et al., 1988). The presence of two glial markers, S-100 and glial fibrillary acidic protein (GFAP), was also reduced substantially in the myenteric plexus of BAC treated rats. The remainder of the treated and control tissues were homogenized and assayed. The concentration of GDNF was twofold higher in the BAC-treated jejunum compared to saline-treated control (Table 5). The concentrations in the saline control tissues are similar to those found in untreated jejunum, shown in Table 3. These data indicate that the GDNF concentrations increase about two fold in homogenates of gut which has undergone a two fold increase in the thickness of smooth muscle and a decrease in myenteric neuron number.

Changes in tissue levels were examined over the course of development. Tissues from embryonic day (E) 17.5, P2 and P21 rats were assayed (Table 2). At E17.5 the kidney contained the highest concentration followed by the intestine and stomach; low concentrations were found in brain, heart, and liver. At P2 the highest concentrations were found in the testis and the kidney. The intestine and stomach had equal concentrations at this stage. At P21 a pattern similar to that in the adult was found. The highest concentrations of GDNF were observed in the intestine, followed by stripped stomach, and trigeminal nerve and sciatic nerve. Fig. 1 shows the GDNF concentrations for brain, intestine, kidney, spinal cord, and stomach plotted against developmental age. Over the course of develop-

Table 4
GDNF concentrations in human jejunum and colon

Tissue	GDNF (pg/mg protein)			
	Colon	Colon	Jejunum	Jejunum
Intact gut	0.472	0.646	0.570	
Stripped gut[a]	0.868	0.911	0.968	0.139
Mucosa	0.225	0.294	0.324	0.044

Each column represents one individual.
[a]Stripped of mucosa.

Table 3
GDNF concentrations in different regions of the adult rat GI tract

Tissue	*n*		GDNF (pg/mg protein)
Esophagus	C	3	0.046 ± 0.043
Stomach	C	3	0.009 ± 0.071
Duodenum	B	3	0.533 ± 0.282
Jejunum	B	3	0.841 ± 0.203
Ileum	B	3	0.686 ± 0.045
Colon	A	3	1.513 ± 0.111

Values are expressed as mean ± SE.
Values with different letters are significantly different at $P < 0.05$.

Table 5
GDNF concentration in adult rat jejunum 12 days after BAC or saline treatment

Treatment	*n*	GDNF (pg/mg protein)
BAC	5	1.609 ± 0.112 *
Saline	4	0.839 ± 0.139

Values are expressed as mean ± SE.
* $P < 0.005$ compared with saline-treated group.

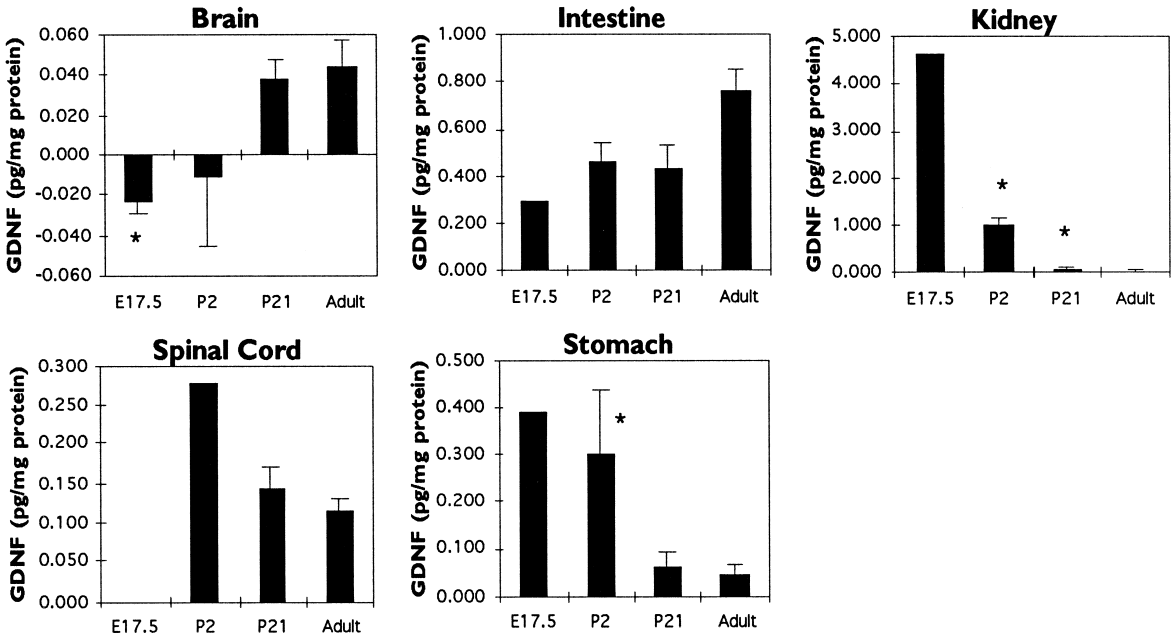

Fig. 1. GDNF levels (mean ± SE) in developing rat tissues. Where error bar is absent, $n = 1$. The asterisks indicate a difference from the adult value at $P < 0.05$ level of significance. Graphs have different scales.

ment the concentrations in the kidney and stomach declined as the age of the animal increased.

4. Discussion

This study was undertaken to determine the levels of GDNF in tissues of the adult and developing rat and in human gut tissue. We have used a two antibody ELISA to measure levels of GDNF immunoreactivity. To date investigators have measured GDNF mRNA in RNAase protection assays or localized it by in situ hybridization (Nostrat et al., 1996; Trupp et al., 1995). Most of the tissues examined have been from developing animals and only a few from mature animals. In order to determine the possible role of GDNF in the adult we have focused on measuring GDNF protein levels. In the adult we find the highest concentrations of GDNF in the intestine. The majority of the GDNF is in the muscular wall but some is associated with the mucosa. Others have found that GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and its receptor Ret (Schuchardt et al., 1994) are required for the development of the ENS, and the high levels found here are supportive of the hypothesis that GDNF may be necessary for the maintenance of the adult ENS. Consistent with this idea is the observation that the GDNF receptor, Ret, is found on adult rat myenteric neurons (Tsuzuki et al., 1995). Although we find abundant GDNF protein in the intestine, the mRNA levels in adult intestine have not been determined. In situ hybridization experiments indicate high levels of signal in the muscular wall of E17–P3 animals (Nostrat et al., 1996). It will be interesting to know the level and lifetime of GDNF mRNA in adult intestine. The high levels of GDNF found in the mucosa are perplexing. Very little or no label is found over the mucosa of postnatal animals studied by in situ hybridization (Nostrat et al., 1996). One possibility is that GDNF is bound by the cells or extracellular matrix of the mucosa. Further work will determine the relationship between GDNF and mucosal cells.

We find that treatment of the tissue extract from small intestine with acid approximately doubles the level of GDNF. However, GDNF was detectable without exposure to acid. Others have found that acid treatment increases the levels of growth factor measured. For instance, (Zettler et al., 1996) found large increases in NGF-immunoreactivity after acid treatment and suggest that this increase results from a dissociation of receptor from the growth factor. These authors suggest that much of the NGF uncovered after acidification was in a complex undergoing retrograde transport in nerve processes. Recently, Okragly and Haak-Frendscho (11) extended these observations and reported that acid treatment increases the amounts of GDNF, neurotrophin-3, and NGF detectable in a number of tissues. They also suggest that the increased amount of GDNF results from acid-induced dissociation of receptor from ligand.

Receptors for GDNF include a soluble glycosylphosphatidyinisitol-anchored escort protein (GDNFR-α) (Jing et al., 1996; Treanor et al., 1996) and a tyrosine kinase membrane protein (RET) (Durbec et al., 1996; Trupp et al., 1996). The acid treatment may dissociate the GDNF from either the RET or GDNFR-α (11). A dissociation of these receptors might increase the amount of GDNF bound

by either the monoclonal or polyclonal antibodies and result in larger GDNF levels. Although the monoclonal antibodies used here are able to recognize GDNF bound to GDNFR-α, as observed by Western blot analysis, the amount of GDNF bound by the monoclonals could be enhanced after dissociation of the GDNFR-α.

The GDNF values obtained in this ELISA depend on the values obtained with a standard protein. We used an unglycosylated, recombinant rat GDNF ($\sim$30 kD) for a standard, while the tissue extracts contain glycosylated, native rat GDNF ($\sim$40 kD). Different affinities of the antibodies for these two GDNF forms could yield error in the absolute values of GDNF, but not in the relative amounts found among the tissues. In addition, the difference in the molecular weights between the native and recombinant GDNF introduces error into the absolute levels of GDNF reported here. Extracts from some tissues had values less than background (negative values). Our interpretation is that proteins from these tissues bind to sites on the plate that would otherwise be bound nonspecifically by the rabbit antibody or secondary antibody.

Although GDNF is enriched in the muscular wall of the intestine, it is not clear whether smooth muscle and/or glia in the ENS are the sites of production in the adult. After treatment with BAC which ablated most of the myenteric plexus and increased the number of smooth muscle cells we find a proportional increase in GDNF concentration. Our results together with in situ hybridization observations in immature rat gut showing label over muscle but absent over enteric ganglia (Nostrat et al., 1996) strongly suggest that the GDNF is produced by smooth muscle cells in the gut. Thus we would predict that tissues with abundant smooth muscle would contain high concentrations of GDNF and most tissues including those with vascular smooth muscle do. However, the values in the stomach are notably low despite the smooth muscle in this organ. We suspect that some factor in this tissue interferes with the extraction of GDNF. The concentration of GDNF in the esophagus was also low but this observation might be expected because of the abundant skeletal muscle in this tissue.

The increase in GDNF after BAC treatment might also explain some previous observations. Dahl et al. (1987) found that the levels of neurotransmitters increase 22–45 days after BAC treatment although myenteric neurons are absent. Subsequent work showed that the submucosal neurons increase in size (See et al., 1988). An increase in GDNF resulting from the increased smooth muscle might be the reason for the hypertrophy of submucosal neurons.

Recent observations suggest glial structures in the ENS produce GDNF. Bar et al. (1997) reported that GDNF-like immunoreactivity was observed in structures resembling glial and Schwann cells in human fetal and adult gut. The increases in GDNF that we observed might result from an increased production by glial cells in the submucosal plexus that remain in the BAC-treated preparation although we feel this possibility is remote. With an ELISA, Bar et al. (1997) found concentrations of GDNF in human gut which were approximately 50% of those we obtained. However, they did not extract in acid and we found that acid treatment doubles the GDNF levels measured. It is possible that GDNF is produced by both glial cells and smooth muscle cells. Additional experiments are necessary to resolve this question.

The sciatic and trigeminal nerve preparations also contain high levels of GDNF. Transection of the sciatic nerve results in increases in GDNF mRNA (Trupp et al., 1995) and GDNFR-α mRNA (Trupp et al., 1996) which appear to be produced by the glial cells within the nerve. Although Trupp et al. (1995) reports very low levels of mRNA in the nerve before transection, we find appreciable levels of GDNF in the intact nerve. Consideration of these data suggest that low levels of mRNA may support the expression of significant levels of protein or that GDNF has a long halflife.

In view of this work, it appears promising that neurotrophic factors may play an important role in the gastrointestinal tract of adult animals. Kuffler (1994) has suggested that growth factors may direct nerve outgrowth during regeneration. In fact, enteric nerves are subjected to constant mechanical stretching and may require a factor such as GDNF to stabilize neuronal function.

Acknowledgements

We are grateful to Mr Jason Benjamin for his measurements on the BAC and control rats and to Mr David Potter for his assistance with the statistical analysis. Supported by NIH Grant NS 31385 and a Grant from the University of Wisconsin Graduate School.

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