Age-Related Loss of GABA-Positive and GABA-Negative Neurons in Neocortical Transplants

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SUMMARY

The numerical density of GABA immunopositive and GABA immunonegative neurons was quantitatively determined in 0, 12, 30 and 90 day-old neocortical transplants, derived from E17 rat embryos and transplanted into adult hosts. It was found that the original, very high neuronal density in the fetal transplant declined steadily after transplantation to the somatosensory cortex of adult rat. The decline in numerical density of GABA-positive neurons, however, was disproportionately larger than that of GABA-negative nerve cells: At 90 days the proportion of GABA-positive cells was 2.3% (in contrast to the 11.8% in the adult host cortex). The density of GABA-negative neurons, on the other hand, remained slightly higher than comparable values in the control cortex. The decline in density of GABA-positive neurons was continuous until the 90th post-transplantation day, while final, close to normal density values of GABA-negative nerve cells were already reached in 30 day-old grafts, with no significant change afterwards.

KEY WORDS

transplantation, neocortex, GABA-immunostaining, numerical density

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INTRODUCTION

We have demonstrated in previous studies /2,3,13/ that in long-term neocortical grafts the proportion of GABA-immunopositive neurons was significantly lower (2.9% of all nerve cells) than in normal cortex (11.8% on average). This decline of GABAergic interneurons in grafts was in strong contrast to the number of GABA-negative neurons which showed a normal or slightly increased density even in 6 month-old intracortical grafts /2/. A similar numerical decrease of the two main GABAergic neuronal classes, the parvalbumin and calbindin-containing nerve cells, has also been found in long-term neocortical grafts /6/. The relevance of these findings is closely related to the observation of the importance of GABAergic inhibitory systems in the normal functioning of neocortical networks /9,11/. Any numerical abnormalities of GABA-cells in neocortical grafts should, therefore, influence the potential of transplanted tissue to restore the function of the damaged area.

Several hypotheses were put forward to explain the selective loss of GABAergic neurons in long-term grafts. Imperfect integration of the graft with host cortex, leading to decreased afferent (and efferent) connectivity is one of the possible factors /1,2; see also 5,7/. A second alternative explanation is that, either due to unfavorable external conditions (i.e. hypoxia) during transplantation /10/ or because some GABAergic cells normally are expressed only postnatally, i.e. after the grafting process /4/, the number of GABA positive neurons is "*ab ovo*" lower than in the normally differentiating cortex. It is equally possible that the loss of GABA-positive nerve cells is not abrupt, but is a gradual process, affected both by selective

embryonic sensitivity and subsequent deficiency in normal afferentation. The aim of this study was, therefore, to determine numerical densities of GABA-positive and GABA-negative neurons in neocortical transplants at various intervals after grafting.

MATERIALS AND METHODS

Neocortical tissue for grafting was taken from embryos of Wistar rats of gestational age 16 to 17 days. A piece of tissue (1.5-2.0 mm^3) was dissected from the cortical anlage, including the provisory somatosensory cortex, and rinsed in cooled Eagle's solution. Altogether, 15 young adult Wistar rats (body weight 150-190 g) were used as recipients. All surgery was performed under pentobarbital (40 mg/kg, i.p.) anesthesia. After opening the skull, the barrel field (ca. 12 mm^3) was removed by aspiration. Subsequently, the embryonic tissue was placed through a slit in the dura into the acutely prepared cortical cavity.

The recipient animals were sacrificed for immunocytochemical examination by overdose of pentobarbital 12 days (5 animals), 30 days (5 animals) and 90 days (5 animals) after surgery. Transcardial perfusion was performed using 2.5% paraformaldehyde + 1% glutaraldehyde fixative in 0.1 M phosphate buffer (pH 7.2). The brain region containing the transplant was dissected by vibratome in serial blocks, 500 μm thick, which were then postfixed in the same fixative, washed in saline, postosmicated (1% buffered OsO_4) and embedded in Araldite.

Consecutive pairs of semithin sections, 1 μm thick, were cut with an ultramicrotome from each block. One section of each pair was stained by toluidine-blue, while the other section was used for postembedding immunocytochemical staining for GABA, according to the method described by Somogyi *et al.* /11/. The a-GABA antibody (made in rabbit) was purchased from the Arnel Products Co., (New York), characterized by Hepler *et al.* /8/.

In addition, pieces from five fetal cortical anlage (gestational age 16-17 days) were also fixed, embedded, cut and processed for immunocytochemistry as described above. The volume of the grafts was determined in toluidine-blue stained serial sections.

For calculation of numerical density of neurons/mm^3 their nuclei were drawn using toluidine-blue stained semithin sections with the help of a camera lucida at 700x magnification. The nuclei of GABA-immunopositive neurons were drawn from adjacent immunostained sections. This procedure was repeated in 10 areas (200 x 200 μm) in each section of a single graft, as well as in the host neocortex (through the whole depth of the cortex, from the pia to the white matter) and in fetal cortical anlage (the cortical plate, including the marginal zone, but not the subcortical plate). The area of the neuronal nuclei was measured by a Hipad digitizing tablet connected to an IBM-PC. Numerical density was calculated using the equation of Weibel and Gomez /14/ for ellipsoids:

$$N_v = (K/\beta)x((N_A)^{3/2}/V_V^{1/2})$$

where: N_A = number of nuclear profiles per unit area;
V_V = the volume (area) fraction;
K and β (coefficients) taken as 1.05 and 1.39, respectively.

The numerical density of GABA-immuno-negative neurons was calculated by subtracting the number of GABA-immunopositive neurons from the total number of neurons.

The N_V data of the "control" cortex — taken from the adult host neocortex of 7-8 month-old animals — as well as the N_V data of the embryonic (E 16/17) cortical tissue were established in an earlier experiment [Bragin *et al.* /3/].

RESULTS

The volume of all grafts exhibited in all cases a gradual increase; compared to the original 1.5-2.0 mm^3 volume, the volume of average grafts had doubled at 30 days, and approximated 10 mm^3 at 90 days.

The fetal neocortical tissue appeared to be densely packed with both GABA-negative and GABA-positive (GABA-ir) nerve cells (Table 1). The neuronal densities were significantly higher (see Table 2), by approximately one order of

TABLE 1
Numerical density (No./mm³) of GABA(+) and GABA(-) neurons in the grafted embryonic, control (adult host SI) cortex and in grafts 12, 30 and 90 days after transplantation

Age (origin) of tissue (transplants)	Density of GABA(+) neurons	Density of GABA(-) neurons	%* of GABA(+) neurons
E-17 embryos**			
Mean ± S.D.	62542 ± 25010	710526 ± 185260	8.1
Control cortex**			
Mean ± S.D.	5605 ± 1353	41708 ± 7816	1.8
12 day-old transplants			
1	3740	88475	4.1
2	6623	95553	6.5
3	9614	101429	8.7
4	2528	90617	2.7
5	3731	97569	3.7
Mean ± S.D.	5247 ± 2869	94729 ± 5236	5.2
30 day-old transplants			
6	840	48539	1.7
7	2768	62629	4.2
8	4749	49862	8.7
9	1752	45168	3.7
10	3117	61678	4.8
Mean ± S.D.	2645 ± 1467	53575 ± 8023	4.7
90 day-old transplants			
11	1912	54287	3.4
12	765	51049	1.5
13	1893	48082	3.8
14	509	51139	1.0
15	897	51945	1.7
Mean ± S.D.	1195 ± 661	51300 ± 2224	2.3

* $\frac{N_V \text{ GABA(+)} * 100}{(N_V \text{ GABA(-)} + N_V \text{ GABA(+)})}$

**5 animals in each group [Bragin *et al.* /3/]

magnitude (GABA(-): 710526 ± 185260 / mm³; GABA(+): 62542 ± 25010 / mm³) compared to the control, adult host cortex (GABA(-): 41708 ± 7816 / mm³; GABA(+): 5605 ± 1353 / mm³). The GABA-ir neurons constituted 8.1% of all nerve cells.

The adult host somatosensory cortex exhibited a relatively even distribution of GABA-ir neurons (Fig. 3) particularly in the 5 inner cortical layers. GABA-positive nerve cells constituted 11.8 % of all neurons in the host cortex [Bragin *et al.* /3/].

In the 12 day-old cortical graft, which did not show the characteristic cortical lamination (Fig. 1), the density of GABA-ir neurons was visibly lower than that in the control cortex. However, the GABA-positive neuronal perikarya appeared to be larger than those in the host cortex. Quantitative measurements clearly showed that, although the density of GABA-ir neurons in 12 day-old grafts was comparable to the control values (5247 ± 2869 versus 5605 ± 1353 / mm³), due to the still high density of GABA-negative neurons (94729 ± 5236

TABLE 2
Statistical comparison* of differences in the numerical densities of GABA(+) and GABA(-) neurons in the grafted embryonic, control (adult host SI) cortex and in 12, 30 and 90 day-old transplants

	Control	12 days o.	30 days o.	90 days o.
GABA(+) neurons				
E17	0.0001<p<0.001	0.0001<p<0.001	0.0001<p<0.001	0.0001<p<0.001
Control		p>0.5 (N.S.)	0.01<p<0.02	0.0001<p<0.001
12 days o.			0.1<p<0.2	0.01<p<0.02
30 days o.				0.05<p<0.1
GABA(-) neurons				
E17	p<0.0001	p<0.0001	p<0.0001	p<0.0001
Control		p<0.0001	0.02<p<0.05	0.02<p<0.05
12 days o.			p<0.0001	p<0.0001
30 days o.				p>0.5 (N.S.)

*Two-sample analyses. The significance levels were calculated by the two-tailed Student's t-test.

Fig. 1: GABA-immunoreactive neurons in 12 day-old cortico-cortical graft. The grafted tissue (G) can be distinguished from the host cortex (H). The GABA-ir cells are intensively labeled in the graft; GABA-positive cells in the cortex near to the border are stained somewhat weaker (arrows). Scale bar: 100 μm.

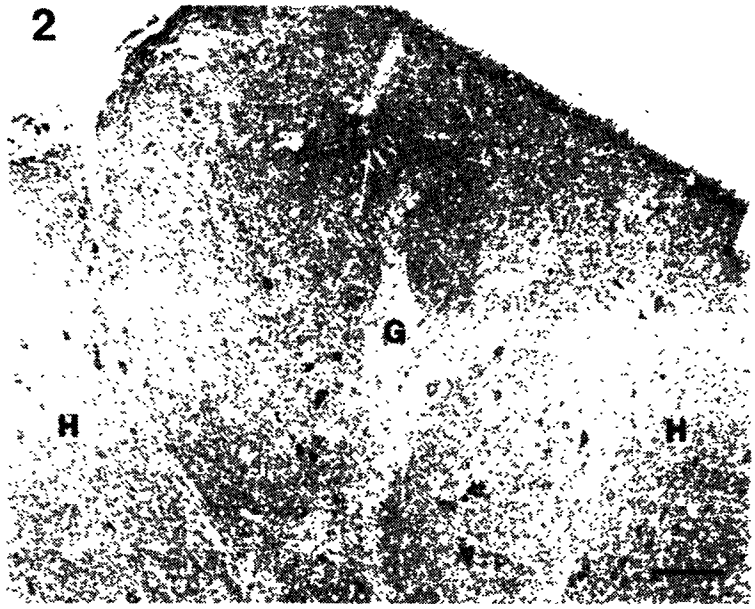

Fig. 2: 90 day-old intracortical transplant. The number of GABA-positive neurons in the graft (G) is lower than in the host cortex (H). Note that the size of GABA-ir neurons in the graft is larger than in the host tissue. Scale bar: 175 μm.

/ mm³), the relative proportion of GABA-ir neurons dropped to a mere 5.2%.

At 30 days the density of both GABA-ir (2645 ± 1476 / mm³) and particularly of GABA-negative (53575 ± 8023 / mm³) nerve cells further decreased. The numerical density of GABAergic neurons dropped to less than half of the control value, while the concentration of GABA-negative neurons, in spite of a conspicuous numerical decline in density, exhibited a significantly ($0.02 < p < 0.05$; Table 2) higher N_V than the control values.

At 90 days (Fig. 2) a further decline in the numerical density of GABA-ir neurons (1195 ± 661 / mm³) and a stabilization of the density values of GABA-negative nerve cells (51300 ± 2224 / mm³), which were still slightly higher than the control values, was observed. The calculated 2.3% for GABA-ir cells of all neurons was only about one fifth of the comparable control value.

Fig. 3: GABA-immunopositive neurons in the somatosensory cortex of adult rat. The GABA-ir cells are relatively evenly distributed in the inner cortical layers and can be well distinguished from the GABA-negative neurons. P: pia; WM: white matter. Scale bar: 100 μm.

DISCUSSION

These observations demonstrate a significant drop in both GABA-positive and GABA-negative neuronal densities in the differentiating intracavity neocortical grafts. This decrease in the numerical densities is obvious (and statistically highly significant) when comparing the N_V data of the E17 embryonal cortices with the N_V values of the grafts. One factor, in fact, which might lead to the observed decline in numerical density of neurons during and/or after transplantation might be hypoxia/anoxia, which is probably also effective during the early period after transplantation, when conditions (e.g. vascularization) are still not optimal for survival of the grafted neuronal population. (This transient period may persist for several days, since in the 6 day-old transplants there was a high number of degenerating nerve cells and cell debris, which made it impossible to evaluate the N_V data at this age in the grafts.) The observed decline in the numerical densities — mainly in the density of the GABA-positive neuronal population — is, most probably, the result of the loss of these cells. At the same time, the ongoing differentiation of the graft as connections are made by the newly formed processes of the surviving nerve cells, results in the enlargement of the neuropil region. This is clearly shown by the increased volume of the grafts, which also contributed to the dilution of cells in the differentiating grafts. The steady decrease of the density of GABA-negative neurons in the graft could be observed until 30 days after transplantation. In the case of the GABA-ir neurons, however, the decline in N_V continued until 90 days. Although this numerical decline of GABA-ir cells occurred simultaneously with the dilution of density of GABA-negative neurons, the loss of GABAergic interneurons was disproportionately larger. In fact, while the density of GABA-negative neurons remained somewhat higher than comparable values in the host cortex, both at 90 days, as observed in the present study, as well as in the 6-8 month grafts /2/, the numerical density of GABA-ir cells was only one fourth to one fifth of the control values. Statistically, this decrease in the numerical densities of GABA-ir cells was not (host cortex compared to 12 day-old transplant) or was only moderately (12 day-old graft compared to 30 day-old and 30 day-old compared to 90 day-old) significant because of the high variability within the age groups. The decrease was highly significant (12 day-old graft compared to 90 day-old) between younger (12 day) and older (90 day) transplants. The decline in N_V of GABA-negative neurons proved to be highly significant in each comparison between the different age groups of the transplants. The decline in density, however, did not occur synchronously in the two neuronal classes: GABA-ir cell number exhibited a steady and continuous decline between 0 day and 90 days, whereas the final density values for GABA-negative neurons were already established at 30 days, with no significant changes afterwards.

Further density changes in older grafts were not examined in the present investigation. In previous studies /2,3/, however, it was observed that the proportion of GABA-positive cells is maintained between 2-3% in 6-8 month grafts.

Another factor which might contribute to the decrease of the GABA-ir neuronal population could be a moderate or missing expression and/or activity of glutamate decarboxylase (GAD), since the development of the GABA synthesizing enzyme is known to depend on the normal innervation of the cortex /7/. The lack of neuronal activity or lack of extrinsic innervation may, therefore, result in reduced levels of GAD and GABA in the transplants at longer survival times. This would mean that the cells are not actually absent, but express low levels of GABA and are consequently counted among the unlabeled cells (the density of which decreases for a shorter period of time).

In conclusion, the present observations demonstrate that in differentiating neocortical transplanted tissue the decline in the density of GABAergic neurons is a time-dependent process, which in long-term transplants results in a relatively low, but stabilized number of GABAergic cells.

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