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Gamma-aminobutyric acid (GABA) receptor binding selectively depleted by viral induced granule cell loss in hamster cerebellum

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Excitatory and inhibitory synaptic interconnections and related neurotransmitters have been more thoroughly studied in the cerebellum than in any other brain region. Of the 5 intrinsic neuronal types in the cerebellar cortex, four (the Purkinje, Golgi, basket and stellate cells) are inhibitory and appear to utilize GABA as their neurotransmitter[4,5,9,11,17–19]. The remaining inhibitory element is composed of noradrenergic fibers which arise from the locus coeruleus and comprise about 1% of the climbing fiber population[3].

There are 3 excitatory elements in the cerebellar cortex, the granule cells which appear to use glutamic acid as their transmitter[21] and the mossy and climbing fibers whose transmitters are unknown. All cerebellar neurons seem to receive GABA containing inhibitory terminals[5]. Those on the granule cell dendrites appear to arise predominantly and probably exclusively from the Golgi II cells.

The density of GABA receptors upon various cell types in the cerebellum may regulate the prevalence of various types of inhibitory synaptic interconnections. Recently, we have identified selective binding of GABA to its postsynaptic receptor sites in the brain[6,7,22]. To ascertain the density of GABA receptors associated with various cell types in the cerebellum, it would be desirable to destroy individual cell types selectively. Several experimental models have been developed to deplete selectively cerebellar granule cells. In the present study we have examined synaptic receptor binding of GABA in the cerebella of hamsters treated with a parvovirus, rat virus, which selectively destroys the rapidly dividing external germinal cells to produce a cerebellum in which only the granule cell population is depleted. We report a selective decline in synaptic GABA receptor binding which parallels the decrease in granule cell number.

Granuloprival hypoplasia of the Syrian hamster cerebellum resulted after intracerebral injection of rat virus strain PRE (HA titer 2^{-12}) into the left hemisphere[21] of

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newborn animals 0–16-h-old. For microscopic confirmation of the desired hypoplasia, the cerebella of the viral treated hamsters (20–45 days after treatment) or their littermate controls were quick frozen on a freezing microtome chuck and sectioned at 6 μm. These sections were fixed to slides, air dried and stained with hematoxylin–eosin. Littermate animals were anesthetized with ether and perfused through the left ventricle with a mixed aldehyde solution composed of 1% paraformaldehyde, 2% glutaraldehyde and 1% acrolein in 0.1 *M* cacodylate buffer containing 669 mg $CaCl_2$ dihydrate/liter. After perfusion, brains were carefully removed from the skulls, bisected and processed for light and electron microscopy as previously described[21].

For determination of [^{3}H]GABA binding to postsynaptic receptors the hamsters were decapitated, their cerebella or cerebral cortices rapidly removed and homogenized for 20 sec with a Brinkmann Polytron in 20 vol. (w/v) of 0.05 *M* Tris-citrate buffer pH 7.1. The homogenate was centrifuged for 10 min at 49,000 $\times$ *g* at 0 °C, and the pellet was frozen at —20 °C. This frozen tissue was assayed for sodium-independent GABA binding according to the procedure described previously[6,7,22]. Aliquots of the frozen tissue (0.8–1.2 mg protein) were incubated in triplicate at 4 °C for 5 min in 2 ml of 0.05 *M* Tris-citrate buffer, pH 7.1 at 4 °C containing 25 m*M* [^{3}H]GABA (New England Nuclear Corp., specific activity 36.7 Ci/mmole). The specific [^{3}H]GABA binding was determined by the amount of the radioactivity that could be displaced by 1 m*M* non-radioactive GABA. After incubation the reaction was determined by centrifugation for 10 min at 48,000 $\times$ *g*, the supernatant fluid was decanted and the pellet rinsed rapidly with 5 ml then 10 ml ice cold distilled water. Bound radioactivity was extracted into 1 ml of Protosol (New England Nuclear Corp.), 15 ml toluene phosphor were added and the radioactivity assayed by liquid scintillation spectrometry at 35% efficiency. The binding of the opiate antagonist [^{3}H]naloxone to opiate receptors[15,16] of [^{3}H]lysergic acid diethylamide (LSD) to serotonin receptor binding sites[1,2], and of [^{3}H]quinuclidinyl benzilate (QNB) to muscarinic cholinergic receptors[20], was determined as described previously. The content of glutamic acid in the tissue was assayed accordingly to Graham and Aprison[8] as described[21].

As observed previously[21], animals inoculated with rat virus after birth and killed between 21 and 40 days of age possess a cerebellum in which approximately 99% of the normal number of granule cells are absent from the internal granular layer. Other neuronal and glial cell types are essentially unaltered when compared to littermate control animals, as described in our previous studies[21]. Since all cerebellar cortical cell types except the granule cells appear to utilize GABA as their neurotransmitter and possess multiple synaptic interconnections, it is particularly important to verify that cell loss after virus treatment is restricted to granule cells. In an extensive light and electron microscopic study[12,13], we found that no more than 10% of basket, stellate, Purkinje or Golgi cells were absent in granuloprival hamsters.

As reported earlier[21], the selective loss of granule cells is accompanied by a 50% fall in endogenous levels of glutamic acid (Table I). Because there is no decline in the endogenous levels of other amino acids examined and because high affinity sodium dependent glutamic acid uptake in synaptosomal fractions of the cerebellum is also markedly reduced[21], these findings agree with the previous suggestion that the neuro-

TABLE I

ENDOGENOUS GLUTAMIC ACID CONTENT AND SODIUM INDEPENDENT [^{3}H]GABA BINDING IN THE CEREBELLUM OF GRANULOPRIVAL HAMSTERS AND THEIR LITTERMATE CONTROLS

Endogenous glutamic acid content of tissue extracts was determined according to Graham and Aprison[8]. Specific sodium independent [^{3}H]GABA binding was determined by incubation of 25 n*M* [^{3}H]GABA (31.7 Ci/mmole) with 1 mg tissue protein in 0.05 *M* Tris–citrate buffer, pH 7.1 at 4 °C and the specific binding is the radioactivity displaced by 1 m*M* non-radioactive GABA. Data are the mean values ± S.E.M. from 4–8 hamsters with each determination conducted in quadruplicate. Values varied less than 15%.

	Endogenous glutamic acid (μmoles/g)	*Specific sodium independent [^{3}H]GABA bound (pmoles/g)*
Experiment 1		
Normal hamsters	10.8 ± 0.5	860 ± 75
Granuloprival hamsters	5.9 ± 0.3*	330 ± 40*
Experiment 2		
Normal hamsters	11.6 ± 0.4	810 ± 105
Partial granuloprival	9.1 ± 0.4**	615 ± 60**
Maximal granuloprival	5.4 ± 0.3*	315 ± 60*

Granuloprival values are significantly lower than values for normal hamsters: * $P < 0.001$, ** $P < 0.05$.

transmitter of the granule cell may be glutamic acid. This profound loss of granule cells is accompanied by a 60–75% decline in specific GABA receptor binding in the cerebellum (Table I). To determine if the depletion of GABA receptor binding is related to granule cell loss, it would be desirable to ascertain if the loss of GABA receptors parallels the decline in granule cells. Granule cell depletion is only partial if rat virus is administered intracerebrally to the hamsters 6–16 h after birth. With the partial depletion of granule cells produced by this regimen, the decline in GABA receptor binding is less marked than in hamsters with a maximal loss of granule cells (Table I).

In receptor binding studies, it is crucial to show that the characteristics of ligand interactions correspond to the known physiology of the synaptic receptor site. Thus, while β-alanine and 2,4-diaminobutyric acid are effective inhibitors respectively of glial and neuronal uptake systems for GABA, neither has substantial GABA-like neurophysiologic activity. Similarly, though glutamic acid has substantial affinity for the GABA synthesizing enzyme glutamate decarboxylase, it has negligible neurophysiologic activity at GABA synapses, whereas bicuculline is a potent GABA receptor antagonist. In both normal and viral-treated hamster cerebella, the relative affinities of GABA and other compounds for the sodium independent [^{3}H]GABA binding sites are similar to those described for the postsynaptic GABA receptor of whole rat brain[6,7,22] (Table II). Thus, half-maximal inhibition of [^{3}H]GABA binding is attained in the hamster cerebellum with about 0.6 μ*M* GABA concentration and about 4 μ*M* concentration of bicuculline. By contrast, half-maximal inhibition requires 70 μ*M* concentrations of β-alanine and no detectable inhibition is obtained with concentrations as high as 1 m*M* of glutamic acid and 2,4-diaminobutyric acid.

TABLE II

SUBSTRATE SPECIFICITY OF SODIUM INDEPENDENT [^{3}H]GABA BINDING TO CEREBELLUM OF NORMAL AND GRANULOPRIVAL HAMSTERS AND RAT BRAIN

Inhibition of [^{3}H]GABA binding by various concentrations of different compounds was determined. ID_{50} values are the concentration of compound which inhibits 50% of specific sodium independent [^{3}H]GABA binding and calculated by log-probit analysis. Values are the means of 2–4 determinations using 4–6 concentrations of each drug in triplicate.

Compound	*ID_{50} (μM)*		
	Cerebellum of normal hamsters	*Cerebellum of granuloprival hamsters*	*Whole rat brain**
GABA	0.61	0.60	0.37
Bicuculline	4.6	3.8	4.0
β-Alanine	72	70	80
Glutamic acid	> 1000	> 1000	—
2,4-Diaminobutyric acid	> 1000	> 1000	> 1000

* Values taken from Enna and Snyder[7].

Accordingly, it appears that the sodium independent GABA binding measured to membrane fractions of hamster cerebellum corresponds to the postsynaptic GABA receptors described previously in rat and monkey brain. Moreover, though the amount of GABA receptor binding is markedly reduced in viral-treated hamsters, the relative affinity of various agents for receptor sites is unaltered.

To determine if the decline in GABA receptor binding represents a change in the affinity of receptor sites for GABA or an alteration in the number of binding sites, we examined sodium independent binding of various concentrations of [^{3}H]GABA to membrane preparations in normal and viral-treated animals in both the cerebellar and cerebral cortex (Fig. 1). In the cerebellum of viral-treated animals there is a 65–70% decline in the number of binding sites, whereas their apparent affinity for GABA is unchanged. The specificity of the viral effect upon cerebellar GABA receptors is indicated by the absence of any change in either receptor number or affinity of GABA receptors in the cerebral cortex in viral-treated animals.

As a further test of the specificity of the change in GABA receptors, we measured receptor binding associated with other neurotransmitters in normal and viral-treated hamster cerebral cortex and cerebellum (Table III). [^{3}H]Naloxone is an opiate antagonist which can be used to label the opiate receptor[15,16] which may be the receptor site for a normally-occurring morphine-like peptide which may function as a neurotransmitter or neuromodulator[10,14]. [^{3}H]LSD can be used to label specific binding sites which appear to be associated with the postsynaptic serotonin receptor[1,2]. [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) is an effective label for the muscarinic cholinergic receptor in the brain[20]. In these series of experiments the marked reduction in synaptic GABA receptor binding in the cerebellum of viral-treated hamsters is confirmed, with no decline in GABA binding demonstrable in the cerebral cortex. Specific

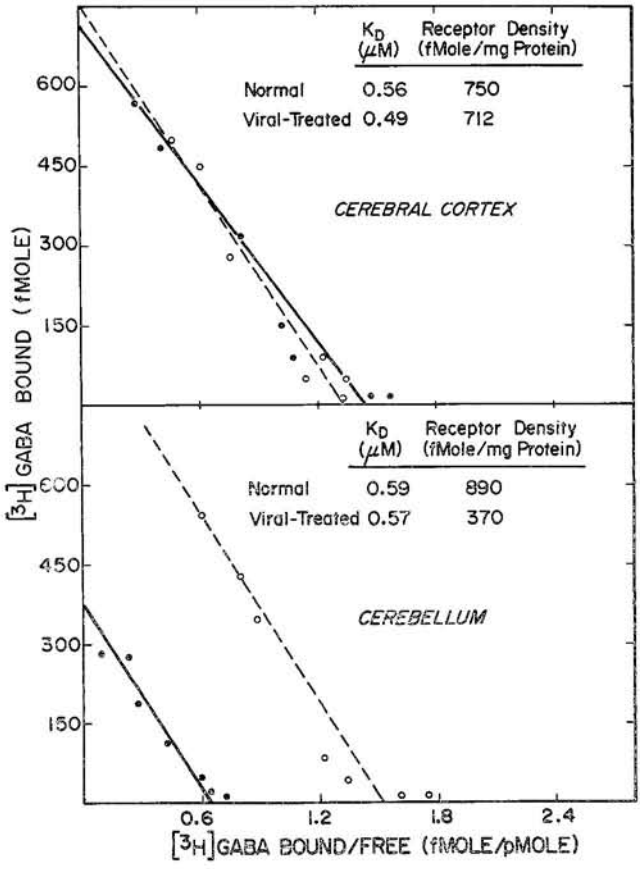

Fig. 1. Scatchard plot of the sodium independent binding of [^{3}H]GABA to cerebral cortex and cerebellum of granuloprival hamsters and their littermate controls. Tissue homogenates (1.0 mg protein/tube) were incubated in 0.05 *M* Tris-citrate buffer, pH 7.1 at 4 °C, containing different concentrations of [^{3}H]GABA. The specific binding was determined as the radioactivity that was displaced by 1 m*M* non-radioactive GABA. The experiment was carried out twice.

TABLE III

RECEPTOR BINDING OF [^{3}H]NALOXONE, [^{3}H]LSD, [^{3}H]QUINUCLIDINYL BENZILATE (QNB) AND [^{3}H]-GABA IN CEREBRAL CORTEX AND CEREBELLUM OF NORMAL AND GRANULOPRIVAL HAMSTERS

Tissue homogenates (1.0 mg protein/tube) were incubated in the presence of 25 n*M* [^{3}H]GABA, 2.5 n*M* [^{3}H]naloxone, 8 n*M* D-[^{3}H]LSD, or 4 n*M* [^{3}H]QNB. The specific binding was determined as the counts/min of the radioactivity bound to 1.0 mg protein that was displaced by 1 mM GABA or 1 μM of naloxone, D-LSD or QNB, respectively. The assays were performed as described previously[2,15,20]. Values are the means ± S.E.M. from 3 hamsters.

	Normal hamsters		*Viral treated hamsters*	
	Cerebral cortex	*Cerebellum*	*Cerebral cortex*	*Cerebellum*
[^{3}H]GABA	1650 ± 120	2605 ± 150	1735 ± 225	1225 ± 157*
[^{3}H]Naloxone	380 ± 45	35 ± 20	370 ± 40	40 ± 25
[^{3}H]LSD	445 ± 30	50 ± 35	470 ± 45	45 ± 20
[^{3}H]QNB	480 ± 65	40 ± 30	465 ± 60	30 ± 25

* Significantly lower than values for cerebellum of normal hamsters $P < 0.001$.

binding of [^{3}H]naloxone, [^{3}H]LSD and [^{3}H]QNB in cerebral cortex and cerebellum is unaltered by viral treatment.

In summary, a selective depletion in granule cells from the hamster cerebellum is associated with a marked loss of postsynaptic GABA receptor binding. The decline in the density of GABA receptors is selective, since there is no change in the cerebral cortex and several other neurotransmitter receptors are not altered by viral treatment.

While the marked loss of postsynaptic binding sites seems to be a direct result of the loss of postsynaptic sites on the granule cells themselves, there are alternative possibilities. The marked imbalance between excitatory and inhibitory activity resulting from the loss of the principal excitatory element in the cortex could result in the failure of the other cell types to elaborate the normal amount of GABA receptors, *i.e.*, with a marked decrease in excitatory input, a cell might, in order to maintain a balance between excitatory and inhibitory input, fail to develop its normal complement of inhibitory receptors.

If the loss of GABA receptors results exclusively from the loss of granule cell postsynaptic surface, it would appear that 70% of the GABA receptors are on the granule cell dendrites themselves. If this is the case, the Golgi-granule synapses could be quantitatively the major inhibitory synapses within the hamster cerebellum, emphasizing the importance of the Golgi cell for inhibitory cerebellar function. Interestingly, the Golgi cell is the only neuron in the cerebellum which receives synaptic terminals from each of the excitatory inputs to the cerebellum, the climbing and mossy fibers. However one must be cautious in attributing the loss of GABA receptors solely to granule cell depletion. In an extensive investigation essentially no loss of cerebellar cells other than granule cells could be detected in virus treated hamsters[12,13]. However, a loss of up to 10% of Purkinje, stellate, basket and Golgi cells could have escaped detection and might contribute to the decline in GABA receptor binding after virus treatment.

Although receptor sites are generally thought to provide an indication of the presence of synapses, one cannot be certain that the number of receptors always reflects the density of synaptic connections. The number of receptor binding sites for a neurotransmitter may vary markedly at different synapses. Moreover, it is possible that receptor density may be independent of the number of synapses so that a cell receiving one nerve terminal of a particular type may possess as many receptors as a cell receiving 100 such nerve terminals. It is even conceivable that cells completely devoid of synaptic input from a particular type of neurotransmitter-specific neuron may still possess receptors for it. Still, the very profound decrease in GABA receptor binding associated with the loss of only one of the many cell-types within the cerebellum suggests that GABA synapses upon granule cells are important for cerebellar physiology. Whether the association of GABA receptors with granule cells reflects a high density of GABA receptors per granule cell or is attributable primarily to the large number of granule cells in the cerebellum is unclear.

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