Cross-reactions among the T antigens of papovaviruses have not been reported previously. Therefore, it was necessary to investigate the cause of the observed reaction between the cultured hamster tumor cells and SV40 T antiserum. The relationship between JC and SV40 T antigens was investigated in PHFG cultures, and it was established that the T antigens of these two viruses are related. Cultures of PHFG were inoculated with a mixture of JC virus and a potent SV40 neutralizing antiserum. Twenty days later, many cells in the culture contained a nuclear antigen that reacted with the SV40 T antiserum in immunofluorescent tests (*6*).

One tumor cell line, HJC-15, started from a mixed malignant glioma of astrocytic and ependymal derivations, was selected for further study. No infectious virus was recovered from this cell line when a sonicated suspension of 5×10^6 cells at the 12th passage level was inoculated into PHFG cell cultures (42-day observation period).

JC virus was rescued from the 14th subculture of HJC-15 cells after fusion with PHFG cells with the use of Sendai virus inactivated with β-propiolactone. Suspensions of 2×10^6 HJC-15 cells were fused with 3×10^6 PHFG cells or 2×10^6 Vero cells with the use of 4000 hemagglutinating units of Sendai virus in 2 ml (*7*). Each fused cell suspension was planted in two plastic flasks (250 ml) in maintenance medium. After 16 days of incubation at 37°C the cells were disrupted by freezing and thawing followed by sonication. A thick suspension of cell debris from the HJC-15 : PHFG fused cultures was layered on PHFG cells in Leighton tubes, while that from the HJC-15 : Vero fused culture was layered on CV-1 cells. These cultures were fixed 21 days later and tested for viral coat protein antigens with immunofluorescent techniques using antiserums against both JC and SV40 viruses. Many cells in the PHFG cultures contained antigen that reacted with JC virus antiserum only, whereas the CV-1 cells were negative with both antiserums.

This initial experiment demonstrates that a human papovavirus (JC), which is associated with a demyelinating disease of the brain, is capable of inducing malignant gliomas in a high percentage of hamsters inoculated intracerebrally as newborns. The majority of these gliomas are of types not previously reported to be induced by papovaviruses in in vivo experiments. In hamsters, SV40 virus induces glial tumors of the ventricular lining only (ependymomas, papillary ependymomas, or plexus papillomas), while polyoma virus induces mesenchymal tumors in the brain (*8*). The rescue of JC virus from hamster tumor cells by fusion with permissive cells is evidence for the continued presence of the JC virus genome in the cultured tumor cells.

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Stimulus-Coupled Secretion of γ-Aminobutyric Acid from Rat Brain Synaptosomes

Abstract. *Synaptosomes treated with radioactive γ-aminobutyric acid can be stimulated to release this substance. The release is maximal within 40 seconds after stimulation and is dependent on calcium. Magnesium and manganese ions, known to block stimulus-secretion coupling processes, depress calcium-dependent release. This release is specific to synaptosomes because microsomal or myelin fractions do not release accumulated γ-aminobutyric acid. The data illustrate a simple in vitro system suitable for analysis of secretion of γ-aminobutyric acid in brain and in addition describe several new aspects of uptake and secretion of this compound at brain nerve endings.*

Understanding the mechanism of synaptic transmission is essential for understanding brain function. Although synaptic transmission is chemical in nature, few chemical techniques are available to directly study the process. In particular we need to understand (i) the nature of the secretory process; (ii) the nature of the transmitters secreted; and (iii) the action of drugs which affect neuronal transmission. Ideally in order to study function at chemical level, it is essential to study the synapse directly. Centrifugation techniques have made it possible to isolate presynaptic endings (synaptosomes) in abundance and in fairly pure fractions from the central nervous system. It has now been demonstrated that the function of synaptic endings can be studied in vitro under a wide range of controlled conditions.

Studies from several laboratories indicate that putative transmitters are released from synaptosomal fractions (*1*, *2*). However, specific criteria required for demonstration of transmitter release have not yet been met. Investigations on neurosecretory activity of isolated synaptosomes require that a number of criteria must be met in order to assume that the observed chemical changes are due to stimulus-induced secretion: (i) The appropriate stimulus should trigger a very rapid efflux of neurotransmitter; (ii) the secretion should be Ca-dependent; (iii) the secretion should be blocked by Mg and other antagonist divalent ions; (iv) the secretion should be specific to synaptosomes; and (v) the Ca-dependent secretion should be distinguished from possible artifacts of the system such as blockage of reuptake and surface exchange. All of these criteria are general properties of stimulus-secretion coupling processes found in various systems in vivo (*3*).

We have characterized a functional synaptosomal system for the release of γ-aminobutyric acid (GABA) which meets all of the criteria listed above. To study transmitter release, synaptosomes

previously treated with radioactive GABA were immobilized on a membrane filter, and the particles were rapidly perfused with media of different compositions. Fractions were collected at 20-second intervals before and after stimulation with various media. This approach provided a rapid profile of transmitter efflux elicited by the various media.

Synaptosomes were purified from rat brain (male Sprague-Dawley rats, 40 to 60 days old) on a Ficoll-sucrose gradient (*4*), washed twice with a modified Ringer solution (*5*), and resuspended in the Ringer solution at a concentration of 1 mg of synaptosomal protein per milliliter. The synaptosomes were incubated with 0.5 μM [^{14}C]GABA (203 mc/mmole; New England Nuclear) for 30 minutes at 25°C in the presence of 1 mM aminooxyacetic acid in order to minimize GABA metabolism (*6*). The synaptosomes were then trapped on a fiber-glass filter system in a Swinnex filter unit and rapidly perfused with media of different compositions. In other experiments as specified, samples were placed on the filter system and incubated for 20-second intervals with various washing and stimulating media. The effluents were then rapidly collected by applying a vacuum. Radioactivity in the effluent was counted in a medium containing Triton and toluene (*7*) and corrected with channels ratio method, while radioactivity in the filters was measured in the same fluid after overnight solubilization in 2 ml of a mixture of 1 percent sodium dodecyl sulfate in 20 mM EDTA. Under conditions of our experiments, 95 percent of the released radioactivity was associated with GABA, as assayed by paper chromatography in two different solvent systems [phenol and water (*8*) and 2-propanol and water (*9*)]. Protein was measured by the method of Lowry *et al.* (*10*).

Stimulation of synaptosomes triggers a very rapid Ca-dependent efflux of transmitter. Measurement of the rate of [^{14}C]GABA efflux from synaptosomes in 20-second increments before and after Ca stimulation in the presence of depolarizing concentrations of K (56 mM) permits an unambiguous demonstration of Ca dependence since Ca is the sole stimulus. Rapid introduction of 2 mM Ca initiates a rapid release of transmitter (Fig. 1). Release begins to take place within the first 20 seconds and is maximal by the first 40 seconds. Considering the rate of flow (0.52 ml/min), and the dead volume in the top of the Swinnex filter unit (3 ml), release appears to begin taking place well within the first 20 seconds after Ca concentrations on the filter are increased. Continuous perfusion with Ca releases 60 percent of the total radioactivity in the first 8 minutes. A further demonstration of the dependence on Ca was provided by the effect of Ca concentration on release. The Ca-stimulated release rose sharply up to 1 mM and became asymptotic near physiological concentrations of Ca. This observation is in agreement with the asymptotic Ca dependence of central nervous system secretion demonstrated in vivo (*11*).

The Ca-dependent release is blocked by Mg and Mn. Magnesium (16 mM) depressed Ca-dependent secretion by 50 $\pm$ 5 percent ($N = 3$). Manganese is as effective as Mg (55 $\pm$ 3 percent; $N = 2$) at a fraction of the concentration (1 mM). Alone Mg has little effect on the rate of GABA efflux (5 $\pm$ 3 percent; $N = 3$). Both Mg and Mn ions are inhibitors of stimulus-secretion coupling processes, and Mn is known to be the more potent inhibitor (*12*).

The release of GABA is specific to synaptosomes. Although myelin and microsomal fractions accumulate GABA, neither of these fractions could be shown to release significant amounts of GABA after stimulation with 2 mM Ca. Whereas synaptosomal fractions release 3 $\pm$ 0.2 percent ($N = 17$) of total radioactive GABA by a 20-second treatment with Ca, myelin releases less than 0.2 percent ($N = 3$), and release from microsomes cannot be detected (less than 0.01 percent; $N = 2$). Likewise homogenates of glial cells grown in tissue culture (C6), HeLa cells, or sympathetic ganglia were found to accumulate GABA, but less than 0.01 percent of total GABA can be released by Ca. Clearly the secretory process is not a general phenomenon of all systems capable of GABA transport whether these are of glial or neuronal origin. Since the major contaminants of the synaptosomal fraction do not secrete GABA, we conclude that synaptosomes are the site of Ca-dependent release of GABA.

In view of the possible affinity of GABA for surface membranes, we investigated likely contributions to release introduced by surface exchange. Synaptosomes were perfused with nonradioactive GABA during washing and Ca-evoked release. Nonradioactive GABA would replace any surface-

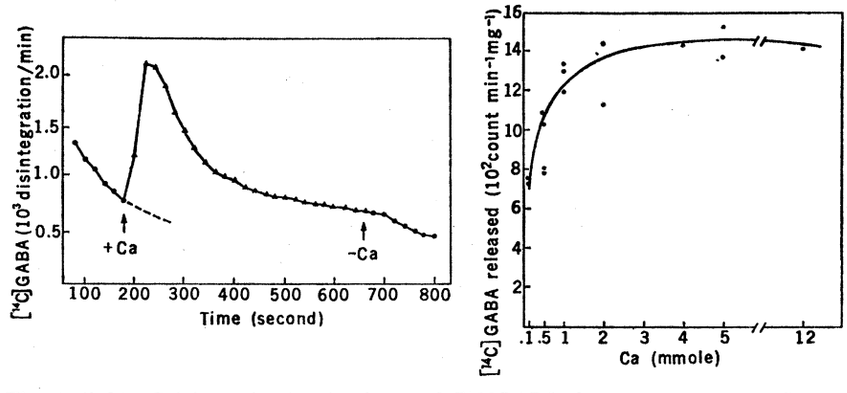

Fig. 1 (left). Calcium-stimulated release of [^{14}C]GABA from synaptosomes. Synaptosomes were plated on a glass fiber–Nucleopore filter system and continuously perfused with Ca-free Ringer solution containing 56 mM K. This solution effectively depolarizes the synaptosomes (*1*). During the perfusion (arrow), 2 mM Ca was introduced. Effluent was collected during 20-second intervals. As shown, the maximum efflux rate occurred within 40 seconds. After 8 minutes, 60 percent of the bound radioactive products was released. A return to Ca-free medium caused a drop in efflux. The positive pressure device used a three-place Harvard syringe pump to force solutions through Millipore 25 mM Swinnex filter holders containing a Whatman 25-mm glass fiber filter stacked over a Nucleopore 0.4-μm filter. Unused solution was pushed through a "dummy" filter unit to maintain constant pressure. Solutions were changed by hand-operated three-way syringe valves. Filtrate outflow and collection were monitored gravimetrically at the immediate conclusion of the experiment. Fig. 2 (right). Effect of external Ca concentration on [^{14}C]GABA release from synaptosomes. Synaptosomes were stimulated by 56 mM K medium during 20-second incubation in the presence of various concentrations of Ca. Maximal release rates were reached between 2 and 3 mM Ca. Data points were compiled from three experiments. The system consisted of a four-place Plexiglas vacuum box that permitted sequential collection of each filtrate. A sliding shelf within the box carried four rows of five filtrate collection vials. Solutions (2 ml) were simultaneously added from above onto four funnel filter units for 20-second incubation periods. Effluents were rapidly collected by applying a vacuum.

bound radioactive GABA. In the presence of nonradioactive GABA, release is not abolished but is slightly reduced, perhaps because of dilution of internal GABA pools or blockage of its own release. Another possible artifact not commonly considered is that the increase in GABA collected on stimulation might arise from blockage of reuptake processes. To study this possibility Ca-dependent release was measured under conditions that inhibit reuptake. Removal of Na ions from the medium and replacement with choline is known to block GABA uptake (*13*). In sodium-free media, Ca-dependent release was little altered. Thus, artifacts from either surface exchange or reuptake blockage do not account for Ca-dependent release.

In summary, we have described a system that rapidly releases GABA from synaptosomes when stimulated by Ca and that meets a number of criteria of stimulus-secretion coupling processes not shown in previous similar studies (*1, 2*). In addition we have shown that (i) the release phenomenon is specific to synaptosomes and not due to several possible artifacts and (ii) exogeneous GABA accumulated by synaptosomes can be released, which means that synaptosomes in vitro possess the capability to package, store, and release neurotransmitters. In this regard, it is particularly interesting that subcellular fractionation has failed to reveal GABA in synaptic vesicles (*14*), possibly owing to the nonphysiological conditions required for isolation. Our system should be suitable for further detailed analysis of packaging, storage, and release of GABA. The rapidity of the measurements makes the system ideal to study the effects of various agents on release of transmitters independent of many metabolic effects. An individual measurement requires only a few micrograms of tissue, and synaptosomal beds previously used (*1*) which require a few milligrams of tissue are not necessary. The system is sensitive enough to be used with specific brain regions and possibly even biopsy samples. At present a simple mammalian system to study the properties of GABA secretion is not available for analysis, and our system appears unique in its potential for studying the secretion properties of this major mammalian inhibitory transmitter.

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Genetic Control of Gonadotrop Differentiation in the Platyfish, Xiphophorus maculatus (Poeciliidae)

Abstract. *A sex-linked gene controlling the age at which the gonadotrops of the pituitary gland differentiate has been discovered in* Xiphophorus maculatus. *Males homozygous for early differentiation become sexually mature between 10 and 16 weeks; those homozygous for late maturation between 22 and 40 weeks. Heterozygotes are intermediate. Since growth rate decreases when testes mature, the two classes of males differ significantly in adult size.*

A sex-linked gene controlling the age at which the gonadotropic zone of the adenohypophysis differentiates has been identified in the teleost *Xiphophorus maculatus* (Guenther) native to southern Mexico and adjacent parts of Central America. Within the "Belize" stock (*1*) males homozygous for the sex-linked factor *Ir* (red iris) reach sexual maturity several weeks prior to males homozygous for the sex-linked factor *Br* (red body). Males homozygous for *Ir* or heterozygous for *Ir* and *Br* (there is no evidence that the two color genes are alleles) were obtained from matings of the type W Y-*Ir*♀ × Y-*Ir* Y-*Br*♂ (*2*). Homozygous *Br* males and heterozygotes originated from the following cross: W Y-*Br*♀ × Y-*Ir* Y-*Br*♂.

Since environmental factors (*3*) can serve as triggering devices for the onset of sexual maturity and since we were interested in measuring the differences in the age of sexual maturation between the two types of males, all fish of a given brood were raised in the same aquarium. Thus each brood served as both control and experimental group. Only pedigree 2964 (born 1/26/72) was separated into two tanks (a and b) when 1 day old because of the large number of offspring (Tables 1 and 2). Intrabrood comparisons are more meaningful than comparisons between groups. Fish were raised in the laboratory equipped with a skylight under standard conditions (*4, 5*).

The anal fin of immature male platyfish is fan-shaped. Under the influence of androgenic hormone from maturing testes, the anal fin transforms into a rodlike gonopodium. This metamorphosis (*6*) requires several weeks and has been divided into six easily recognizable stages. The structure of the anal fin can be used as a precise index of sexual maturity.

Within the Belize stock, homozygous *Ir* males become sexually mature before their *IrBr* sibs and these in turn mature at a younger age than homozygous *Br* males (Table 1). Within the five broods of pedigrees 2828, 2918, and 3030 the difference between the two classes of males is absolute. In the four groups of pedigree 2964 only one *Br Br* male (2964-11, mature at 18 weeks) differentiated as early as any of its *IrBr* sibs. Overlap between broods and pedigrees is primarily related to the number of fish raised per liter of water.

Since the pituitary gland controls testicular maturation and, indirectly, gonopodial differentiation (*7*), we examined both the hypophyses and the testes of *Ir Ir* and *Ir Br* males (pedigree 2828, born 2/27/71). In addition to eight females this sibship consisted of five *Ir Ir* and ten *Ir Br* males. At 9 weeks of age, the anal fins of the homozygous *Ir* males and of one *Ir Br*