Further results obtained with pairing of the "kindling effect" and the "transfer experiment"

JOHN GAITO

York University, 4700 Keele Street, Downview, Ontario M3J 1P3

Previous experiments indicated that the development of clonic convulsions could be retarded by intraperitoneal injections of brain homogenate supernatant from male Wistar rats which had convulsed via amygdaloid electrical stimulation if two or more brain equivalents were used. Six experiments were conducted employing intracerebral injections. Although significant differences appeared in one experiment, the overall effect was one of no difference between experimental (E) and control (C) recipients. However, the mean number of trials for first clonic convulsion was greater for E and C than for E donors, and for recipients in the previous intraperitoneal experiments, suggesting possible disruption of brain function with this type of injection. Thus, two further experiments were conducted with male Wistar rats using the intraperitoneal route of injection and separating the homogenate into two fractions: molecular weight less than 500, molecular weight greater than 500. In the fraction greater than 500 molecular weight, E mean value to first clonic convulsion was greater than that for C, confirming results of the previous experiments.

The kindling paradigm (Goddard, McIntyre, & Leech, 1969) was instituted in this laboratory several years ago to determine brain chemical events which accompany the behavioral changes. In one set of experiments (Gaito, Hopkins, & Pelletier, 1973), the electrophoretic pattern of prealbumin range proteins specific to the nervous system were evaluated in rats during the kindling process. No significant differences in patterns were found for rats at different stages in this process.

In another set of experiments, the effects of brain homogenate supernatant from kindled (E) and nonkindled (C) rats on the kindling rates of recipients was evaluated (Gaito & Gaito, 1974). All recipients were run 24 h after injection. The results showed that a "retardation effect" was present when rats were injected intraperitoneally (IP) with supernatant from kindled rats, if the amount injected was equivalent to that from two or more brains. The recipient mean number of trials to first clonic convulsion (CC) were: E, 14.3 for 42 rats; C, 8.9 for 26 rats. The means when one-brain amounts were used were: E, 13.9 for 34 rats; C, 12.2 for 16 rats. These means were not significantly different. With all experiments combined, the means were: E, 14.2 for 76 animals; C, 10.2 for 42 rats. In all experiments, the mean was approximately 10 for donor rats.

This report describes further experiments using the kindling and transfer paradigms.

METHODS AND RESULTS

The brain coordinates used for electrode implantation were the same as in earlier experiments (.5 mm posterior to bregma, 4.5 mm from midline, 8.5 mm from skull). In previous

experiments (Gaito, Hopkins, & Pelletier, 1973, histological analyses indicated that the tip of the electrode was in the amygdala. In the present experiments, Wistar strain male rats (90 to 130 days old at the beginning of the experiment) had bipolar electrodes implanted into the amygdala (Nichrome wire with trimel coating, .127 mm in diam, dipped on time in Epoxylite). Electrodes were inserted into the right or left hemisphere according to a random process. All electrodes were inspected for electrical adequacy before insertion. All recipients were coded and run by an experimenter who was unaware of the designation of each rat.

Experimental Series 1

The previous experiments used the IP injection route, and only a small fraction of the injected material would reach the brain. A more efficient method would be to inject the material directly into the brain.

Ten donor rats from six litters had electrodes implanted unilaterally in the amygdala. Six or more days later, the kindling procedures began. Five experimental rats (E) were stimulated at approximately 12-h intervals by a 60-Hz sine wave of 100 microA intensity (peak to peak) for 60 sec from a Lafayette sine wave stimulator. A response was scored as a clonic convulsion only if the convulsion continued after termination of the current. On the 10th CC trial, at 10 min after the initiation of the trial, the rats were sacrificed by decapitation. The brain was rapidly removed; the olfactory bulbs, cerebellum, and brainstem parts below the cerebellum were deleted; the remainder was used for injections. Each brain was inspected visually to determine the position of the electrode. This was accomplished by two cuts: a vertical slice at the site at which the electrode entered the brain, perpendicular to the longitudinal fissure, and a similar one along the longitudinal fissure. Then the anterior portion of the hemisphere was removed to provide a coronal view of the electrode location in both the removed (anterior) portion as well

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Table 1
Data for Recipients in Experimental Series 1

		Number	Mean: First CC
Experiment 1	E	6	19.3
	C	6	10.2
Experiment 2	E	15	14.9
	C	12	16.7
Experiment 3	E	11	17.3
	C	8	18.0
Combined	E	32	16.6
	C	26	15.5

Note—Significant differences, $p < .05$ only in Experiment 1. E = recipients injected with material from donors that had convulsed; C = injection from nonstimulated donors. Approximately 30%, 41%, and 20% of a brain per injection was used in Experiments 1, 2, and 3, respectively.

as the intact (posterior) portion. The electrode made a thin, easily discerned, line from the point of entry to its location subcortically. The brains were stored at -60°C until used.

Five controls (C), littermates of the E rats, were connected to stimulator lead wires but received no stimulation. The Cs were sacrificed and treated in the same manner as the Es.

The E brains were homogenized together in physiological saline. A similar procedure was used with C brains. The homogenates were shaken over night at 2°C and centrifuged at $20,000 \times g$ for 1 h. The homogenate supernatants ("soluble protein" fraction) from each of the E and C rats were pooled separately, 6 ml for each. Each sample was run through an Amicon Ultrafiltration cell with a UM 05 membrane. This membrane allows sample components below a molecular weight of 500 to pass through. The 6 ml was reduced to 1.7 ml by this procedure. The material less than 500 molecular weight was discarded.

Recipient rats (12 from the 6 litters from which the donors had been taken) had electrodes implanted unilaterally in the amygdala. A plastic grommet was cemented to the skull over the hemisphere opposite to that in which the electrode was implanted. The grommet was constructed such that when a 26-ga. $\frac{1}{2}$ -in. needle was inserted into the grommet, the tip of the needle was in the lateral ventricle. The skull was drilled slightly 2.0 mm from the midline, and the center of the grommet was over this spot. (Tracking dye used with a few rats previously indicated that the injected material spread over the entire brain.)

Six or more days later, each recipient was lightly anesthetized with ether and received 100 microliters of supernatant (the equivalent of approximately 30% of a brain) through the grommet. Stimulation began 24 h later at an intensity of 50 microA at approximately 12-h intervals.

The data for the dependent variable, first trial of CC, which was sensitive in previous experiments in discriminating between E and C rats is indicated in Table 1. The value for each E in the six pairs was greater than for the C littermate. A Pairs by Treatments analysis of variance indicated that the mean for the E group was significantly greater than that for C ($F = 10.92$, $df = 1/5$, $p < .05$).

A second experiment was conducted in the same fashion but with 15 E and 12 C recipients, each receiving approximately 41% of a brain in the injection. No significant differences between E and C occurred in the mean number of trials to first CC (Table 1).

A third experiment with 11 E and 8 C rats receiving approximately 20% of a brain showed the same results as in Experiment 2 (Table 1).

When the results of the three experiments are combined, the means are essentially identical at approximately 16. This value is in sharp contrast to the mean for 27 E donors in these experiments, 10.6; in the previous experiments using the IP route of injection, the mean value for E donors was approximately 10 also, as was the mean value for C recipients as well. This discrepancy suggests that injection directly into the brain may disrupt brain conditions such that any tendency to retard the onset of clonic convulsions by the supernatant fraction from E donors may be obscured.

Experimental Series 2

Because the brain injection used in Experimental Series 1 suggested a disruptive brain function effect, a second set of experiments were conducted using a different route of intracerebral injection, through the eye socket into the lateral ventricle. The recipients were lightly anesthetized and injected with 100 microliters of the supernatant of molecular weight greater than 500. Three experiments were conducted which were similar to those of Experimental Series 1 in every detail except in route of injection. Although the mean of E was greater than for C in the first experiment, this difference

Table 2
Data for Recipients in Experimental Series 2

		Number	Mean: First CC
Experiment 1	E	10	17.6
	C	9	11.6
Experiment 2	E	11	15.3
	C	9	17.1
Experiment 3	E	16	16.3
	C	19	16.9
Combined	E	37	16.3
	C	37	15.6

Note—Approximately 25%, 32%, and 29% of a brain per injection was used in Experiments 1, 2, and 3, respectively.

was not significant statistically at a p level of .05 (Table 2). In the remaining two experiments, the means were not different (Table 2). Combining the three experiments produced a mean for E and C of approximately 16. Combining the six intracerebral injection experiments also produced a mean of approximately 16 for E and C.

The mean values for C recipients in this series of experiments was, likewise, greater than about 10 which was obtained in the IP injection experiments.

Experimental Series 3

The six experiments using an intracerebral route of injection raised a question as to the authenticity of the retardation effect obtained in the previous IP experiments. Thus, two further IP experiments were conducted.

In these experiments, the equivalent of 2.5 brains per injection was used with each recipient. The supernatant from the homogenate was separated by a UM 05 membrane into two equal fractions of approximately 15 ml for E and C separately and injected into four groups of recipients: E, M.W. < 500; C, M.W. < 500; E, M.W. > 500; C, M.W. > 500. In each experiment, there were 10 sets of four littermates, two for each fraction. Each recipient received 3 ml IP under light ether anesthetization.

The overall analysis of variance procedure was a three dimensional design (Litters by Treatments by Fractions). However, it was hypothesized that an interaction of treatments and fractions would occur (i.e., $E \neq C$ for only one fraction). This result usually would suggest the use of separate analyses for each fraction (Gaito, 1973). Therefore, the separate analyses (Litters by Treatments) were conducted without attempting the overall analysis. The CC data are shown in Table 3.

For each experiment and for each fraction, the mean differences between E and C were not great enough to reject the null hypotheses at a p level of .05, although, in both experiments, E tended to be greater than C in the M.W. > 500 fraction. However, when the two experiments were combined to provide 20 pairs for each fraction, the mean for E was significantly greater than C for the fraction containing material of molecular weight greater than 500 ($F = 5.17$, $df = 1/19$, $p < .05$). The differences for the other fraction were not statistically significant. Furthermore, the mean trial of first CC values were lower than in the intracerebral injection experiments and consistent with the results of previous IP experiments.

DISCUSSION

Although there was a tendency for the E mean to be greater than the C mean in one experiment in each of

Table 3
Data for Recipients in Experimental Series 3

		E	C
Experiment 1	< 500	11.8	17.1
	> 500	17.8	12.3
Experiment 2	< 500	12.9	8.9
	> 500	14.3	10.0
Combined	< 500	11.9	13.4
	> 500	16.1	11.2

Note—Significant differences, $p < .05$ only for the fraction of M.W. > 500 for the combined experiments, E vs. C.

the intracerebral series, the overall effect was one of equality. Considering that the mean trial of first CC in these experiments was greater than that which was obtained in the IP series (3), that obtained in the previous IP experiments (Gaito & Gaito, 1974), and that for donors in these and previous experiments, it appears that the intracerebral injection may be disrupting brain function such that the overall threshold to convulse is increased. The previous IP experiments and those in Experimental Series 3 indicate that the "retardation effect" is a minimal one which is not great enough to provide statistical significance in each experiment (although in almost every experiment in which two or more brain amounts have been used, the E mean is greater than the C mean). Thus with an increased threshold occurring as a result of the brain injection, the minimal "retardation effect" is obscured.

The results of Experimental Series 3 were consistent with our expectation. Although the fraction of M.W. < 500 contains a number of biologically active compounds (including some hormones, peptides with up to approximately five amino acids, other small molecules), the main constituents are water and sodium chloride. Thus, we did not expect this fraction to produce a retarding effect. However, the other fraction containing polypeptides, proteins, hormones, and various other molecules would be a logical candidate to be responsible for the retardation. Research is now underway to isolate and identify the specific characteristics of the molecule which is producing this negative transfer effect.

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