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Sensitivity of Cerebellar Purkinje Neurons to Neurotransmitters in Genetically Epileptic Rats[1].

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ABSTRACT

Epilepsy is a state of neuronal hyperactivity which may be caused by an altered relationship between inhibitory and excitatory influences on neurons. We have conducted experiments using microiontophoresis with *in situ* extracellular recording of anesthetized rat cerebellar Purkinje neuron activity to determine the sensitivity of these neurons to neurotransmitters in a genetic model of epilepsy. Quantitative evaluations of agonist-induced changes in activity were carried out by using poststimulus time histograms. Current-response curves were generated and linear regression analysis was performed to evaluate changes in responsiveness and sensitivity between control and genetically epilepsy-prone-9 rats. The current required to produce 50% inhibition of activity by GABA was 2.6-fold higher in the genetically epilepsy-prone-9 rats compared to control. In contrast, the amount of current required to produce 50% inhibition by norepinephrine was not significantly different between groups. There also was no significant change in cerebellar neuron sensitivity to the excitatory transmitter, glutamate. The lack of an alteration in sensitivity and responsiveness to norepinephrine or glutamate suggests that the hyperexcitability of neurons may be associated with a specific subsensitivity of the GABAergic system. Such a specific subsensitivity to γ-aminobutyric acid would, therefore, yield a more excitable state of the neuron and may contribute to the development of the hyperactivity observed in epilepsy.

The epilepsies are characterized by neuronal hyperactivity which may be created by an altered responsiveness to a variety of different unknown stimuli. Hyperexcitability of neurons may be due to: 1) an enhanced sensitivity to excitatory neurotransmitters; 2) a reduced sensitivity to inhibitory neurotransmitters; 3) an alteration in transmitter metabolism; or 4) a combination of any of the above.

In these studies, a genetic model of epilepsy was utilized, the GEPR. A maximally seizure prone rat (GEPR-9) exhibits full tonic-clonic seizures to audiogenic stimuli whereas its genetic control exhibits no seizure activity to audiogenic stimuli (Jobe *et al.*, 1973). We have investigated the sensitivity of cerebellar Purkinje neurons in control rats and GEPR-9s to various chemical stimuli.

There are several reasons for studying the cerebellum: 1) changes in its involvement in motor activity have been observed during seizures (Dam, 1982); 2) the biochemical differences in neurotransmitter levels in the GEPRs have been well characterized in the cerebellum (Laird *et al.*, 1984; Dailey *et al.*, 1991); and 3) the anatomical and physiological pathways in this brain area have been well defined.

The biochemical differences observed in neurotransmitter levels in the cerebellum of the GEPR (Dailey *et al.*, 1989, 1991) have focused our investigation of differences in sensitivity of Purkinje neurons to the endogenous neurotransmitters. The cerebellum contains terminals of the noradrenergic pathway which originate from cell bodies located in the locus ceruleus. The terminals impinge upon the Purkinje cells directly and release NE (Ottoson, 1983), which decreases spontaneous Purkinje cell firing (Moises *et al.*, 1980). GABA is the major inhibitory neurotransmitter present in the cerebellum. This transmitter is released from terminals of basket cells in the cerebellum which act directly upon the Purkinje cells to decrease spontaneous activity (Ottoson, 1983). Granule cells, also located in the cerebellum, release the major excitatory neurotransmitter, glutamic acid. Glutamic acid increases Purkinje cell firing (Ottoson, 1983).

A fine balance between excitatory and inhibitory influences exists throughout the central nervous system. Any alteration in this delicate balance could lead to the neuronal hyperexcitability which is characteristic of the seizure-prone state. The purpose of this study was to examine if alterations in neuronal sensitivity to these identified neurotransmitters exist in seizure-prone GEPR-9s compared to their genetic controls which exhibit no seizure activity to audiogenic stimuli (Jobe *et al.*, 1973).

Methods

The animals were obtained from Dr. Philip C. Jobe (University of Illinois at Peoria, Peoria, IL) who performed the initial task of deter-

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ABBREVIATIONS: GEPR, genetically epilepsy-prone rat; NE, norepinephrine; GABA, γ-aminobutyric acid; C.I., confidence interval.

mining their seizure susceptibility. GEPR-9s and their genetic controls were utilized in these experiments. All animals weighed between 250 and 400 g. The animals were anesthetized with 1.25 g/kg of urethane i.p., intubated and allowed to breath spontaneously. Body temperature was monitored constantly by rectal probe and maintained at 37°C by heating pad.

Electrophysiological recording. The activity of cerebellar Purkinje neurons was recorded as described by Taylor *et al.* (1978). Briefly, the cisterna magnum was opened and allowed to drain. The skull covering the cerebellar vermis and the dura mater was removed. The exposed cerebellum was covered by 3% agar in distilled water to prevent drying of the brain surface. Purkinje neurons were identified by their characteristic climbing fiber and simple spike discharge rates (Murphy and Sabah, 1970). Cells with regular discharge rates between 20 and 50 Hz were used for study.

Extracellular electrophysiological recordings from cerebellar Purkinje neurons were conducted through one barrel of a 5-barrel multibarrel pipette. Multibarreled micropipettes were pulled to a fine tip using a vertical micropipette puller (Narshige USA, Inc., Greenvale, NY). One barrel, used for recording, was filled with 5 M NaCl and generally had resistances between 1.5 to 4 megohms. Three of the four remaining barrels were filled with drugs, and the fourth was used as the balance barrel. The balance barrel, containing 3 M NaCl, neutralizes any excess current at the tip of the micropipette (Curtis, 1964, 1972). Drug solutions were: (−)-NE (0.5 M, pH 5.0), GABA (1.0 M, pH 4.0) and glutamic acid (0.5 M, pH 7.5) (Sigma Chemical Co., St. Louis, MO).

The electrophysiological signals were amplified using a high impedance amplifier and filtered (−3 db at 300 and 5000–6000 Hz). The signal was monitored on an oscilloscope and action potentials of a single neuron separated from background noise by a window discriminator (Medical Systems, Inc., Great Neck, NY) which converted each discriminated action potential to a constant voltage pulse. The constant voltage pulses were led to a ratemeter which integrated the activity over time, usually at one second epochs, and displayed the integrated activity on a stripchart recorder.

Commuter analysis of neuronal responses. The computer analysis of neuronal activity utilizing poststimulus time histograms was performed according to Freedman *et al.* (1975). Briefly, each poststimulus time histogram is a collection and summation of constant voltage pulses over a defined time period after a synchronization pulse ("start" pulse). Drug ejection was accurately timed to be delivered after the synchronization pulse using a crystal clock. Activity was then summed over several consecutive sweeps to decrease the variability among trials and improve quantification of responses. Increases and decreases in neuronal activity for each cell at different ejection currents were analyzed with the aid of a computer. Each histogram consisted of three consecutive sweeps with each sweep lasting 32.768 sec. A number of histograms were constructed on the same cell using varying ejection currents of the same drug. Computer analysis was utilized to determine the extent of excitation or inhibition produced by the compound for each ejection current in the series of histograms on a single neuron.

The principle underlying the analysis program is the comparison of the average counts per bin in a user defined control period with the counts per bin during drug application. By using the same bins for each successive histogram on the same cell allows quantification of the percentage of change in response for different currents. In this way, a current-response relationship was established for a given compound on each cell.

The current-response curves generated from the histograms were used to calculate the current required to produce 50% of the maximal inhibition for GABA and NE. Such a value has been used previously to quantify neuronal sensitivity to iontophoretically applied drug (Simmonds, 1974). Responses to glutamate were expressed as percentage of increase in basal firing rate.

Statistical analysis. Utilizing values from the individual current-response curves lying between 20 and 80% of maximum, mean linear regression lines and their 95% C.I.s were calculated and drawn. Slopes of the lines were calculated and compared for parallelism to evaluate significant differences. If linear regression analysis suggested that a difference in the current-response relationship might exist, the geometric mean IC_{50} values (Fleming *et al.*, 1972) were compared between GEPR-9s and controls using Student's *t* test. The mean for the individual slopes of the current-response curves for the GEPR-9s and controls was also compared using Student's *t* test.

Comparisons between groups were made at the level of the geometric mean effective current needed to produce 50% inhibition of activity (IC_{50}) for GABA and NE. Because the current-response relationship is similar to that of dose- or concentration-response relationships, analysis should account for the log-normal distribution of mean equieffective concentrations (Fleming *et al.*, 1972). Calculations and comparisons of these values between GEPR-9 and controls permitted a determination of the level of change in sensitivity of Purkinje neurons to a given drug. The 95% C.I. for each IC_{50} is also included. Mean IC_{50} values were considered to be significantly different when $P \leq .05$.

Results

Shown in figure 1 are the current-response curves to iontophoretically applied GABA obtained from four different cerebellar Purkinje neurons in one control animal. These curves are typical of those observed from other control animals. There appears to be little variation among responses to GABA by each neuron to similar currents.

The current-response curve for one of the above neurons was generated from the sequential histogram series shown in figure 2A. Although the histograms are presented sequentially by current intensity, they were generated in random order during the recording session. This was performed to reduce the impact of sensitization or desensitization on the response of the neuron to drug application. The bar above each response (inhibition) denotes the time over which that current of GABA was ejected. The percentage of inhibition, calculated as described under "Methods," is indicated in the middle of the response in each histogram. As predicted, increasing current intensity leads to greater inhibition.

The histograms illustrated in figure 2B were constructed similarly in a GEPR-9. Comparing the percentage of response with the current applied between the histograms in control (fig. 2A) with those of the GEPR-9 (fig. 2B), one observes an apparent subsensitivity of the cerebellar Purkinje neurons in the GEPR-9 to GABA. This is better illustrated by the mean regression lines constructed from all the individual current-

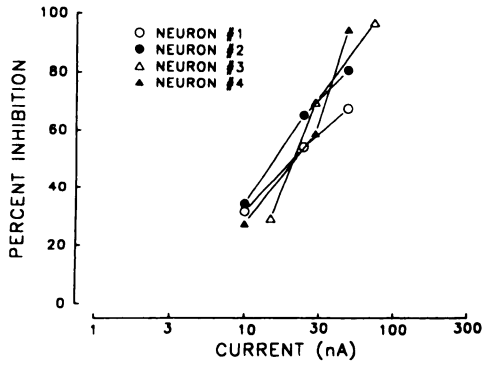

Fig. 1. Current-response curves of cerebellar Purkinje neurons from a control rat to iontophoretically applied GABA. Each curve represents the current-response relationship for a single neuron from the same animal. Histograms illustrated in figure 2A were derived from neuron #3. Note the remarkable consistency of responses obtained to the same agonist on different cells.

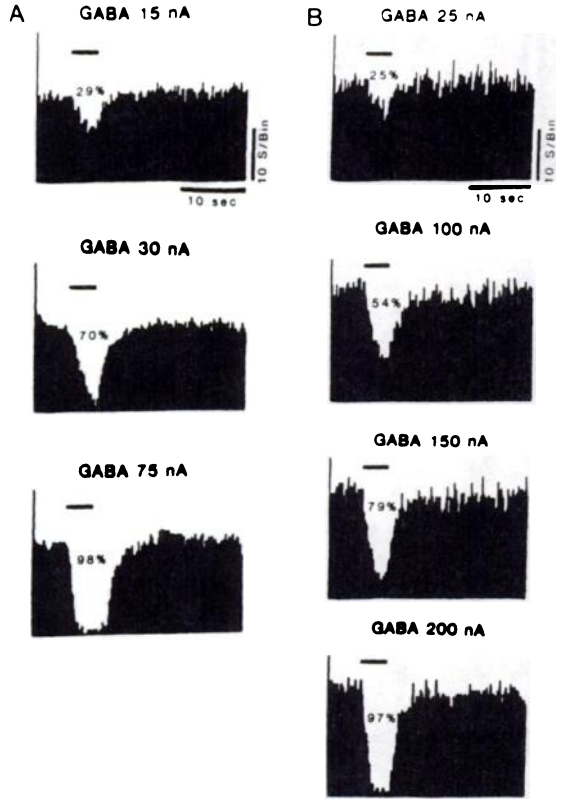

Fig. 2. Sequential poststimulus time histograms representing the responses of a single cerebellar Purkinje neuron to microiontophoretically applied GABA. A. control rat. B. GEPR-9. The current (nanoamperes) applied is indicated above each histogram, and the time of application represented by the bar. Numbers within the response period represent the calculated percentage of inhibition induced by this iontophoretic current of GABA. For comparative purposes, the calculated response period was identical among the different histograms for a given neuron. *Note*: The response period outlasts the duration of drug application and is directly related to the magnitude of the current applied.

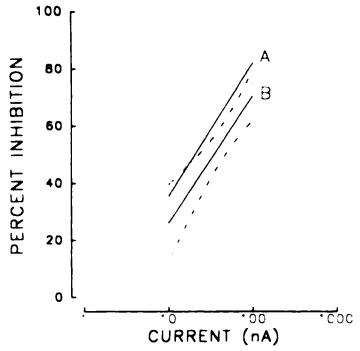

Fig. 3. Mean regression lines of responses from cerebellar Purkinje neurons in controls (A) and GEPR-9s (B) to iontophoretically applied GABA. The *n* value for controls equals 33 cells from 9 animals and for the GEPR-9s equals 32 cells from 16 animals. Statistical comparisons of the geometric mean log IC_{50} values and slopes are presented in table 1. Also indicated are the 95% C.I.s for each regression line with short dashed lines corresponding to line A and long dashed lines corresponding to line B.

response curves obtained in a number of experiments (fig. 3) between control animals and GEPR-9s. The relationship demonstrates a rightward shift of the mean regression line for GEPR-9s compared to controls. The slopes were not significantly different from each other suggesting that the shift was a parallel rightward shift of the current-response relationship.

The calculated mean log IC_{50} (inhibitory current [nanoamperes] producing 50% inhibition) for the controls is 1.40 ± 0.07 and for the GEPR-9s is 1.82 ± 0.08 (table 1). The antilog of the mean log current translates to a current producing 50% inhibition equal to 25.19 nA (95% C.I., 18.6–34.1) for controls and 65.79 nA (95% C.I., 46.6–92.8) for GEPR-9s. The difference in the mean log IC_{50} was statistically significant ($P < .05$). The 2.6-fold rightward shift was calculated as the ratio of the mean IC_{50} value for the GEPR-9s to the mean IC_{50} value for the controls. This rightward shift demonstrates a nearly 3-fold reduction in sensitivity of cerebellar Purkinje neurons to GABA in the GEPR-9 as compared to control.

No significant difference in sensitivity to NE was observed between GEPR-9s and controls. Figure 4 illustrates the mean regression lines for NE applied to cerebellar Purkinje neurons in the two animal strains. The slopes were not significantly different. The mean log IC_{50} for NE in the GEPR-9 was 2.00 ± 0.13 which translates to a current of 100.03 nA (95% C.I., 53.4–187.3) whereas, in controls, the mean log IC_{50} was 1.99 ± 0.05 which equals 96.72 nA (95% C.I., 74.4–125.8) (table 1). The mean log IC_{50} values were not significantly different.

Mean regression lines and their 95% C.I.s for glutamate are illustrated in figure 5. There was no significant difference between the control and GEPR-9 current-response relationships for glutamate, and the slopes were not significantly different (fig. 5). Geometric mean equieffective currents for glutamate-induced excitation were determined. An increase in

TABLE 1

Sensitivity of Purkinje neurons to iontophoretically applied GABA and norepinephrine in control and GEPR-9 rats.

Group	Mean Log IC_{50} (±S.E.M.)	Geometric Mean IC_{50} (nA) (95% C.I.)	Ratio Exp/Ctl[a]	Mean Slope (%/nA) (±S.E.M.)
GABA Control (33, 9)[b]	1.40 ± .07	25.19 (18.6–34.1)	2.61	96.4 ± 7.9
GEPR-9 (32, 16)[b]	1.82 ± .08**	65.79 (46.6–92.8)		120.3 ± 12.5
NE Control (20, 10)[b]	1.99 ± .05	96.72 (74.4–125.8)	1.03	168.2 ± 17.5
GEPR-9 (19, 9)[b]	2.00 ± .13	100.03 (53.4–187.3)		124.3 ± 14.9

[a] Ratio EXP/CTL represents the magnitude of the shift IC_{50} values.
[b] Numbers in parentheses, number of cells and the number of animals, respectively.
** Significantly different from Controls at $P < .01$, Student's *t* test.

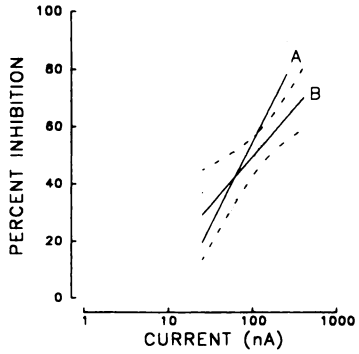

Fig. 4. Mean regression lines of cerebellar Purkinje neuron responses to iontophoretically applied NE in controls (A) and GEPR-9s (B). The *n* value was equal to 20 cells from 10 animals in controls and 19 cells from 9 animals in GEPR-9s. Statistical comparisons of the mean log IC_{50} values and slopes are presented in table 1. Also indicated are the 95% C.I.s for each regression line with short dashed lines corresponding to line A and long dashed lines corresponding to line B.

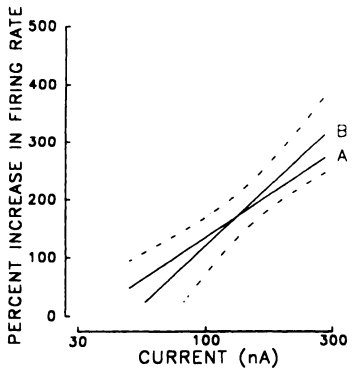

Fig. 5. Mean regression lines of the relationship between current and responses of cerebellar Purkinje neurons in controls (A) and GEPR-9s (B) to iontophoretically applied glutamate. The *n* value is equal to 17 cells from 9 animals in controls and 17 cells from 7 animals in GEPR-9s. Also indicated are the 95% C.I.s for each regression line with short dashed lines corresponding to line A and long dashed lines corresponding to line B.

firing rate of 150% above basal activity was chosen as the level for comparison. The calculated values (EC_{150}) were selected because they represent a level of excitation for an individual neuron which is greater than a doubling of the rate. Variations in maximum excitatory capability between neurons precluded use of values based on percentage of maximum. The mean log EC_{150} for glutamate in GEPR-9s was 2.09 ± 0.06 which translates to a geometric mean EC_{150} of 123.7 nA (95% C.I., 89.9–170.1). Similarly calculated values for controls were 2.03 ± 0.08 for the mean log EC_{150} which represents a geometric mean EC_{150} of 108.1 nA (95% C.I., 72.1–161.9).

Mean basal firing rate was calculated for the population of neurons studied. There was no significant difference in the mean basal firing frequency of firing for Purkinje neurons between control (mean frequency = 23 ± 2 Hz [n = 43]) and GEPR-9 (mean frequency = 26 ± 3 Hz [n = 54]).

Discussion

The alteration in sensitivity to GABA but not to NE and glutamate represents a very specific change in Purkinje neuron sensitivity of the GEPR. The difference in sensitivity (table 1) cannot result from a change in basal activity because no difference in mean basal rate of Purkinje cells was observed in this study. The reported mean basal frequencies of 25 and 23 Hz for control and GEPR-9, respectively, are consistent with mean basal activity reported for Purkinje cells in other studies (Murphy and Sabah, 1970). A similar reduction in sensitivity to GABA has been observed in the inferior colliculus (Faingold *et al.*, 1986a,b). These authors also observed a quantitatively similar reduction in sensitivity to flurazepam. However, the action of benzodiazepines requires endogenous GABA (DeFeudis, 1990) so that the investigations by Faingold *et al.* (1986a,b) did not address the specificity of the change in sensitivity as was evaluated in the present study (table 1; figs. 3, 4 and 5).

The degree of specificity of this sensitivity change permits elimination of certain mechanisms which have been shown to be responsible for the development of adaptive sensitivity changes in other excitable tissues (Fleming and Westfall, 1988). These identified alterations include: 1) changes in electrical properties of target cells; 2) alterations in second messenger systems common to several receptors; and/or 3) an alteration in receptors for a single agonist/neurotransmitter.

The highly specific change in sensitivity to GABA argues against the possibility that a change in membrane potential underlies the change in sensitivity. If such a change was present, then sensitivity should be altered to any agonist whose mechanism of action requires alterations in membrane electrical properties (*cf.* Fleming and Westfall, 1988; Johnson and Fleming, 1989). The action of both glutamate (Cotman and Monaghan, 1987) and GABA (Bormann, 1988) involve mechanisms which are either voltage-dependent or lead to changes in resting membrane potential. A change in resting potential of the cell membrane would be expected to evoke opposing alterations in sensitivity to excitatory and inhibitory agonists as has been observed in the myenteric plexus/longitudinal smooth muscle from guinea pigs made tolerant to morphine (Johnson and Fleming, 1989; Johnson *et al.*, 1978; Taylor *et al.*, 1988). The reduction in sensitivity to GABA, therefore, would be accompanied by an increase in sensitivity to glutamate if the mechanism underlying the alteration in sensitivity was a change in membrane electrical properties.

An equally unlikely mechanism for the observed reduction in sensitivity to GABA is a change in second messengers which function as convergent intracellular mediators of multiple drug and transmitter action. The drugs and transmitters utilized in this study possess actions mediated by separate receptors which are coupled to different transduction systems. For example, Siggins *et al.* (1971) have demonstrated that NE responses in the cerebellum are mediated *via* an increase in cyclic AMP. Thus, the *beta* adrenoceptor which is activated by NE is coupled to adenylyl cyclase. In contrast, the actions of GABA are mediated primarily by activation of $GABA_A$ receptors coupled to chloride channels (Harris and Allan, 1985; Schwartz, 1989; Bormann, 1988) or $GABA_B$ receptors coupled to cyclic GMP (*cf.* Wood, 1991). Similarly, the glutamate responses are mediated by one of at least three different types of receptors, all of which are coupled to ion channels (Cotman and Monaghan, 1987). The fact that each agonist used utilizes a different second messenger clearly suggests that the specific reduction in responsiveness to GABA must occur as a result of an alteration in the activation/transduction system for that transmitter substance.

The most likely site of change which could be responsible for the observed subsensitivity to GABA resides at the level of the GABA receptor or its transduction mechanisms. In the rat cerebellum, the action of GABA on Purkinje neurons is mediated by $GABA_A$ receptors (Kaneda *et al.*, 1989; Parfit *et al.*, 1990). Because $GABA_A$ receptors are an integral part of the chloride channel (Schwartz, 1989; Bormann, 1988), the possibility exists that reduced responsiveness to GABA could be due to either a decrease in the number of $GABA_A$ receptors or a reduced activation of the chloride channel by GABA. Support for this idea is found in the reduction in chloride ion conductance which has been observed in cerebral cortex (J. W. Dailey, personal communication). In addition, Faingold *et al.* (1986a,b) have reported reduced sensitivity of inferior collicullar neurons to both GABA and flurazepam. These two compounds rely on similar changes in chloride ion conductance for their action. These data and those of the present study suggest that an alteration in the number of $GABA_A$ receptors or the GABA-mediated increase in chloride conductance may exist at all levels of the neuraxis in the GEPR-9. Interestingly, Booker *et*

al. (1986) reported a 1.7-fold increase in the number of $GABA_A$ receptors (determined by using [^{3}H]muscimol binding) in the GEPR-9. These authors also found no alteration in affinity of the GABA receptor (*i.e.*, no change in K_d) and concluded that the increase in receptor number may represent an adaptive response to reduced turnover. Such a compensatory increase in receptor number would normally lead to greater responses of target cells to exogenously applied compounds rather than the reduction in responsiveness observed in this study.

A final possibility to be considered is an alteration in the disposition of the neurotransmitter. An increased ability of neurons and/or glia to remove the transmitter from the biophase could lead to a reduced sensitivity which would be specific for GABA. Because alterations in uptake of GABA might also be expected to reciprocally affect glutamate levels, one might expect opposing changes in neuronal sensitivity to glutamate concomitant with alterations in sensitivity to GABA. The reduced sensitivity observed in this study was specific for GABA. Whereas the present data do not distinguish between an alteration in receptor and/or transduction mechanism and an alteration in disposition, the latter type of mechanism is not a common form of cellular response to changes in the environment. Additional studies are required to differentiate between these possibilities.

The data provided from this study demonstrate that cerebellar Purkinje neurons in GEPR-9 animals are subsensitive to GABA with no change in responsiveness to either NE or glutamate. Such a selective reduction in sensitivity to the inhibitory neurotransmitter, GABA, may alter the fine balance which normally exists between excitatory and inhibitory influences in favor of greater excitability. The most likely possibilities to account for this change in sensitivity include either a change in receptor number, receptor coupling, disposition of the normal transmitter or a combination of these changes. Among the potential mechanisms, the most probable mechanisms accounting for these observations relate to either the $GABA_A$ receptor or the coupling of this receptor to the chloride channel.

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