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## Involvement of GABA<sub>A</sub> receptors in the outgrowth of cultured hippocampal neurons

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Whereas GABA is a major inhibitory neurotransmitter in the adult central nervous system, recent experiments performed in our laboratory have shown that the activation of GABA<sub>A</sub> receptors in the hippocampus leads to excitatory effects during the early post-natal period. The possible consequence of a depolarizing effect of GABA was assessed on the neuritic outgrowth of embryonic hippocampal neurons in culture. No morphological alterations were observed when hippocampal neurons were cultured for three days in the presence of muscimol, a GABA<sub>A</sub> receptor agonist. In contrast, the neuritic outgrowth of cultured hippocampal neurons was profoundly affected by the presence of bicuculline in the culture medium. In the presence of this GABA<sub>A</sub> receptor antagonist neurons displayed a reduction in the number of primary neurites and branching points, resulting in a concomitant decrease of the total neuritic length. Thus, this study suggests that GABA, acting on GABA<sub>A</sub> subtype of receptors, is able to affect the development of the hippocampus.

$\gamma$ -Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the adult mammalian CNS; it interacts with two pharmacologically distinct types of receptors: the GABA<sub>A</sub> receptor is sensitive to muscimol as an agonist and to bicuculline as an antagonist whereas the GABA<sub>B</sub> receptor recognizes baclofen as an agonist and is blocked by phaclofen (see ref. 23 for a review). GABA<sub>A</sub> receptors activate a channel permeable to Cl<sup>-</sup> ions; in the adult hippocampus this activation induces an hyperpolarization and an increased conductance, thus leading to a reduction in the cell excitability [23]. We have recently shown that a totally different situation prevails in the hippocampus during the first post-natal week. At this early stage, the activation of GABA<sub>A</sub> receptors leads to excitatory effects and mediates virtually all the excitatory drive in hippocampal cells [3]. At this stage bicuculline blocks all spontaneous and evoked activity whereas it will generate paroxysmal discharges later on. The mechanism underlying this abrupt change is a change in  $E_{Cl^-}$  [28]. The functional significance of excitatory effects of GABA in early postnatal life and the abrupt shift which occurs by the end of the first post-natal week have not yet been clarified. Neurotransmitters can be released from the growth cone of growing neurites and affect neuronal

development [13]. Glutamate is able to modulate the outgrowth of hippocampal neurons in culture [13] and several observations suggest that this effect is mediated by a depolarization and a Ca<sup>2+</sup> influx [10]. The possible functional role of a depolarizing action of GABA on the hippocampus during the early postnatal period was explored on cultured hippocampal neurons. We have previously shown that such cultures comprise pyramidal, stellate and bipolar neurons; a high percentage of these cells are GABA immunoreactive [21]. We presently report that bicuculline reduces the outgrowth of hippocampal neurons. A preliminary portion of this work was presented under an abstract form [2].

Cultures of hippocampal neurons were issued from 18-day-old Wistar rat embryos and prepared as previously described [1]. Cells were grown onto polylysine at a low density in a defined medium without serum. Two hours after seeding the drugs were added at the following final concentrations: muscimol (Cambridge Res. Biochem.) 10<sup>-5</sup> M; bicuculline methochloride (Tocris) 2 × 10<sup>-4</sup> M. After 3 days, the cultures were processed for silver staining and morphometric analysis (with a computer-assisted image analyzer, Starwise, IMSTAR, France). Statistical comparisons were made using the  $\chi^2$ - and the Colin-White bilateral ranking tests.

Hippocampal neurons are easily identified in culture (Fig. 1) since neuritic processes are clearly visible as early

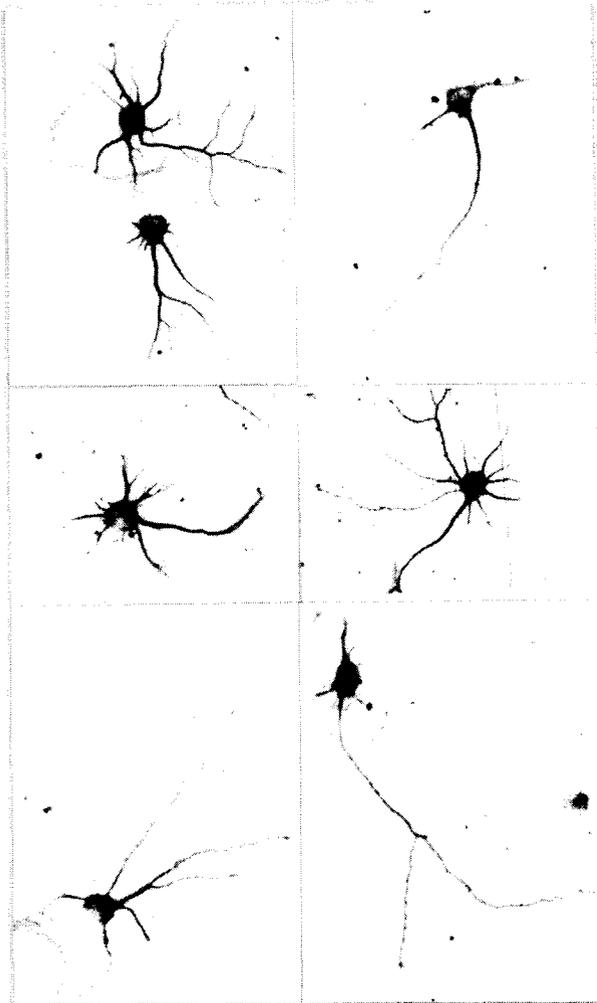


Fig. 1. Hippocampal neurons grown for 3 days on polylysine in a chemically defined medium. Since cells were plated at a low density, neurons were taken from several fields and the pictures were juxtaposed.

as 24 h after seeding. Fig. 2 represents the percentage distribution of primary neurites (starting from the cell soma, Fig. 2A) and branching points (Fig. 2B), respectively. The two distributions did not differ significantly when hippocampal neurons were grown in the absence or in the presence of the GABA<sub>A</sub> agonist, muscimol ( $10^{-5}$  M). On the other hand when the GABA<sub>A</sub> antagonist bicuculline ( $2 \times 10^{-4}$  M), was added to the culture medium morphological alterations of hippocampal neurons were noticed (Fig. 2): the median value of the number of primary neurites decreased from 5.2 to 4.0 and the one for branching points from 3.8 to 1.5, giving a 25% and a 60% reduction respectively. The total neuritic length (i.e. the sum of the neurite lengths for each neuron) was compared in cumulative frequency distribution plots (Fig. 3). The distribution did not differ when the cultures were grown in the absence or in the presence of muscimol ( $10^{-5}$  M). In the presence of bicuculline ( $2 \times 10^{-4}$  M) the

distribution curve was shifted towards lower values indicating that all neurons responded to bicuculline with an average 50% decrease of their total neuritic length as compared to the controls (Fig. 3). Moreover the graphical representation shows that the data for control or muscimol treated cells best fit with two straight lines (Henry's line); this suggests that there are two log-nor-

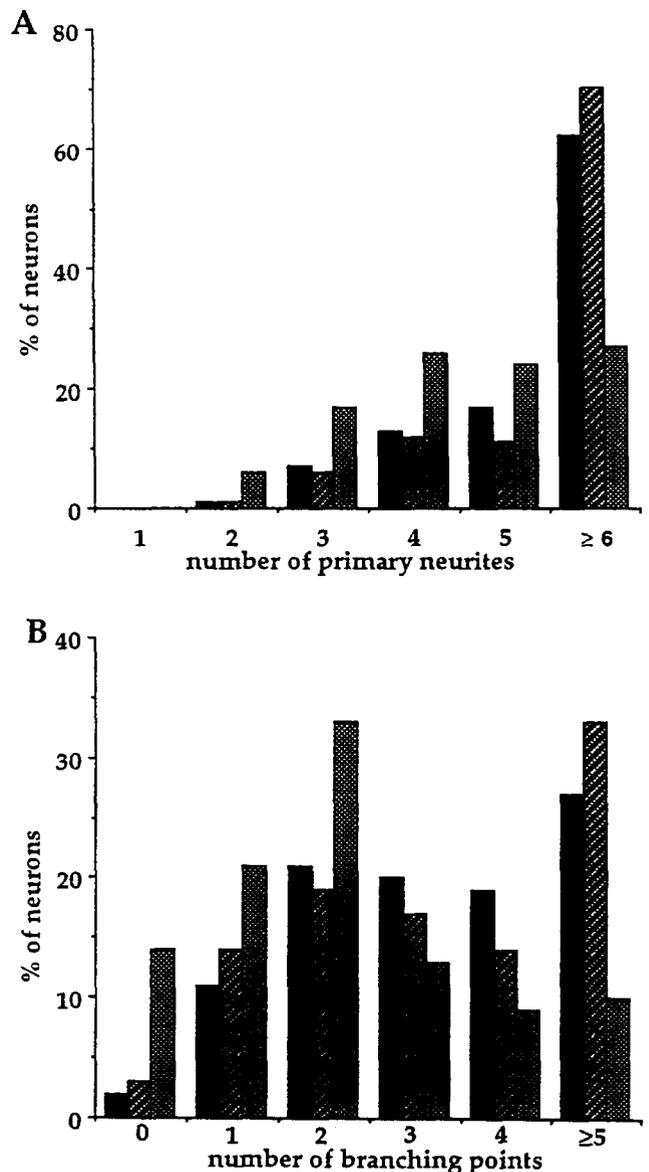


Fig. 2. Effects of pharmacological agents on the number of primary neurites (A) and branching points (B). Cells were dissociated after a trypsin digestion and were plated on polylysine. Hippocampal neurons were grown for 3 days in the absence or in the presence of the pharmacological agents. For each culture condition, 150 neurons were sampled through 3 dishes and analysed. Data are given as percentages of neurons having  $n$  primary neurites (A) or  $n$  branching points (B). Filled columns: no treatment; hatched columns: muscimol,  $10^{-5}$  M; stippled columns: bicuculline,  $2 \times 10^{-4}$  M. A significant change in the distribution of primary neurites or branching points was found only when bicuculline was added ( $\chi^2$  test with  $P < 0.001$ ).

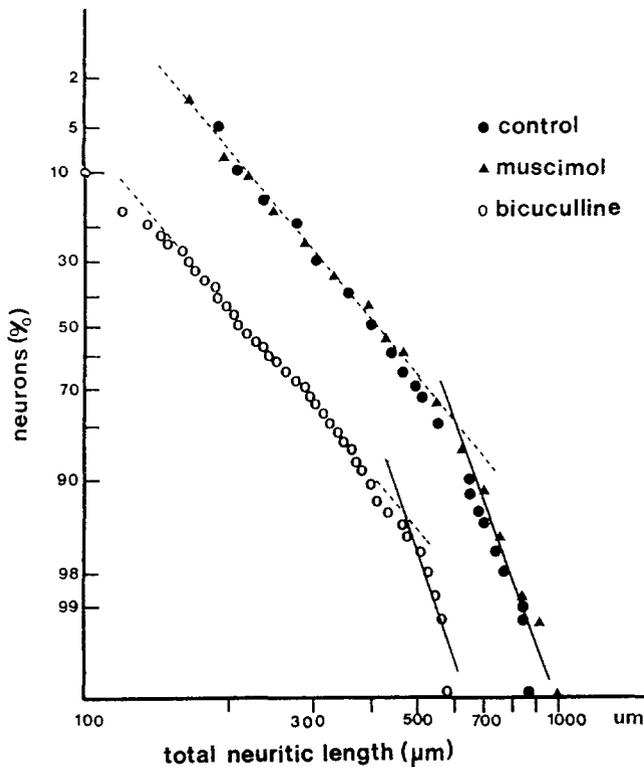


Fig. 3. Effect of various pharmacological treatments on the total neuritic length of cultured hippocampal neurons. Plot of the cumulative frequency distribution of the population of hippocampal neurons (ordinate) versus the total neuritic length of the neurons in  $\mu\text{m}$  (abscissa). Using a Gaussian scale for the ordinate and a logarithmic scale for the abscissa, a log-normal distribution appears as a straight line (Henry's line). Neurons were grown for 3 days in the absence or in the presence of the following agents: muscimol ( $10^{-5}$  M), bicuculline ( $2 \times 10^{-4}$  M). For each condition 150 neurons taken from 3 dishes were analysed. When the two distributions (control and bicuculline treatment) are compared using the Colin-White bilateral ranking test (NF X06-065), a value of 8.4 is obtained for the  $U$  criterium. Thus the distribution of the total neuritic lengths observed after bicuculline treatment is significantly different (with a probability greater than 99%) from that obtained with untreated cells.

mal distributions reflecting the existence of two populations of neurons which differ according to their neuritic outgrowth. However the two log-normal distributions are similarly affected by the bicuculline treatment as shown by the parallel displacement of the straight lines (Fig. 3).

Most of our experiments were performed with dissociated cell cultures obtained through enzymatic digestion followed by mechanical dissociation of the embryonic tissues. In order to check the possible interference brought by the dissociation procedure, hippocampal cell suspensions were obtained through mechanical dissociation without a trypsin digestion. In addition, the nature of the substratum was modified by adsorbing serum onto polylysine-coated dishes before cell seeding;

such a procedure fosters neuritic outgrowth. For all these conditions, cultures were grown with or without bicuculline ( $2 \times 10^{-4}$  M) and the total neuritic length was measured. For each protocol, the median value (i.e. the neuritic length achieved by 50% of the neuronal population) observed after bicuculline treatment is reduced by 20–50% as compared to untreated cells (Table 1). The significance of this reduction was assessed by comparing the distribution of the total neuritic length in both conditions using the Colin-White bilateral ranking test. In all cases, the  $U$  criterium value is greater than 2.58. Thus the distribution observed after bicuculline treatment is always different from that of untreated cells, with a probability greater than 99%.

The present study indicates that  $\text{GABA}_A$  receptors may modulate the neuritic outgrowth of cultured rat hippocampal neurons. In the presence of bicuculline neurons have a lower number of primary neurites and branching points with a concomitant reduction in the total neuritic length. The concentration of bicuculline required to produce morphological alterations of neuritic outgrowth ( $2 \times 10^{-4}$  M) is higher than the one which blocks  $\text{GABA}$ -mediated currents. Bicuculline was added only once at the beginning of the culture period and might have been partly inactivated during the 3 days period of culture. On the other hand, the specific  $\text{GABA}_A$  receptor

TABLE 1

EFFECTS OF VARIOUS CULTURE PROTOCOLS ON THE TOTAL NEURITIC LENGTH OF CONTROL AND BICUCULLINE-TREATED CELLS

Four experimental conditions were tested: (i) no trypsin digestion and no serum coating (polylysine alone); (ii) no trypsin digestion and serum coating; (iii) trypsin digestion and no serum coating; (iv) trypsin digestion and serum coating. Cells were grown for 3 days in the absence or in the presence of  $2 \times 10^{-4}$  M bicuculline. For each condition, the total neuritic length was measured on 150 neurons randomly taken. The last experimental condition (\*) was assessed in a separate experiment. For each protocol, the total neuritic length distribution observed after bicuculline treatment was found to be significantly different (by using the Colin-White test) with a probability greater than 99% from that of untreated cultures.

Cell culture protocol	Total neuritic length (median value, $\mu\text{m}$ )	
	Control	Bicuculline
No trypsin digestion		
No serum coating	478	381 (-21%)
Serum coating	516	265 (-49%)
Trypsin digestion		
No serum coating	405	210 (-48%)
Serum coating*	294	188 (-37%)

agonist, muscimol ( $10^{-5}$  M), did not modify the neuritic patterns. The lack of effect of muscimol could be due to the release in the culture medium of endogenous GABA or GABA-like substances that would compete with muscimol. Indeed previous studies including ours, indicate that the density of GABAergic neurons is much higher in cultures [9, 21] than in vivo [26].

Earlier studies have also suggested that GABA promotes the neurite outgrowth and differentiation of different types of neurons in culture [8, 15, 16, 20, 24] but the underlying molecular mechanisms are unknown. Cytosolic free calcium is known to play an important role for the growth cone motility and neuritic elongation [5, 10]. An elevation in intracellular calcium concentration may originate from an increase in calcium influx through voltage-sensitive calcium channels or receptor-channel complex as well as from intracellular pools of calcium [17]. Several observations indicate that the growth cone motility is strongly dependent on calcium influx through voltage-sensitive calcium channels activated by depolarizing neurotransmitters [10, 13] like glutamate [14, 18, 19].

An earlier study from our laboratory has shown that GABA provides the majority of the excitatory drive during the first post-natal week in the hippocampus [3, 4]. Bicuculline blocks all the spontaneous synaptic activity whereas GABA or GABA<sub>A</sub> agonist depolarize immature pyramidal neurons. Only after post-natal day 6, does GABA become inhibitory. We therefore propose that GABA by depolarizing immature pyramidal cells will increase the intracellular calcium concentration via the activation of calcium voltage-dependent channels and thus will promote the morphological development of these neurons. In agreement with this hypothesis, recent studies have shown that GABA depolarized immature cortical neurons on slices [12] and cerebellar granule cells in culture [6] during a restricted period of development, and induced a persistent increase (for several minutes) in the intracellular calcium concentration [6, 27]. The rise in calcium concentration induced by GABA in developing neocortex was blocked by Ni<sup>2+</sup>, supporting the activation of voltage-gated calcium channels [27].

In the hippocampus, GABAergic interneurons divide and differentiate prior the pyramidal neurons [11] and immature GABAergic synapses are observed early in postnatal development [22]. Since developing neurons require an excitatory drive and a rise in Ca<sup>2+</sup>, GABAergic interneurons are in a unique situation to modulate the differentiation and synaptogenesis on the pyramidal cells. Indeed spontaneous depolarizing potentials mediated by GABA [3] probably released from growth cone [25], may provide the calcium influx necessary for the morphological development of the pyramidal neurons at

an early developmental stage when excitatory connections are poorly developed [7].

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