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# SHORT COMMUNICATION
# Nitric oxide sensitive depolarization-induced hyperpolarization: a possible role for gap junctions during development

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## Abstract

Electrical coupling is a widespread feature of developing neuronal circuits and it contributes to the generation of patterned activity. In the developing rat hippocampus, release of GABA by coactive hilar interneurones generates widespread synchronized activity. Here it is shown that hilar interneurones strongly rectify in the outward direction when depolarized. This depolarization-induced hyperpolarization, abolished by gap junction uncouplers, is modulated by nitric oxide. This phenomenon might represent a current-shunting mechanism of the excess current by providing functional inhibition at a developmental stage when GABA is excitatory. Spatial buffering of the current might represent an osmotic mechanism for growth and differentiation.

## Introduction

Although γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain, during development it excites and depolarizes neurones through $GABA_A$ receptors, by an outward flux of $Cl^-$ (Serafini *et al.*, 1995; Kaneda *et al.*, 1995; Chen *et al.*, 1996). In newborn rat hippocampi, spontaneous release of GABA is detected as large synaptic events, named giant GABAergic potentials (GGPs; Ben Ari *et al.* 1989; reviewed in Cherubini *et al.*, 1991). GGPs originate in the hilus from electrically coupled interneurones, paced by an inward rectifier current, and then propagate successively to CA3 and CA1 fields (Strata *et al.*, 1997).

In the present study, aimed at characterizing the membrane properties of electrically coupled hilar interneurones, we show that membrane depolarization elicits a strong voltage-dependent hyperpolarization. This phenomenon, modulated by nitric oxide (NO), is also detectable in tetrodotoxin (TTX) and is blocked by gap junction uncouplers. This finding suggests that it may be related to electrical synapses. Parts of this work have been presented elsewhere (Strata *et al.*, 1995; Strata, 1996).

## Materials and methods

### *Electrophysiology*

Hippocampal slices from P1–P17 Wistar rats were prepared as described (Strata *et al.*, 1997). Slices (600 μm thick) were cut and placed at room temperature in artificial cerebrospinal fluid (ACSF), of the following composition (mM): NaCl 126, KCl 3.5, $NaH_2PO_4$ 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 25 and glucose 11, equilibrated with 95% $O_2$ and 5% $CO_2$. Microelectrodes were filled with 3 M KCl. Resting membrane potentials ranged from –51 to –67 mV. Unless otherwise specified, data are expressed as means ± SD

### *Histology*

Microelectrodes (70–120 MΩ) were filled with a solution containing 2–3% biocytin (Sigma) or 1–2% neurobiotin (Vector). Biocytin or neurobiotin were injected by passing 0.8–1 nA hyperpolarizing and depolarizing pulses alternatively (500 ms, 1 Hz), for at least 10 min. Slices were then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 6 h. After postfixation slices were washed (4 × 15 min) in FITC buffer (150 mM NaCl, 10 mM Hepes, pH 7.4) and then incubated with fluorescinated avidin (Vector) diluted (1 : 500) in FITC buffer for 1 h. Slices were then placed on gelatinized slides, briefly air dried and mounted in mounting-shield (Vector).

## Results

We have recently reported that by injecting biocytin in a hilar cell a constellation of surrounding neurones was also stained (see Strata *et al.*, 1997; Fig. 1A). A similar result was obtained when neurobiotin was injected; on average the number of cells stained

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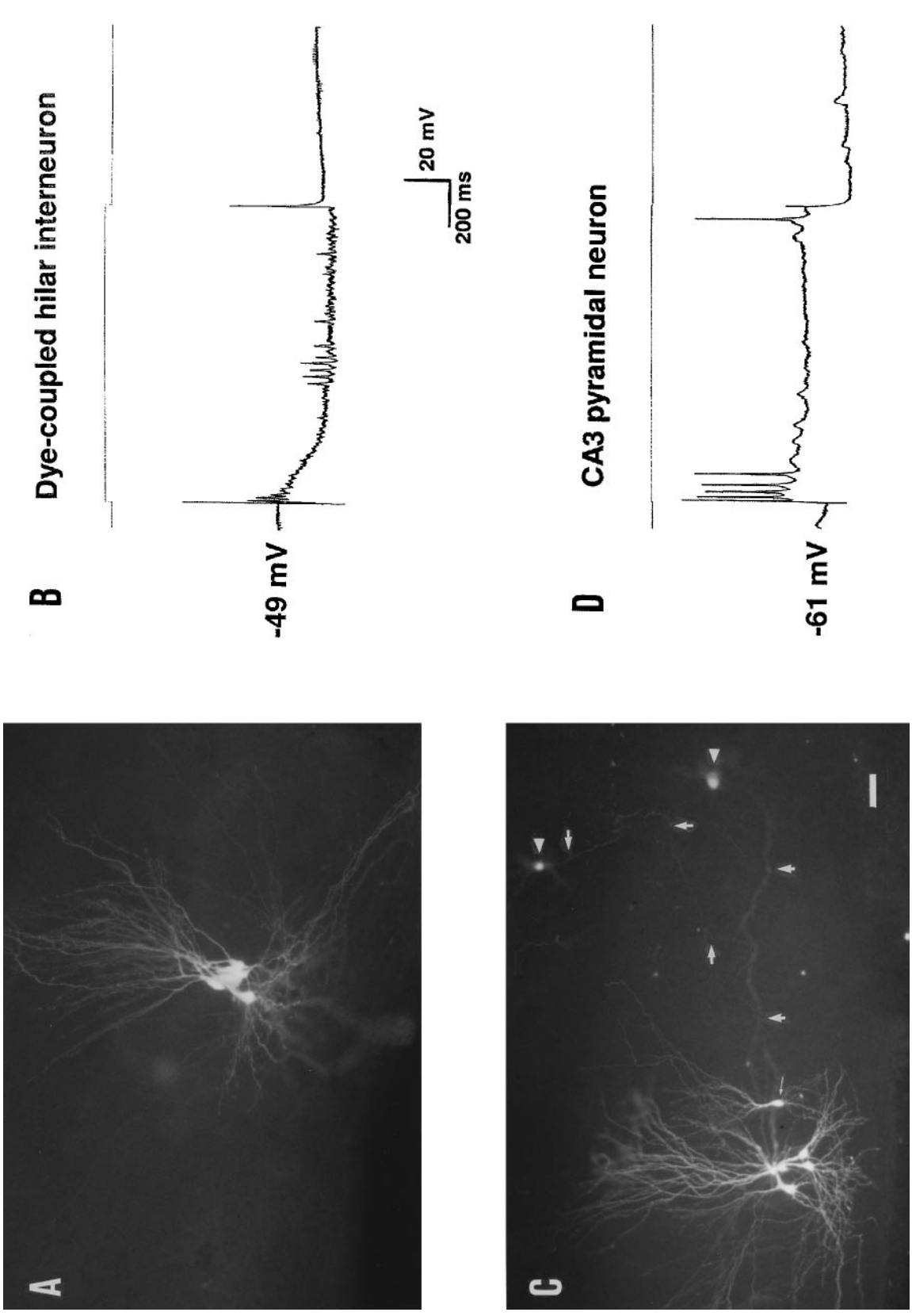


FIG. 1. Depolarization-induced hyperpolarization (DIH) is present in hilar but not in CA3 pyramidal neurones. (A) A cluster of dye coupled (biocytin) hilar interneurones, from a P6 rat hippocampus. (B) A depolarizing current pulse of +2 nA (upper trace) injected into one of the hilar interneurones shown in (A), elicited a strong hyperpolarization, which persisted throughout the pulse. The presence of 'spikelets' observed at the peak of the steady state hyperpolarization is remarkable. (C) An heterogeneous population of neurones was revealed by neurobiotin injected into a P1 CA3 pyramidal neurone. Neurobiotin spread to adjacent pyramidal neurones and pyramidal basket cells within the CA3 itself (small white arrow) but also to interneurones localized in the hilus (arrowheads) and connected through long processes (white arrows). Clusters of coactive neurones may represent the functional unit that contribute to GGPs propagation. Scale bar = 50 μm. (D) A depolarizing electrotonic potential elicited, in a P1 CA3 pyramidal neurone, by a current pulse of + 0.4 nA (upper trace). The firing was characterized by an accommodative burst of action potentials.

by a single injection was 5.6 ± 2.3 (range 3–11; $n = 15$). By contrast, in the CA3 region only neurobiotin injections revealed heterogeneous clusters of pyramidal cells and interneurones, also localized in the hilus, whereas biocytin labelled a single cell (see Strata *et al.*, 1997). By injecting neurobiotin the average number of cells stained was 4.6 ± 2.3 (range 2–8; $n = 5$).

When hilar neurones were depolarized from their resting membrane potential by a current pulse (34 out of 46 neurones tested) or by a slow current ramp (6/7), a strong membrane hyperpolarization was induced that reset the membrane potential either at resting level or below (Fig. 1B). This depolarization-induced hyperpolarization (DIH) could be elicited also when depolarizing current pulses were delivered in the presence of TTX (1 μM; Fig. 2A,E), indicating that sodium spikes were not required for its induction ($n = 7$). Moreover DIH was not blocked by either 4-aminopyridine (4-AP, 1 mM; Fig. 2A) or a low concentration of tetraethylammonium (1 mM, not shown), thus excluding the involvement of some potassium conductance in the hyperpolarization. DIH was never observed in dye-coupled CA3 neurones ($n = 5$). A depolarizing current pulse in pyramidal neurones (+0.4–+0.5 nA) elicited action potentials (mean 5.67 ± 1, $n = 9$) in accommodative trains (Wong & Prince, 1981) followed by an after hyperpolarization (Fig. 1D).

To ascertain whether DIH was related to electrical coupling, we tested the effect of the gap junction uncouplers octanol (0.5 mM) and halothane (2%). As shown in Figure 2(B), DIH was reversibly abolished by octanol ($n = 11$), and a recovery was observed within 15 min. DIH was also abolished by halothane ($n = 3$) leading to an increase in the input resistance, as calculated by depolarizing current steps (Fig. 2E,F). A partial recovery was obtained after 45 min of wash.

In turtle retinal horizontal cells (Miyachi *et al.*, 1990) and in rat sensorimotor cortex (Rörig & Sutor, 1996) gap junctions are modulated by NO. To probe whether this unconventional messenger might regulate the DIH, we tested the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). As shown in Figure 3(A), bath application of L-NAME (1 mM) reversibly inhibited DIH. The effect was rapid in onset (3–5 min) and a full recovery was obtained after a 15-min wash. The NO scavenger haemoglobin (10 μM) reduced DIH as well (Fig. 3B). As shown in Figure 3(C) DIH was not affected by the NO natural precursor, L-arginine (0.3–1 mM, $n = 14$). A similar result was obtained by applying sodium nitroprusside (SNP, 1 μM, $n = 2$; Fig. 3D), known to increase NO activity (Schuman & Madison, 1994).

It has been proposed that the voltage sensor, which is responsible for voltage sensitivity of gap junctions, can be controlled by modifying the ion composition of the extracellular space (Bennet *et al.*, 1991). When the external concentration of chloride was lowered (from 132 mM to 69.8 mM; $n = 3$; isethionate substitution; Fig. 3E), or devoided of potassium ($n = 2$; not shown), DIH was readily abolished.

Finally, DIH was developmentally regulated. In Figure 3(F,G) are shown the slightly accommodative firing of a P14 hilar interneurone and the histogram of hilar neurones displaying DIH at different ages, respectively.

## Discussion

The present experiments provide the first demonstration that:
**1** single injections of neurobiotin, instead of biocytin (323 vs. 372.5 (Da), revealed clusters of dye-coupled neurones in the CA3 region as well;
**2** the V/I curve of electrically coupled hilar interneurones strongly rectified in the outward direction when depolarized;
**3** DIH was abolished by gap junction uncouplers and modulated by the diffusible messenger NO.

Coupling is widespread during development, thus providing the outlines of functional architecture in the brain (for recent reviews see Kandler & Katz, 1995; Bruzzone & Ressot, 1997). Junctional communication can serve to synchronize the electrical activity of given neuronal groups, thus contributing to the generation of patterned activity (Yuste *et al.*, 1992; Peinado *et al.*, 1993; Penn *et al.*, 1994). In the CA3 region pyramidal neurones displayed homologous coupling to other principal cells and to GABAergic interneurones located in both the CA3 field and the hilus. It is therefore possible that the developing hippocampal cortex is probably organized in functional clusters (or domains) like those described in the neocortex (Yuste *et al.*, 1991) and in the retina (Penn *et al.*, 1994). Moreover, the presence of backprojecting collaterals from the CA3 to the hilus, reported also in mature hippocampi, may represent a rudimentary functional connectivity that contributes to the high correlation of spontaneously occurring GGPs in the CA3 and in the hilus (Strata *et al.*, 1997). Thinner interconnections permeable to neurobiotin but not to biocytin, are consistent with the different electrophysiological features of CA3 and hilar neurones.

To date, the main features correlated with the presence of electrical coupling are: (i) electrotonic coupling (Christie *et al.*, 1989); (ii) fast prepotentials (MacVicar & Dudek, 1981); (iii) short latency depolarizations in rat hippocampus (Taylor & Dudek, 1982), developing neocortex (Connors *et al.*, 1983), and nucleus accumbens (O'Donnel & Grace, 1993); (iv) and increase in neuronal input resistance when gap junctions are uncoupled (Rörig *et al.*, 1996).

In the hilar region of the mature hippocampus several cell types have been morphologically identified (Amaral, 1978). Their major electrophysiological trait is to fire in a non-accommodative fashion upon depolarization (Buhl *et al.*, 1994; Scharfman, 1995). Moreover, some exhibit a decrease in input resistance with depolarization indicating an outward rectification of the membrane (Misgeld & Frotscher, 1986). Different behaviour was observed in neonatal hilar interneurones: these exhibited a strong voltage-dependent DIH. This was also detected in TTX, thus suggesting that it was generated within the recorded neurone. Similarly, during the development of myotubes, electrotonic potentials are reduced during constant depolarization (Obata, 1974). Moreover, a particular combination of chimeric connexins expressed by *Xenopus* oocytes shows similar electrophysiological properties (Bruzzone *et al.*, 1994).

Neurones yielding this rectification were dye coupled as morphologically demonstrated by biocytin or neurobiotin injection. Although we failed to record from pairs of dye coupled cells, it is fair to assume that DIH was due to intercellular communication as it was abolished by gap junction uncouplers. In these conditions the V/I relationship became symmetrical in the depolarizing and hyperpolarizing direction (Fig. 2F).

The conductance of some gap junctions is gated by voltage in a manner similar to that of voltage-dependent channels. Moderate changes in resting potential would markedly affect junctional conductance (Bennet *et al.*, 1991). In our case, depolarization would open gap junctions, shunt the current, and thereby provide a functional inhibition of the syncytium at an age when GABA is excitatory (Cherubini *et al.*, 1991). When neurones are hyperpolarized and gap junctions closed, a higher input resistance would facilitate the response to a given input. Moreover, it is also

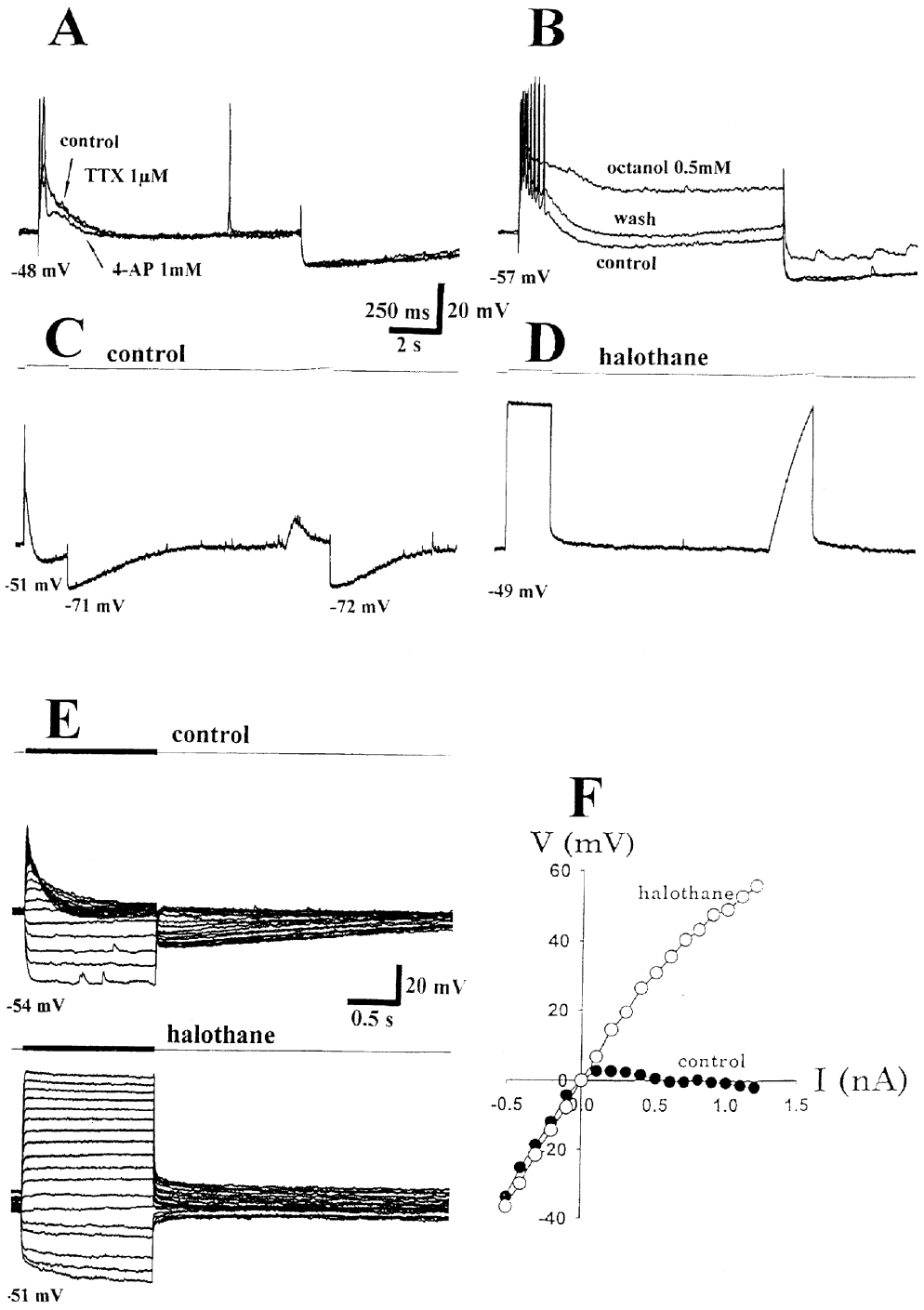


FIG. 2. The gap junction blockers octanol and halothane abolish depolarization-induced hyperpolarization (DIH). (A) In a P5 neurone a depolarizing step of current (+ 0.6 nA) elicited DIH that was blocked neither by tetrodotoxin (TTX) nor by 4-aminopyridine (4-AP). (B) Octanol reversibly blocked DIH in a P6 neurone (current pulse + 0.6 nA). To note that the undershoot at the end of the current step is also reduced. (C) The injection of a sustained depolarizing current pulse (+ 1.2 nA, 2 s; upper trace), in a P4 hilar cell, elicited a strong membrane hyperpolarization followed at the end of the pulse by a pronounced undershoot (– 71 mV) of the membrane potential. DIH was produced by injecting a slow depolarizing current ramp of the same magnitude (+ 1.2 nA, 2 s; right part of the trace). When the membrane potential reached – 36 mV, the rectification was triggered in the absence of action potentials. It is worth noting, that although the membrane rectification during the application of the ramp did not attain the same value observed during the application of the sustained current pulse, the undershoot, occurring at the end of the ramp, reached the same membrane potential level (– 72 mV). (D) DIH was blocked within 2 min by halothane 2% bubbled in the solution. (E) In the upper panel electrotonic potentials evoked in TTX by hyperpolarizing and depolarizing current pulses (from – 0.5 to 1.2 nA). DIH increased with increasing current pulse amplitude (see also F filled circle). In the lower panel DIH was completely abolished by the gap junction blocker halothane. The input resistance of the cell was unchanged when measured by hyperpolarizing steps of current. (F) V/I plot showing the behaviour of the membrane potential of the cell shown in (E) before (filled circles) and during (open circles) superfusion with halothane. Panels (C) to (F) have been obtained from the same neurone.

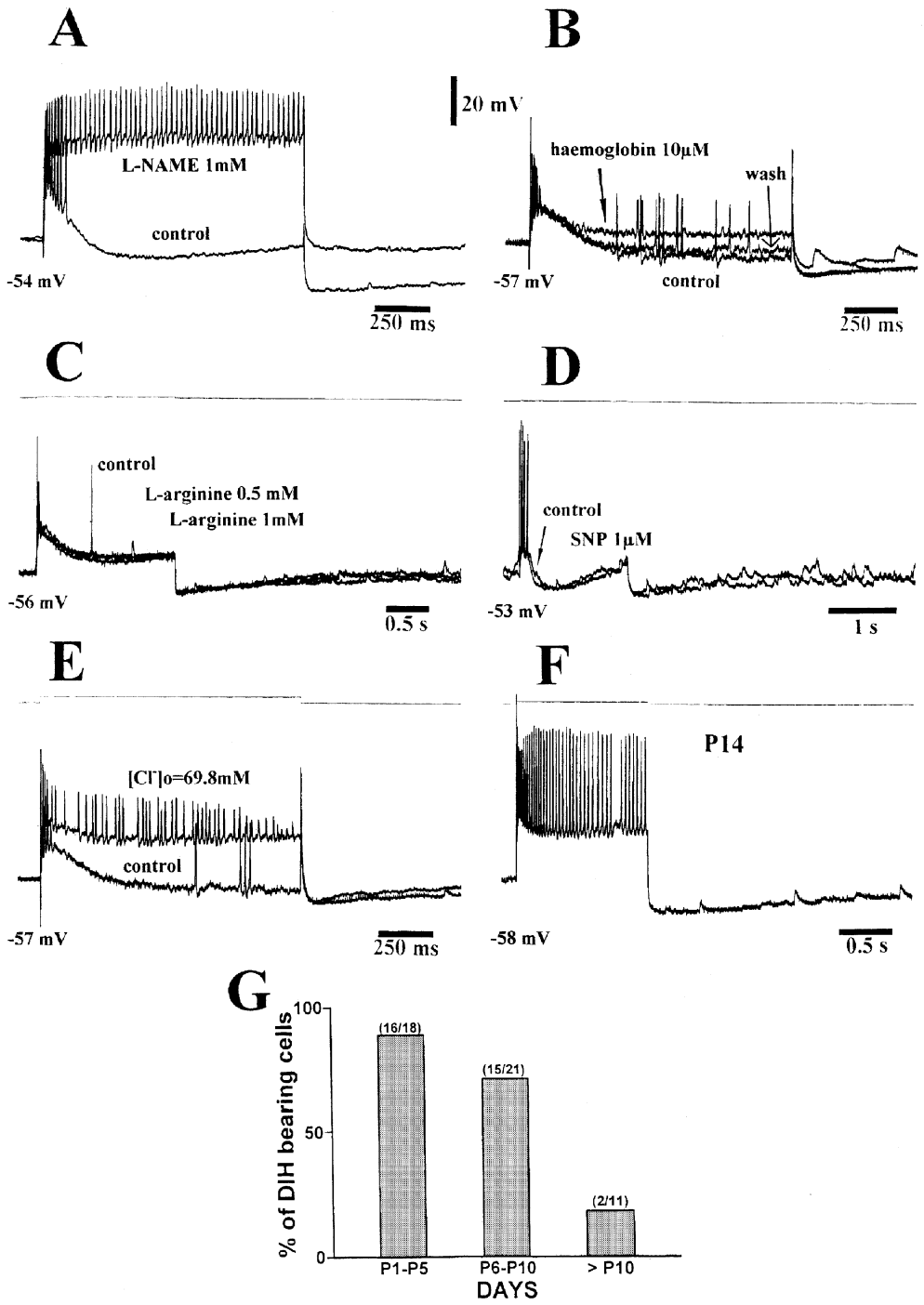


FIG. 3. Depolarization-induced hyperpolarization (DIH) is regulated by nitric oxide (NO). A. In a P6 hilar interneurone, the nitric oxide synthase (NOS) antagonist Nω-nitro-L-arginine methyl ester (L-NAME) blocked DIH, thus modifying its firing pattern. Note that, in the presence of L-NAME, the cell was able to fire a train of action potentials without any sign of accommodation. It is noteworthy, that, in the presence of L-NAME, also the amplitude of the undershoot, following the current pulse was reduced in amplitude (current pulse + 0.6 nA). (B) The NO scavenger haemoglobin partially reduced DIH (P7; 2 nA). (C) In a P5 neurone, DIH was not affected by L-arginine 0.5–1 mM (+ 0.4 nA). (D) DIH elicited in a P6 neurone was not modified by SNP (+ 0.2 nA). (E) In a P7 neurone, by lowering the external concentration of $Cl^-$ from 132 mM to 69.8 mM, DIH was inhibited and the neurone fired in a non-accommodative fashion. Also to be noted in this case is the presence of spikelets during the steady state of DIH (test pulse 2 nA). (F) In a P14 neurone, a depolarizing step of current (+ 0.8 nA) elicited a train of action potentials, with sign of slight accommodation. (G) Histogram showing the number of cells bearing DIH vs. the age. 88.9% of the neurones between P1 and P5 displayed DIH, 71.4% of those between P6 and P10, whereas it was found only in 18.2% of the cases, in animals older than P10.

possible that gap junctions can be gated by modified concentrations of both $[Cl^-]_o$ and $[K^+]_o$.

Finally, gap junctions are regulated by NO. This unconventional signalling molecule is involved in many developmental processes such as cerebellar granule cells migration and differentiation, and refinement of synaptic connectivity (Tanaka *et al.*, 1994; Wu *et al.*, 1994; Cramer & Sur, 1994; Wang *et al.*, 1995). During the development of hippocampal circuitry a weak reaction for NOS has been observed in the hilar region and is attributable to developing granule cells (Bredt & Snyder, 1994). The activity-dependent production of NO makes it the most suitable modulator of an oscillator that alternates periods of activity and inactivity. Therefore, NO might on the one hand inhibit an excessive activity through its action on the pacemaker current (Strata *et al.*, 1995) and on the other hand facilitate coactivation between hilar interneurones. GABAergic activity is associated with a temporary increase of $[K^+]_o$ (Barolet & Morris, 1991). By acting on gap junctions, NO might also facilitate the spatial buffering of $K^+$ ions (Orkand *et al.*, 1966; see also Newman, 1985) that in turn might induce changes in the extracellular osmolarity and associated water flux. These changes in osmolarity may produce swelling at sites of maximal activity and shrinking at the remote site of smaller activity (Lux *et al.*, 1986). In this combination of swelling and shrinking, fine tuned by the activity, the morphology of the growing neurone will be the resultant of the whole activity that has involved the neurone itself during the period of its growth. In addition, the voltage gradient generated by ion movements might regulate the spatio-temporal dynamics of $Ca^{2+}$ transients, important for neurite outgrowth and differentiation (Gu & Spitzer, 1995). An analogous mechanism of cell growth and differentiation is well described in developing plants (Alberts *et al.*, 1994).

In conclusion, the combination of gap junctional communications and GABA-mediated $Cl^-$ dynamics may represent important physiological mechanisms leading to plastic changes, such as growth and differentiation, occurring during development (see also Strata, 1996).

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## Abbreviations

| | |
|---|---|
| DIH | depolarization-induced hyperpolarization |
| GABA | γ-aminobutyric acid |
| GGPs | giant GABAergic potentials |
| L-NAME | Nω-nitro-L-arginine methyl ester |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| TTX | tetrodotoxin |
| SNP | sodium nitroprusside |
| 4-AP | 4-aminopyridine |

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