

# Progesterone Synthesis and Myelin Formation by Schwann Cells

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Progesterone is shown here to be produced from pregnenolone by Schwann cells in peripheral nerves. After cryolesion of the sciatic nerve in male mice, axons regenerate and become myelinated. Blocking either the local synthesis or the receptor-mediated action of progesterone impaired remyelination. Administration of progesterone or its precursor, pregnenolone, to the lesion site increased the extent of myelin sheath formation. Myelination of axons was also increased when progesterone was added to cultures of rat dorsal root ganglia. These observations indicate a role for locally produced progesterone in myelination, demonstrate that progesterone is not simply a sex steroid, and suggest a new therapeutic approach to promote myelin repair.

Steroids regulate important neuronal functions in developing and adult brain either by intracellular receptor-mediated effects on the transcription of specific genes or by acting directly on the neuronal membrane (1). Pregnenolone, progesterone, and their reduced or sulfated metabolites (termed neurosteroids) are synthesized within the central nervous system by glial cells (2) and may modulate neurotransmission (3). We now show that progesterone is also synthesized by Schwann cells in the peripheral nervous system, where it promotes myelin formation during nerve regeneration.

The progesterone biosynthetic pathway is summarized in Fig. 1A. The immediate precursor of progesterone is pregnenolone, which accumulates in large quantities in rodent sciatic nerve (4). Progesterone was also abundant in this peripheral nerve (Fig. 1B). In adult male mice, the concentration of progesterone was  $10.5 \pm 1.1$  ng per gram of tissue in sciatic nerve (mean  $\pm$  SEM,  $n = 4$ ) and only  $1.5 \pm 0.2$  ng/ml in plasma,  $1.9 \pm 0.2$  ng/g in kidney,  $1.2 \pm 0.1$  ng/g in liver, and  $3.8 \pm 0.7$  ng/g in heart [ $P < 0.001$  for all tissues compared to sciatic nerve by Dunnett's multiple comparison tests after analysis of variance (ANOVA)].

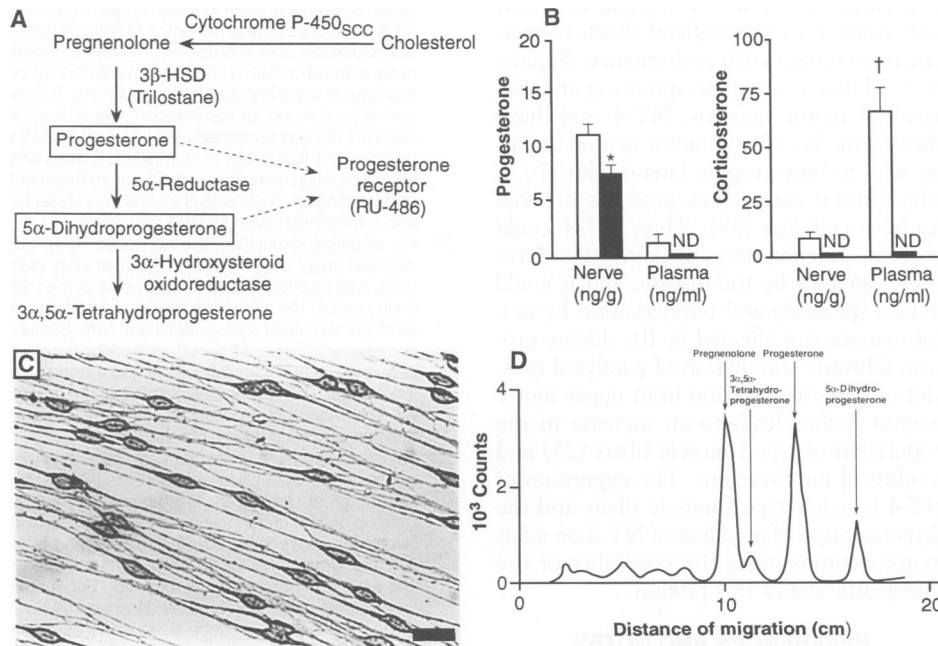
To determine whether progesterone in peripheral nerves originates from the circula-

tion or is synthesized locally, we measured progesterone concentrations in male mice after removal of the steroidogenic endocrine glands (5). Concentrations of progesterone remained high in sciatic nerve ( $7.5 \pm 0.4$  ng/g,  $n = 4$ ), but became undetectable in plasma, 5 days after castration and adrenalectomy (Fig. 1B). Because progesterone is rapidly cleared from nervous tissues

(6), these observations suggest that the steroid is synthesized within the sciatic nerve. This conclusion was further supported by the observation that the concentration of corticosterone, a steroid hormone of adrenal origin, was much higher in plasma ( $67.3 \pm 9$  ng/ml,  $n = 4$ ) than in sciatic nerve ( $7.1 \pm 0.9$  ng/g) of control mice and that it decreased almost to zero after castration and adrenalectomy (Fig. 1B).

Schwann cells were shown to be a source of progesterone. Pure cultures of Schwann cells prepared from rat dorsal root ganglia (DRG) expressed the  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase isomerase enzyme (3 $\beta$ -HSD) (Fig. 1C) and produced significant amounts of [ $^3$ H]progesterone and its reduced metabolites, 5 $\alpha$ -dihydroprogesterone and 3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone, when incubated with [ $^3$ H]pregnenolone (Fig. 1D).

The production of neurosteroids appears to be a feature of myelinating glial cells in the peripheral and central nervous systems; we have previously shown that oligodendrocytes also synthesize steroids (7). Thus, neurosteroids—and, in particular, progesterone, which is frequently (but empirically) used in defined culture media for cells of the nervous system (8)—may play an important role in myelination. To investigate this possibility, we measured neurosteroids



**Fig. 1.** (A) Pathways of neurosteroid biosynthesis in glial cells indicating enzymes and their inhibitors (parentheses). SCC, side-chain cleavage. 3 $\beta$ -HSD,  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase isomerase. (B) Concentrations of progesterone and corticosterone in sciatic nerve and plasma of sham-operated adult male mice (open bars) and of mice subjected to castration and adrenalectomy 5 days previously (solid bars) (5). Data are means  $\pm$  SEM of four samples (24). \* $P < 0.05$  versus intact animals (Student's  $t$  test). † $P < 0.001$  versus nerve of intact animals (Student's  $t$  test). ND, not detectable. (C) Rat Schwann cells isolated from DRG cultures and stained with antibodies to rat 3 $\beta$ -HSD (25). Scale bar, 20  $\mu$ m. (D) Metabolism of pregnenolone by rat Schwann cells in culture. The thin-layer chromatogram shows that tritiated [ $^3$ H]pregnenolone (100 nM) was converted to progesterone ( $14.3 \pm 0.6$ ), 5 $\alpha$ -dihydroprogesterone ( $3.9 \pm 0.2$ ), and 3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone ( $2.6 \pm 0.1$  pmol per microgram of DNA per 24 hours) (means  $\pm$  SEM) (26).

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and myelin formation in vivo, in the regenerating sciatic nerve of male mice after cryolesion (9). In response to local freezing, axons and their accompanying myelin sheaths degenerate quickly within the frozen zone and in the distal segment (Wallerian degeneration) (10). However, the basal lamina tubes remain intact and provide an appropriate environment and support for rapid regeneration of the damaged nerve fibers. In the frozen zone, Schwann cells start to myelinate the regenerating fibers within 1 week. Two weeks after injury, myelin sheaths reach approximately one-third of their final size.

If pregnenolone and progesterone stimulate the formation of new myelin sheaths, increased concentrations of these neurosteroids might be expected within the regenerating sciatic nerve during the process of myelination. Indeed, concentrations of pregnenolone and progesterone remained high (5 to 10 times the plasma values) within the distal part of the sciatic nerve 7 and 15 days after lesion (Table 1). When steroid concentrations were expressed as nanograms per gram of tissue, values did not change significantly in response to the lesion. However, because the mass of the nerves increased as a result of Schwann cell proliferation and edema (11), the amounts of pregnenolone and progesterone per nerve, as expressed in picograms per centimeter, were increased; the increases were more marked 15 days after lesion, when Schwann cells are actively myelinating the regenerating axons (Table 1).

The role of progesterone in myelin repair was assessed by inhibiting its synthesis or action. Repeated local applications of 100

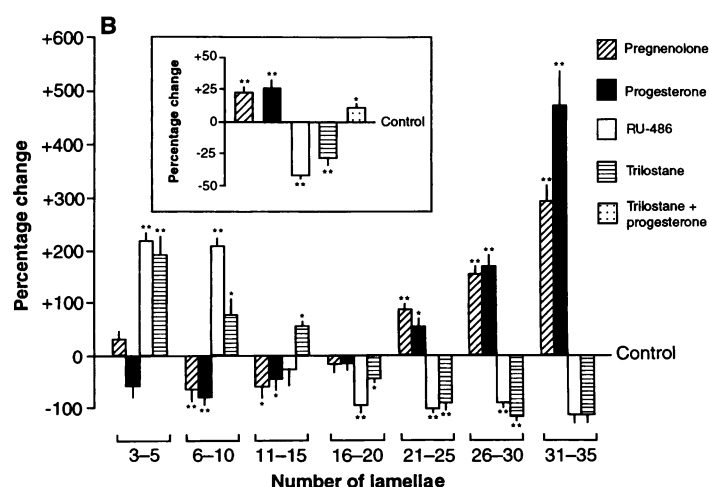
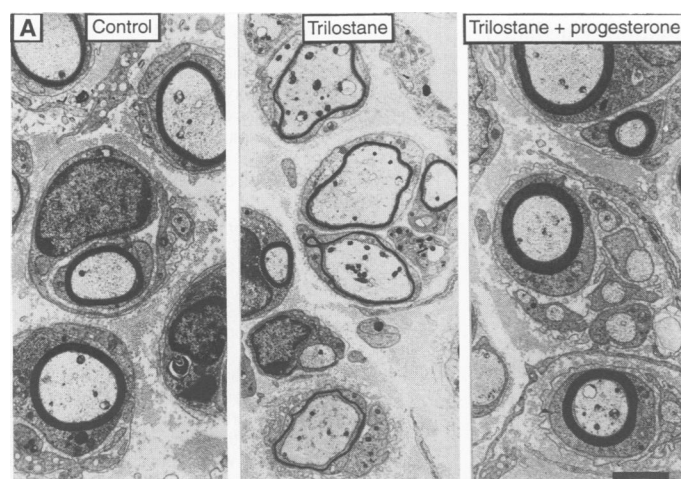
$\mu\text{g}$  of trilostane, an inhibitor of the conversion of pregnenolone to progesterone, or of RU-486, a potent competitive antagonist of progesterone at the receptor level, decreased the thickness (number of lamellae) of myelin sheaths in cross sections measured by electron microscopy 15 days after lesion (Fig. 2, A and B) (12). The inhibitory effect of trilostane was not attributable to toxicity because it could be reversed by the simultaneous administration of progesterone. Myelin sheaths were thicker in nerves treated with trilostane plus progesterone than in control nerves (Fig. 2B, inset), suggesting that remyelination may be enhanced by high doses of neurosteroids. Indeed, application of 100  $\mu\text{g}$  of either pregnenolone or

progesterone close to cryolesioned sciatic nerves significantly increased the thickness of the myelin sheaths measured after 15 days (Fig. 2B, inset). The structure and compaction of the myelin sheaths formed in response to either neurosteroid were morphologically normal when examined by electron microscopy. The effects of the neurosteroids and of their inhibitors were particularly apparent when myelin sheaths were classified according to the number of lamellae formed. Both pregnenolone and progesterone increased the proportion of myelin sheaths with large numbers of lamellae ( $>20$ ), whereas in the presence of progesterone inhibitors only thin myelin sheaths with few lamellae were observed (Fig. 2B).

**Table 1.** Concentrations of pregnenolone and progesterone in sciatic nerve and plasma 7 and 15 days after cryolesion of the sciatic nerve in male mice (9). Fourteen nerves were pooled for each sample. Data are means  $\pm$  SEM of four samples.

Day	Concentration	Sciatic nerve		Plasma	
		Sham	Lesioned	Sham	Lesioned
Pregnenolone					
7	pg/cm	21.4 ± 1.5	25.3 ± 0.4	0.6 ± 0.1	0.5 ± 0.1
	ng/g	6.9 ± 0.2	5.7 ± 0.5		
15	ng/ml				
	pg/cm	18.2 ± 1.4	29.1 ± 2.3**		
	ng/g	5.5 ± 0.1	5.9 ± 0.4		
	ng/ml			0.6 ± 0.2	0.7 ± 0.1
Progesterone					
7	pg/cm	32.0 ± 3.0	38.0 ± 2.3*	1.4 ± 0.3	1.5 ± 0.1
	ng/g	10.3 ± 0.7	8.7 ± 0.9		
	ng/ml				
	pg/cm	28.9 ± 3.2	38.9 ± 2.1**		
15	ng/g	8.5 ± 0.9	8.9 ± 0.6		
	ng/ml			1.3 ± 0.1	1.4 ± 0.2

\* $P < 0.05$ , \*\* $P < 0.001$  versus the corresponding sham-operated controls (Student-Newman-Keuls post hoc comparison after one-way ANOVA).



**Fig. 2.** Role of neurosteroids in the formation of myelin sheaths. The thickness of myelin sheaths (number of lamellae) was quantified by electron microscopy of cross sections of sciatic nerves from male mice 15 days after cryolesion (12). (A) Effect of trilostane in the absence (center panel) or presence (right panel) of progesterone on the thickness of myelin sheaths, relative to that in control nerves (left panel). Scale bar, 2  $\mu\text{m}$ . (B) Effects of neurosteroids

and their inhibitors on the number of lamellae of myelin sheaths, classified in increments of five. (Inset) Effects of neurosteroids and inhibitors on the mean number of lamellae per myelin sheath. Data are means  $\pm$  SEM of five animals and are expressed as a percentage of control (baseline). \* $P < 0.05$ , \*\* $P < 0.01$  versus the corresponding control (Dunnett's multiple comparison tests after one-way ANOVA).

The percentage of myelinated fibers in the regenerating nerves (75 to 85%) was not affected by the steroid environment, suggesting that progesterone may not initiate myelination but rather stimulates ongoing myelination. We detected no correlation between axonal diameter and the size of myelin sheaths either in control or in treated nerves. In addition, axonal diameter 15 days after lesion was not affected by trilostane or RU-486, but was decreased by progesterone [control,  $2.0 \pm 0.07 \mu\text{m}$ ; progesterone,  $1.6 \pm 0.09 \mu\text{m}$  (means  $\pm$  SEM,  $n = 4$ ,  $P < 0.05$  by Student's  $t$  test)]. These data suggest that progesterone promotes myelination independently of axonal growth. Such an effect was also apparent in cultures of rat DRG. After 4 weeks in culture, neurite elongation and Schwann cell proliferation had ceased and Schwann cells myelinated the sensory axons in the presence of serum and ascorbic acid (13). At this stage, a physiological concentration of progesterone (20 nM) added daily to the culture medium for 2 weeks did not further increase the area occupied by the neurite network, the density of neurites, or the number of Schwann cells (14). However, the total length of myelinated axons (control,  $3540 \pm 183$ ; progesterone,  $19,710 \pm 1319 \mu\text{m}/\text{mm}^2$ ) (Fig. 3) and the number of myelin segments [control,  $21 \pm 2.4$ ; progesterone,  $122 \pm 18$  segments per square millimeter of culture (means  $\pm$  SEM of five culture dishes, each containing 10 DRG

explants,  $P < 0.001$  by Student's  $t$  test)] were increased sixfold by progesterone. The length of individual myelin segments was not affected by the neurosteroid [control,  $168 \pm 12$ ; progesterone,  $161 \pm 10 \mu\text{m}$  (means  $\pm$  SEM)].

A relation between neurosteroids and myelin formation was also suggested by the low concentrations of pregnenolone and progesterone in the sciatic nerves of Trembler mice, which are severely hypomyelinated and have a dominant mutation affecting the peripheral myelin protein PMP22 (15). The pregnenolone concentration was  $20.6 \pm 3.2$  ng per microgram of DNA in wild-type mice and  $1.3 \pm 0.8$  ng/ $\mu\text{g}$  in Trembler mice. The progesterone concentration was  $42.5 \pm 5.4$  ng/ $\mu\text{g}$  in wild-type animals and  $7.2 \pm 2.5$  ng/ $\mu\text{g}$  in Trembler mice (means  $\pm$  SEM,  $n = 4$  for all experimental groups,  $P < 0.001$  for both steroids when compared to wild-type mice by Student's  $t$  test). Whether the reduced concentrations of neurosteroids are a cause or a consequence of the defect in myelination remains to be investigated.

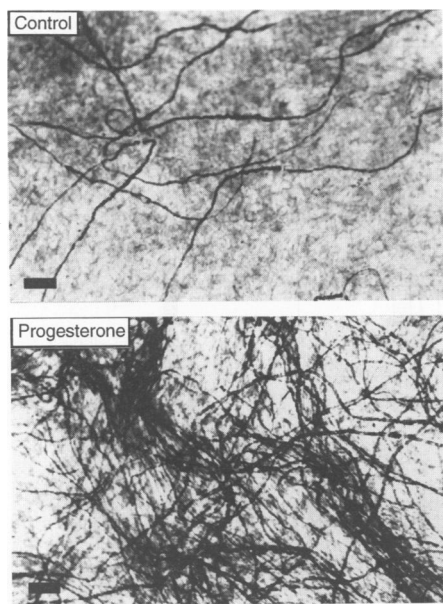
At least two mechanisms may account for the effects of progesterone on myelin formation. Progesterone produced by Schwann cells may act on adjacent neurons and activate the expression of neuronal signaling molecules required for myelination (16) or the transfer of phospholipids from axons to myelin (17). Alternatively, progesterone may function as an autocrine trophic factor and directly enhance the formation of new myelin sheaths. Indeed, Schwann cells contain an intracellular receptor for progesterone, as shown by binding studies, immunocytochemistry, and reverse transcription and polymerase chain reaction analysis (18). The marked inhibitory effect of RU-486 suggests that the progesterone-induced increase in myelination is mediated by this receptor. Progesterone may induce the synthesis of myelin proteins in Schwann cells; we have recently shown that expression of myelin basic protein and 2',3'-cyclic nucleotide-3'-phosphodiesterase is increased by progesterone in cultures of rat oligodendrocytes (19). Whether these effects of progesterone are mediated at the transcriptional or posttranscriptional level (20) remains to be determined. The high concentrations of progesterone in intact adult nerves also indicate a role for this neurosteroid in the slow but continuous renewal of peripheral myelin (21).

It is conceivable that progesterone is synthesized de novo from cholesterol by Schwann cells, because pregnenolone, the direct precursor of progesterone, is present at high concentrations in rodent sciatic nerve (4) (Table 1) and can be synthesized from 25-hydroxycholesterol by Schwann cells in culture (4). Thus, both its synthe-

sis and function in the nervous system render progesterone, a classical sex steroid, an active neurosteroid (22). Given that high concentrations of neurosteroids are also present in human sciatic nerve (23), our observations may lead to new pharmacological approaches for the treatment of demyelinating diseases.

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5. Three-month-old male mice (OF1; Iffa Credo, L'Arbresles, France) were subjected to castration and adrenalectomy under anesthesia [ketamine (3.5 mg per kilogram of body mass) and acepromazine (3.7 mg/kg)]. Anesthetics were injected intraperitoneally and Vetranquil (3.7 g/kg). We used males for all the in vivo studies because of their consistently low plasma progesterone concentrations.
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9. Animals were deeply anesthetized as described (5). The sciatic nerve of the right leg was exposed and lesioned with a copper cryode (diameter, 0.5 mm) that had been dipped in liquid nitrogen and was repeatedly applied to the upper part of the nerve. The extent of the lesion was  $\sim 1$  mm [B. Ferzaz, H. L. Koenig, A. Ressouches, *C. R. Acad. Sci. (III) (Paris)* **309**, 377 (1989)]. In sham-operated animals, the sciatic nerve was simply exposed by cutting the surrounding muscles. Success of the lesion was verified by monitoring paralysis of the right leg. Seven or 15 days after surgery, nerve sections (1.5 cm long starting from the site of lesion) and plasma samples were collected for radioimmunoassay (RIA) of pregnenolone and progesterone.
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11. Sciatic nerves of mice were weighed 7 or 15 days after cryolesion [day 7: sham-operated mice,  $4.6 \pm 0.2$ ; lesioned mice,  $6.8 \pm 0.5$ . Day 15: sham-operated mice,  $5.1 \pm 0.3$ ; lesioned mice,  $7.4 \pm 0.4$  mg per nerve (means  $\pm$  SEM,  $P < 0.01$  for both days compared to sham-operated mice by Student's  $t$  test)].
12. Steroids or their inhibitors (100  $\mu\text{g}$  in 50  $\mu\text{l}$  of sesame oil) were applied to the lesioned site immediately after surgery (day 0) and on days 5, 10, and 14. Control mice were treated with vehicle alone. The lesioned zones of the nerves were removed 15 days after cryolesion and prepared for electron microscopy. We analyzed nerves at this stage because most regenerated axons (75 to 85%) are myelinated and myelin sheaths are approximately one-third of their final size, allowing any increase or decrease in the size of myelin sheaths in response to steroids or their inhibitors to be observed. The proportion of myelinated fibers and the number of myelin lamellae per sheath were directly counted on the fluorescent



**Fig. 3.** Effect of progesterone on myelin formation in DRG cultures. Cells were cultured for 2 weeks in myelination-promoting medium (27) in the absence (**top**) or presence (**bottom**) of 20 nM progesterone. Myelinated fibers were stained with Sudan black and the number of myelin segments was determined (28). Only myelinated fibers are visible on these photographs. Scale bars, 40  $\mu\text{m}$ .

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  14. Addition of progesterone (dissolved in ethanol; final concentration of ethanol, 0.1%) daily to a final concentration of 20 nM to culture medium for 2 weeks did not increase the area occupied by the neurite network extending around DRG explants (control,  $20.6 \pm 1.4$ ; progesterone,  $19.7 \pm 1.2$  mm<sup>2</sup>), the density of neurites (control,  $422 \pm 24$ ; progesterone,  $397 \pm 35$  mm/mm<sup>2</sup>), or the number of Schwann cells (control,  $2500 \pm 193$ ; progesterone,  $2625 \pm 104$  cells per mm<sup>2</sup>), measured after staining the explants with toluidine blue. Measurements were made with an imaging system (Biocom, RAG version 2). Results are means  $\pm$  SEM of five culture dishes, each containing 10 DRG explants.
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  24. Steroids (pregnenolone, progesterone, and corticosterone) in sciatic nerve and plasma were measured by RIA. Fourteen nerves were pooled for each sample. The RIA procedures have been described and validated (4) [C. Corpéchoy *et al.*, *Endocrinology* **133**, 1003 (1993)]. The progesterone antiserum cross-reacts with 5 $\alpha$ -dihydroprogesterone (32%); therefore, our assay includes both progestins, which are potent activators of gene expression via the intracellular progesterone receptor [R. Rupprecht *et al.*, *Neuron* **11**, 523 (1993)].
  25. Rat Schwann cells were prepared from DRG cultures as described (13), with minor modifications. During the first week, DRG explants were cultured in Dulbecco's modified Eagle's medium-Ham's F12 (50:50, v/v) containing 10% fetal bovine serum and nerve growth factor (30 ng/ml), and were treated for 3 days with the antimetabolic agent cytosine arabinoside (10  $\mu$ M) to eliminate all cells other than sensory neurons and Schwann cells. Subsequently, the explants were grown for an additional 3 weeks in defined serum-free medium (8). The outgrowth of axons induces Schwann cell growth [J. L. Salzer and R. P. Bunge, *J. Cell Biol.* **84**, 739 (1980)], resulting in the generation of large numbers of surrounding Schwann cells. The ganglia were then cut out of the cultures with a microscalpel. The remaining pure Schwann cells were stained as described [S. M. Hsu, L. Raine, H. Fanger, *J. Histochem. Cytochem.* **29**, 577 (1981)] with a polyclonal rabbit antiserum to a synthetic peptide common to the four known isoforms of rat 3 $\beta$ -HSD (22).
  26. Schwann cells from DRG explants were harvested by exposure to trypsin and seeded in new culture dishes. After 48 hours, the cells were incubated in triplicate with 100 nM [7-<sup>3</sup>H(N)]pregnenolone (NEN, 925 GBq/mmol) at 37°C for 24 hours. Steroids were extracted from the incubation medium with ethyl acetate:isooctane (1:1, v/v) and separated by thin-layer chromatography (TLC) on silica gel plates developed once in chloroform:ethyl acetate (4:1, v/v). Quantitation of radioactive areas on TLC plates was performed with an automatic TLC linear analyzer. Steroids were further characterized by high-pressure liquid chromatography on a reversed-phase C<sub>18</sub> octadecylsilane column and by recrystallization (4) [Y. Akwa *et al.*, *J. Cell Biol.* **121**, 135 (1993)].
  27. Neuron-Schwann cell cultures of rat DRG were prepared as described (25). After 4 weeks in defined medium, cells were cultured for an additional 2 weeks in myelination-promoting medium [defined medium containing 10% fetal bovine serum and L-ascorbic acid (50  $\mu$ g/ml)], in which Schwann cells differentiate rapidly and myelinate the DRG axons. Ascorbic acid is necessary for the Schwann cells to assemble a basal lamina, a prerequisite for myelination (13).
  28. Myelinated fibers were stained with Sudan black after fixing the cultures with paraformaldehyde (4%) and osmium (0.1%) [N. Kleitman, P. M. Wood, R. P. Bunge, in *Culturing Nerve Cells*, G. Banker and K. Goslin, Eds. (Bradford Books, London, 1991), pp. 337-377]. Myelin formation was quantified as described (13) with an imaging system (Biocom, RAG version 2). Electron microscopic studies confirmed that the myelin formed in vitro was morphologically normal.
  29. We thank Roussel-Uclaf for antisera to corticosterone (Ab 524) and progesterone (Ab 1093); J. Fiet for the antiserum to pregnenolone; B. Delespierre for technical assistance; C. Corpéchoy for help with the RIAs; V. Meremans for help with the analysis of axon diameters; and J.-C. Lambert and L. Outin for editorial assistance. Supported in part by the Association Française contre les Myopathies.

19 December 1994; accepted 22 March 1995

## Reliability of Spike Timing in Neocortical Neurons

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It is not known whether the variability of neural activity in the cerebral cortex carries information or reflects noisy underlying mechanisms. In an examination of the reliability of spike generation using recordings from neurons in rat neocortical slices, the precision of spike timing was found to depend on stimulus transients. Constant stimuli led to imprecise spike trains, whereas stimuli with fluctuations resembling synaptic activity produced spike trains with timing reproducible to less than 1 millisecond. These data suggest a low intrinsic noise level in spike generation, which could allow cortical neurons to accurately transform synaptic input into spike sequences, supporting a possible role for spike timing in the processing of cortical information by the neocortex.

Neurons transmit information by transforming continuously varying input signals into trains of discrete action potentials. The coding scheme used in this process is an unresolved issue that is critical to computational theories of brain function. Codes that utilize spike timing (1, 2) can make more efficient use of the capacity of neural connections than those that simply rely on the average rate of firing (3). The simplest spike-timing code would be one output pulse for each input pulse, but synaptic currents in the cortex are too small and intracellular recordings in vivo look very noisy (4). Furthermore, cortical activity is characterized by highly irregular interspike intervals in both spontaneous (5) and stimulus-evoked conditions (6). These observations have led some to conclude that only statistical averages of many inputs carry useful information between cortical neurons (7). Another possibility, which we explore here, is that cortical neurons may respond reliably to relatively weak input fluctuations. Irregularity in spike timing may then reflect the presence of information. This is possible only if the intrinsic noise within neurons is small. Although some earlier

studies have suggested that neurons may have low intrinsic noise (8, 9), others have argued to the contrary (10).

The aim of the present report was to determine directly the temporal precision with which cortical neurons are capable of encoding a stimulus into a spike train. A rat cortical slice preparation was chosen so that the state of a single neuron and its input could be well controlled experimentally (11). Somatic whole-cell recordings were made in the current-clamp configuration (12), and spike trains were elicited with current injected through the recording electrode, near the presumed site of generation of action potentials (13). We assessed reliability by repeatedly presenting the same stimulus and evaluating the consistency of the evoked spike sequences.

First, repetitive firing was evoked with flat (dc) current pulses (0 to 250 pA, 0.9 s; Fig. 1A). The variability of spike counts from trial to trial was small [coefficient of variation (CV) =  $0.10 \pm 0.13$ ; mean  $\pm$  SD;  $n = 10$  cells]. However, the small variances in interspike intervals (ISIs) summed to increase the desynchronization of corresponding action potentials over the course of the stimulus. The first spike of each train was tightly locked to the onset of the pulse (SD =  $0.62 \pm 0.25$  ms;  $n = 8$ ), whereas the timing of the last spike in the train was highly variable (SD =  $31 \pm 19$  ms;  $n = 8$ ). Thus, responses to flat pulse stimuli indicate reliability of spike count or average firing

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