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FUNCTIONS OF THE CEREBRAL CORTEX AND CHOLINERGIC SYSTEMS IN SYNAPTIC PLASTICITY INDUCED BY SENSORY PRECONDITIONING

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ABBREVIATIONS

ACh	:	acetylcholine
AChE	:	acetylcholinesterase
AD	:	Alzheimer's disease
AMN	:	atropine methyl nitrate
ATS	:	atropine sulfate
CCx	:	cerebral cortex
DFP	:	diisopropylfluorophosphate
NMDAr	:	NMDA receptor
NOS	:	nitric oxide synthase

SUMMARY

This thesis provides evidence to support the hypothesis that synaptic plasticity in the primary somatosensory cortex is a cellular correlate of associative learning, that the process depends upon acetylcholine and that only certain cortical neurons display this plasticity.

In a first series of experiments, single-unit recordings were carried out in the barrel cortex of awake, adult rats subjected to whisker pairing, an associative learning paradigm where deflections of the recorded neuron's principle vibrissa were repeatedly paired with those of a non-adjacent one. On average, this form of sensory preconditioning increased the responses of a recorded unit to the stimulation of the non-adjacent vibrissa. In contrast, following explicitly unpaired control experiments, neuronal responsiveness decreased. The effect of pairing was further enhanced by local, microiontophoretic delivery of NMDA and the nitric oxide synthase inhibitor L-NAME and reduced by the NMDA receptor competitive antagonist AP5. These results and the fact that the influence of the pharmacological agents on neuronal excitability were either transient (limited to the delivery period) or simply absent indicated that the somatosensory cerebral cortex is one site where plasticity emerges following whisker pairing.

In subsequent experiments, using a similar conditioning paradigm that relied on evoked potential rather than single-unit recordings, increases in the responses of cortical neurons to the non-adjacent whisker were blocked by atropine sulfate, an antagonist of muscarinic cholinoreceptors. Administration of normal saline or atropine methyl nitrate, a muscarinic antagonist that did not cross the blood-brain barrier, instead of atropine sulfate, did not affect plasticity. Analysis of the behavioral state of the animal showed that the changes observed in the evoked potential could not be attributed to fluctuations in the behavioral state of the animal.

By combining the results described in this thesis with data found in related literature, the author hypothesizes that whisker pairing induces an acetylcholine-dependent form of plasticity within the somatosensory cortex through Hebbian mechanisms.

RÉSUMÉ

L'être humain se distingue du reste des créatures par sa capacité d'apprendre et de mémoriser un nombre incroyable de tâches. Que des patients atteints de la maladie d'Alzheimer puissent perdre ce don, ou des enfants encéphalopathes ne jamais l'acquérir, sans qu'aucun traitement ne leur soit disponible représente une tragédie pour ces personnes et leurs familles ainsi qu'une perte incalculable pour la société. Dans ce contexte, il n'est pas étonnant que l'étude des mécanismes de l'apprentissage et de la mémoire mobilise beaucoup de ressources humaines, physiques et financières dans la communauté scientifique. Cette thèse a été donc centrée sur la fonction d'apprentissage associatif dans le nécortex et ses rapports avec les noyaux cholinergiques du prosencéphale basal, sachant que ces deux structures atteignent leur développement maximal chez l'humain et subissent des processus dégénératifs caractéristiques dans la maladie d'Alzheimer.

Une première série d'expériences impliquant des enregistrements unicellulaires et la microiontophorèse a été effectuée sur le cortex somatosensoriel de rats adultes et éveillés. Ces animaux ont été soumis à un paradigme d'apprentissage associatif où des déflexions de la vibrisse principale activant le neurone à l'étude et communément désignée S2 étaient appariées ou non à celles d'une vibrisse non-adjacente (S1). L'appariement consistait en une stimulation de S1 couplée à celle de S2 avec un intervalle de 300 ms entre les deux stimulations. Ce "pré-conditionnement sensoriel" a été utilisé pour 61 enregistrements unicellulaires. Dans certains cas, l'appariement a eu lieu sous l'effet de trois agents pharmacologiques déjà connus pour leur propriétés modulatrices de l'apprentissage, de la plasticité des champs récepteurs et de la potentialisation à long terme: le NMDA, antagoniste du récepteur glutamatergique ainsi nommé (n = 8), l'AP5, antagoniste compétitif du récepteur NMDA (n = 17) et le L-NAME, un inhibiteur de l'enzyme de synthèse de l'oxide nitrique (n = 13). Les situations témoin ont consisté en des stimulations non appariées des vibrisses (contrôles cognitifs) ou l'administration des produits

pharmacologiques sans pré-conditionnement (contrôles pharmacologiques). Nous nous sommes principalement intéressés aux changements de réponse à la stimulation de S1 consécutifs à son appariement avec S2. Ces changements ont été mesurés sous forme d'un rapport entre les activités évoquées avant et après l'appariement, la réponse à l'activation non couplée de S2 servant de référence.

En moyenne, l'appariement des vibrisses a augmenté la réponse à S1 de 7%. Cet effet est apparu significativement différent de la diminution de 17% observée en l'absence de couplage, lors des contrôles cognitifs. Il ne pouvait pas être attribué à des fluctuations de l'état d'éveil de l'animal parce que l'analyse de l'électromyogramme, enregistré simultanément, a montré que les périodes d'augmentation de l'activité musculaire étaient peu fréquentes (moins de 4% d'une expérience complète en moyenne) et survenaient au hasard. L'accroissement des réponses à S1 induit par l'appariement s'est trouvé accentué par l'application microiontophorétique de NMDA (35%) et de L-NAME (27%) durant l'appariement, mais diminué par celle de l'AP5 (1%). L'AP5 et le NMDA affectaient aussi l'activité neuronale spontanée, mais durant la période d'administration seulement. Les changements de réponse à la stimulation S1 ne correspondaient donc pas à un effet excitateur ou inhibiteur résiduel des substances pharmacologiques puisqu'ils duraient plus longtemps que les périodes de conditionnement et de microiontophorèse.

En tenant compte de l'ensemble de ces résultats, nous avons conclu que l'appariement des stimulations de deux vibrisses non adjacentes augmente la réponse des neurones corticaux de 24% par rapport aux situations témoin où les stimulations des deux vibrisses sont indépendantes l'une de l'autre. Cette augmentation peut être modulée par l'application locale d'agents qui agissent sur les récepteurs NMDA ou l'enzyme de synthèse de l'oxyde nitrique. Nous en déduisons que le cortex somatosensoriel est un site primaire de plasticité synaptique au cours du pré-conditionnement sensoriel.

Au cours d'une seconde série d'expériences, nous avons cherché à démontrer que la plasticité induite dans le cortex somesthésique dépendait de l'action de l'acétylcholine sur des récepteurs muscariniques. Des potentiels évoqués ont été enregistrés simultanément dans plusieurs régions du cortex somatosensoriel de rats adultes et éveillés avant, durant et après cinq procedures distinctes. Durant une procédure d'appparіement, 50 déflexions de la première vibrisse (S1) ont été couplées à celle de la seconde, non adjacente (S2). Dans les expériences témoin, les stimulations de S1 et S2 ont été présentées dans un ordre aléatoire et à intervalles prolongés. Dans les trois autres situations, l'appariement des deux vibrisses eut lieu 30 min après l'injection intra-péritonéale d'une solution de 0.5 ml de salin (NaCl 150 mM), de sulfate d'atropine (ATS 100 mg/kg dans du NaCl 150 mM) ou de méthyl nitrate d'atropine (AMN 100 mg/kg dans du NaCl 150 mM).

Lorsque l'appariement des deux vibrisses a eu lieu seul, en présence de salin ou par suite de l'administration d'AMN (un antagoniste muscarinique qui ne traverse pas la barrière hémo-encéphalique), les réponses à la stimulation de S1 étaient généralement augmentées. Une augmentation a été également observée à partir de neurones situés au pourtour du champ récepteur cortical de la vibrisse S2. Par contre, en présence d'ATS ou par suite d'un contrôle cognitif, les réponse à la stimulation de S1 ont diminué. Le salin, l'AMN et l'ATS sont restés sans effets sur l'amplitude des potentiels évoqués par S1 en l'absence d'appariement des vibrisses. L'analyse des électroencéphalogrammes et électromyogrammes enregistrés simultanément a permis d'éliminer les variations de l'état d'éveil de l'animal comme source possible des changements de réponse à S1. L'ensemble des résultats a mené à la conclusion que l'activation des récepteurs cholinergiques est nécessaires à l'induction de la plasticité synaptique survenant dans le cortex somatosensoriel lors de la stimulation appariée de vibrisses, et que cette action est indépendante de la modulation de l'excitabilité cellulaire ou de la transmission synaptique en cours.

Les données électrophysiologiques ainsi recueillies chez le rat éveillé et soumis à un paradigme de pré-conditionnement sensoriel permettent donc de conclure que le cortex cérébral est bien un site primaire de plasticité synaptique dépendante de l'acétylcholine lors de l'apprentissage associatif. Etant donné la nature associative du paradigme utilisé, le modèle de covariance de Hebb représente un mécanisme plausible conduisant à l'émergence de cette forme de plasticité corticale. Ces résultats nous apprennent également qu'un traitement efficace de la maladie d'Alzheimer devra pallier aux atteintes du cortex cérébral et des noyaux cholinergiques tout au moins et ne pourra se contenter d'agir sur l'une ou l'autre de ces structures. Une approche expérimentale comparable à celle-ci pourrait éventuellement servir à tester les effets cellulaires des agents pharmacologiques destinés au traitement de maladies comme la démence sénile de type Alzheimer.

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GENERAL INTRODUCTION

A major part of what defines human beings and distinguishes them from other living creatures is their ability to learn and remember a large and diverse array of tasks, ranging from noticing simple associations between two stimuli, a function also present in lower mammals and invertebrates, to mastering complex mathematical calculations or performing neurosurgery. Thus, it is not surprising that attempts to understand the cellular mechanisms of learning and memory have been a primary field of research in neuroscience for over a century. The issue has been gaining even more significance lately since, on one side, cognitive deficits are being recognized as major consequences of a variety of common pediatric neurological and psychiatric disorders (for instance epilepsy or attention-deficit disorders) and, on the other, Alzheimer's disease (AD) is becoming an increasing source of morbidity and mortality in the continuously aging population of the western world.

THEORETICAL AND EXPERIMENTAL BACKGROUNDS

The main challenge for the neurobiology of learning and memory is determining how the central nervous system (CNS) acquires information about its environment and preserves it when the source of such information is no longer present. To simplify this complex task, neuroscientists have focused on the simpler forms of learning, traditionally divided into two groups: non-associative and associative. Associative learning, which includes sensory preconditioning, classical conditioning and instrumental (or operant) conditioning, has held a privileged position since Pavlov's pioneering work on conditioning of the salivation reflex in the dog at the beginning of the century. Among the various structures regularly implicated in associative learning, the cerebral cortex (CCx) and the cholinergic system originating in the nucleus basalis of Meynert (NBM) have attracted special attention for two main reasons. First, their development

reaches its peak in humans (see Wenk 1993). Second, in Alzheimer's disease (AD), they both consistently undergo distinctive degenerative processes, the extent of which has been correlated with the severity of the cognitive deficits that characterize this disease (Albert 1996; Esiri 1989; Hof and Morrisson 1994; Levey 1996; Woodruff-Pak et al. 1996).

Synaptic plasticity in the CCx can be induced, either in vivo or in vitro in adult animals, by different protocols, all hypothesized to reflect mechanisms that underlie some aspect of learning. Some rely on direct electrical stimulation of sensory pathways or of cell bodies. For instance, associative LTP occurs in slices of neocortex following high-frequency stimulation of thalamocortical or intracortical pathways (Artola et al. 1990; Brocher et al. 1992a,b; Hess et al. 1996; Kirkwood and Bear 1994). Similar results are obtained by stimulating thalamocortical fibers in vivo at high frequencies (Lee and Ebner 1992). Moreover, pairing depolarization of neocortical neurons by electrical or pharmacological means with electrical or natural stimulation of an afferent sensory pathway leads to potentiation of the responses elicited by that stimulated pathway (Cruikshank and Weinberger 1996; Fregnac et al. 1992, 1994; Shulz and Fregnac 1992). Changes in neocortical neuronal excitability and responsiveness to a peripheral sensory stimulus have also been described following a wide variety of sensory conditioning and deprivation paradigms such as habituation to repeated whisker stimulation (Welker et al. 1992), classical conditioning of a tone (Oleson et al. 1974; Bakin and Weinberger 1990; see Weinberger and Diamond 1987), tactile discrimination training (Recanzone et al. 1992a,b,c,d) and deafferentation by peripheral nerve transections (Merzenich et al. 1983).

Several lines of evidence suggest a important role of ACh in associative learning and more generally in plasticity. First, ACh can modulate neuronal excitability and enhance the responsiveness of cortical neurons to afferent stimuli for periods of time that outlast their immediate presence (Gusev and Myasnikov 1995; Metherate et al. 1987, 1988a,b). Second, although sensory stimulation alone, as is the case in sensory preconditioning, can enhance ACh

release (Kurosawa et al. 1992), the level of ACh furhter increases in various regions of the cerebral cortex (CCx) during learning (Butt et al. 1997). Changes in the firing of suspected NBM cholinergic neurons have also been reported following learning (Pirch et al. 1991; Richardson and DeLong 1991; Wilson and Rolls 1990a,b). For instance, Wilson and Rolls (1990a,b) recorded from the NBM of trained monkeys to perform go/no-go visual discrimination and serial visual recognition memory tasks with or without a delayed response period. They identified two types of neurons. The first one responded on the basis of the novelty of the stimulus while firing in the second group was related to the reinforcement value of the stimulus. The responses of these reinforcement-related neurons (RRN) were dissociated from the sensory properties of the stimulus, motor responses and possible mechanisms aimed at restoring homeostatic equilibrium as the animals were not water or food-deprived. Third, cholinergic antagonists or lesions of the NBM prevent or, at least, delay the acquisition of various visual (Butt and Hodge 1995) and tactile (Jacobs and Juliano 1995) discrimination tasks in addition to whisker pairing (Baskerville et al. 1997; Maalouf et al. 1998 b). These deficits are reduced by the administration of cholinergic agonists or acetylcholinesterase (AChE) inhibitors (Muff et al. 1993; Myers et al. 1996). Finally, the severity of memory impairment in AD has been correlated with the extent of NBM degeneration (Albert 1996; Levey 1996; Poirier et al. 1995).

Despite the efforts invested until now, our understanding of the functions of the CCx and the role of acetylcholine (ACh) in synaptic plasticity induced by associative learning remains incomplete. Part of the problem arises from the fact that learning produces long-term changes in neuronal firing at all the levels of the CNS, including the spinal cord (Wolpaw), cochlear nucleus (Oleson et al. 1984), the magnocellular geniculate nucleus (Weinberger and Diamond 1987), the mediodorsal thalamic nucleus (Delacour 1984, Oyoshi et al. 1996), the hippocampus (Berger et al. 1983; Delacour 1982; Sanchez-Andres and Alkon 1991), the amygdala (see Stevens 1999; Weinberger 1998), the hypothalamus (Ono et al. 1992), the red nucleus (Ito and Oda 1994), the

basal forebrain cholinergic nuclei (Pirch 1993; Pirch et al. 1992; Wilson and Rolls 1990) and locus coeruleus (Sara et al. 1994; Vankov et al. 1995). As a consequence, it has been difficult to determine whether plasticity occurrs within the cerebral cortex or is passively transmitted from subcortical structures such as the thalamus, brainstem nuclei or spinal cord. To complicate the situation even further, several brain regions have been found to be necessary for the acquisition but not for the recall of information whereas others display plasticity following the acquisition of certain tasks but damaging them does not affect learning. The NBM is at the center of such controversies. Finally, several studies have recently contested the classical role of ACh in learning, arguing that it is primarily involved in the modulation of attention (Muff et al. 1993; Sarter and Bruno 1997; Voytko 1996). Thus, previous findings raise several questions about the roles of the CCx and of ACh in synaptic plasticity following learning. Are changes in firing of neocortical cells following learning stored in the CCx or merely reflections of changes occurring in other areas of the brain? Are these changes sufficient for learning or are they rather a part of similar phenomena occurring throughout the CNS? Is ACh necessary for such processes to occur and, if so, what is its function relative to other neuromodulators such as norepinephrine, serotonin or dopamine? Does it influence learning directly or through the modulation of attention? Is it involved in the acquisition or the retention of new tasks?

Various additional questions remain unanswered as well, starting with the cellular and biochemical mechanisms underlying plasticity in a learning situation. Conditions necessary for the establishment of a learned association between two stimuli have been described by Hebb (1949). According to his covariance hypothesis, the establishment of a learned association depends on an increased correlation in the activities of the neuronal groups activated by the two stimuli involved in that association. This situation takes place in the presence of 1) contiguity and 2) contingency between the two stimuli. At the cellular level, the covariance hypothesis assumes that the pathways activated by both stimuli converge at one or several levels within the CNS.

Consequently, when presynaptic activity, originating from the first stimulus, is repeatedly paired with increased postsynaptic activity resulting from the subsequent presentation of the second stimulus, synapses linking the two elements are potentiated. In contrast, the lack of contiguity and contingency between the two stimuli or, in other terms, the absence of correlation between pre- and postsynaptic activity leads to synaptic depression (see Cruikshank and Weinberger 1996). At the molecular level, one mechanism put forward on several occasions to explain the enhanced neural responsiveness resulting from repeatedly pairing two stimuli is long-term potentiation (LTP). In fact, this process is believed by many to be the neural correlate of learning and memory. Evidence for this hypothesis is however rather indirect. It relies in part on the fact that pharmacological agents known to block LTP (for example NMDA receptor antagonists; see Bliss and Collingridge 1993; Madison et al. 1991) also decrease performance in learning tasks (Davis et al. 1992; Shapiro and Caramanos 1990; Shapiro and O'Connor 1992). Moreover, the paradigms used to induce some forms of LTP comply with the rules set by Hebb's covariance hypothesis. This thesis provides new information on some some of these issues.

PLAN OF THE THESIS

The experiments reported rely on sensory preconditioning to study synaptic plasticity in the mystacial vibrissae representation of the CCx (the posteromedial barrel subfield or barrel cortex; Woolsey and Van der Loos 1970) of adult, awake rats. As was originally shown by Delacour et al. (1987), repeatedly pairing the deflections of two non-adjacent whiskers leads to changes in the subsequent responses of cortical neurons to the stimulation of one or the other of the paired vibrissae. In the first section, microiontophoretic delivery of pharmacological agents previously shown to modulate LTP is used to manipulate these changes locally within the

posteromedial barrel subfield, thereby providing evidence that the CCx is a primary site of synaptic plasticity in associative learning. An associated hypothesis in that paper is that LTP-like mechanisms are behind the changes in neuronal responses. In the second series of experiments, the involvement of ACh in these processes is investigated with a similar pharmacological approach. Simultaneous recordings from different barrels provide further insight into the cellular effects of sensory preconditioning. The effects of constantly increased levels of ACh on neocortical neurons are reported in the third chapter. This situation occurs when drugs that enhance cholinergic activity are given to patients with AD. Unfortunately, constantly elevated ACh levels differ from the time and space-dependent physiological release of ACh in the normal brain. In the general discussion, related findings are reviewed, a cellular model is offered to summarize preferred hypotheses and the implications for the treatment of AD are discussed. References are made to an analysis currently in progress and consisting in an attempt to correlate the electrophysiological properties of the cortical neurons recorded in the previous experiments with their ability to display plasticity following sensory preconditioning. The goal of this study is to provide an easy way to differentiate and recognize neocortical neurons and thus help to understand the anatomy of the cellular circuits expressing plasticity.

CHAPTER 1

Effects of D-AP5 and NMDA microiontophoresis on associative learning in the barrel cortex of awake rats

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BRAIN RESEARCH

Research report

Effects of D-AP5 and NMDA microiontophoresis on associative learning in the barrel cortex of awake rats

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Abstract

Experiments involving single-unit recordings and microiontophoresis were carried out in the barrel cortex of awake, adult rats subjected to whisker pairing, an associative learning paradigm where deflections of the recorded neuron's principle vibrissa (S2) are repeatedly paired with those of a non-adjacent one (S1). Whisker pairing with a 300 ms interstimulus interval was applied to 61 cells. In 23 cases, there was no other manipulation whereas in the remaining 38, pairing occurred in the presence of one of three pharmacological agents previously shown to modulate learning, receptive field plasticity and long-term potentiation: *N*-methyl-D-aspartic acid (NMDA) ($n = 8$), the NMDA receptor antagonist AP5 ($n = 17$) or the nitric oxide synthase inhibitor L-nitro-arginine-*N*-methyl-ester (L-NAME) ($n = 13$). Non-associative (unpaired) experiments ($n = 14$) and delivery of pharmacological agents without pairing ($n = 14$) served as controls. Changes in neuronal responsiveness to S1 following one of these procedures were calculated and adjusted relative to changes in the responses to S2. On average, whisker pairing alone yielded a 7% increase in the responses to S1. This enhancement differed significantly from the 17% decrease obtained in the non-associative control condition and could not be attributed to variations in the state of the animals because analysis of the cervical and facial muscle electromyograms revealed that periods of increased muscular activity, reflecting heightened arousal, were infrequent (less than 4% of a complete experiment on average) and occurred randomly. The enhancement of the responses to S1 was further increased when whisker pairing was performed in the presence of L-NAME (27%) or NMDA (35%) whereas AP5 reduced it to 1%. During the delivery period, NMDA enhanced both neuronal excitability and responsiveness to S1 whereas AP5 depressed them. However, the effects of both substances disappeared immediately after administration had ended. L-NAME did not affect the level of ongoing activity and responses to S1 significantly. From these data, we concluded that, since the changes in the responses to S1 lasted longer than the periods of both whisker pairing and drug delivery, they were not residual excitatory or inhibitory drug effects on neuronal excitability. Thus, our results indicate that, relative to the unpaired controls, whisker pairing led to a 24% increase in the responsiveness of barrel cortex neurons to peripheral stimulation and that these changes were modulated by the local application of pharmacological agents that act upon NMDA receptors and pathways involving nitric oxide. We can infer that somatosensory cerebral cortex is one site where plasticity emerges following whisker pairing.

Keywords: L-NAME; Unit-recording; Whisker pairing; Unpaired control; Principle vibrissa

1. Introduction

Learning and memory disorders are frequent clinical manifestations of neurological pathologies involving the cerebral cortex and represent a major field of research in clinical neuroscience as even a mild impairment of these higher brain functions can interfere with a patient's personal, social and professional life. Changes in neocortical neuronal excitability and responsiveness to a peripheral sensory stimulus occur following a wide variety of sensory conditioning paradigms such as habituation to repeated whisker stimulation [101], classical conditioning of a tone [9,70,100] and tactile discrimination [76–79]. Plasticity can also be induced in slices of neocortex [7,17,46,52] or in vivo [23,58] by high-frequency stimulation of thalamocortical or intracortical pathways. Finally, pairing depolarization of neocortical neurons by electrical or pharmacological means with electrical or natural stimulation of an

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afferent sensory pathway leads to potentiation of the stimulated pathway [24,38,39,91]. What has remained unclear up to now, however, is whether the changes in neocortical responsiveness that are described in vivo following learning occur within the cerebral cortex, are transmitted from subcortical regions or are a combination of both.

Recent studies involving sensory conditioning [98] and altered tactile experience [31–33,37] have tried to determine whether plasticity in these situations actually occurs within the cerebral cortex or is passively transmitted from subcortical regions. In the present paper, the mechanisms by which the cerebral cortex contributes to the formation, storage and retrieval of memories were investigated in an associative learning situation. Awake rats were subjected to a sensory conditioning paradigm originally described by Delacour et al. [30]. After single neurons were isolated in the somatosensory 'barrel' cortex [103], deflections of their principal whiskers were repeatedly paired with deflections of a non-adjacent whisker. In some cases, the whisker-pairing session was combined with microiontophoretic application of one of three pharmacological agents known to affect both learning [22,26,51] and LTP [16,61,86]: the NMDA receptor (NMDAr) competitive antagonist D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), the NMDAr agonist *N*-methyl-D-aspartic acid (NMDA), and the nitric oxide (NO) synthase inhibitor L-nitro-arginine-*N*-methyl-ester (L-NAME).

The interest in associative learning relates to the facts that it is a basic form of learning that occurs constantly in the daily life of any living creature and that some forms of associative learning are affected in Alzheimer's disease [102]. In this context, the mystacial vibrissal system of the rat offers several advantages. First, it provides an opportunity to test associations between stimuli belonging to the same modality. Second, associations between the deflections of distinct vibrissae undoubtedly result from the whisking movements that rats perform during the active exploration of their natural environment, making whisker pairing a behaviorally relevant paradigm [20,47,55]. Third, vibrissal architecture facilitates the administration of precisely localized and controlled stimuli. Fourth, whisker pairing induces changes in the responsiveness of cortical neurons very rapidly (less than 50 pairings; [30]). At the same time, microiontophoresis allows local delivery of drugs to a few neurons surrounding the recording area, thereby limiting the site of action to a few neurons surrounding the pipette and providing the opportunity to study the mechanisms activated by whisker pairing in the somatosensory cortex. Thus, to our knowledge, our experimental setting is the first that combines electrophysiological recordings of neocortical single-units with local pharmacological treatments in a learning situation involving natural sensory stimuli. Our results offer strong evidence for the need of cortical NMDAr and NO synthase in the neuronal plasticity observed in the cerebral cortex following whisker pairing.

2. Methods

2.1. Surgical preparation and adaptation to restraint of animal to be studied awake

All procedures were approved by the local committee on animal care and respected the standards established by the Canadian Council of Animal Care. Adult male Sprague–Dawley rats (350–450 g; 400.9 ± 31.3, Mean ± S.D.), housed individually with food and water ad libitum, were anaesthetized with intraperitoneal injections of 35 mg/kg sodium pentobarbital (65 mg/ml in an aqueous propylene glycol base with 2% benzyl alcohol; Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Supplements were added as required to maintain areflexia until the end of the surgery. Following administration of the antibiotic enrofloxacin (50 mg/ml in *N*-butyl alcohol and water, 0.05 ml i.m.; Baytril, Chemargo, Etobicoke, Ontario, Canada), the eyes were covered with baby oil (Johnson & Johnson) and a local anaesthetic (0.3 ml of lidocaine chlorhydrate 20 mg/ml; Xylocaine, Astra Pharmaceuticals, Mississauga, Ontario, Canada) was injected under the scalp. A few minutes later, the scalp was excised and the temporal muscles reflected. Two teflon-insulated stainless-steel wires were placed over the cervical and facial muscles to monitor electromyographic activity (EMG) and eleven stainless-steel screws (Model MF-5182, Bioanalytical Systems, West Lafayette, IN) were inserted through holes drilled in the skull at the following positions (in millimeters): anteroposterior to bregma (AP) +3, mediolateral to midline (ML) ±3; AP −10, ML −3, 0, +3; on the lateral edges: AP +2 and −8. Finally, two parallel steel tubes (6 cm long, 1.5 mm external diameter) placed 12 mm apart and secured by two shorter tubes (1.5 cm long) were attached to the screws with dental acrylic. The bone overlying barrel cortex remained exposed and was temporarily covered with silicone (Reprosil, Dentsply International, Milford, DE, USA). This frame served to immobilize the rat's head in a restraint box that held the recording apparatus (Fig. 1).

The animal was allowed a minimum of 48 h to recover from surgery and was then trained to sit calmly in the restraint box during three to five sessions of increasing length (from 30 min to 3 h). During the second or third session, dental acrylic was added to the frame to provide additional stability and to accustom the rat to having the experimenter work around it.

One day prior to recording, the rat was re-anesthetized with sodium pentobarbital. The branches of the facial nerve that control vibrissal movement were transected to reduce movements of the vibrissa relative to the mechanical stimulation [88]. A craniotomy was performed over barrel cortex (in mm from bregma: AP −1 to −4, ML 4 to 7) and the opening was blocked with silicone until the following day.

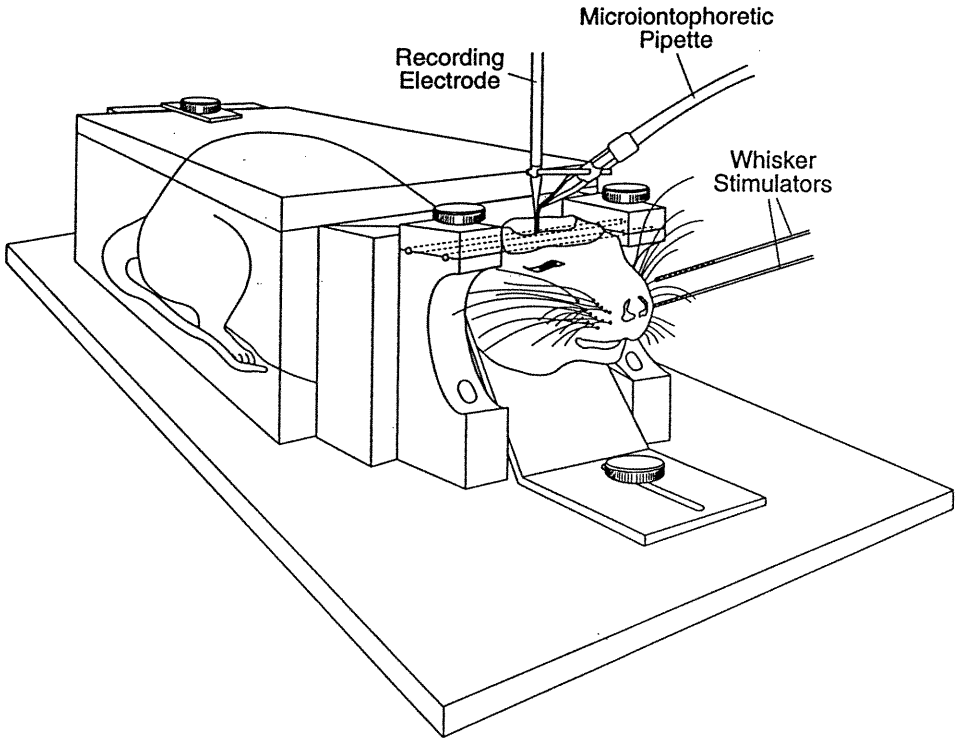


Fig. 1. Rat in restraining box. The head was held stationary by the horizontal steel bars fixed to the skull with dental acrylic. Not shown are the posts mounted on each side of the platform which supported the microdrive and its microelectrode. The two whiskers chosen for the experiment were inserted into the ends of hollow tubular grass glued to the membranes of miniature speakers (not shown).

2.2. Preparation of the electrodes

Following methods summarized by Godwin [43], tungsten wires (6 cm long; 250 μm diameter) were etched over 2 cm by electrolysis, glued inside glass pipettes (10 cm long; 1.2 mm external and 0.68 mm internal diameters; World Precision Instruments, Sarasota, FL) with 2-methyl-cyanoacrylate and then pulled with a vertical electrode puller (Narishige, Tokyo, Japan) to leave approximately 2 μm of metal tip exposed. Those having an impedance of 2 to 5 MΩ at 1000 Hz were used. Two-barrel iontophoresis pipettes containing microfilaments (1.2 mm external and 0.68 mm internal diameters; World Precision Instruments) were pulled and the tips broken to form a 2 to 4 μm outer diameter opening. The pipettes were bent 1 cm from the tip to an angle of 30 to 60° over an alcohol burner. One barrel was filled with 0.15 M NaCl (to be used for compensation currents) and the second one with either 50 mM D-AP5 (Research Biochemicals International, Natick, MA), 50 mM NMDA (Research Biochemicals International) or 0.5 mM L-NAME (Sigma, St. Louis, MO). All solutions were adjusted to a pH between 7 and 8. Silver wires were inserted into the upper ends of each barrel and sealed with low melting-point wax. The lower ends of the recording electrode and the iontophoresis pipettes were positioned under a microscope and glued with nail varnish, leaving the tips exposed and separated by less than 15 μm. The shafts of the electrode and the pipettes were joined with low melting-point dental impression compound.

2.3. Electrophysiological recording, whisker stimulation and microiontophoresis

Single-unit activity was filtered (band-pass filters set from 300 Hz to 3 kHz) and amplified (50,000 $\times$) with a preamplifier (Model 113, Princeton Applied Research, Princeton, NJ) and an amplifier (Tektronix Model AM502 and TM504, Beaverton, OR). Action potentials were discriminated with a window discriminator (Model, RAD-II-A, Winston Electronics, San Francisco, CA) before being sent through a laboratory computer interface (Model CED 1401 plus, Cambridge Electronic Design, Cambridge, UK) to a personal computer. Digital events signaling the time of occurrence of action potentials (with a 100 μs resolution) were recorded simultaneously with the EMG activity using the Spike 2 software package (CED, Cambridge, UK). The shapes of action potentials were collected at the beginning and end of each recording session with the SIGAVG program (CED, Cambridge, UK) to ensure that the same neuron was recorded during the whole experiment and to allow subsequent classification of the cells.

Whisker stimulation was accomplished with a piece of tubular grass glued to a light-weight plastic ring over the membrane of a miniature speaker (5 mm in thickness and 25 mm in diameter). Stimulation parameters were under

the control of the Spike 2 program. A single stimulus consisted of 25 vertical, sinusoidal deflections at 30 Hz. The total duration was 750 ms with an amplitude of 3 mm.

Drug administration was controlled by a microiontophoretic pump (Model BH-2, Medical Systems, Greenvale, NY). Ejection and retention currents were respectively (in nA) −10 and +5 to +15 (8.2 ± 4.8; Mean ± S.D.) for AP5 and +90 and −5 for L-NAME. These values were chosen on the basis of effective currents employed in other studies [6,72,84]. Retention currents for NMDA were set between +15 to +30 nA (24.6 ± 11.0) to eliminate leakage. Delivery of the drug was achieved by a 10–15 nA reduction of the retention current. If a small increase in firing was not observed online as a result, ejection currents of −10 nA were used. The saline-filled pipette enabled the use of compensation currents.

2.4. Experimental procedures

After opening the dura mater with the tip of a 30 G needle, the electrode was positioned 0.1 to 0.2 mm below the cortical surface. The exposed brain area was covered with agar (4% in NaCl 0.9%) to prevent drying and to reduce tissue movement. Twenty minutes later, the electrode was advanced until a well-isolated neuron was identified. If an obvious and reproducible response could be evoked by whisker stimulation, the principal whisker was identified and a non-adjacent whisker was chosen that did not belong to the same row or same column as the principal whisker. The experimental protocol was then initiated and comprised five steps (see Fig. 2). First, the non-adjacent whisker was stimulated (stimulus S1) 10 or 20 times. Second, the principal whisker was stimulated (stimulus S2) 10 or 20 times. These recordings of baseline responses to the stimuli were followed by one of five experimental paradigms: the pairing procedure in the absence of any pharmacological treatment, the pairing procedure combined with microiontophoretic application of either AP5 (AP5 procedure), NMDA (NMDA procedure) or L-NAME (L-NAME procedure) or the unpaired procedure.

The pairing procedure consisted of 50 paired presentations of S1 and S2, the end of S1 preceding the beginning of S2 by 300 ms. When drug administration was involved,

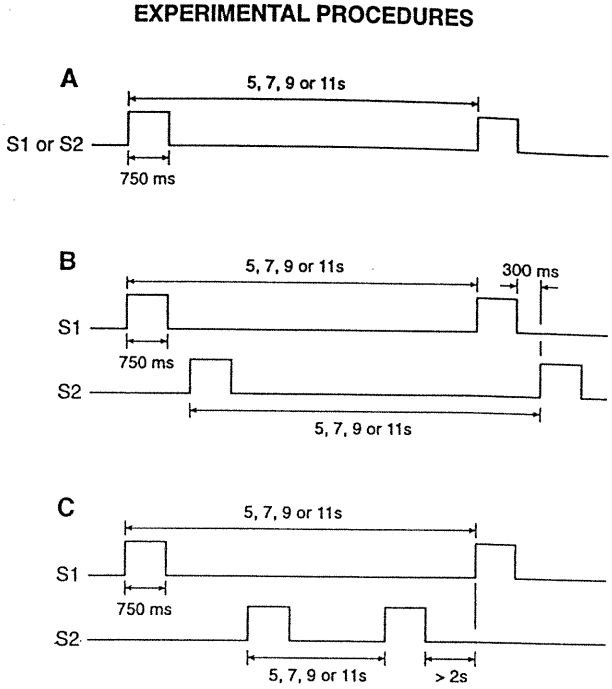
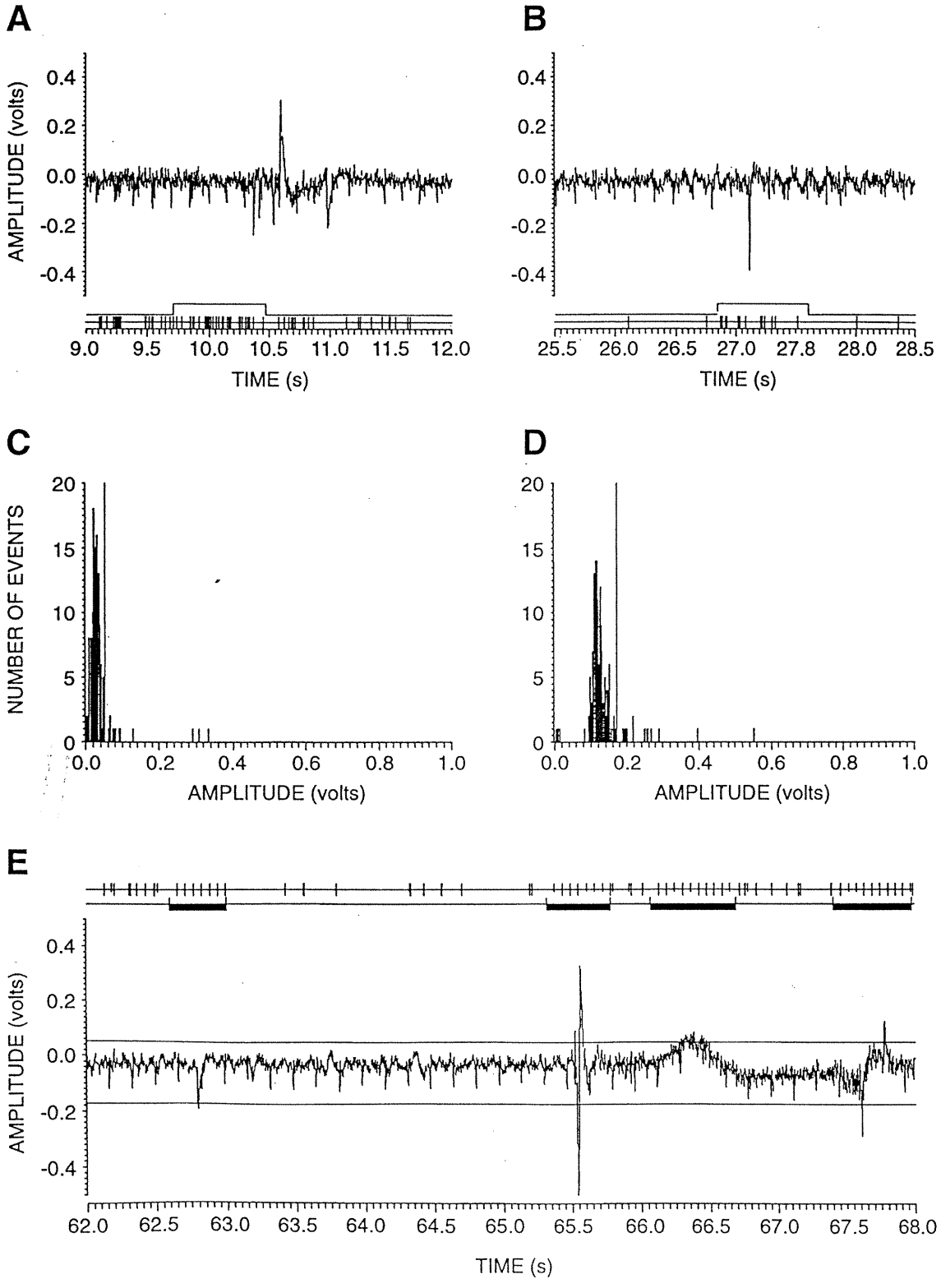


Fig. 2. Experimental paradigms. (A) Single vibratory stimuli were delivered at 30 Hz. Each lasted for 750 ms. They were presented at variable intervals of 5, 7, 9 or 11 s first to the whisker S1 for 10 or 20 times, then to the whisker S2 for the same number of times. (B) Pairing consisted of temporal contiguity and contingency of the stimuli applied to both whiskers. During each of 50 paired presentations, stimulation of S2 followed the stimulation of S1 with a 300 ms delay. (C) In the non-associative control, the number of stimuli was the same but a consistent relation was avoided. Following this, S1 and S2 were stimulated again, separately for 10 or 20 times each. The responses to S2 were used to estimate whether or not the excitability of the neuron had remained unchanged for the period of the study.

a 2 to 5 min delay was inserted between the start of drug ejection and that of whisker pairing to allow saturation of the recording site by the substance in use. During the control procedure, each stimulus was given 50 times independently of the other, the interval between S1 and S2 always being larger than 2 s and each stimulus occurring not more than twice sequentially. At the end of the experimental paradigm, 10 or 20 presentations of S1 were given followed by 10 or 20 presentations of S2. An additional series of experiments was performed to test the effects of

Fig. 3. Procedure for removal of movement-related artifacts from neural activity. Examples of the data collected are presented in (A) and (B). The upper trace represents a digital record of the amplified analog EMG signal. The movement artifacts are seen as large negative and positive deflections. The line below indicates the time of stimulus application and shows that some artifacts occurred during stimulation. The bottom line, just above the time scale, shows the output of the digital amplitude discriminator with the contributions generated by the artifacts. (C,D) Frequency histograms of the voltage peaks identified in each 0.5 s segment across the entire EMG record (upper line in (A) and (B), respectively) were built. The threshold for recognition of artifacts (indicated by the long vertical line) was set at a level that excluded the signals to the right of the first series of four successive empty bins on histograms built separately for positive and negative deflections. (E) Periods containing artifacts (marked in the middle trace with black rectangles, above the EMG record) were removed and replaced with spikes representing the mean frequency of activity across the record. The top trace shows uniform spike trains in the intervals that previously contained artifacts. Notice that this procedure affecting less than 4% of the data decreased the probability of obtaining significant changes in the responsiveness of the neurons tested.

the pharmacological agents used on the firing pattern of the recorded neurons. They started with 10 presentations of S1 followed by 10 presentations of S2. Drug delivery was then initiated and the same whiskers were stimulated again, each during 10 trials. In all the experiments, the interval between two successive stimulations applied to the same whisker varied constantly and was randomly chosen among four preset values of 5, 7, 9 or 11 s to minimize the possibilities of a conditioning to time [29]. Recording sessions lasted 2–4 h and were terminated when the animals demonstrated growing impatience with restraint by frequent movements.



2.5. Data analysis

EMG activity or other mechanoelectrical artifacts related to movements sometimes interfered with the recording of the neural activity. An approach similar to spike amplitude discrimination was used to remove such artifacts from the digitized record. The EMG record was divided into blocks of 0.5 s. The maximum positive and negative amplitudes within each block were determined and frequency distributions of both extremes were built (Fig. 3). The excessively larger amplitudes fell on the right side of the distribution and were separated by several empty bins from the rest of the data. The threshold for amplitude discrimination was set at a value greater than the fourth successive empty bin separating these large values from the rest of the distribution. During periods where the EMG exceeded this threshold, the record of the neural activity was removed and replaced by the mean frequency of the cell being studied. This procedure avoided the possibility that spontaneous movements could contribute artifacts erroneously interpreted as spikes in the neural record. It affected less than 4% of the digital record.

Once an acceptable five-part sequence of data was obtained for a cell, it was processed to determine if the pairing procedure had produced any significant change in the spike activity of the neuron. Peristimulus time histograms (PSTH; 30 ms bin width) were built for each sequence. The size of a response to whisker stimulation

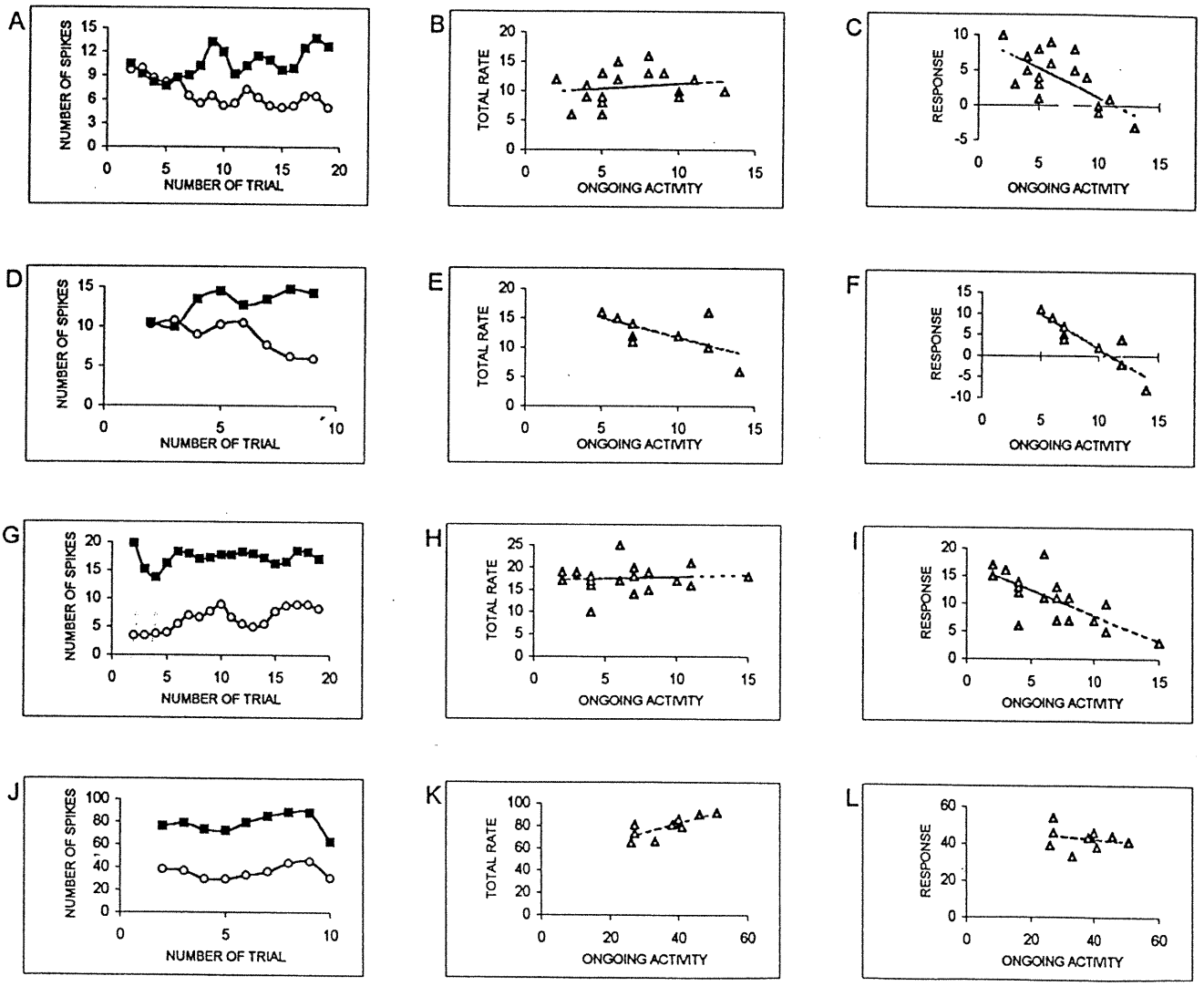


Fig. 4. Selection of the appropriate measure of neuronal responsiveness. All neurons displayed spontaneous activity. The responses (squares) to whisker stimulation were usually an increase in the rate of spiking over the ongoing level (opened circles). Two examples are presented for both S1 (A and D) and S2 (G and J). Notice that from one trial to the next, the rate of spontaneous activity fluctuated but the magnitude of the response did not always follow the same trend. When the total response was plotted against the level of ongoing activity, there was no consistent relationship between the two measures in 75% of the cases studied, as shown in (B) for S1 and in (H) for S2. The infrequent situation where a relationship was found is illustrated for S1 (E) and S2 (K). In contrast, when the ongoing activity was subtracted from the total response to obtain a measure of the neural activity provided by whisker stimulation, that measure was correlated with the rate of ongoing activity in 80% of cases (C,F,I). For these reasons, the difference measure was rejected and the data were expressed as the total number of spikes collected during the stimulus interval.

was quantified by counting the total number of spikes during the 750 ms stimulation period. This measure was found to be independent of the level of spontaneous activity in 75% of the cases studied. In contrast, we did not use the incremental number of spikes added by the stimulus because when the number of spikes in the 750 ms of spontaneous activity preceding the stimulus was subtracted from the total number of spikes generated during the stimulus, a significant correlation ($p < 0.05$) was found between the adjusted spike count and the level of spontaneous activity in 80% of the recordings (Fig. 4).

To compare the effects of the different protocols, normalized measures were calculated by expressing the responses to whisker stimulation following a particular procedure as percentages of change relative to the baseline responses preceding that procedure. Changes in individual cells were considered significant when the type I error was lower than 0.01 with a paired t-test. However, to properly analyze time series data, it was necessary to form a model that predicted the temporal evolution of the neuronal activity in the absence of any manipulation before one could detect a significant deviation from expectation. Because the nervous system is characterized by slow trends in neuronal excitability, it was not obvious that the level of neuronal excitability would remain constant for the duration of the five-part test. We addressed this problem by using the magnitude of the response to the principle whisker as a measure of neuronal excitability and as an indicator of any temporal trend in the excitability of the cell being studied. In those cases where a significant trend was observed in the responses to the principle whisker (S2), the responses to S1 collected following pairing were corrected by the percentage of change observed in the responses to S2. The magnitude and direction of the relationship between the responses to S1 and those to S2 were taken into account. For each experiment the change (ΔS1) in response to S1 caused by pairing was calculated with different equations, corresponding to different conditions as follows:

if $R^1_{S2} = R^2_{S2}$ (t – test: $p > 0.05$), then

$$\Delta S1 = 100\left(R^2_{S1} - R^1_{S1}\right)/R^1_{S1};$$

if $R^1_{S2} \neq R^2_{S2}$ (t – test: $p \leq 0.05$), then

$$\Delta S1 = 100\left(R^2_{S1} - R^1_{S1}\right)/R^1_{S1}\left[1 + \left(R^2_{S2} - R^1_{S2}\right)/R^1_{S2}\right];$$

where R_{S1} and R_{S2} were responses to stimulation of S1 and S2, respectively before (R^1_{S1} and R^1_{S2}) and after (R^2_{S1} and R^2_{S2}) the pairing. Such procedure did not affect the sign of ΔS1; it only affected its value.

To compare responses of cells from one experimental paradigm with those from another, the percentages of change (both significant and non-significant) in the responses to S1 were pooled by experimental group and subjected to an unpaired t-test and an F-test.

To evaluate the extent and duration of the drug effects on neuronal excitability, analyses similar to those performed on the responses to whisker stimulation were applied to the 750 ms of ongoing activity preceding the onset of a stimulus. The rates of spontaneous activity during and immediately after the period of drug delivery were expressed as percentages of change relative to the level preceding the beginning of the ejection. These normalized measures were averaged across cells belonging to the same treatment group and compared with a t-test to the pairing procedures. Finally, since EMG activity is correlated with arousal and motor activity, the behavioral state of the animal during an experiment was analyzed by quantifying the proportion of time when the amplitude of the EMG exceeded the threshold previously set for the elimination of artifacts. An analysis on trend was then performed on the series of extremes to determine whether high amplitude activity occurred randomly throughout an experiment or was concentrated in a specific period.

2.6. Histology

At the end of a successfully completed experiment, a small lesion was placed at the recording site by passing 10 μA of positive current through the electrode for 20 s. Any further data collection from the same animal was done at cortical sites several barrels away. At the end of the final recording session, the animal was deeply anesthetized with an intraperitoneal dose of sodium pentobarbital. The thoracic cage was opened and the animal was perfused through the aorta with 500 ml of phosphate-buffered saline (pH 7.3) followed by 500 ml of 4–10% solution of paraformaldehyde. After the brain was removed from the skull, the frontoparietal region was blocked for either tangential or frontal sections and placed in 0.8 M sucrose for at least 48 h. The sections were cut with either a freezing microtome or a cryotome at a thickness of 40–80 μm and mounted on chrom–alum coated slides for staining with cresyl-violet. Lesion sites were identified from the Nissl-stained sections allowing 63 cells to be located in the histology. The cells were assigned to a cortical lamina based upon the characteristics of the surrounding tissue. As well, the histology allowed us to confirm that the neurons studied were located within the posteromedial barrel subfield.

3. Results

3.1. General description of the recorded neurons

The 116 cells that form the basis for this report were recorded from 37 adult rats. Cells were selected for their ability to respond to deflections of one or more vibrissae on the contralateral face. The population of neurons was studied for 20.0 ± 11.3 min (Mean ± S.D.). The time that a single unit was held generally exceeded the amount required to complete the study and the cell was abandoned to initiate a search for the next one. In those cases where the cell was lost before the termination of data collection,

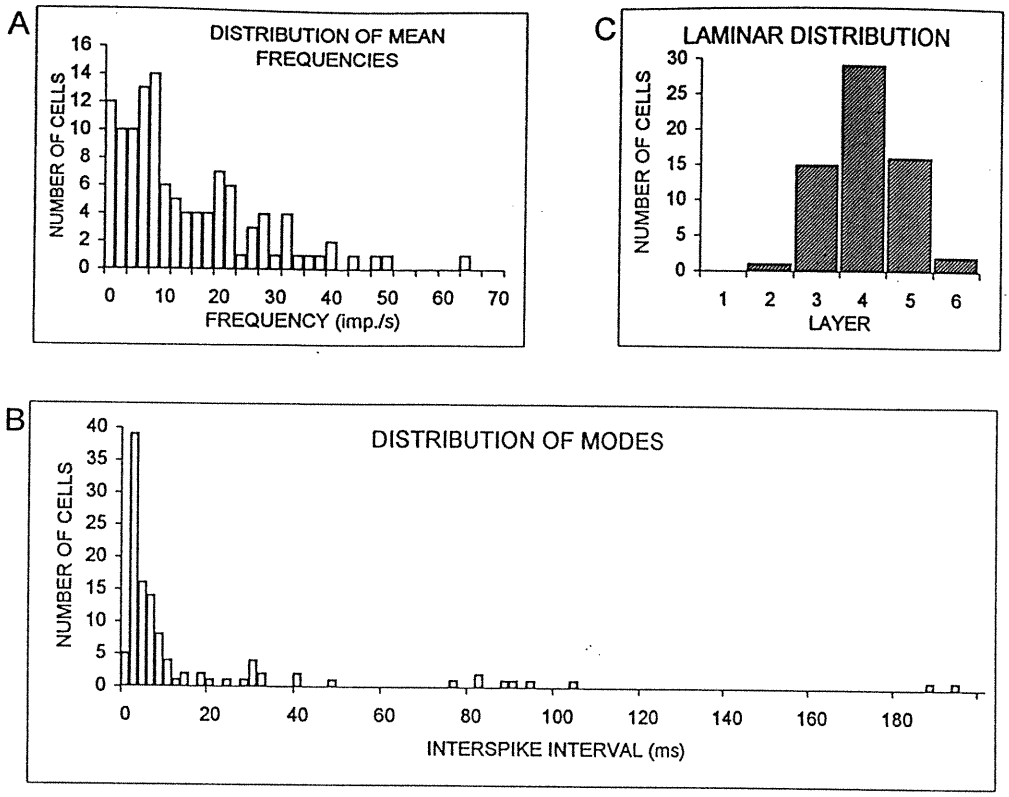


Fig. 5. Characteristics of the sample of neurons studied. (A) The mean frequencies of ongoing activities ranged from less than 2 imp./s to as high as 65 imp./s but averaged 10 imp./s showing a skewed distribution. (B) The modal value of the interspike intervals tended to be very short. This suggests that many neurons fired in bursts. (C) Most cells were isolated in layer IV and all but a few came from laminae III, IV and V.

the final data sets were carefully inspected for the loss of spikes from the record or any other evidence of abnormal behavior attributable to injury.

Spontaneous activity was present in all cells studied. The average discharge rate was 14.5 ± 12.7 spikes/s but the distribution was highly skewed (Fig. 5A). Most cells had a spontaneous activity of 10 Hz or less with the median being 10 Hz. The preferred interspike interval of most neurons lay between 2 and 4 ms (Fig. 5B). Such distributions of spontaneous activity are characteristic of the somatosensory cortex where rates greater than 20 Hz are infrequent [83]. When interspike interval histograms were prepared for any particular cell, they usually showed the mean frequency to be made up of very short and very long intervals, an indication that most cells displayed bursts in their discharge pattern. These attributes are discussed in the following paper [59]. Here it is interesting to note that cells isolated in different layers had different ongoing frequencies; the cells of layer IV had a rate that was 50% greater than the average whereas in layers II and VI, the rates tended to be much lower than the average. These observations are comparable to those reported by Salimi et al. [83] in the somatosensory cortex of waking cats. Most cells were isolated in the middle layers (Fig. 5C). Fewer cells in the superficial and deeper layers could be driven by deflection of the vibrissae. The laminar distribution of the recording sites as identified by lesions in tangential and frontal sections is presented in Fig. 5C.

Table 1
Number of neurons studied in each experimental condition, average duration of recording and average spontaneous rate of firing

Experimental condition	Number of neurons	Average duration of recording (min)	Rate of ongoing activity (spikes/s)
Pairing alone	23	15.9 ± 1.9	13.7 ± 12.11
Pairing + AP5	17	26.4 ± 7.0	16.6 ± 12.4
Pairing + NMDA	8	30.5 ± 8.4	19.6 ± 16.5
Pairing + L-NAME	13	38.8 ± 5.2	17.7 ± 17.6
Control	14	27.1 ± 4.7	21.4 ± 12.2
AP5	6	7.0 ± 0.0	9.3 ± 9.6
NMDA	4	7.7 ± 1.5	11.5 ± 7.2
L-NAME	4	8.0 ± 0.0	4.9 ± 2.8
Total	89	19.96 ± 11.3	14.5 ± 12.7

Table 2
Row and column of mystacial vibrissae pad where the vibrissa serving as S2 was located

			1	2	3	4	5	6	7	8
α		A	2	1	2	1				
β	1	B	1	5	6	12	5			
γ	1	C	1	2	9	16	2			
δ	1	D	3	4	10	8				
		E	6	3	1			1		

The numbers in the body of the table indicate the number of times a vibrissa served as S2. C4 was the vibrissa used on the left most frequently as S2. The numbers across the top indicate rostralcaudal position within the vibrissal row. Along the left side, the greek letters indicate the vibrissae located on the caudal margin of the mystacial pad and the roman letters indicate the vibrissal row within the mystacial pad.

One of the five experimental protocols was administered to individual neurons and data sets were completed for the number of cells shown in Table 1. Pairing was successfully carried out in 61 cells. In 23 cases there was no other manipulation and in the other 38 cases pairing occurred in the presence of a pharmacologically active substance. Non-associative control experiments were performed on 14 cells and another 14 were used as controls for drug effects. In 27 cases the cell was lost before the five-step pairing procedure was completed, but data from these cells was useful for some of the analyses reported in the following paper [59]. Different whisker combinations were used in experiments performed on the same rat. The two whiskers used in a single experiment were separated by 1 to 5 (2.5 ± 0.9; Mean $\pm$ S.D.) columns and 0 to 3 (1.9 ± 0.8) rows. In euclidian coordinates, the distance between the whiskers ranged from 1.4 to 5.4 (3.2 ± 0.7) whiskers. Table 2 indicates the number of times each whisker was used as S2.

3.2. The pairing procedure

After isolation of a single unit, the principal vibrissa (S2) was attached to one stimulator and a second vibrissa, (S1) separated by several intervening vibrissae, was attached to another stimulator. Data collection began with 10 or 20 presentations of a 0.75 s vibration applied to S1. The response to S1 was much less obvious than for S2. After a baseline of responsiveness was established for both S1 and S2, the two were paired for 50 trials. The effect of the pairing was assessed by repeating the stimulation of S1 10 or 20 times followed by an equivalent number of stimulations of S2 alone. Before making this assessment, the responses were corrected for any non-stationarity in the responses to S2 (see Section 2). This correction, performed in 55 of the 116 cells, reduced the possibility that we might attribute the observed effects on responses to S1, to an unrelated, overall shift in neuronal excitability. Fig. 6 illustrates a case where both the responses to S1 and S2 increased significantly by 65% and 64%, respectively (paired t-test; $p < 0.01$) following pairing. However, after the responses to S1 were corrected by the amount of the increase in the response to S2, the change in S1 (8%) was below the level of significance. Of the 23 neurons studied in this paradigm, five showed a significant enhancement ($76.4 \pm 5.5\%$; Mean $\pm$ S.E.M.; Table 3) in their responses to S1. An example is seen in Fig. 7 where the responses to

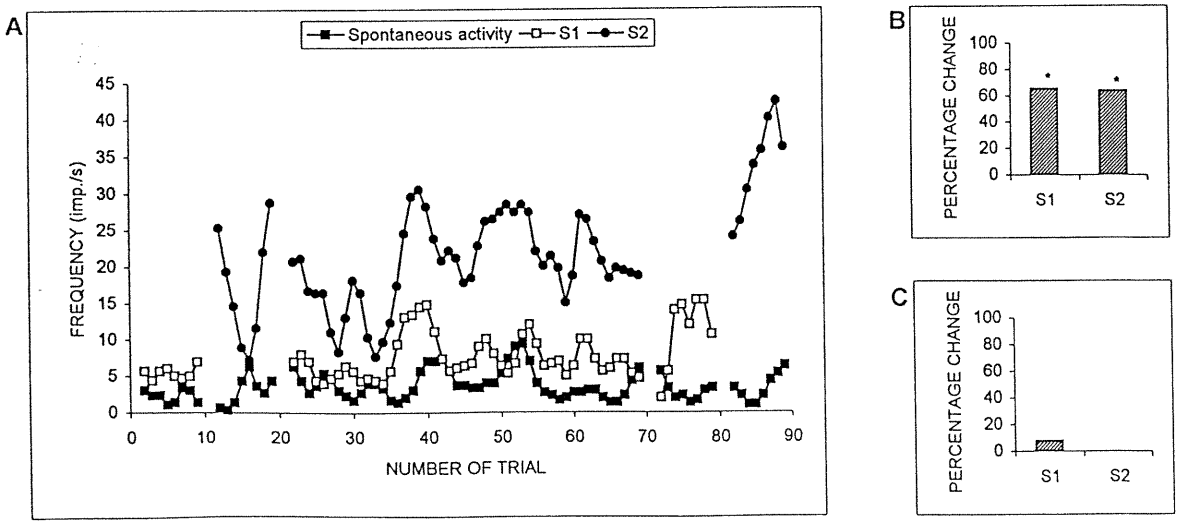


Fig. 6. Changes in the responsiveness to whisker stimulation of a single cell following pairing. (A) The variability in the responsiveness of the neuron to individual stimuli confounded the analysis. Large fluctuations occurred in the total responses to S1 and S2 and in the levels of ongoing activity. However, it is readily apparent that responses to both S1 and S2 changed with time. (B) When the responses after pairing were compared to the responses before pairing, the responses to S1 and S2 increased significantly by more than 60%. (C) When the responses to S1 were corrected by the amount of change in the responses to S2 which might reflect a change in overall cortical excitability, only a small, insignificant effect (8%) of pairing remained.

Table 3
Magnitudes of the average change observed for each experimental condition (Mean ± S.E.M)

Experimental condition	Increased	No change	Decreased	Group average
Pairing alone	76.4 ± 5.5 (5)	−1.6 ± 1.7 (14)	−55.0 ± 0.9 (4)	6.6 ± 3.6 (23)
Pairing + AP5	28.7 ± 2.9 (2)	21.4 ± 3.2 (10)	−51.5 ± 1.2 (5)	1.0 ± 5.5 (17)
Pairing + NMDA	69.1 ± 4.2 (4)	44.7 ± 2.0 (2)	−41.0 ± 1.6 (2)	35.0 ± 8.0 (8)
Pairing + L-NAME	97.7 ± 7.0 (3)	17.8 ± 1.1 (8)	−39.4 ± 3.9 (2)	27.0 ± 5.3 (13)
Control	33.3 (1)	0.9 ± 0.9 (7)	−47.0 ± 1.3 (6)	−17.3 ± 2.9 (14)
AP5	− (0)	−3.9 ± 1.8 (4)	−35.3 ± 2.9 (2)	−7.8 ± 11.7 (6)
NMDA	52.9 ± 2.9 (2)	11.5 ± 0.6 (2)	− (0)	32.2 ± 5.9 (4)
L-NAME	58.2 (1)	4.6 ± 1.0 (3)	− (0)	18.5 ± 9.5 (4)

The number of neurons contributing to each mean is indicated in parentheses.

S1 increased by 21%. The responses to S2 remained statistically unchanged. In four different cells, the responses to S1 decreased significantly (−55.0 ± 0.9%) whereas in the remaining 14, there were no significant changes.

3.3. The effects of pharmacological agents on the changes in neuronal responsiveness induced by whisker pairing

3.3.1. D-AP5

To test the hypothesis that the changes induced by whisker pairing depend upon NMDA receptors, the same experiment was performed in 17 cells that were subjected to the NMDA receptor antagonist D-AP5. Drug delivery was limited to the duration of the pairing. Seven out of 17 cells showed a significant change in the responses to S1. Five of these cases showed a decrease (−51.5 ± 1.2%, Mean ± S.E.M.) while the remaining two showed a 28.7 ± 2.9% increase. More commonly (10/17), responsiveness was not significantly altered when this antagonist was present (Fig. 8).

3.3.2. NMDA

The administration of NMDA increased the proportion of neurons exhibiting enhanced responses to S1; in 4 out of 8 cells, the average increase was 69.1 ± 4.2% (Mean ±

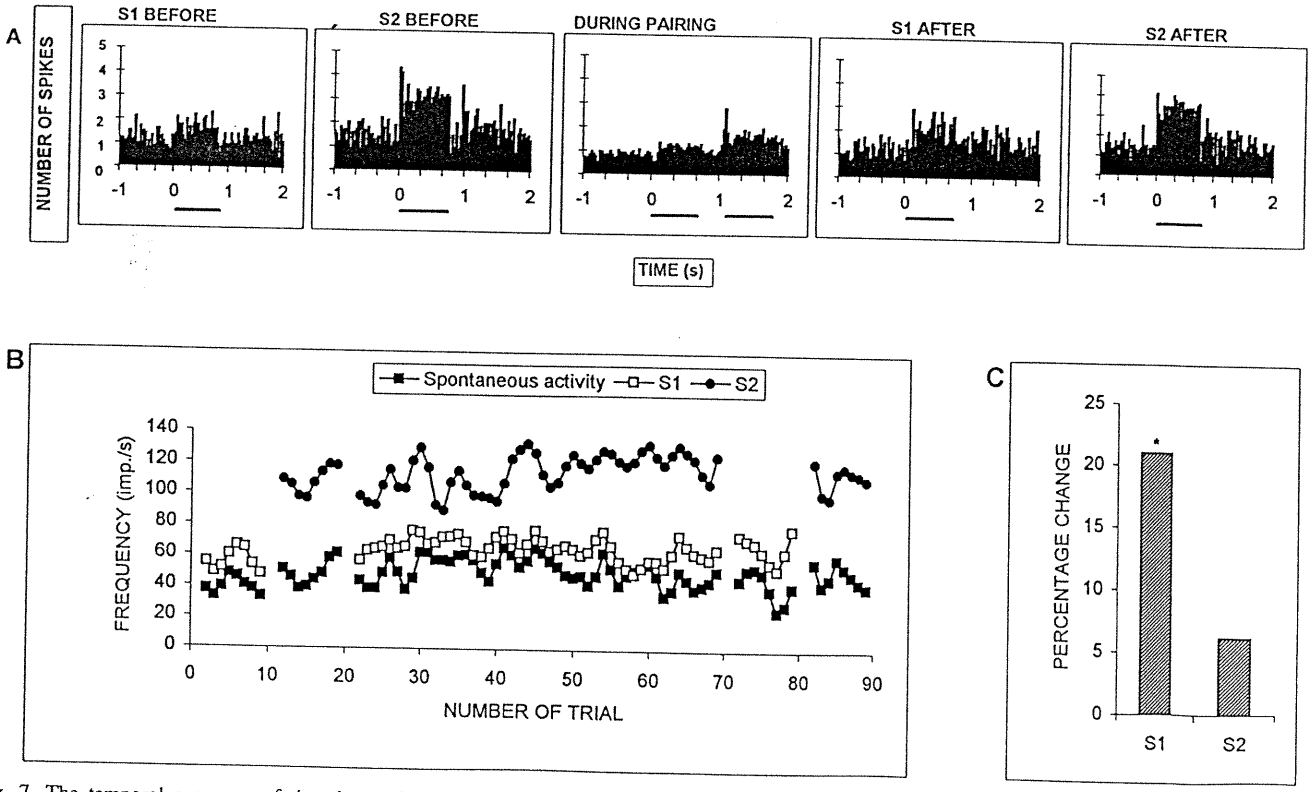


Fig. 7. The temporal sequence of the observations obtained from a single cell subjected to the pairing procedure. (A) Peristimulus time histograms represent 10 summated trials (50 for pairing, in the middle histogram). (B) Firing frequencies in individual trials across time. (C) In this case, the responses to S1 increased by more than 20% whereas there was no significant change in responses to S2.

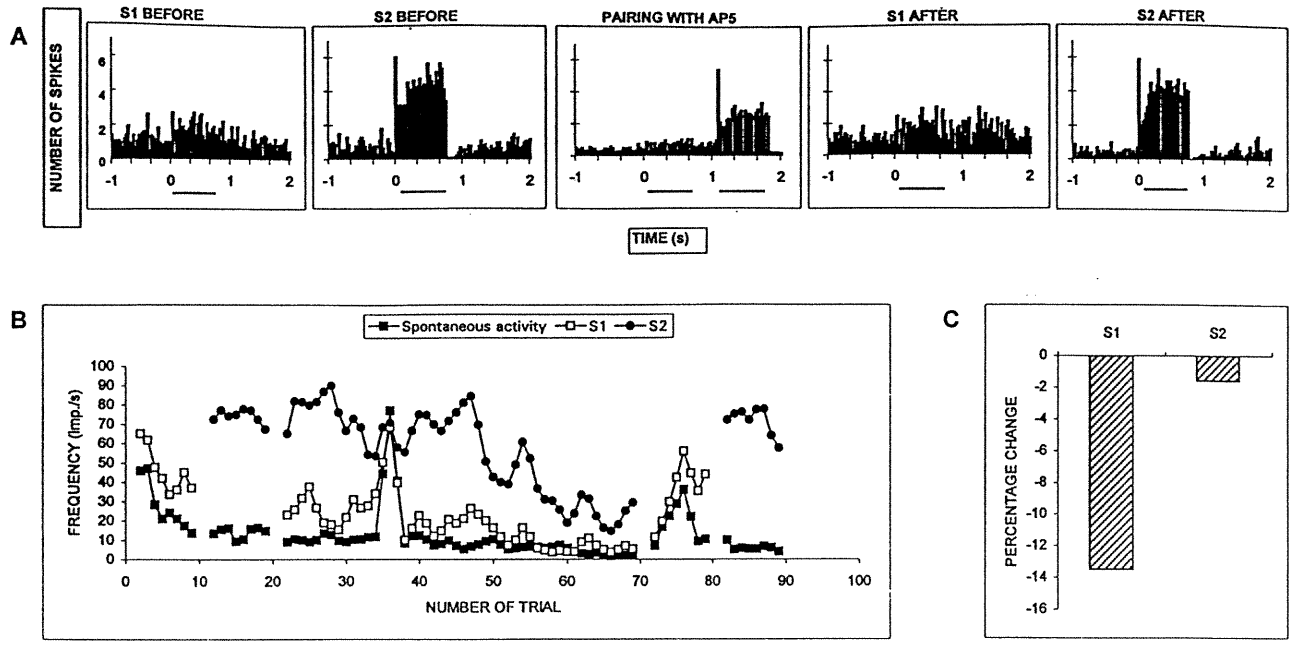


Fig. 8. Illustration of an experiment in which pairing in the presence of AP5 did not produce any significant changes in the responses to either S1 or S2. (A) Peristimulus time histograms of 10 trials (50 with pairing) in each of the 5 stages of the experiment. (B) Firing frequency during the responses to S1 and S2 as a function of the trial number. There were important fluctuations in the rate of spontaneous activity measured just before the stimulus was presented as well as fluctuation during the responses. (C) The percentage of change following pairing was not significant for either S1 or S2.

S.E.M.). Reduced responses to S1 ($-41.0 \pm 1.6\%$) were seen in only two cases. In two others, the changes were not significant.

3.3.3. L-NAME

With the concentrations and microiontophoresis currents used in our experiments, the effects of L-NAME on

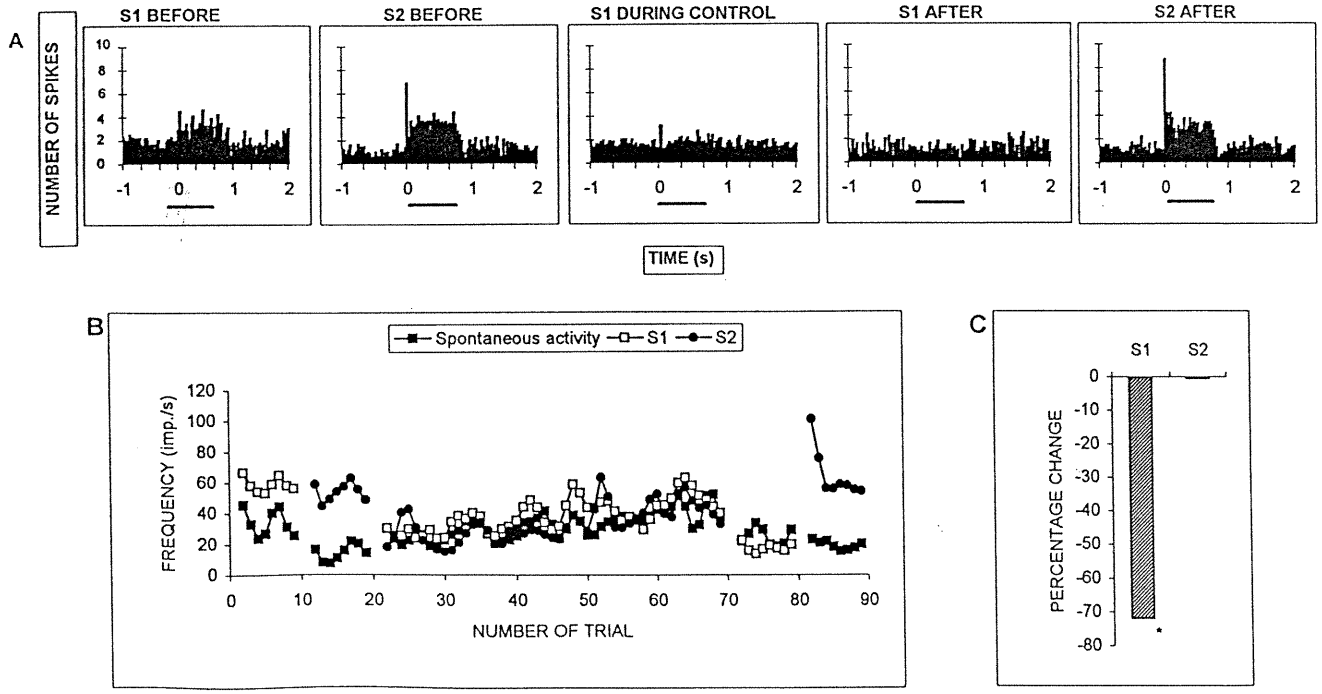


Fig. 9. The non-associative control procedure. (A) Peristimulus time histograms of each of the five stages of the experiment. The responses to S1 decreased to a level below that of the ongoing activity as if an active inhibition of the cell had emerged. The responses to S2 did not change. (B) Firing frequency for the spontaneous activity preceding each stimulation and the firing frequency observed during each stimulation as a function of the trial number. (C) The responses to S1 decreased by more than 70% with no change in responses to S2.

pairing were similar but less pronounced than those of NMDA. When pairing occurred during delivery of L-NAME, the ratio of increased over decreased responses to S1 was only slightly higher than with pairing alone but the size of the enhancement was greater. Out of 13 neurons, three showed increased responses to S1 (97.7 ± 7.0%, Mean ± S.E.M.) whereas two displayed a decrease (−39.4 ± 3.9%). The remaining eight were not significantly affected by the procedure.

3.4. Control experiments

3.4.1. Non-associative control

The control procedure differed from pairing by not having any consistent relation between S1 and S2. The main result of delivering vibratory stimuli to the two whiskers in this temporally independent manner was a significant decrease of the responses to S1 (−47.0 ± 1.3, Mean ± S.E.M.) in 6 out of 14 cases (Fig. 9). Increased responses (33.3%) were seen in only one experiment. Seven neurons did not show any significant changes.

3.4.2. Pharmacological controls

To test the effects of the pharmacological agents without the influence of pairing, responses to whisker stimulation were recorded for the same cell before and during delivery of one of the substances (Table 3; last 3 rows). With the drugs alone, significant changes within a group were unidirectional whereas combining whisker pairing with drug administration led to both increased and decreased responses to S1. Responses to S1 were only reduced by AP5 (−35.3 ± 2.9% in 2 out of 6 cases, Mean ±

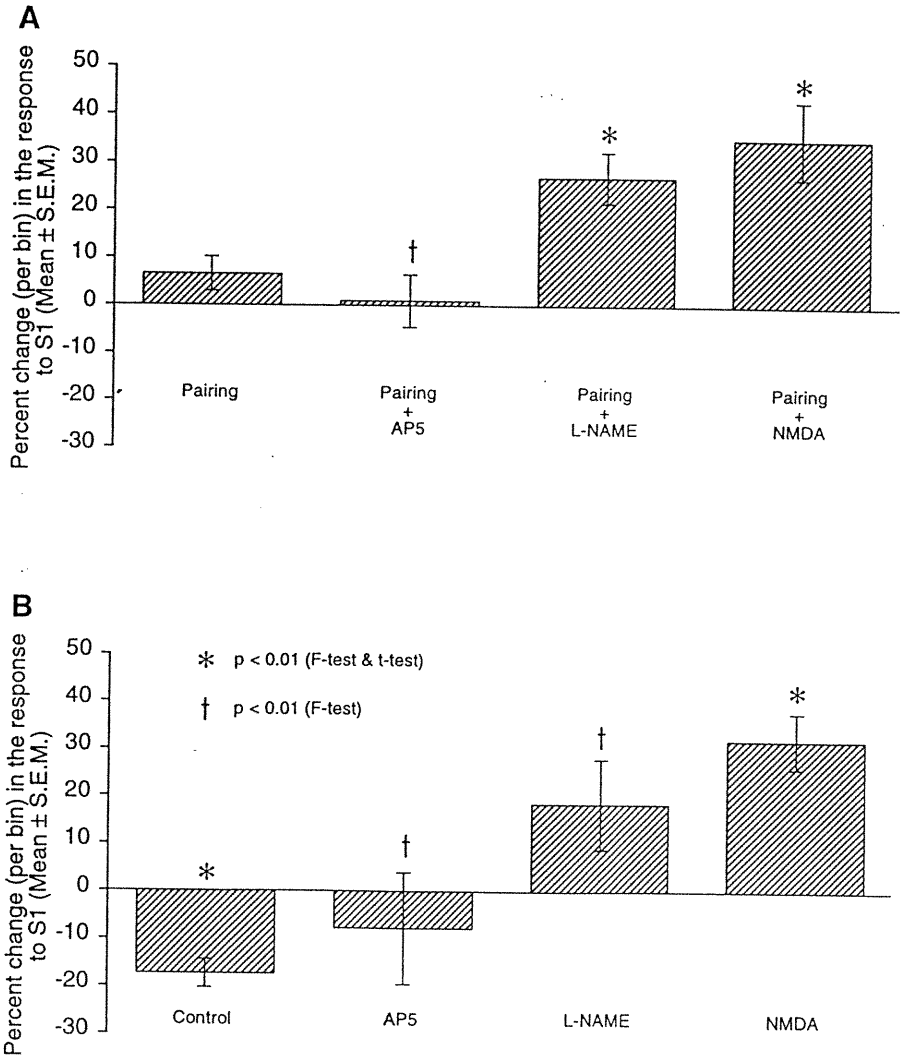


Fig. 10. Average changes (Mean ± S.E.M.) in the population responses for each experimental condition. (A) The associative procedure (pairing) led to increases in responsiveness to S1 whereas AP5 administration during pairing (Pairing + AP5) prevented any changes. In contrast, the responsiveness caused by pairing was enhanced by the concurrent delivery of either L-NAME (Pairing + L-NAME) or NMDA (Pairing + NMDA). (B) The non-associative control procedure (Control) led to a decline in the responsiveness to S1. In the presence of AP5 alone (AP5), responses to S1 were reduced. L-NAME and NMDA (L-NAME and NMDA, respectively) enhanced them. The responsiveness measured in each condition was compared to pairing with a *t*-test (*) and an *F*-test (†).

S.E.M.) and only enhanced by NMDA (52.9 ± 2.9% in 2 out of 4 cases) and L-NAME (58.2% in one experiment).

3.5. Average values of changes in neuronal responsiveness to S1 obtained in each experimental condition

It is reasonable to presume that any change in the behavior of an animal following conditioning depends upon the net change in the responsiveness produced in populations of neurons influenced by a conditioning procedure. Following this logic, we averaged the changes in the responses to S1 for all cells belonging to the same experimental condition and interpreted these averages as indicators of the behavior of the neuronal population in the affected region of cortex.

The average values, obtained by combining the results of cells without significant effects and those with significant changes, showed significant differences between each experimental condition ($p < 0.05$) and the pairing procedure (Fig. 10). The pairing condition resulted in a modest increase (6.6 ± 3.6%) in responsiveness to S1 that was significantly different from the decline (−17.3 ± 2.9%,

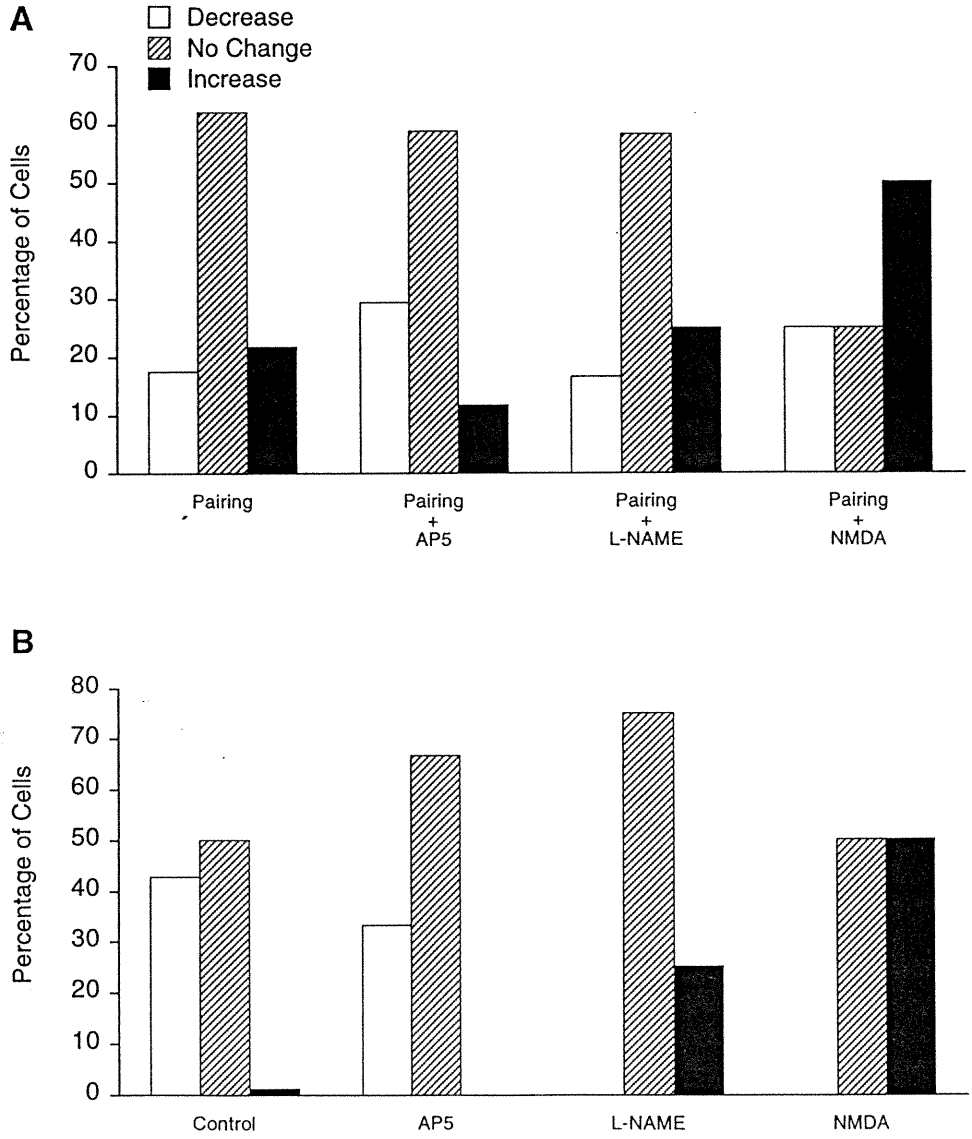


Fig. 11. The proportion of cells showing a significant change (decrease or increase) or no change in responsiveness to S1 in each experimental condition. (A) Following pairing, unchanged responses were the more common behavior, with smaller and almost equal numbers of cells showing either declines or enhancements in responsiveness. AP5 administration during pairing (Pairing + AP5) produced more declines and fewer enhancements, while the proportion of cells with no change remained almost the same. During pairing in the presence of L-NAME (Pairing + L-NAME), the proportion of increased responses was modestly higher than with pairing alone. However, delivery of NMDA during pairing (Pairing + NMDA) led to a significant reduction in the number of cells with unchanged responses to S1 with twice as many enhancements as declines. (B) Following the non-associative control procedure the most common change was a reduced response to S1 (Control). The effect of AP5 alone (AP5) on the proportion of cells in each response category was comparable to its counterpart with pairing except, that no enhanced responses were found. During L-NAME and NMDA delivery (L-NAME and NMDA, respectively), only unchanged or increased responses to S1 occurred.

Mean ± S.E.M.) observed in the control condition. Thus, relative to the unpaired controls, pairing led to a 24% increase in the responses to S1. In the absence of pairing, NMDA and L-NAME enhanced responses to S1 during the delivery period by 32.2 ± 5.9% and 18.5 ± 9.5%, respectively. This effect was larger when the drug was given during pairing and persisted after microiontophoresis had ceased and the pairing had been completed; it reached 35.0 ± 8.0% for NMDA and 27.0 ± 5.3% for L-NAME. In contrast, responses to S1 were depressed by 7.8 ± 11.7% during application of AP5 alone and virtually no change (1.0 ± 5.5%) occurred when pairing was performed in the presence of AP5. The results are illustrated in Figs. 10 and 11 and summarized in Table 3. What is readily apparent is that the differences in the averages of each experimental group could be attributed mainly to the relative proportions of cells showing either an increase or decrease in responsiveness (Fig. 11).

3.6. Effects of pharmacological agents on neuronal excitability

Changes in the rates of spontaneous activity during and following the delivery of AP5, NMDA and L-NAME alone were expressed as percentages of change relative to the pre-delivery levels. These normalized measures were then compared (*t*-test: $p < 0.05$) to equivalent ones in the pairing group to determine the drug effects on neuronal excitability. During delivery, the spontaneous firing rate of neurons subjected to pairing in the presence of NMDA was significantly higher (12%; $p < 0.05$) than for those exposed to pairing alone. The effect of AP5 ejection during pairing was dramatically affected by the level of EMG activity. In the group of experiments where the drug delivery occurred while the EMG activity was low and stable ($n = 14$), the ejection of AP5 during pairing significantly decreased the rate of ongoing activity in comparison with pairing alone (−10%). Once that group was extended to include the data from three additional experiments where the EMG activity during the drug delivery was elevated, the average effect of AP5 administration became a statistically significant increase (25%). The normalized rate of spontaneous activity during the delivery of L-NAME was not significantly different from that of pairing alone. Immediately after the ejection had ceased, all significant differences disappeared. From these results, we concluded that the pharmacological agents we used affected neuronal excitability only during the delivery period.

3.7. State of the animals

The state of the animal was evaluated by monitoring EMG activity across time. Periods during which the amplitude of the EMG was excessively high had been used previously to remove artifacts from the digitized spike record (see Section 2) and covered, on average, 3.1 ± 0.7% (Mean ± S.E.M.) of a complete experiment. Subsequently, the EMG amplitude was plotted against time in each experiment. Statistical analysis applied to the slope of the time series revealed that there were no significant trends ($p > 0.05$), suggesting that the variations in the amplitude of the EMG were randomly distributed throughout a recording.

4. Discussion

4.1. Summary of the results

We have described a series of experiments investigating the role of the cerebral cortex in associative learning. Our results indicate that, on average, the responses of neurons in barrel cortex to the deflection of a non-principal whisker (S1) were enhanced when stimulation of this non-principle whisker was repeatedly paired with that of the principal whisker (S2). Such an enhancement could not be attributed to changes in the state of the animal or to changes in the overall excitability of the cell. Analysis of the EMG revealed that periods of increased muscular activity, reflecting heightened arousal, were infrequent, averaging less than 4% of a complete experiment and occurred randomly. In addition, the measure we used to identify changes in the responses to S1 compensated for fluctuations in the overall excitability of the recorded neuron. More precisely, when responses to S2 varied significantly, the responses to S1 were adjusted by the amount of the change observed in the responses to S2. This correction was justified by the presumption that any change in neuronal responsiveness would affect S1 and S2 equally [54,56].

The enhancement of the responses to S1 was increased further when whisker pairing was performed in the presence of NMDA and L-NAME and reduced when AP5 was used. In contrast to whisker pairing, the non-associative control procedure markedly depressed the cortical response to S1. During the delivery period, NMDA enhanced both neuronal excitability and responsiveness to S1 whereas AP5 reduced them. The effects of both substances disappeared immediately after administration had ended. L-NAME had no significant effect upon either the level of ongoing activity or upon the responses to S1. Therefore, since the observed changes in the responses to S1 lasted longer than the periods of both whisker pairing and drug delivery, we concluded that they were not residual excitatory or inhibitory drug effects but rather were the result of changes in synaptic efficacy at the cortical level.

4.2. The role of NMDA receptors

The involvement of NMDAr in synaptic plasticity has been demonstrated in a very large number of experiments. The administration of NMDAr antagonists or blockers

prevents the induction of LTP in several regions of the central nervous system [1,8,16,52,61,82], reorganization of receptive fields following sensory deprivation [25,42,49] and learning of various tasks [26,51,89,90]. The cation channel associated with an NMDAr receptor is blocked by a magnesium ion when the cytoplasmic membrane is at its resting potential. The activation of NMDAr requires two conditions: (1) interaction with an agonist and (2) depolarization of the membrane on which the receptor is located. The simultaneous occurrence of these two factors allows the opening of the channel and the subsequent emergence of synaptic plasticity [16,61,82]. As a consequence, AP5 and NMDA, by respectively decreasing or increasing the probability of activation of NMDAr, can modulate the extent of plastic changes induced by whisker pairing.

4.3. The role of nitric oxide

The opening of NMDA channels facilitates the entry of calcium (Ca^{2+}) in the postsynaptic cell. Ca^{2+} activates several protein kinases which, in turn, increase the production of second messengers [16,61,82]. These eventually stimulate the production of retrograde messengers that diffuse through the extracellular space towards the presynaptic cell where they can increase the release of glutamate into the synaptic junction. NO has been proposed as one of these retrograde messengers [64,67,82] and its importance has been further reinforced by a possible involvement in some forms of learning [12,22]. The mechanisms by which NO can increase neurotransmitter release are currently being investigated and potential targets include protein kinase C and cytosolic ADP-ribosyltransferase [86]. In view of these findings, it was unexpected that, in our experiments, inhibition of NO synthase by L-NAME enhanced, on average, the responses of barrel cortex neurons to S1. However, several possible explanations exist. NO has been shown to reduce currents flowing through NMDA channels [72,86]. Thus, it has the ability to influence the many processes that depend on Ca^{2+} influx through NMDA channels, such as synaptic plasticity. NO might then support a feedback control mechanism which serves to reduce calcium entry resulting from the activation of the NMDAr, protecting the cell from such processes as glutamate neurotoxicity. Consistent with this idea is the fact that NO synthase in the cerebral cortex is localized in inhibitory interneurons [40,41]. These cells are activated by whisker stimulation [93,94,101] and might synthesize NO as a result. A possible effect of L-NAME is, as a consequence, a reduction of inhibitory transmission, which has been shown to be required by several paradigms leading to LTP induction [8,46,52]. On a different level, several studies have reported an absence of NO implication in plasticity and learning or LTP [10,11,52]. This fact has led to the suggestion that the requirement for NO might depend on the species, the sensory modality, the learning paradigm or the stimulation protocol used to induced LTP [86]. As well, there are other diffusible substances such as CO that may serve as retrograde messengers. Such substances could be playing a role here.

4.4. Specificity of pharmacological effects

The microiontophoretic technique offers two main advantages. First, it limits the delivery of pharmacological agents to a few cells surrounding the recording site. Thus, in our preparation, extracortical sites of drug action can be easily excluded. Second, it eliminates systemic side effects that could result from an intravenous, intraperitoneal or subcutaneous injection. This is particularly important in the case of L-NAME which can alter the state of the autonomic nervous system through its vasoconstricting effects [65]. Unfortunately, microiontophoresis cannot eliminate a weakness of all studies investigating plasticity with pharmacological means, namely, how to distinguish the effects of pharmacological agents on plasticity from those on normal synaptic transmission or on the intrinsic firing properties of the cell. In our experiments, we are faced with the fact that application of AP5 and NMDA alone affected both averaged neuronal excitability and evoked responses to S1 in ways very similar to the combination of pairing with drug delivery. These results do not contradict the Hebbian hypothesis nor the conclusion that plasticity occurs within barrel cortex. However, they raise the possibility that pharmacological modulation of the excitability of neurons simultaneously subjected to sensory stimulation induces plasticity independently of whisker pairing [19]. This issue was raised by Cruikshank and Weinberger [25] when they reviewed two studies dealing with the effects of AP5 on shifts in ocular dominance following monocular deprivation. In one study, Bear et al. [13] suggested that the lack of an ocular dominance shift in the presence of AP5 resulted from a specific action on plasticity itself. In contrast, Rauschecker et al. [75] concluded that AP5 prevented reorganization by reducing post-synaptic responses.

We offer two arguments that our experiments resulted in a phar-macological manipulation of plasticity rather than of normal synaptic transmission. The first argument relies on the observation that drug application alone produced changes in one direction only (increases in the case of NMDA and decreases for AP5) whereas, when combined with pairing, the changes included both increases and decreases in responses to S1. Second, the average responses for each treatment group were clearly greater when combining pairing with delivery of pharmacological agents than when applying drugs alone. The situation was simpler in the case of L-NAME because it did not alter neuronal excitability and its effects on responsiveness to sensory stimulation were only significant when associated with pairing. Thus, based on this result and the previously mentioned arguments, we suggest that the changes in the responses to S1 were induced by the combined effects of

whisker pairing and pharmacological agents acting upon mechanisms of plasticity.

4.5. The cerebral cortex as a site of plasticity in whisker pairing

In our experiments, D-AP5 and NMDA were administered by microiontophoresis, a technique that limited their effects to the cells surrounding the injection site. It follows that the targets of these drugs were located within the barrel cortex or, more precisely, the barrel serving S2. Based on these data, we propose that the somatosensory cerebral cortex is one site where plasticity emerges following whisker pairing. Our results are supported by several studies.

Diamond et al. [32] trimmed all but two adjacent whiskers (D2 and either D1 or D3) in rats that were subsequently allowed to live for 1–3 days before being anaesthetized and prepared for single-unit recording. This manipulation resulted in a form of whisker pairing that enhanced the responses of neurons in D2 to the adjacent intact whisker relative to the trimmed whiskers. To demonstrate the cortical origin of this plasticity, they offered two arguments. First, the plastic changes affected the portions of the neuronal responses that occurred more than 10 ms after the onset of whisker deflection [32]. Second, plasticity appeared in non-granular layers above layer IV [33]. Although these observations would argue against plasticity in the principal trigeminal nucleus and the ventral posteromedial thalamus (VPM) or at thalamocortical synapses from the VPM to layer IV, they cannot exclude possible changes in synapses formed by the VPM with neurons in other layers (for example Vb) or by axons of neurons in subcortical regions such as the posterior thalamic nucleus that relay signals from the periphery at latencies longer than 10 ms [2–5,35,50,53,66,68,69,73,74,97].

Fox [37] reached similar conclusions with a paradigm involving altered tactile experience. All but one vibrissa (D1) from one side of the face of newly born rats were repeatedly trimmed. Sixty days later, the stimulation of D1 elicited larger than normal responses from neurons located in barrels surrounding the one served by D1. These responses could be prevented by acute microlesions placed in the D1 barrel suggesting that they depended on information relayed through the barrel served by D1 rather than a subcortical region. However, a different interpretation would be that the lesion eliminates a source of excitation which is necessary for the emergence of plasticity or for its expression in another brain area. Finally, Wang et al. [98] showed that, in primates, repeated synchronous stimulation of the distal phalanges induced a remodelling of the cortical representations of these fingers in area 3b. The representations became fused implying that neurons in this area had acquired new receptive fields. This reorganization was not evident in the lateral division of the ventral posterior thalamus.

4.6. The covariance hypothesis as a possible mechanism for plasticity induced in the cerebral cortex by whisker pairing

We believe that the mechanisms underlying part of the changes in cortical responsiveness induced by whisker pairing are consistent with the covariance hypothesis originally described by Hebb [45] and that the activation of the NMDAr occurs when Hebb's rule of covariance is fulfilled. The covariance rule can be divided into two parts [24] which are described separately in the following two paragraphs.

The first part of the covariance hypothesis states that repeated pairing of presynaptic activity with increased postsynaptic activity enhances synaptic transmission between the afferent fibers and the postsynaptic element. These conditions are satisfied by whisker pairing. The neurons located in the barrel serving S2 represent the postsynaptic element. Stimulation of S1 activates afferent fibers, releasing glutamate that subsequently interacts with NMDAr located on the postsynaptic membrane. At almost the same time, the postsynaptic membrane is depolarized by the stimulation of S2. Thus, whisker pairing provides the conditions required for the opening of NMDA channels and the emergence of plastic changes. Note that increased spiking in response to whisker stimulation generally does not last more than a few milliseconds after the end of stimulus delivery. However, recordings of evoked potentials and imaging techniques with voltage-sensitive dyes clearly show that increased post-synaptic activity elicited by whisker stimulation can last up to 500 ms, which is longer than the 300 ms interstimulus interval used during pairing [54,60]. Additional factors that might contribute to the activation of NMDA receptors are neuromodulators such as acetylcholine, norepinephrine and serotonin [17,34,56,87,92]. Acetylcholine (ACh) release is increased during learning, in part due to an arousal of the animal by sensory stimulation [48] but perhaps also by the learning context [18]. It could facilitate the activation of NMDA receptors by increasing postsynaptic activity through the inactivation of several potassium outward currents and the activation of a non-selective cation inward current [15,17,57,61,63].

The second part of the covariance hypothesis indicates that either repeated pairing of presynaptic activity with decreased postsynaptic activity or increased postsynaptic activity while the presynaptic element is inactive depresses synaptic transmission between the afferent fibers and the postsynaptic element. These conditions correspond to the 'pre not post' and 'post not pre' rules, respectively, as described by Cruikshank and Weinberger [25]. We propose that this phenomenon occurs during the non-associative control procedure and that it can be interpreted in two different ways. First, decreased cortical responsiveness might simply reflect habituation as a result of continuous presentation of a sensory stimulus without appropriate

reinforcement [21,96,104]. Groves and Thompson [44] proposed that habituation could result from the interaction of two distinct processes: habituation itself and an initial period of sensitization. The magnitude and duration of sensitization depends upon the intensity of the reinforcing stimulus [95]. The second possibility is conditioned inhibition, where the stimulation of S1 signals a decreased probability of the presentation of S2 [80,81].

4.7. Effects of whisker pairing on single-unit responses to S1

We have concentrated our discussion thus far on averaged effects of whisker pairing. However, as our data show, individual neuronal responses to S1 could increase, decrease or remain unchanged following pairing. Averaging all these results produced the frequently reported conclusion that conditioning of a sensory stimulus (S1 in our case) enhances cortical responsiveness to the conditioned stimulus in question [62,100]. In this context, the fact that certain neurons exhibit decreased or unchanged responses to S1 following pairing cannot be accounted for by the covariance hypothesis and deserves additional comments.

First of all, several studies have consistently reported that a proportion of the cells recorded during a sensory conditioning paradigm do not demonstrate any plasticity [38,39,100]. On one hand, this could represent a failure of the animal to learn. On the other, the amount of time required by these neurons to exhibit plastic changes might be longer than the recording time. At the same time, the lack of a detectable change in spike frequency does not exclude modifications in subthreshold postsynaptic potentials. Another possibility, however, is that some types of neurons in the primary somatosensory cortex do not undergo plasticity to preserve a fixed representation of the basic properties of a stimulus [36].

A failure of the animal to learn the relationship between S1 and S2 may also take the form of decreased responses to S1, as habituation develops during the course of the experiment due to factors like a lack of motivation or distraction by different stimuli. From a different perspective, decreased responses to S1 could also reflect a necessary effect of conditioning for two possible reasons. First, depression of the responses of certain classes of neurons, inhibitory interneurons for example, can lead to potentiation of the responses of different types of neurons. Second, this depression might act as a feedback control system that provides stability to cortical neural networks in a way similar to the role hypothesized above for NO.

4.8. Criticisms and future experiments

In the preceding paragraphs, we have tried to describe the mechanisms by which whisker pairing induces plasticity within the cerebral cortex in accordance with Hebb's hypothesis of covariance. The arguments were based on the fact that the pharmacological agents we used were applied locally and, whether they influenced the level of postsynaptic activity or acted specifically on synaptic plasticity by preventing the detection of increased covariance by the postsynaptic cell, they were able to modulate the magnitude of the observed plastic changes. Unfortunately, we cannot eliminate the possible induction of plasticity by pharmacological presynaptic modulation of the increased or decreased covariance between the presynaptic and postsynaptic elements. As illustrated in Fig. 12, the pharmacological agents might modulate presynaptic activity directly or through neurons surrounding the postsynaptic cell. An additional pathway could involve any remote neuron that receives input from the cells affected by the drugs and feeds back to the presynaptic terminal. Such neurons could be located in another area of the cerebral cortex or in any one of many subcortical regions reciprocally connected to the sensory cortical areas and known to display plasticity such as the thalamus [28,71] or hippocampus [14,27,85]. This last possibility is particularly important because it would argue at the same time against a cortical locus of plasticity.

However, if the interpretations that we offer are valid, they only provide proof for the necessity of increased covariance for the induction of plasticity by whisker pairing. Testing the sufficiency of covariance would require

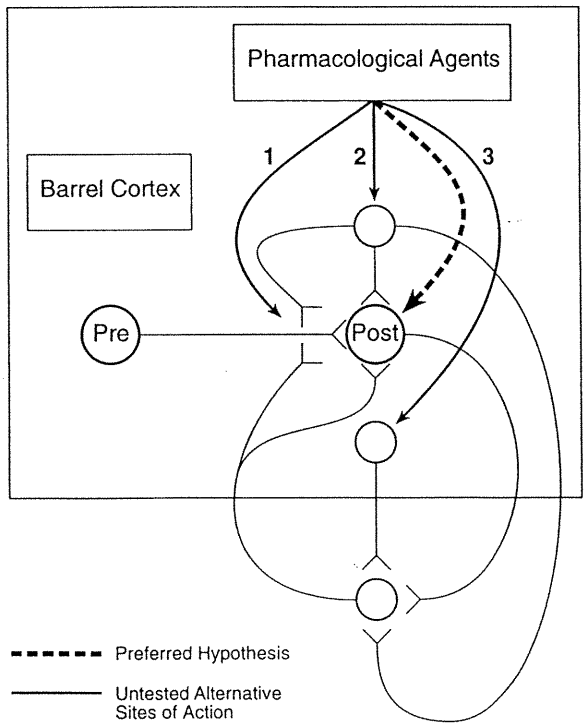


Fig. 12. Although we suspect that our drug treatments have affected the postsynaptic cell, there are three other possible mechanisms by which pharmacological agents might modulate presynaptic activity and, subsequently, plasticity, independently of their effects on the postsynaptic cell: (1) direct presynaptic action, (2) through neurons surrounding the cell being recorded, and (3) through a remote neuron that receives input from the cells affected by the drugs and feeds back to the presynaptic terminal.

precise control of postsynaptic activity, for example with direct juxtacellular current application as was done by Fregnac et al. [38,39] and Shulz and Fregnac [91] in visual cortex or by Cruikshank and Weinberger [24] in auditory cortex. A possible experiment would then be to perform whisker pairing while depolarizing or hyperpolarizing a neuron in the barrel serving S2. The use of a third whisker that is not a part of any conditioning would also be necessary to determine whether the changes in the responses to S1 following pairing are specific to the paired whisker or extend to others as part of a generalized increase in postsynaptic excitability.

4.9. Conclusion

We have shown that repeatedly pairing the stimulations of two whiskers, S1 and S2, increases the responses of neurons in the barrel serving S2 to the deflection of S1 and that this enhancement is modulated by local, microiontophoretic application of pharmacological agents that act upon NMDAr and NO synthase. We have also argued that the results were not due to long-lasting, non-specific effects of the drugs on neuronal excitability and have consequently concluded that NMDAr and NO are necessary for the induction of plasticity in barrel cortex by whisker pairing. However, completing the investigation of the sites where plasticity occurs following associative learning in general would ultimately require the recording of neurons located inside and outside the cerebral cortex during similar tasks, as was done by Oleson et al. [70], Weinberger [99], and Weinberger and Diamond [100] in the auditory system and Wang et al. [98] in the somatosensory system.

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CHAPTER 2

Blockade of cholinergic receptors in rat barrel cortex prevents long-term changes in the evoked potential during sensory preconditioning

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- *Conception du projet.*
- *Expérimentation*
- *Analyse des données.*
- *Rédaction de l'article.*

Blockade of Cholinergic Receptors in Rat Barrel Cortex Prevents Long-Term Changes in the Evoked Potential During Sensory Preconditioning

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Maalouf, M., A. A. Miasnikov, and R. W. Dykes. Blockade of cholinergic receptors in rat barrel cortex prevents long-term changes in the evoked potential during sensory preconditioning. *J. Neurophysiol.* 80: 529–545, 1998. We offer evidence that acetylcholine (ACh) is involved in the emergence of functional neuronal plasticity induced by whisker pairing. Evoked potentials were recorded within the barrel cortex of awake, adult rats before, during, and after one of five paradigms. In the pairing procedure, each of 50 deflections of a whisker (S1) was followed 150 ms later by the deflection of a second whisker (S2). The explicitly unpaired control procedure differed by the lack of contiguity and contingency between the stimulation of S1 and S2. In the three remaining groups, pairing was performed 30 min after an intraperitoneal injection of either 0.5 ml of saline (150 mM NaCl), 100 mg/kg of atropine methyl nitrate (0.5 ml of AMN in saline), or 100 mg/kg of atropine sulfate (0.5 ml of ATS in saline). Changes in responsiveness to S1 were compared with, and adjusted by, changes in responsiveness to stimulation of S2. Changes in potentials evoked by S1 were interpreted as a change in neuronal excitability occurring when the first innocuous stimulus systematically predicted the appearance of the second innocuous stimulus. When whisker pairing was performed alone or in the presence of either saline or AMN (a blocker of muscarinic cholinoreceptors that does not cross the blood-brain barrier, BBB), responses to S1 increased, whereas, in the presence of ATS (blocker of muscarinic cholinoreceptors that does cross the BBB) or following the explicitly unpaired control, they decreased. The effects of saline, AMN, and ATS on the evoked potential without vibrissae pairing were opposite to those observed when these substances were injected and pairing occurred. Analysis of the behavioral state of the animal showed that the changes observed in the evoked potential could not be attributed to changes in behavioral state. The changes in responsiveness to S1 induced by whisker pairing were independent of neuronal excitability, did not occur in the absence of contingency and contiguity between S1 and S2, were blocked by the muscarinic receptor antagonist ATS, but not by blockade of muscarinic modulation of normal synaptic transmission. Thus activation of muscarinic cholinoreceptors within the CNS were a necessary condition for this form of neuronal plasticity.

INTRODUCTION

As a result of increased longevity, the number of people affected by Alzheimer's disease (AD) has been increasing steadily, provoking massive efforts to understand its neurobiological basis and to find effective treatments for this disease. AD is characterized by a slowly progressing deterioration of higher brain functions, including learning and memory. Early postmortem studies of patients with AD revealed that the nucleus basalis of Meynert (NBM), an important structure for learning and memory, displays characteristic histopathologic lesions associated with extensive cell loss.

The NBM is the major source of cortical acetylcholine (ACh) (Johnston et al. 1981; Mesulam 1989). Several lines of evidence support an important role for ACh in learning and, more generally, in neuronal plasticity: the levels of ACh increase in various regions of the cerebral cortex (CCx) during learning (Butt et al. 1997); ACh can modulate neuronal excitability and enhance the responsiveness of cortical neurons to afferent stimuli for periods of time that outlast its presence (Kotlyar and Ovcharenko 1978; Metherate et al. 1987, 1988a,b); changes in the firing of suspected NBM cholinergic neurons has been reported during learning (Pirch et al. 1991; Richardson and DeLong 1991; Wilson and Rolls 1990a,b); cholinergic antagonists or lesions of the NBM prevent or, at least, delay the acquisition of various learned tasks (Butt and Hodge 1995; Jacobs and Juliano 1995); these deficits are reduced by the administration of cholinergic agonists or acetylcholinesterase (AChE) inhibitors (Muff et al. 1993; Myers et al. 1996); and the severity of memory impairment in AD has been correlated with the extent of NBM degeneration (Albert 1996; Levey 1996; Poirier et al. 1995).

More recently, however, the significance of cholinergic systems for learning and memory and the effects of their disruption in AD has been questioned. Although there is now general agreement that ACh is not necessary for retention, its role in acquisition of memories is the subject of considerable controversy in view of several findings: the decreased learning performance induced by NBM lesions or cholinergic antagonists could be explained by the lack of specificity of such procedures and the possible side effects on processes more directly related to attention rather than to memory formation (Blokland 1996; Hagan et al. 1986; Voytko 1996; Whishaw 1989; Whishaw and Petrie 1988); the success of cholinergic pharmacotherapies (the best developed approach being the use of AChE inhibitors) in treating cognitive deficits has been very limited (see Enz et al. 1993; Giacobini 1993); and the cholinergic hypothesis of learning and memory dysfunction in AD does not take into account the lesions reported in various other regions equally important for learning and memory, most importantly the CCx, a major source of afferents to the NBM and, at the same time, its main target (Esiri 1989; Hof and Morrisson 1994).

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The aim of the present study is to examine the nature of the interaction between ACh and neuronal plasticity in the somatosensory CCx and thereby its significance for learning and memory. We describe experiments involving whisker pairing, an associative learning paradigm consisting of repeatedly pairing two mystacial vibrissae that is believed to induce plastic changes within the vibrissae representation of the primary somatosensory "barrel" cortex (Maalouf et al. 1998). Evoked potentials were collected from several barrels simultaneously before, during, and after awake, but restrained, adult rats were subjected to whisker pairing in the absence of or after intraperitoneal injection of either saline, the muscarinic antagonist atropine sulfate (ATS) or atropine methyl nitrate (AMN), an analogue of ATS that does not cross the blood-brain barrier. Our results suggest that systemic administration of ATS prevents the emergence of synaptic plasticity within barrel cortex during whisker pairing.

METHODS

Surgical preparation of animal to be studied awake

All procedures were approved by the local committee on animal care; they respected the standards established by the Canadian Council of Animal Care. Adult male Sprague-Dawley rats (350–550 g; 471.3 ± 57.4 g, mean ± SD), housed individually with food and water ad libitum, were anesthetized with intraperitoneal injections of 35 mg/kg pentobarbital sodium (65 mg/ml in an aqueous propylene glycol base with 2% benzyl alcohol; Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Supplements were added as required to maintain areflexia until the end of the surgery. An antibiotic was administered systemically (enrofloxacin 50 mg/ml in N-butyl alcohol and water, 0.05 ml im; Baytril, Chemargo, Etobicoke, Ontario, Canada) and the eyes of the animal were covered with baby oil (Johnson and Johnson). After the injection of a local anesthetic (0.3 ml of lidocaine hydrochloride 20 mg/ml; Xylocaine, Astra Pharmaceuticals, Mississauga, Ontario, Canada), a central portion of the scalp was excised and the temporal muscles reflected. Two teflon-insulated stainless-steel wires were placed over the cervical muscles to monitor the electromyogram (EMG). Three stainless steel screws to which teflon-insulated stainless steel wires were soldered were inserted in the skull. The first two were used to record the electro-oculogram (EOG; +5 to +6 mm anterior to the bregma, at the right edge of the skull) and electroencephalogram (EEG; over the right parietal lobe: 2.5 mm posterior to the bregma and 2 mm lateral to the midline) respectively. The third one (over the left orbital sinus: +5 to +6 mm anterior to the bregma and 2 mm lateral to the midline) served as a reference. Eight additional stainless steel screws were inserted posteriorly to lambda and on the lateral sides of the skull to help anchor the attachments.

A craniotomy was performed over the left hemisphere from +3 to −3 mm, anteroposterior (AP) and 2 to 5 mm mediolateral (ML) to the midline, and a small portion of the dura was removed. Two arrays of 4, 25 μm-diameter, formvar-insulated nichrome wires (A-M Systems,, Everett, WA) were inserted successively into the left hemisphere between −2 and −3.5, AP, and 3 and 4.5, ML, with vertical and horizontal angles of 35–45°. The tips of the electrodes (1–2 MΩ impedance at 1 kHz) were separated by ≤0.5 mm. During the penetration, multiunit activity from all four electrodes (connected together) was monitored continuously. The insertion of the electrode assembly was terminated when neurons responding to the deflection of one or several vibrissae were encountered. The array was then stabilized with dental acrylic cement. After insertion of the second four-electrode assembly, the exposed brain was covered with low-melting–point wax and more dental acrylic was used to cover the exposed skull and attachments. Two parallel stainless steel tubes secured by two shorter tubes were imbedded in the dental acrylic. This rectangular arrangement of tubes was fixed to the recording apparatus where it served to stabilize the head and restrain the animal during the experiment (Maalouf et al. 1998).

Electrophysiology and experimental protocols

Animals were allowed 72 h to recover from surgery before being placed in the recording apparatus for one to four practice sessions of ≤1–2 h to adapt them to restraint. Before the first experiment, multiunit activity from each electrode was briefly recorded (filter set at 300–3,000 Hz) to identify the principal whisker that drove the neurons sampled by that electrode. Evoked potentials were filtered (band-pass filter set at 1.6–70 Hz) and amplified at 10,000–30,000 with a Neurofax electroencephalograph (5–15 μV/mm Nihon Kodhen, Tokyo). Filtering at band-pass of 1–100 Hz and an amplification of 5,000 (AM 502 & TM 504, Tektronix, Beaverton, OR) was used for the processing of the EEG, EMG, and EOG signals. All signals were digitized with a CED 1401 *plus* laboratory computer interface (Cambridge Electronic Design, Cambridge, UK) at a resolution of 2,000 samples/s (sps) for evoked potentials and at 500 sps for the EEG, EMG, and EOG.

At the beginning of the experiment, two whiskers were selected. The one stimulated first during pairing (S1) was always rostral to the other (S2). In addition, S2 had to be the principal whisker of one of the barrels that we could record from. Each stimulus consisted of a discrete, 5-ms–long, 3-mm horizontal deflection of the whisker. The stimulating device driving each of the whiskers consisted of a miniature speaker with one end a light-weight tube of dried grass glued to the membrane. The stimulated whisker was inserted into other end of the tube and stayed there for the duration of experiment. S1 and S2 were each stimulated separately during 20 trials to establish baseline responses (Fig. 1, *A* and *B*). After that, the animal was injected intraperitoneally, while still in the recording box, with 0.5 ml of either 150 mM NaCl (saline), the solution of ATS (100 mg/kg; Research Biochemicals International, Natick, MA) in saline, or the solution of AMN (100 mg/kg; Sigma, Saint-Louis, MO) in saline. Thirty minutes later, a new baseline was established by stimulating first S1, then S2, during 20 trials each (Fig. 1, *C* and *D*). Whisker pairing then was performed 50 times, the interstimulus interval between S1 and S2 being 150 ms (Fig. 1*E*). After that, 20 deflections of S1 followed by 20 deflections of S2 were presented again (Fig. 1, *G* and *H*). This final set of independent stimuli for S1 and S2 allowed evaluation of changes in the responses of each whisker that might have been brought about by their pairing. The intertrial interval varied constantly in all sections of the protocol between four preset values (5, 7, 9, or 11 s) to minimize a possible conditioning to time (Delacour and Houcine 1987).

In six preliminary experiments involving whisker pairing alone as well as in the explicitly unpaired controls, recording of the first baseline for each whisker was omitted and the animals did not receive any injections. In unpaired controls, the interstimulus intervals were 2.5, 3.5, 4.5, or 5.5 s (half the intertrial intervals used during in the pairing procedures) and each whisker could be stimulated up to five times in a row (Fig. 1*F*).

Cortical responses to whisker stimulation

To calculate the averaged response over several presentations, we selected and combined only evoked potentials with similar

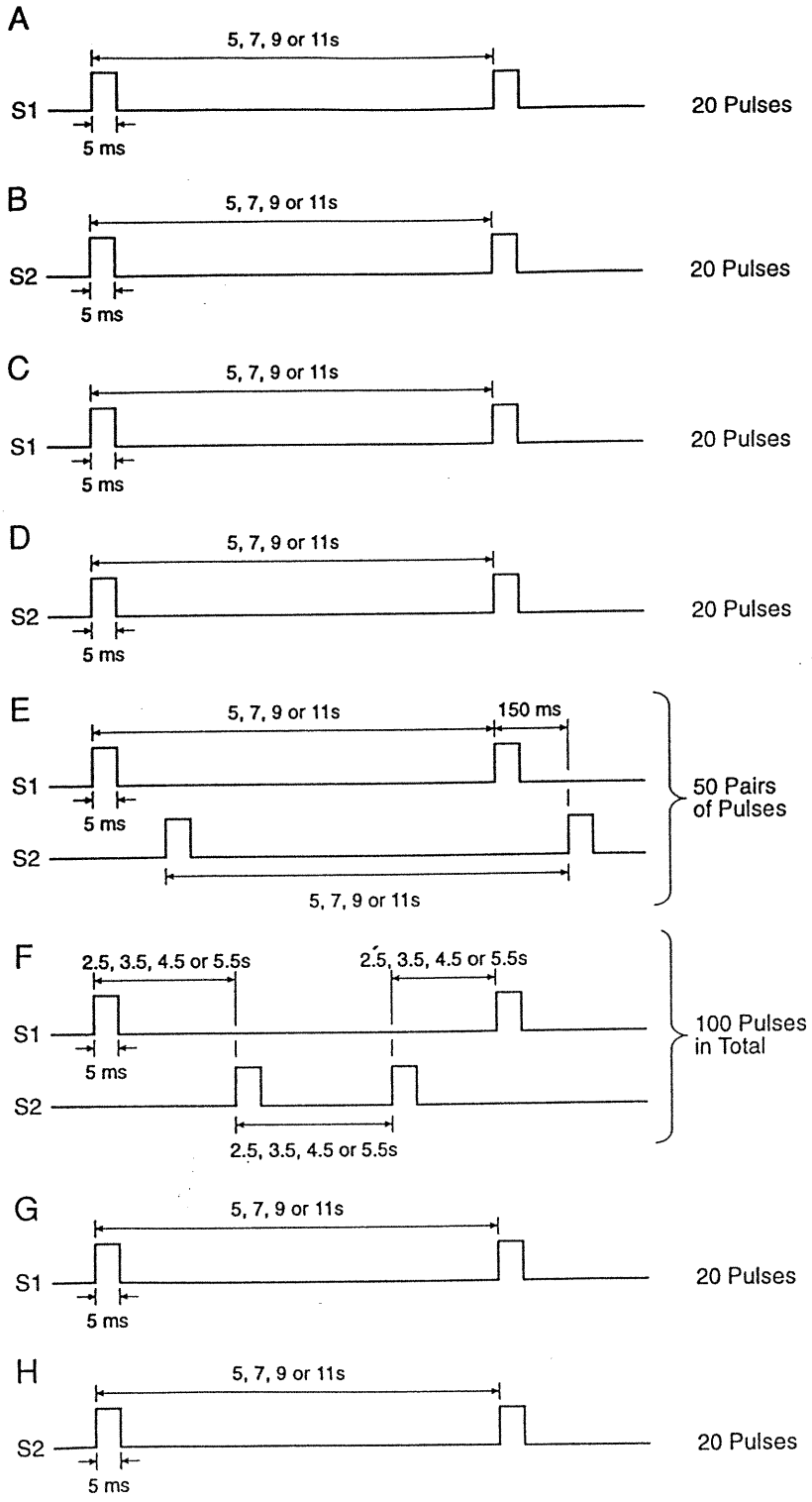

FIG. 1. Experimental paradigms. *A* and *B*: initial measures of responsiveness; whisker stimulation consisted of 10 or 20 single, 5-ms–long horizontal deflections of the whisker S1 (*A*) followed by the same number of deflections of whisker S2 (*B*). Stimuli were separated by intervals of 5, 7, 9, or 11 s arranged in pseudorandom sequences (*C* and *D*) drug tests; whisker stimulation in patterns identical to those shown on *A* and *B* but performed 30 min after the intraperitoneal injection of either saline, atropine sulfate (ATS), or atropine methyl nitrate (AMN). *E*: pairing; during pairing, stimulation of S1 preceded the stimulation of S2 by 150 ms but pairings still occurred after a pseudorandom interval of 5, 7, 9, or 11 s. *F*: explicitly unpaired controls; concomitent stimulation of S1 and S2. This paradigm differed from pairing by the lack of a structured temporal relationship between stimulation of S1 and S2. In these experiments, the stimulations of one whisker or the other occurred every 2.5, 3.5, 4.5, or 5.5 s, but deflection of the same whisker could not occur more than 5 times in a row. *G* and *H*: final measure of responsiveness; repeated whisker stimulation, identical to the one shown in *A* and *B* used to obtain a measure of the effects of pairing.

shapes. The pattern that occurred most frequently was chosen for analysis; evoked potentials contaminated by artifacts related to movements of the animal were eliminated. On average, 53.7 ± 2.1% (mean ± SE) of the recorded potentials were used. There were no statistically significant differences among the various treatment groups in terms of number of evoked potentials chosen for analysis [1-way analysis of variance (ANOVA) and Kruskal-Wallis ANOVA on ranks; $P < 0.05$] nor was there any correlation (linear regression analysis $P > 0.05$) between the values obtained for each group and the percentage of potentials eliminated.

Evoked potentials from up to seven different electrodes could be recorded while simultaneously monitoring the EEG, EMG, and EOG. Deflection of a single whisker could elicit evoked potentials in several barrels. Our observations suggested that the more distant was the whisker from the whisker giving the largest response (prin-

TABLE 1. *Percentages of evoked potentials used in each treatment group*

Group	Evoked Potentials, %
Pairing	52.3 ± 14.3
Pairing + saline	53.8 ± 13.3
Pairing + AMN	50.2 ± 8.3
Pairing + ATS	61.6 ± 20.4
Control	50.4 ± 6.8

Values are means ± SD. Averaging across all groups, the percentage of evoked potentials used was 53.7 ± 2.1 (Mean ± SEM) of all the recordings. The treatment groups did not differ statistically from each other based on the one-way analysis of variance ($P = 0.51$) and Kruskal-Wallis analysis of variance on ranks ($P = 0.85$).

cipal whisker), the smaller and the less consistent was the response it produced.

State of the animals

To evaluate the possibility that the observed changes in amplitude of the evoked potentials were affected by the behavioral state of the animal, the power spectra of the EEG (1–30 Hz) and EMG (1–100 Hz) were calculated using Fourier transformation. The transforms were represented as 100-bin histograms, and a cluster analysis was performed on the histograms calculated for each trial of each experiment to identify cases having EEGs and EMGs spectra of distinctive profiles. The power spectra were built with a Spike 2 software (Cambridge Electronic Design) and were clustered with SPSS 4.0 (SPSS, Chicago, IL) software using the average linkage-between-groups method applied on squared Euclidian distances

TABLE 2. *Number, time schedule, and design of experiments performed in each animal*

	Animal									
Order of the Experiment	*M95*	*M89*	*M94*	*M88*	*M90*	*M93*	*M96*	*M91*	*M92*	
									First 5 Experiments	Last 4 Experiments
n	1	2	2	3	4	4	4	5	5	4
1										
Whiskers (S1 and S2) Simple experimental condition*	D3-E2 AMN	E3-E2 P	E3-E2 ATS	E3-E1 P	D4-E2 C	E4-E3 ATS	E4-E3 AMN	D5-E3 C	D3-E1 ATS	D3-E1 S
Complex experimental condition*	P-AMN		P-ATS			P-ATS	P-AMN		P-ATS	P-S
Time between consecutive experiments, h		(24)	(24)	(24)	(72)	(24)	(24)	(24)	(24)	(24)
2										
Whiskers (S1 and S2) Simple experimental condition*		C3-E2 P	D3-E2 ATS	C4-E1 P	D4-E2 C	E4-E2 ATS	D4-D2 AMN	D5-D4 C	D3-E2 ATS	D4-E2 ATS
Complex experimental condition*			P-ATS			P-ATS	P-AMN		P-ATS	P-ATS
Time between consecutive experiments, h				(24)	(24)	(24)	(24)	(24)	(24)	(24)
3										
Whiskers (S1 and S2) Simple experimental condition*				E4-E2 P	D3-E2 P	C4-E3 ATS	D4-E3 AMN	C5-E3 C	D4-E1 ATS	C2-E1 S
Complex experimental condition*						P-ATS	P-AMN		P-ATS	P-S
Time between consecutive experiments, h					(24)	(48)	(24)	(24)	(24)	(24)
4										
Whiskers (S1 and S2) Simple experimental condition*					D3-E2 C	D4-E3 ATS	D4-D3 AMN	D5-E3 S	D4-E2 ATS	D3-E2 S
Complex experimental condition*						P-ATS	P-AMN	P-S	P-ATS	P-S
Time between consecutive experiments, h								(24)	(6)	
5										
Whiskers (S1 and S2) Simple experimental condition*								D5-E3 S	C2-E1 ATS	
Complex experimental condition*								P-S	P-ATS	
Time between consecutive experiments, h									(48)	

Thirty-four experiments were conducted in nine different animals. The number of experiments per animal ranged from one to nine, the average being ± 2.3 (mean ± SD). * C, control; P, pairing; S, saline; ATS, atropine sulfate; AMN, atropine methyl nitrate.

between the values in each bin of the spectral distribution histogram (see Norusis 1990). The EOG was used as an indicator of rapid eye movement (REM) sleep.

Data analysis

To quantify the effects of the various experimental protocols, two different approaches were used. In the first one, the percentage of change in the amplitude of the first component of evoked potentials was calculated for S1 and S2. Then the measures obtained for S2 were subtracted from those of S1

$$\Delta A = 100(R^2_{S1} - R^1_{S1})/R^1_{S1} - 100(R^2_{S2} - R^1_{S2})/R^1_{S2} \qquad (1)$$

where R_{S1} and R_{S2} were the amplitude of responses to stimulation of whiskers S_1 and S_2, respectively, before (R^1) and after (R^2) the pairing. The adjusted percentages of change in the responses to S1 (ΔA) were averaged across all experiments belonging to the same treatment group. Significant differences between the groups were distinguished with *t*-test and Mann-Whitney rank-sum test ($P < 0.05$). The advantage of this approach is that it allowed us to measure the effects of whisker pairing on S1 and S2 separately.

Using the second approach, ratios calculated by dividing the responses to S1 by those to S2 were obtained before and after pairing. These were subtracted from one another as follows

$$\Delta R_n = R^2_{S1}/R^2_{S2} - R^1_{S1}/R^1_{S2}$$

where R was the same as in (*Eq. 1*). As was done with the first method, differences of ratios (ΔR) were averaged across all experiments belonging to the same treatment group and the resulting measures were compared with *t*-test and Mann-Whitney rank-sum test ($P < 0.05$).

Both of these methods favor the detection of changes that are of different magnitudes in S1 and S2. The rationale for this

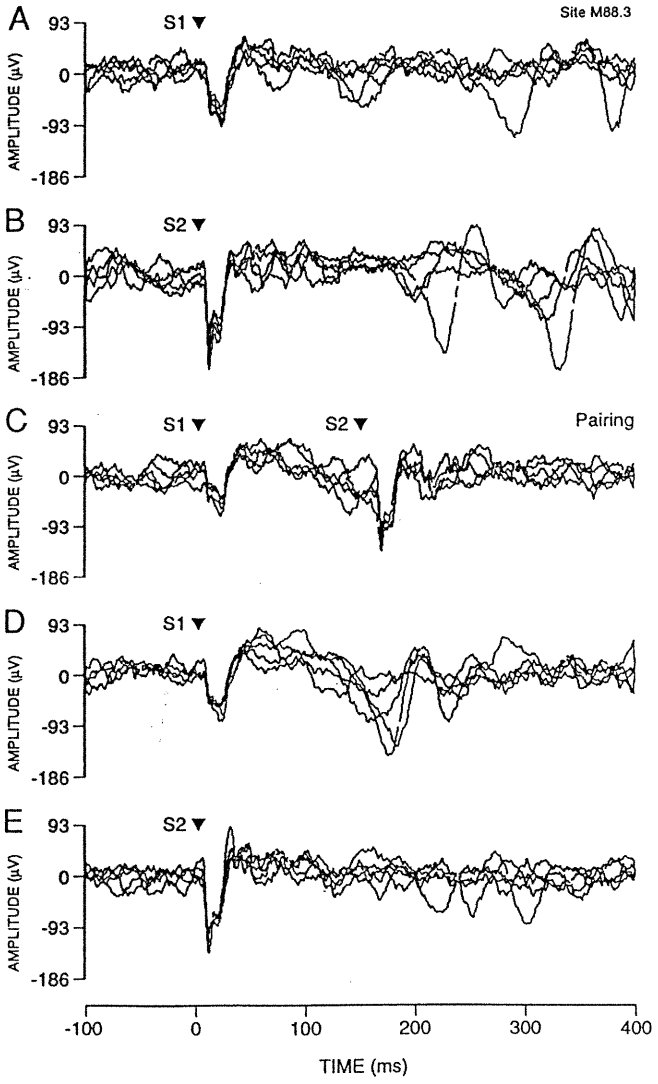

FIG. 2. Changes attributable to pairing in the responses recorded from barrel for which S2 is the principal whisker. Five waveforms before (*A* and *B*), during (*C*), and after (*D* and *E*) pairing are illustrated. Amplitude of the 1st component (at the moment in time when the absolute minimum occurred) of the potentials evoked by stimulation of S1 decreased by 5.7% after pairing and by 27.1% for S2. Substracting the change in responses to stimulation of S2 from those of S1 yielded a 21.4% increase in the relative size of the responses to S1 (see also Fig. 4*A*). ▼, time of whisker deflection.

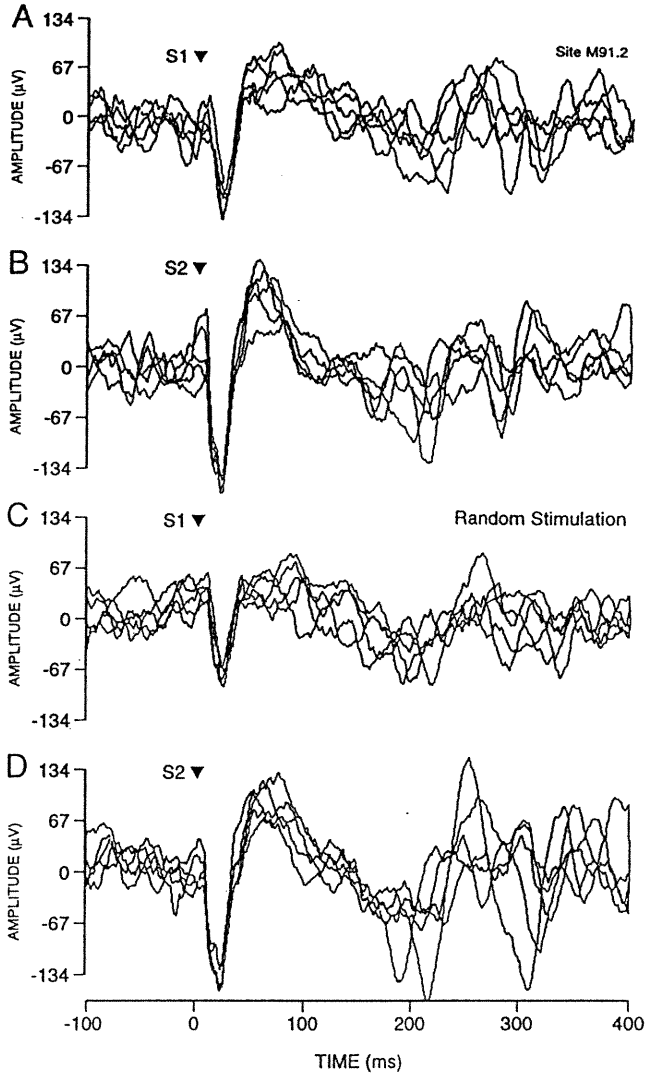

FIG. 3. Explicitly unpaired control procedure. After repeated, unpaired whisker deflections (▼), the responses to stimulation of S1 recorded in the barrel for which S2 is the principal whisker, decreased by 1.5%. However, after adjustment for the 10.1% increase seen in the responses to S2, responses to stimulation of S1 relative to the changes in those of S2, decreased by 11.6% (see also Fig. 4*B*). Five traces are shown for each experimental condition.

approach was based on evidence in the literature suggesting that this conditioning paradigm changes the relationship between responses to S1 and S2. As well, these relative measures minimized any generalized change in excitability that affected all areas of somatosensory cortex.

The effects of saline or drug adminstration without pairing were evaluated by comparing the amplitude of the first component of the evoked potentials measured 30 min after the drug injection with the first components collected before the injection with *t*-test and Mann-Whitney rank-sum test ($P < 0.05$).

Using a cluster analysis, recordings having markedly different evoked potential profiles, when compared with the overall sample, also were excluded. These criteria, albeit stringent, excluding ~50% of the potentials, prevented the analysis of waveforms contaminated by artifacts and spontaneous fluctuations of the EEG

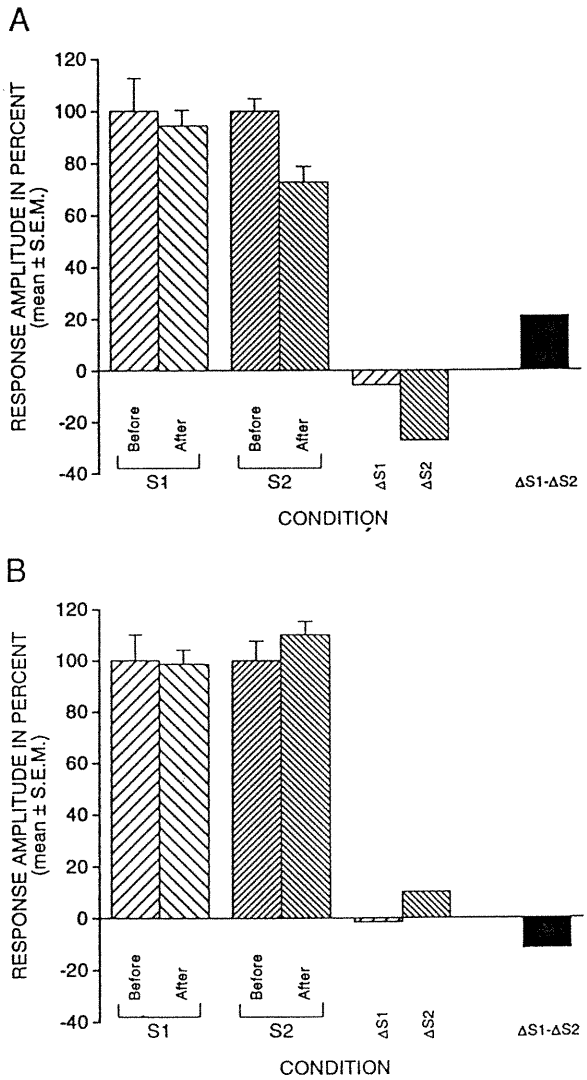

FIG. 4. Normalized amplitude of responses to stimulation of S1 and S2 before and after conditioning when a 5-ms deflection of S1 was followed within 150 ms by a 5-ms deflection of S2 for 50 trials (*A*) and when the control procedure was administered where the 5-ms deflections of S1 and S2 were not related (*B*). In both panels, the values in the 3rd column represent the difference between the responses before and after stimulation of the whiskers. Most informative measure, however, was the net difference between these changes in response to S1 and to S2 (in this study, we always subtracted the changes in ΔS2 from the changes in ΔS1 (*4th column*). *A*: Site M88.3. *B*: Site M91.2.

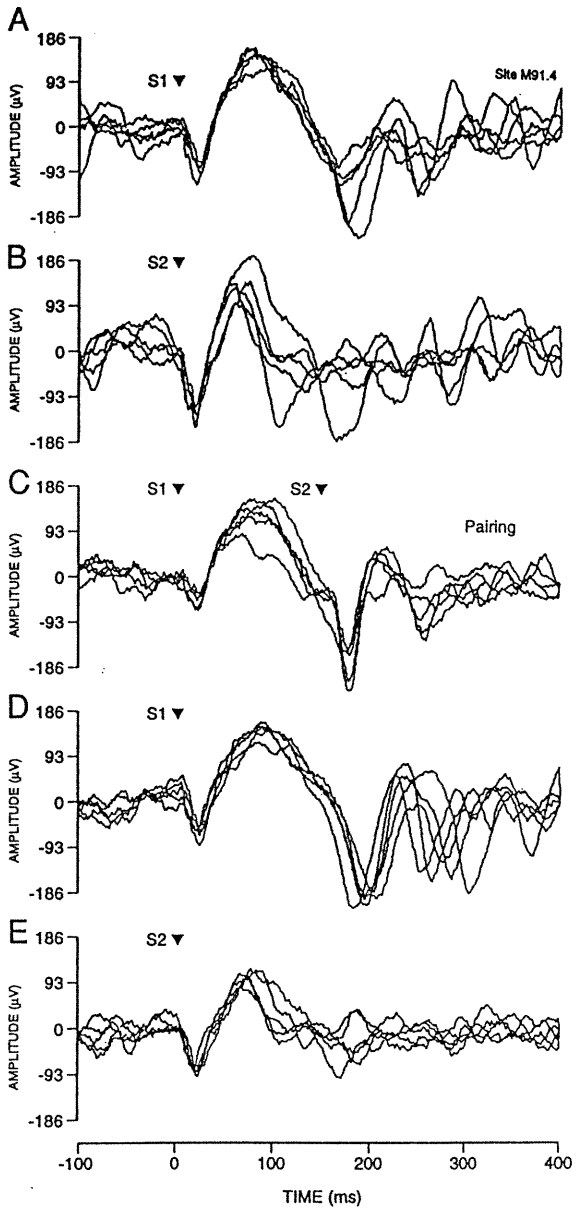

FIG. 5. Effects of pairing after the intraperitoneal injection of saline. These responses were recorded from a barrel for which S2 is the principal whisker. Responses to stimulation of S1 decreased by 7.7% and responses to stimulation of S2 decreased by 21.4%. Adjusted difference represents a 13.7% increase (see also Fig. 7*A*). Saline was injected 30 min before the 1st recording of responses to S1 shown on the graph. ▼, moments of stimulation; only 5 sample traces are shown for each experimental situation.

(Table 1). The identical approach was used for all treatment groups and both one-way ANOVA ($P = 0.51$) and Kruskal-Wallis ANOVA on ranks ($P = 0.85$) revealed no statistically significant difference in our exclusion procedures among groups.

Histology

At the end of the last experiment, an electric current (10 μA for 15 s) was passed through three or four of the electrodes to mark the recording sites. The animal then was anesthetized deeply with an intraperitoneal dose of pentobarbital sodium. The thoracic cage was opened, and the animal was perfused through the aorta with

500 ml of phosphate-buffered saline (pH 7.3) followed by 500 ml of phosphate-buffered 4–10% solution of paraformaldehyde. The brain was removed from the skull and placed in 0.8 M sucrose for ≥48 h. Frontal sections were cut with a cryotome at a thickness of 50 μm and mounted on chrom-alum coated slides for staining with cresyl violet.

RESULTS

The results were obtained from 34 experiments performed on nine rats. The number of experiments per animal ranged from one to nine, the average being 3.8 ± 2.3 (mean ± SD; Table 2). The explicitly unpaired control procedure was applied in 6 of the 34 cases. The remaining ones involved whisker pairing either without any injection or after the intraperitoneal administration of saline, AMN or ATS (Table 3).

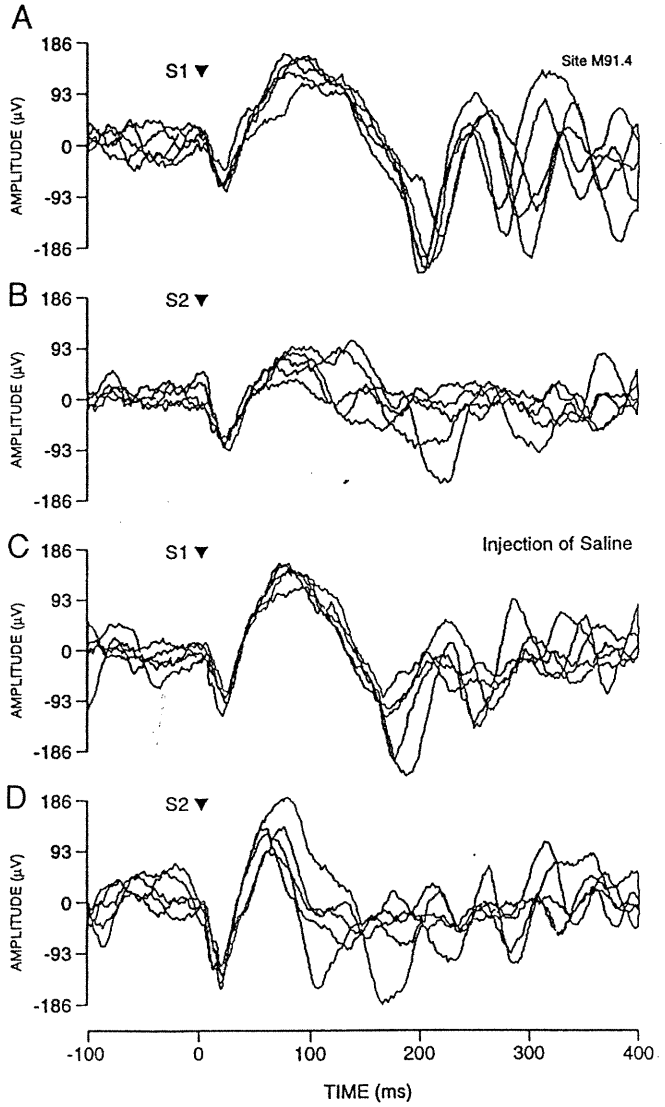

FIG. 6. Effects of saline injection without pairing. Responses to stimulation of S1 recorded from a barrel for which S2 is the principal whisker after intraperitoneal injection of saline increased by 6.8%. The responses to stimulation of S2 increased by 37.3%. The difference was a 30.5% decrease (see also Fig. 7B), without a dramatic change in the shape of the potential. ▼, moments of stimulation; only 5 sample traces are shown for each experimental condition.

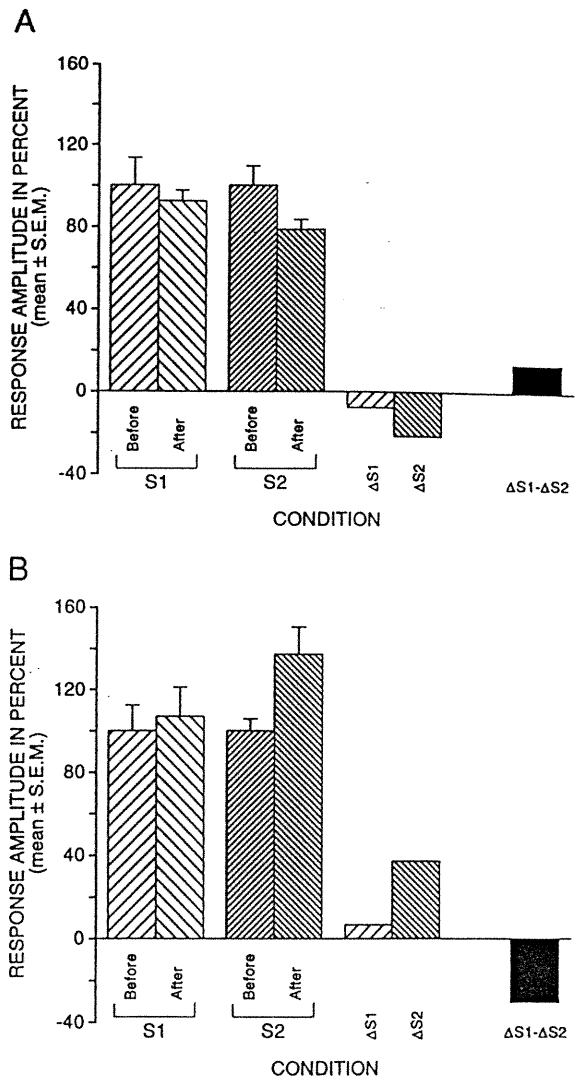

FIG. 7. Saline injection with (*A*) and without (*B*) pairing. *First 2 columns*: average, normalized response to stimulation of S1 and S2 before and after pairing. Difference for S1 (ΔS1) and S2 (ΔS2) were negative, and the net effect (ΔS1 − ΔS2) was +13.7%, whereas without pairing the net effect was −30.5%. Pattern of changes in general reproduces the one, shown in Fig. 4, *A* and *B*, indicating that injection of a neutral agent does not affect the results of pairing. *A* and *B*: Site M91.4.

Three animals were subjected to more than one type of protocol to verify whether the effects of different treatments could be reproduced in the same animal. The first animal was exposed to two sessions of explicitly unpaired whisker stimulation followed by pairing and then a control session. The second animal was subjected to three control sessions followed by three pairing experiments with injections of saline. The third one received nine pairing sessions, the first five and the seventh in the presence of ATS and the remaining ones with the injection of saline. In total, 18 experiments were performed in rats that were subjected to more than one type of treatment. However, a rat was never subjected to more than one treatment per day. The group where pairing was performed after injection of AMN was the only one where rats were only exposed to a single procedure.

Different whisker combinations were used, however, S1 always was rostral to S2. On average, S1 and S2 were separated by 1.5 ± 0.6 columns and 1.0 ± 0.6 rows. In Euclidian coordinates, the distance between them was 1.9 ± 0.6 whiskers.

Effects of whisker pairing

In six experiments, whisker pairing was performed during 50 trials where the stimulation of S1 preceded the stimulation of S2 by 150 ms.

ADJUSTED PERCENTAGES OF CHANGE IN THE RESPONSES TO S1. On average, the responses to S1 and S2 decreased by 5.9 ± 7.4 and 16.1 ± 8.9% (mean ± SE), respectively, after pairing. Figures 2 and 4*A* illustrate the situation where responses to S2 decline more than those to S1 (4 of the 6 cases). In one of the remaining cases, responses to S1 dropped more than S2, whereas in the other, responses to stimulation of S1 and S2 both climbed, however, the increase was larger for responses to S1.

Because the change in the responses to S2 was taken as a reference, it was subtracted from the change in the responses to S1, and the residual change was used to estimate the effect of the experimental manipulation. These differences were averaged across all experiments within a group. The result was positive, suggesting that whisker pairing produced an increase in the amplitude of S1 with respect to S2 (10.3 ± 4.9%). This was also true after injection of either saline (5 experiments showing a 14.5 ± 6.4% average increase; Figs. 5 and 6*A*; Fig. 7 shows responses with and without pairing) or AMN (5 experiments showing a 14.3 ± 6.1% average increase; Table 4 and Figs. 8 and 10*A*; see also Fig. 9). With the *t*-test ($P < 0.05$), the means of the three groups did not differ from each other, but each was significantly higher than the decrease (6 experiments; −7.2 ± 5.0%) observed in the explicitly unpaired controls (Figs. 3 and 4*B*). The results of the Mann-Whitney rank-sum test confirmed this outcome; pairing alone, pairing after saline injections and pairing after AMN injections were significantly different from the explicitly unpaired controls. The results are summarized in Fig. 14 and Tables 4 and 5.

TABLE 3. *Number of experiments performed in each treatment group and the number of rats they were obtained from*

Procedure	Experiments	Rats
Recordings from S2		
Pairing	6	3
Pairing + saline	5	2
Pairing + AMN	5	2
Pairing + ATS	12	3
Control	6	2
Recordings from S1		
Pairing	5	2
Control	4	2
Recordings from barrels serving unstimulated whiskers		
Pairing	5	3
Control	13	2

AMN, atropine methyl nitrate; ATS, atropine sulfate.

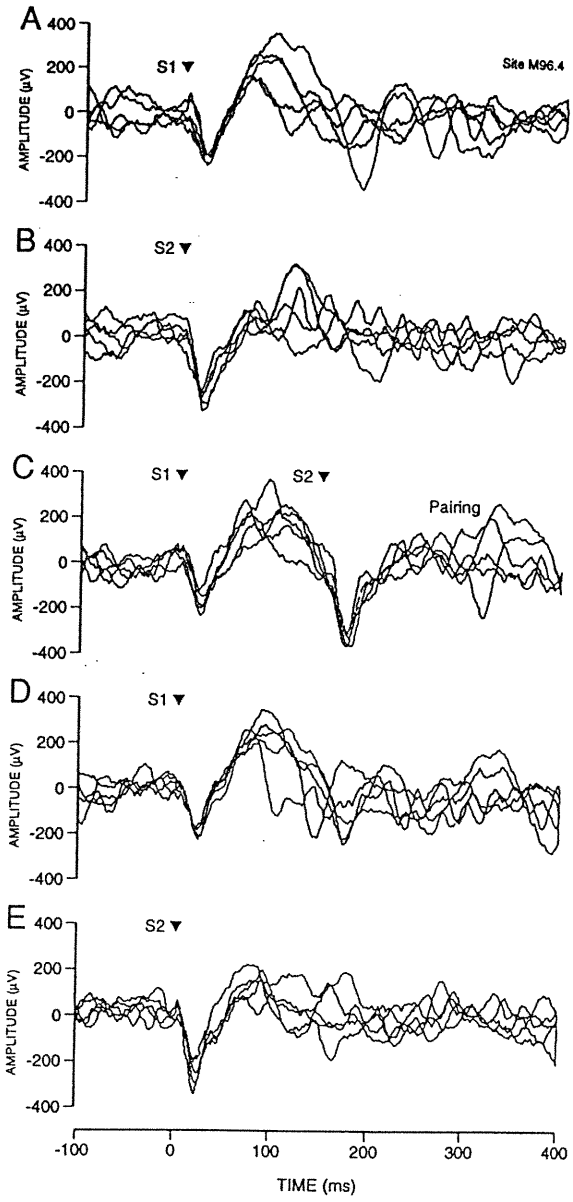

FIG. 8. Responses observed in the barrel for which S2 is the principal whisker before (*A* and *B*) and after (*D* and *F*) pairing (*C*) in the presence of an intraperitoneal injection of AMN 30 min before the pairing. Responses to stimulation of S1 increased by 9.3%, whereas those to S2 decreased by 8.1%, leading to a corrected increase of 17.4% in the responses to stimulation of S1 (see also Fig. 10*A*). ▼, moments of stimulation; 5 traces are shown for each panel.

When available, data collected from barrels neighboring the barrel for which S2 was the principal whisker (i.e., S1 or other, unstimulated whiskers) showed the same tendency seen in barrels related to S2; pairing increased responses to stimulation of S1 relative to S2, whereas control procedure provoked their decrease (Table 6). However, these differences were smaller and were statistically significant only when the data from all the experiments in the barrels for which S1 was the principal whisker and other unstimulated whiskers were combined.

DIFFERENCE OF RATIOS (ΔR). The ratio of the response to S1 over the response to S2 (R_{S1}):(R_{S2}) increased after

whisker pairing alone (0.14 ± 0.05; mean ± SE) or after the intraperitoneal injection of saline (0.12 ± 0.06) or AMN (0.10 ± 0.04), whereas the ratio decreased after the explicitly unpaired control procedure (−0.05 ± 0.04). The differences between the pairing and control groups were statistically significant with both the *t*-test and Mann-Whitney rank-sum test ($P < 0.05$; Tables 4 and 5).

Applying the preceding analyses to data collected from barrels for which S1 is the principal whisker or other, unstimulated whiskers yielded results similar to those obtained for the barrels for which S2 was the principal whisker. The (R_{S1}):(R_{S2}) ratio increased after pairing and decreased after the explicitly unpaired control (Table 6), however, the pairing and control groups differed significantly from one another only when the data collected from all of these off-focus regions was combined, again suggesting that the effects of pairing were smaller in the barrels for which S2 was not the principal.

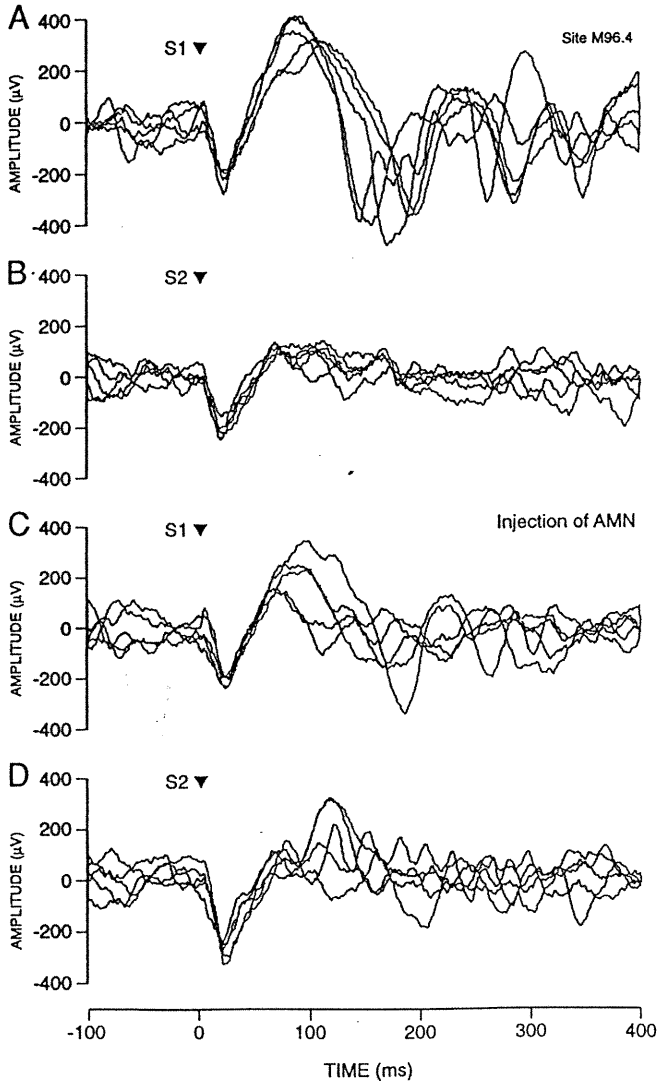

FIG. 9. Responses recorded from a barrel for which S2 is the principal whisker before (*A* and *B*) and after (*C* and *D*) intraperitoneal injection of AMN. Amplitude of the responses to stimulation of S1 decreased by 5.0%, and those evoked by stimulation of S2 increased by 3.3%, resulting in a corrected decrease of 8.3% in the response to S1 (see also Fig. 10*B*). ▼, moments of stimulation; 5 sample traces are shown for each experimental situation.

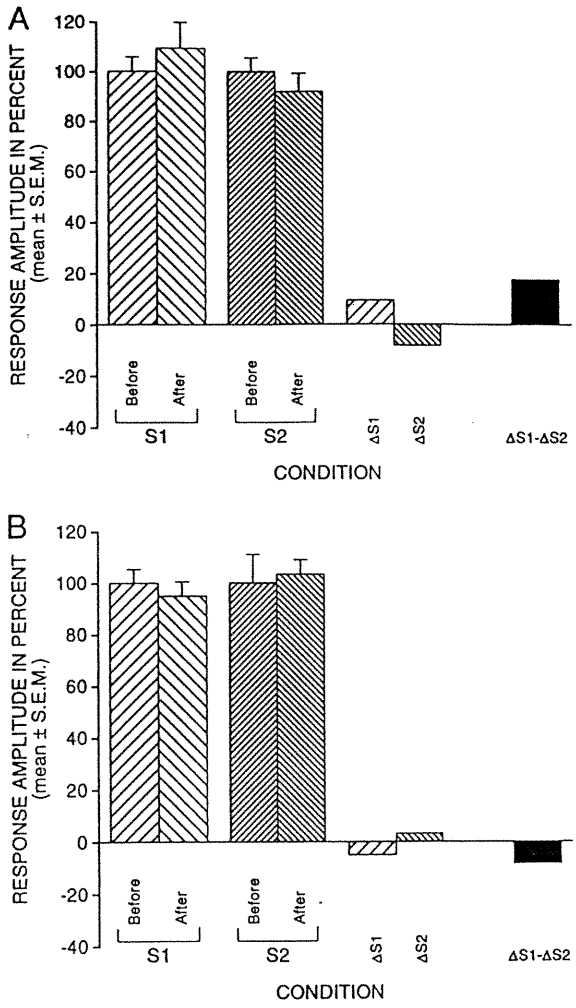

FIG. 10. AMN injection with (*A*) and without (*B*) pairing. *First two columns*: average, normalized response to stimulation of S1 and S2 before and after treatment. With pairing, the difference between the changes in S1 and S2 was +17.4%, whereas without pairing that difference was −8.3%. Net effect was similar to injections of saline with and without pairing (Fig. 7). *A* and *B*: Site M96.4.

Effects of saline, AMN and ATS on the responses to whisker stimulation before pairing

Observations of the waveforms recorded during the experiments suggested that the injected drugs alone affected the amplitude of the evoked potential. This was most evident with ATS. To evaluate these effects, evoked potentials were recorded 30 min after the injection of saline (Figs. 5 and 7*B*), AMN (Figs. 9 and 10*B*) or ATS (Figs. 12 and 13*B*; see also Fig. 11), and the amplitudes of the first component were normalized relative to the baseline established at the beginning of the experiment. In all three groups, the potentials generated by stimulation of both S1 and S2 were greater after the injections but only ATS caused a significant increase. However, the changes attrib-

TABLE 4. *Summary of the results from recordings in the cortex for which S2 is the principal whisker for each experimental condition*

Procedure	Percentage Change			Ratio of S1 Over S2		
	To S1	To S2	To S2 Adjusted for S1	Before	After	Difference
Pairing	−5.9 ± 7.4	−16.1 ± 8.9	10.3 ± 5.0	0.87 ± 0.11	1.01 ± 0.14	0.14 ± 0.05
Pairing + saline	6.3 ± 8.7	−8.2 ± 10.9	14.5 ± 6.4	0.73 ± 0.06	0.85 ± 0.05	0.12 ± 0.06
Pairing + AMN	20.7 ± 12.6	6.4 ± 7.9	14.3 ± 6.1	0.83 ± 0.04	0.93 ± 0.05	0.10 ± 0.04
Pairing + ATS	3.8 ± 8.5	12.9 ± 9.1	−9.1 ± 8.2	0.74 ± 0.06	0.70 ± 0.10	−0.04 ± 0.08
Control	−5.1 ± 8.2	2.1 ± 4.2	−7.2 ± 5.0	0.71 ± 0.05	0.66 ± 0.07	−0.05 ± 0.04

Values are means ± SE.

utable to the treatment were opposite to the changes induced by pairing. For instance, the adjusted responses to stimulation of S1 decreased by 17.4 ± 11.1% 30 min after the intraperitoneal injection of AMN but increased by 14.3 ± 6.1% after pairing. In contrast, ATS enhanced responses to stimulation of S1 by 16.9 ± 13.0%, whereas pairing under the effects of this drug led to a 9.1 ± 8.2% reduction (Table 7; Figs. 7*B*, 10*B*, and 13*B*). Further because the effects of the drugs were similar for S1 and S2, average adjusted changes in the responses to stimulation of S1 did not show any significant effects of the drugs with either the *t*-test or Mann-Whitney rank-sum test.

Effects of ATS on whisker pairing

ADJUSTED PERCENTAGES OF CHANGE IN THE RESPONSES TO S1. Whisker pairing subsequent to ATS administration (12 experiments), produced increases of 3.8 ± 8.5 and 12.9 ± 9.1% in S1 and S2 respectively (Figs. 12 and 13*B*). After adjustment of S1 for changes in S2, S1 decreased by 9.1 ± 8.2%. This change was significantly ($P < 0.05$, *t*-test) different from the increases observed when pairing followed injections of saline or AMN (Table 4). It should be noted that ATS also affected the variability of the evoked potential; the amplitude of the changes in the responses to S1 varied considerably more when ATS was a part of the protocol, ranging from a 60% decrease to a 57% increase, whereas the maximum deviation was <35% for all the other groups (Fig. 14).

DIFFERENCES OF RATIOS (ΔR). Whisker pairing after the injection of ATS led to a 0.04 ± 0.08 (mean ± SE) decrease in the $(R_{S1}):(R_{S2})$ ratio. This differed significantly from the ratios obtained for the pairing alone and for pairing after injection of AMN (Mann-Whitney rank-sum test; Tables 4 and 5). Winsorization with 1 degree of freedom (Winer 1971) was subsequently applied to the data from pairing after ATS on the hypothesis that the *t*-test was not significant because of the influence of outliers (see Fig. 14). This correction produced a significant difference based on *t*-tests and Mann-Whitney rank-sum tests for all comparisons of the ATS group with the other pairing groups.

State of the animal

The state of vigilance of the animals was evaluated through continuous monitoring of the EEG, EMG, and EOG. Preliminary inspection of the EEG and EMG confirmed our observation that the animals did not sleep during an experiment. As a consequence, the EOG, an indicator of REM sleep, was not analyzed further. In each experiment, cluster analysis of power spectra from the EEG and EMG signals obtained during stimulus delivery was used to search for changes in the level of wakefulness that might have influenced our results at different phases of an experiment.

Using Euclidian distances between pairs of power spectra

TABLE 5. *Statistical evaluation of the significant differences between the various experimental conditions*

Procedure	Pairing + Saline		Pairing + AMN		Pairing + ATS		Control	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Pairing	0.52	0.61	0.52	0.61	1.91	0.07	2.49	0.03*
	—	—	—	—	—	0.03*	—	0.02*
	0.27	0.80	0.57	0.58	1.82	0.09	3.10	0.01*
Pairing + saline			0.02	0.98	2.16	0.04*	2.69	0.03*
			—	—	—	0.06	—	0.03*
			0.23	0.83	1.63	0.12	2.49	0.04*
Pairing + AMN					2.18	0.04*	2.75	0.02*
					—	0.04*	—	0.02*
					1.51	0.15	3.15	0.01*
Pairing + ATS							0.14	0.89
							—	—
							0.16	0.88

Evaluation applied to the adjusted percentages of change in the responses to S1 (*t*-test, 1st line; Mann-Whitney rank-sum test where available, 2nd) and to the difference of $(R_{S1}):(R_{S2})$ ratios (*t*-test, 3rd line) in the barrels for which S2 is the principal whisker. * $P < 0.05$.

TABLE 6. *Summary of the results for the pairing and control procedures in the barrels for which S1 or none of the whiskers stimulated during an experiment were the principal whiskers*

Procedure	Number of experiments	Percentages of Change: Percentage of change in the responses to S1	Percentages of Change: Percentage of change in the responses to S2	Ratio of S1 over S2: Adjusted change in the responses to S1	Ratio of S1 over S2: Before	Ratio of S1 over S2: After	Ratio of S1 over S2: Difference of ratios
Barrels serving S1							
Pairing	5	−5.1 ± 7.6	−13.7 ± 13.1	8.6 ± 6.9	1.17 ± 0.08	1.31 ± 0.05	0.14 ± 0.10
Control	4	−12.7 ± 5.3	−0.5 ± 1.5	−12.2 ± 6.1	0.87 ± 0.03	0.77 ± 0.05	−0.11 ± 0.06
Barrels serving unstimulated whiskers							
Pairing	5	34.0 ± 27.2	1.9 ± 10.3	32.0 ± 17.7	0.77 ± 0.20	0.87 ± 0.18	0.10 ± 0.16
Control	13	1.3 ± 5.3	9.3 ± 4.7	−7.9 ± 6.0	0.87 ± 0.23	0.80 ± 0.20	−0.07 ± 0.21

Values are means ± SE.

plotted in 100-dimensional space, >90% of the trials were shown to have similar frequency distributions; <10% differed by 1 or 2 orders of magnitude. When the distances were ordered and plotted on a semilogarithmic scale of distance versus rank-order, they formed a smooth, gradually increasing progression until near the end when there was a sharp increase to much larger values. The minority of trials during the experiment that contributed to these larger values were interpreted as cases where the animal had been in a different behavioral state.

Figure 15 shows the analysis from one experiment. The distances on the *left* are small relative to one another suggesting that they arose from cases where the power spectra were nearly identical. In the *long midportion* of the graph, the distances were approximately of the same length and thus formed a group having similar characteristics. By contrast, the rapidly rising arm on the right side of the figure represents distances that are 1–2 orders of magnitude larger than the majority. Based on this analysis, we chose to exclude all trials where the power spectra differed from the majority by a factor of ≥10. Because we know that during the majority of the data collection period the animals were alert but resting, the minority of cases when the animal was in some other state (perhaps excessively active) should not seriously alter our conclusions, and exclude the possibility that our results could be attributable to an effect of changes in behavioral state.

Histology

Recording sites ($n = 61$) were identified in frontal sections of eight animals. These were located in or near layer IV; 34% were actually in layer IV and almost equal numbers were obtained from layers III (23%) and V (20%). Fewer data were obtained from layers II (11%) and VI (12%). This laminar distribution is close to the one reported by Maalouf et al. (1998) with single-unit recordings, suggesting that our data are derived from a similar neuronal populations at similar depths to the neurons studied by single-unit methods.

DISCUSSION

Our experimental procedure offers several advantages: *1*) whisker pairing, a behaviorally relevant sensory conditioning paradigm (see Carvell and Simmons 1996; Hutson and Masterton 1986; Kossut 1992), is combined with electrophysiological recordings, histological identification of the laminar distribution of recording sites and the use of pharmacological agents that allowed us to distinguish the effects of a muscarinic antagonist on the CNS from those on the peripheral nervous system; *2*) evoked potentials reflect a summation of membrane currents from a large population of neurons many being below the threshold necessary to generate action potentials and therefore providing information that cannot be not be revealed by recordings of action potentials (see Martin 1991); *3*) the behavioral state of the animal, as revealed by the EEG, EMG, and EOG, can be correlated with the probability of emergence of plasticity (see Cruikshank and Weinberger 1996).

The data described in this paper confirm with a different method, the conclusion of previous single-unit studies by Maalouf et al. (1998) that whisker pairing increases neuronal responsiveness to stimulation of S1 relative to S2 in the barrel for which S2 is the principal whisker. In addition, we report that similar changes in nearby barrels were smaller, implying a localization of the plastic changes to the cortex for which S2 is the principal whisker, and that the cortical plasticity emerging after whisker pairing depends on the action of ACh on muscarinic receptors in the CNS.

Cortical responses to whisker stimulation

The deflection of a single whisker provokes a neuronal response in the barrel cortex of awake or lightly anaesthetized rats consisting of three phases: an initial, prompt excitation, followed by inhibition, and then a longer lasting excitation (Ebner and Armstrong-James 1990). In more deeply anesthetized animals, the third component does not appear, and the response is frequently limited to a single excitatory phase, although the inhibitory phase has been recorded intracellularly (Carvell and Simons 1988). Similar patterns were observed recently with imaging techniques that rely on voltage-sensitive dyes (Kleinfeld and Delaney 1996).

The origins of the late components (≤500 ms) of the evoked potential remain relatively unknown. Several proposed mechanisms hypothesize a role for the multiple reciprocal connections that sensory areas form with various corti-

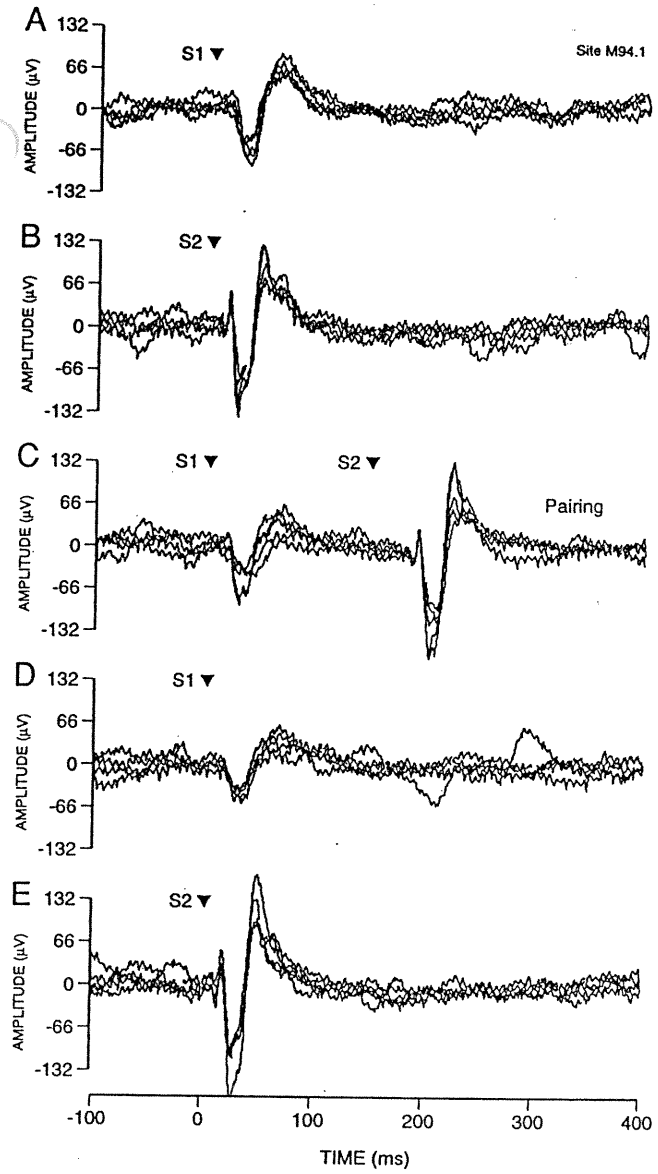

FIG. 11. Effects of pairing after the intraperitoneal injection of ATS. ATS was applied 30 min before pairing of S1 and S2. Waveforms of potentials evoked by S1 and S2 before are shown in *A* and *B*, respectively. Two whiskers were paired 50 times (*C*). After pairing, the amplitude of the responses evoked by S1 decreased by 6.5% and the responses to S2 increased by 10.4% (*D* and *E*, respectively). Average, corrected response (by substracting changes in S2 from those in S1) was a 16.9% decrease (see also Fig. 13*A*). ▼, moments of stimulation; 5 sample traces are shown for each panel.

cal and subcortical regions such as the prefrontal cortex (Desmedt and Tomberg 1995), the hippocampal formation (Eichenbaum et al. 1996; Fair 1992), and the thalamus (Paré and Llinas 1995). These re-entrant circuits (Edelman 1993) might extend the persistence of cortical representations of sensory stimuli and could therefore underlie some form of working memory, defined by Baddeley (1995) as a temporary information storage system necessary for the performance of cognitive tasks such as learning. The 150-ms interval between S1 and S2 allowed sufficient time to engage such circuits in this experiment.

Effects of whisker pairing on cortical responses to whisker stimulation

Our recordings in the cortex for which S2 is the principal whisker clearly show that whisker pairing, either alone or after systemic injection of saline or AMN, enhanced the difference in responses to stimulation of S1 relative to S2 whereas the explicitly unpaired control procedure decreased it. After pairing (Fig. 4), reactivity to the second signal in the pair declined (see also Diamond and Weinberger 1989; Lennartz and Weinberger 1992; Rescorla 1988; Simons 1983, 1985). This change can be interpreted as a neural representation of the fact that the second stimulus would follow the first one; the sensitivity to the second (harmless) stimulus was adjusted accordingly. On the other hand, when the first signal did not predict the appearance of the second

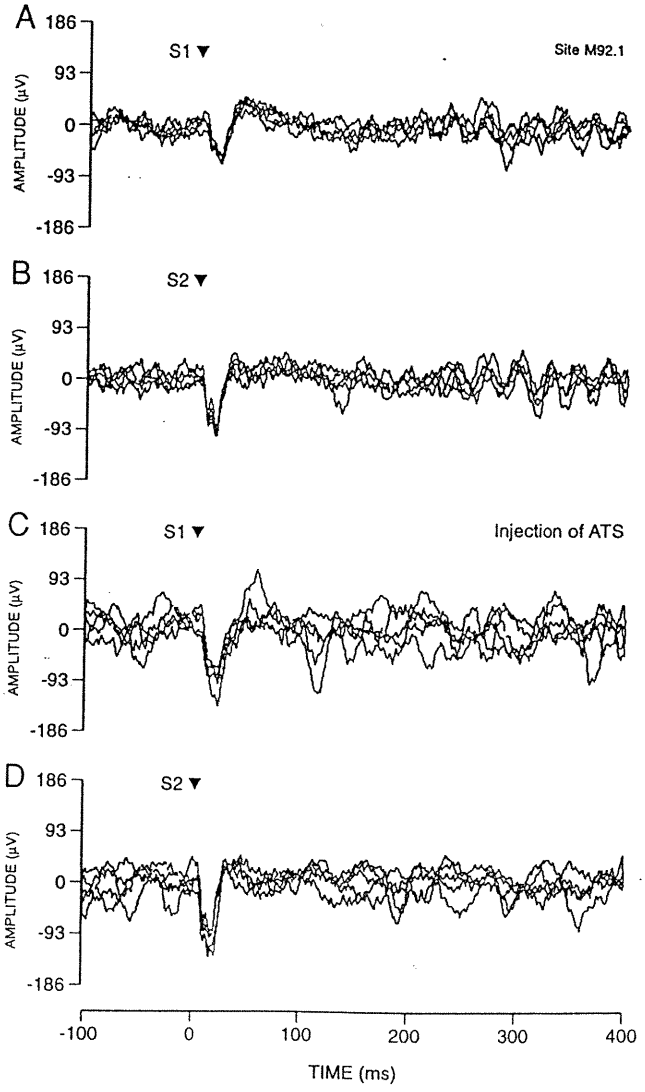

FIG. 12. Changes in the responses recorded from a barrel for which S2 is the principal whisker before (*A* and *B*) and after (*C* and *D*) intraperitoneal injection of ATS. Amplitude of the 1st component of the potentials evoked by stimulation of S1 and S2 increased by 18.6 and 25.0%, respectively. Corrected response to stimulation of S1 was a 6.4% decrease (see also Fig. 13*B*). ▼, moments of stimulation; 5 sample traces are shown for each panel.

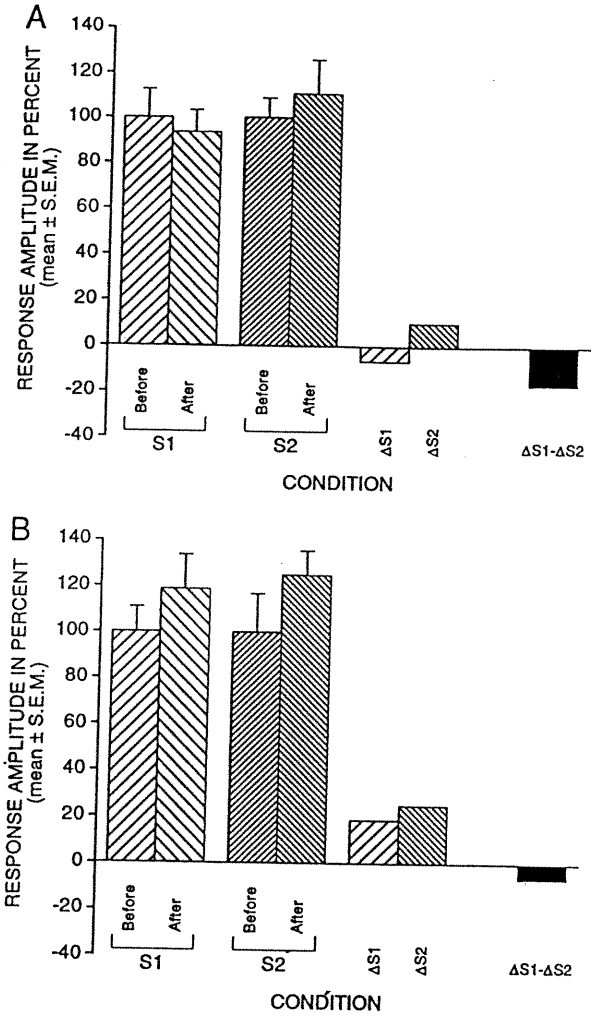

FIG. 13. ATS injection with (*A*) and without (*B*) pairing. *First two columns*: average normalized response to stimulation of S1 and S2 before and after treatment. Net difference after pairing was −16.9%, whereas without pairing that difference was −6.4%. Pattern of changes in responses to both whiskers during pairing (*top*) in general reproduces the one shown in Fig. 4*B* when the stimuli were applied randomly. *A* and *B*: Site M94.1.

one as in the explicitly unpaired procedure, the cortical sensitivity to S2 changed in opposite direction.

That the injection of saline was followed by an even more pronounced growth in responses to stimulation of S2 in the same direction as that produced by the explicitly unpaired control suggests that the change in these two conditions could be related to the degree of arousal caused by the procedure itself.

These results confirm previous findings by Maalouf et al. (1998), who recorded single-unit activity in the barrel cortex of awake rats and found that responses to stimulation of S1, when averaged across several neurons, were enhanced by whisker pairing and were reduced after unpaired presentations of stimuli to S1 and S2. Siucinska and Kossut (1996) reached similar conclusions about classical conditioning in barrel cortex in the barrel cortex of mice from a study involving desoxyglucose; classical conditioning caused an enlargement of the metabolically active region serving as the conditioned stimulus (CS). Bakin and Weinberger (1990) described similar changes in neuronal responses from the auditory cortex of guinea pigs undergoing classical conditioning with a tone serving as a CS.

Maalouf et al. (1998) suggested that the plasticity induced in barrel cortex by whisker pairing obeys the covariance rule originally described by Hebb (1949). The covariance rule can be divided into two parts: when presynaptic activation is synchronized repeatedly with increased levels of postsynaptic activation, the synapses linking the two elements are potentiated and when there is no correlation between pre- and postsynaptic activities, synaptic efficacy is reduced (see Cruikshank and Weinberger 1996). The results described in this study consolidate the hypothesis of Maalouf et al. (1998). During pairing, the interstimulus interval was 150 ms so the stimulus to S2 was presented when the activity of the recorded neurons was still clearly enhanced by the previous stimulation of S1 (Fig. 2*C*). This was not the case in the explicitly unpaired controls.

The conditions set by the rules of covariance were met in several regions of barrel cortex during pairing since deflections of S1 and S2 both activated neurons in several barrels. Responses to stimulation of S1 were enhanced significantly by pairing relative to the control procedure in the barrel serving S2 (see RESULTS and Table 6). In surrounding areas, only a pooled sample from several vibrissae allowed us to detect a significant change. We attribute this weaker effect in the surround to the fact that postsynaptic activity provoked by stimulation of S2 is significantly higher in the barrel corresponding to S2 and is more consistent there than in the surrounding ones.

Effects of muscarinic antagonism on whisker pairing

The increase in cortical responsiveness to whisker stimulation induced by the pairing procedure was prevented by

TABLE 7. *Effects of saline, AMN and ATS on the responses to whisker stimulation recorded from the barrels for which S2 is the principal whisker*

		Percentages of Change		Ratio of S1 over S2			
Procedure	Number of experiments	Percentage of change in the responses to S1	Percentage of change in the responses to S2	Adjusted change in the responses to S1	Before	After	Difference of ratios
Saline	5	11.2 ± 6.3	22.7 ± 9.8	−11.6 ± 10.9	0.78 ± 0.05	0.73 ± 0.06	−0.05 ± 0.08
AMN	5	7.8 ± 16.5	25.2 ± 17.8	−17.4 ± 11.1	0.94 ± 0.03	0.83 ± 0.04	−0.11 ± 0.07
ATS	12	68.1 ± 21.8	51.2 ± 16.0	16.9 ± 13.1	0.66 ± 0.04	0.74 ± 0.06	0.08 ± 0.06

Values are means ± SE.

the muscarinic antagonist ATS but not AMN, an analogue that does not cross the blood-brain barrier. In spite of the greater variability in the responses observed after ATS injection, it was evident that the cortex no longer adjusted to the predictive value of S1 followed by S2. When the blocker of muscarinic cholinoreceptors could not cross the blood-brain barrier, the responsiveness to the second (harmless) stimulus was still capable of being adjusted, offering support of the idea that ACh plays a role in cortical neuronal plasticity (Dykes 1997).

In the absence of pairing, the effects of ATS on responses to stimulation of S1 relative to S2 were not significantly different from those of a control condition (saline injection) and from AMN. However, because these changes in the opposite direction were significant, it suggests that cholinergic mechanisms are not the only mechanism of plasticity in sensory cortex. Based on these data, we suggest that ACh is necessary for the plastic changes induced by associative learning at the cortical level and that this function is independent from its influence on normal synaptic transmission. However, even though these results confirm a conclusion derived from the previous work on the importance of ACh for learning (see INTRODUCTION), they do not solve the controversy of whether ACh affects learning and memory directly or through an increase in attention that might have been provoked by the paired stimuli. This question has been raised by several studies that showed that enhanced cortical cholinergic release was correlated with sensory stimulation or increased attentional processing (Inglis and Fibiger 1995; Sarter and Bruno 1997) as well as with learning (Butt et al. 1997).

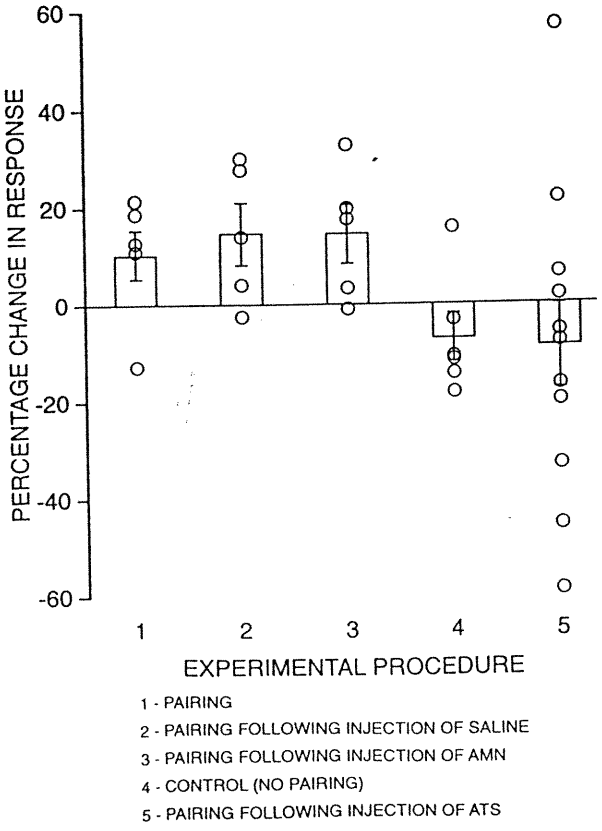

FIG. 14. Summary of the responses to stimulation of S1 recorded in the barrels for which S2 is the principal whisker for each experimental condition. O, net value of change in response from each experiment. Bars show the group mean and SE. Note the much greater variability in the amplitude of the evoked potentials when the animal had received ATS.

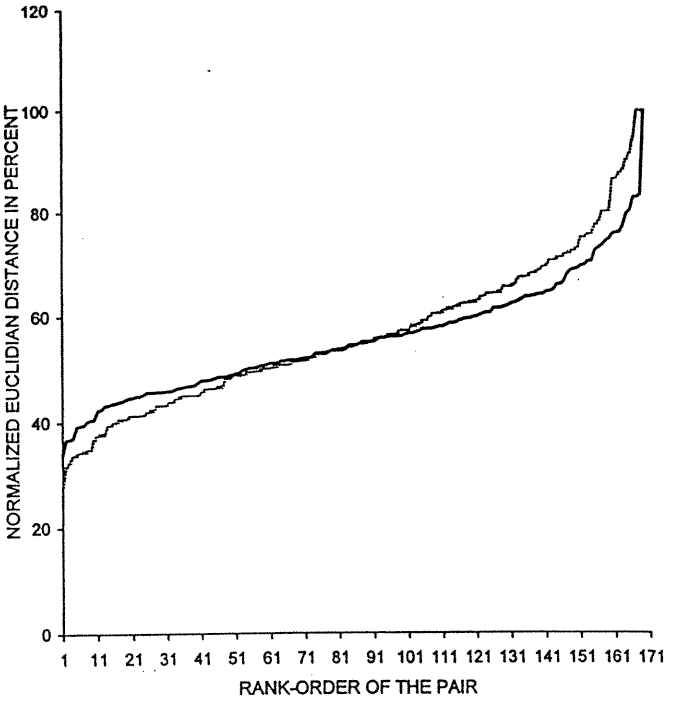

FIG. 15. Rank-ordered Euclidian distances between 170 pairs of spectral histograms in 100-dimensional (each histogram consisted of a 100 bins) space, expressed on a logaritmic scale (*y* axis) and normalized by the maximum value observed. ——, rank-ordered distances for the power spectra of the electroencephalogram (EEG); – – –, respective measures for electromyogram (EMG). Both curves form a smooth, gradually increasing progression until near the end when there was a sharp increase toward much larger values. Smaller distances (*left*) were interpreted as arising from the cases where the power spectra and, therefore, the levels of EEG and EMG activity of the animal were nearly identical, while the sharply greater distances (*right*) indicated that the animal was in a different behavioral state than the majority of the sample. *x* axis represents the rank-order of the measured distances, a measure that does not relate to the actual position of the trial during the recording session. Site M92.5.

Our data are not incompatible with any of these views, and we propose that ACh is involved in both attentional processing of sensory stimuli and in the mechanisms of plasticity underlying learning (see Dykes 1991, 1997). First of all, ACh has been shown, on the one hand, to take part in the generation of sensory-evoked potentials, particularly the late components (Meador 1995; Sannita 1995) and, on the other hand, to be necessary for working memory in the prefrontal cortex (Granon and Poucet 1995) and the hippocampal formation (Myers et al. 1996). Hasselmo and his colleague (Hasselmo 1995; Hasselmo and Bower 1993) have suggested that ACh shifts hippocampal circuits toward a state that is appropriate for acquiring new inputs and prevents stored information from interfering with learning by selectively depressing transmission in intrinsic fibers while not affecting responses evoked by afferent fiber stimulation. Interestingly, Inglis and Fibiger (1995) found that ACh re-

lease in the prefrontal cortex and the hippocampus is increased during sensory stimulation, and Butt et al. (1997) showed a further increase during tactile learning.

ACh most probably accomplishes these functions through its interactions with postsynaptic muscarinic receptors. It can decrease neuronal adaptation and lengthen the duration of action potentials in pyramidal cells by decreasing several potassium (K^+) outward currents, including the voltage-dependent noninactivating I_M, the fast-inactivating I_A, and the calcium (Ca^{2+})-activated K^+ current responsible for slow afterhyperpolarization (Bernardo and Prince 1982; Madison et al. 1987; McCormick 1993). Moreover, it activates a voltage-dependent cation nonselective inward current (Haj-Dahmane and Andrade 1996) and has been shown to reduce inhibition by enhancing K^+ outward currents in GABAergic interneurons (see McCormick 1993). In contrast on the presynaptic side, ACh decreases the amplitude of synaptic potentials, presumably by activating inhibitory receptors on glutamatergic presynaptic terminals (Hasselmo 1995; Hasselmo and Barkai 1995; Hasselmo and Bower 1993). This would explain why cortical responses to whisker deflection are enhanced in the presence of atropine, whereas those evoked by stimulation of the hindpaw a few milliseconds after electrical stimulation of the basal forebrain are reduced (Verdier et al. 1995).

These same mechanisms that decrease neuronal adaptation and increase action potential duration also can facilitate long-term potentiation (LTP), a form of plasticity believed by many to be activated by learning and to underlie memory storage. Because action potentials last longer under the influence of ACh, the probability and duration of opening of *N*-methyl-D-aspartate (NMDA) channels and probably voltage-dependent Ca^{2+} channels are increased, leading in turn to the entry of larger amounts of Ca^{2+} into the postsynaptic cell (Brocher et al. 1992; Segal 1992). This rise in cytoplasmic Ca^{2+} also may be achieved by muscarinic potentiation of phosphoinositol turnover, resulting in the synthesis of inositol triphosphate, which subsequently enhances the release of Ca^{2+} from intracellular stores (Jones 1993). The rise in intracellular Ca^{2+} concentration ultimately would lead to the activation of the cascade of second-messenger events leading to synaptic plasticity. Consistent with this model are the findings of Verdier et al. (1995), who demonstrated that pairing electrical activation of the basal forebrain with hindpaw stimulation in anesthetized rats leads to a long-term enhancement (over an hour) of cortical responsiveness to the tactile stimulus and that this enhancement requires the activation of NMDA receptors. In addition, the data presented in this paper indicate that the muscarinic antagonist atropine is sufficient to block an NMDA-dependent form of cortical plasticity (Maalouf et al. 1998).

Tenable hypotheses

Thus ACh might facilitate the emergence of plasticity in the CCx at two levels: by enhancement of working memory via extension of a period of time during which external stimuli provoke their neural representation within the cerebral cortex and by directly affecting plasticity through the facilitation of the opening of postsynaptic NMDA channels and increasing the intracellular levels of Ca^{2+} that promote induction of LTP. Because a cholinergic deficit would prevent the facilitation of these two processes, it might be at these levels that the absence of ACh has an important role in production of cognitive impairments that characterize Alzheimer's disease. Treatment directed to correction of cholinergic malfunction, however, should take into account the transient temporal needs for ACh as well as the fact that degeneration also occurs in other neuromodulatory systems involved in learning and memory.

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CHAPTER 3

Evidence for homeostatic adjustments of rat somatosensory cortical neurons to changes in extracellular acetylcholine concentrations produced by iontophoretic administration of acetylcholine and by systemic diisopropylfluorophosphate treatment

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EVIDENCE FOR HOMEOSTATIC ADJUSTMENTS OF RAT SOMATOSENSORY CORTICAL NEURONS TO CHANGES IN EXTRACELLULAR ACETYLCHOLINE CONCENTRATIONS PRODUCED BY IONTOPHORETIC ADMINISTRATION OF ACETYLCHOLINE AND BY SYSTEMIC DIISOPROPYLFLUOROPHOSPHATE TREATMENT

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Abstract—We describe the responses of single units in the awake (24 cells) or urethane-anesthetized (37 cells) rat somatosensory cortex during repeated iontophoretic pulses (1.0 s, 85 nA) of acetylcholine, both before and after systemic treatment with the irreversible acetylcholinesterase inhibitor diisopropylfluorophosphate (i.p., 0.3–0.5 LD_{50}). The time-course of the response to acetylcholine pulses differed among cortical neurons but was characteristic for a given cell. Different time-courses included monophasic excitatory or inhibitory responses, biphasic (excitatory–inhibitory, inhibitory–excitatory, excitatory–excitatory, and inhibitory–inhibitory), and triphasic (excitatory–excitatory–inhibitory, inhibitory–inhibitory–excitatory, and inhibitory–excitatory–inhibitory) responses. Although the sign and time-course of the individual responses remained consistent, their magnitude fluctuated across time; most cells exhibited either an initial increase or decrease in response magnitude followed by oscillations in magnitude that diminished with time, gradually approaching the original size. The time-course of the characteristic response to an acetylcholine pulse appeared to determine direction and rate of change in response magnitude with successive pulses of acetylcholine. Diisopropylfluorophosphate treatment, given 1 h after beginning repeated acetylcholine pulses, often resulted in a gradual increase in spontaneous activity to a slightly higher but stable level. Superimposed on this change in background activity, the oscillations in the response amplitude reappeared and then subsided in a pattern similar to the decay seen prior to diisopropylfluorophosphate treatment.

Our results suggest that dynamic, homeostatic mechanisms control neuronal excitability by adjusting the balance between excitatory and inhibitory influences within the cortical circuitry and that these mechanisms are engaged by prolonged increases in extracellular acetylcholine levels caused by repeated pulses of acetylcholine and by acetylcholinesterase inhibition. However, this ability of neurons in the cortical neuronal network to rapidly adjust to changes in extracellular levels of acetylcholine questions the potential efficacy of therapeutic treatments designed to increase ambient levels of acetylcholine as a treatment for Alzheimer's disease or to enhance mechanisms of learning and memory. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: oscillatory pattern, Alzheimer's disease, acetylcholinesterase inhibition.

Because Alzheimer's disease (AD) is a progressive, neurodegenerative disorder associated with a pronounced cholinergic deficiency characterized in part by a loss of basal forebrain cholinergic neurons and a corresponding loss of cholinergic transmission in the neocortex,[7,10,61] pharmacological treatment has traditionally focused on restoring brain acetylcholine (ACh) levels. There are three basic strategies: increasing the availability of ACh precursors (e.g., choline or lecithin), administering muscarinic receptor agonists (e.g., arecoline) or providing acetylcholinesterase (AChE) inhibitors (e.g., physostigmine) that prevent the degradation of ACh at the synapse.[14,16,25] Unfortunately, these treatments have shown only modest benefits[3,19] as effective therapies in the treatment of AD;[8] some shortcomings of cholinergic drug treatments include an inadequately short duration of drug action, a narrow therapeutic window of effective doses, and the rapid development of tolerance.[3] The failure to develop adequate drug treatments for AD and related dementias suggests that cholinergic replacement therapies may be incapable of providing significant, long-lasting amelioration of the cognitive impairments

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; DFP, diisopropylfluorophosphate; E, excitatory; EEG, electroencephalographic; EMG, electromyographic; I, inhibitory.

associated with this disease.[19,27] Although cholinergic replacement therapies have met with only limited therapeutic success, the neurobiological mechanisms that are responsible for limiting this success are as yet only poorly understood.

An understanding of the mechanisms governing the response to cholinergic drug treatments depends partly on an understanding of the neuronal response to ACh. Previously, electrophysiological studies have shown that administration of single pulses of ACh on to cortical pyramidal cells induces a slow, excitatory response.[34,38,41,42,48] The excitatory response to ACh in these cells is sometimes preceded by an inhibitory phase[37] attributable to the rapid but transient excitation of nearby GABAergic interneurons by ACh.[48] In the cortex, these effects involve muscarinic receptor (mAChR) activation, a process that suppresses several potassium conductances and activates a voltage-dependent, non-selective current in both hippocampal and cortical pyramidal neurons.[5,22,46,48] These influences of ACh contribute to its neuromodulatory properties, which include its ability to increase the responsiveness of cortical neurons to sensory inputs,[12,39,49,50] and to promote the long-term enhancement of synaptic connections that are active while ACh is present at the synapse.[2,21,23,29–31,40,59]

With few exceptions,[28,32,35,36,47] the effects of long-term or repeated administration of ACh have not been evaluated at the cellular level even though the efficacy of cholinergic drug therapies will ultimately depend upon their long-term influence on neuronal function. Unless the cholinergic enhancement of cellular responsiveness is enduring, it is unlikely that cholinergic drug treatment will provide long-lasting benefits in hypocholinergic syndromes such as AD.

The purpose of the following experiments was to examine the responses of single units in the awake or urethane-anesthetized rat somatosensory cortex during the repeated iontophoretic administration of ACh. Using this approach, we sought to describe the responses of cortical neurons to prolonged pulsatile, local increases in extracellular ACh levels. Additionally, we examined the responses of these same neurons following global changes in the ambient level of extracellular ACh caused by the systemic administration of the irreversible AChE inhibitor, diisopropylfluorophosphate (DFP).

EXPERIMENTAL PROCEDURES

Preparation of animals studied acutely under urethane anesthesia

Male Sprague–Dawley rats ($n = 20$; Charles River, St-Constant, Québec, Canada) weighing 399 ± 52 g were anesthetized with urethane (1.6 g/kg, i.p.; Sigma, St Louis, MO, U.S.A.). Urethane supplements (10% of original dose) were subsequently added to maintain a state where the hindlimb withdrawal reflex was weak but present. After induction of general anesthesia, a local anesthetic (0.3 ml lidocaine hydrochloride 20 mg/ml; Astra, Mississauga, Ontario, Canada) was injected under the scalp and the eyes were covered with baby oil (Johnson and Johnson, Montréal, Québec, Canada). After placing the animal in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, U.S.A.) on a feedback-controlled heating pad, where body temperature was maintained at 37°C, the scalp was incised and the right temporal muscle reflected. A craniotomy was then performed to expose the somatosensory cortex serving the hindlimb (AP = 0.0 mm to −2.0 mm, bregma; ML = 1.0 mm to 3.0 mm, midline).

Preparation of animals studied while awake

Male Sprague–Dawley rats ($n = 5$; Charles River, St-Constant, Québec, Canada) weighing 486 ± 35 g were anesthetized with i.p. injections of 35 mg/kg sodium pentobarbital (Somnotol: 65 mg/ml in an aqueous propylene glycol base with 2% benzyl alcohol; MTC Pharmaceuticals, Cambridge, Ontario, Canada). Sodium pentobarbital levels were supplemented as necessary to maintain areflexia until the end of the surgery. Presurgical treatment was the same as for animals studied under anesthesia except for the additional administration of an i.m. injection of 0.05 ml of the antibiotic enrofloxacin (Baytril: 50 mg/ml in N-butyl alcohol and water; Miles Canada, Etobicoke, Ontario, Canada).

Following incision of the scalp and reflection of both temporal muscles, two silver-wire electrodes, insulated with plastic tubing except for the 0.3 mm diameter balls of silver exposed at their ends, were placed over the cervical muscles to monitor electromyographic (EMG) activity. A subcutaneous catheter consisting of plastic tubing (1 mm o.d.) attached to a stainless steel needle (30 G; Becton Dickinson and Co., Franklin Lakes, NJ, U.S.A.) was then installed subcutaneously for subsequent DFP administration. Finally, six hooks were fashioned from stainless steel needles (23 G; Becton Dickinson and Co., Franklin Lakes, NJ, U.S.A.) bent to form a "J" shape at one end, with the sharp tip removed. These hooks were inserted in the skull through holes drilled on the lateral edges of the cranium. When imbedded in dental acrylic, these formed a solid base for attaching two parallel steel tubes (6 cm long, 1.5 mm external diameter) placed 12 mm apart and secured by two shorter tubes (1.5 cm long) soldered together; this frame served to immobilize the head of the rat in the restraint box. The bone overlying the somatosensory cortex remained exposed and was covered with a layer of bone wax (Ethicon, Peterborough, Ontario, Canada).

The animal was allowed a minimum of 24 h to recover from surgery and was then trained to sit calmly in the recording box with four to eight sessions of progressively increasing length (3–15 min, 30 min, 1 h, and 1 h 30 min). One day prior to recording, the rat was anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and a craniotomy was performed over the hindlimb representation. This opening was blocked with bone wax until the following day. These animals were studied two to four successive days before being given DFP on the day of the terminal experiment that ended with an overdose of anesthetic and perfusion with phosphate-buffered formalin. During the training and the recording sessions all efforts were made to minimize animals suffering and to reduce the number of animals used; all experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Preparation of the electrodes

Following methods similar to those summarized by Godwin,[17] tungsten wires (80 μm diameter) were etched by electrolysis over a length of 2 cm, inserted into glass pipettes (1 mm o.d., 0.5 mm i.d.; A-M Systems, Everett,

WA, U.S.A) and glued in place with 2-methyl-cyanoacrylate. These were pulled in a vertical electrode puller (Model PE-2, Narishige, Tokyo, Japan) to leave approximately 2 μm of exposed metal at the tip. Those having an impedance of 1–4 MΩ at 1 000 Hz were retained. Iontophoresis pipettes containing microfilaments (1.5 mm o.d., 0.86 mm i.d.; A-M Systems, Everett, WA, U.S.A.) were pulled and the tips carefully broken to form an opening of 4–6 μm (o.d.). The pipette was bent 1 cm from the tip to approximately 45° by heating it over a flame of an ethyl alcohol burner and then it was filled with 1 M ACh (pH = 4). A silver or tungsten wire was inserted into the upper end and sealed with low melting-point wax. The electrode and the iontophoresis micropipette were positioned under a microscope to ensure that the tips of the tungsten electrode and the micropipette were separated by 15 μm or less. The assembly was then glued together with nail varnish along the adjoining lengths of the electrode and micropipette except at their tips. Finally, the shafts of each pipette were joined with dental impression compound.

Electrophysiological recordings and microiontophoresis

After opening the dura mater with the tip of a 30-G needle, positioning the electrode over the cortex, and embedding the electrode in a layer of agar covering the surface of the cortex, the electrode was advanced with a microdrive (Model 3301R, World Precision Instruments, Sarasota, FL, U.S.A.) until a well-isolated, spontaneously active neuron was identified. Standard electrophysiological equipment was used to record from single cells. The combined amplification of a preamplifier (Princeton Applied Research, Model PAR 113, Princeton, NJ, U.S.A.) and an amplifier (Tektronix, model TM504, Beaverton, OR, U.S.A.) was × 10,000. A band-pass filter was set to allow recording of signals between 300 Hz and 10 kHz. Action potentials were discriminated from background noise using a window discriminator (Winston Electronics Company, Model ARD-II-A, San Francisco, CA, U.S.A.) before being sent through a laboratory computer interface (Cambridge Electronic Design, model CED 1401 Plus, Cambridge, U.K.) to a personal computer. Digital events signaling the action potentials were collected using commercially available software ("Spike 2", Cambridge Electronic Design (CED), Cambridge, U.K.) that also recorded the electroencephalographic (EEG) and EMG activity from the awake rats. Action potential shapes were monitored on-line by using a digital oscilloscope (Tektronix, Model 2201, Beaverton, OR, U.S.A.) and recorded with the SIGAVG program (CED, Cambridge, U.K.) at the beginning and end of each recording session.

Time of onset and duration of ACh administration were controlled by the software in the Spike 2 programming environment, and ACh delivery was accomplished by a microiontophoresis pump (Medical Systems Corporation, Model BH-2, Greenvale, NY, U.S.A.). An ejection current of 85 nA and a retention current of −2 nA were used. Pulses of ACh (1 s pulse duration) were administered every minute in some animals or every 2 min in others, for an average duration of 61 min.

Diisopropylfluorophosphate treatment

One hour after a stable baseline of neuronal responses was obtained, DFP was injected through the indwelling catheter in the awake animals or subcutaneously in the anesthetized ones. The dosage used was either 0.3 or 0.5 times the LD_{50} (14.5 μmol/kg[58]). Periodic ACh delivery and data collection were continued for two more hours. At the end of the experiment, rats received lethal doses of sodium pentobarbital (80 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and brains were removed. The neocortex was then prepared for assay of cortical AChE following routine procedures (see Ref. 58).

Statistical analyses

The measure of responsiveness used was the area of the curve that exceeded the average level of ongoing activity by two standard deviations. The area was a more reliable response than the peak amplitude because it was an integral of the excitation produced over time rather than a single, instantaneous measure. In these calculations, the beginning and the end of the response to ACh were defined as the points where the smoothed response crossed the level of one standard deviation above the mean (mean + σ) on the smoothed curve. The statistical model being used was a time-series wherein the null hypothesis was that there should be no changes with time.

RESULTS

Overview of the data sample

Data were obtained from 77 cells recorded in 25 rats. Sixteen cells (21%) showed no change in rate of impulse activity during ACh administration, leaving 61 cells (79%) that were responsive to ACh and therefore provided the basis for our subsequent observations. Of these cells, 37 cells were recorded in 20 urethane-anesthetized rats and 24 cells were recorded in five awake, undrugged rats. All cells chosen for study showed spontaneous activity. The majority of cells were found in the deeper cortical layers, at an average depth of 1.27 ± 0.38 mm below the surface of the cortex; stable recordings were reliably obtained from these deeper cells, which were readily isolated from background activity. The average duration of the electrophysiological recording in cells responsive to ACh was 70 ± 66 min. Their average rate of spontaneous activity was 14.2 ± 10.9 imp./s, but this rate varied considerably (0.40–50.6 imp./s) prior to ACh administration; the majority of cells studied had relatively low activity, with a few showing much higher rates. Figure 1 shows the recording site for a representative cell, the relative consistency of recording quality across the course of the experiment, an autocorrelogram, and an interval histogram of the data from the same cell.

Characteristic responses to iontophoretic administration of acetylcholine

As Fig. 2 illustrates, cells typically showed a readily recognizable response to the iontophoretic administration of a single pulse of ACh; these responses were reflected both in the raster displays of action potentials and in the peristimulus histograms derived from those raster displays. Combining the neuronal response to several ACh pulses resulted in peristimulus histograms (50 ms/bin) that suggested a relatively consistent or characteristic response to ACh; nevertheless, these histograms showed considerable variability from bin to bin (Fig. 3). The characteristic response to ACh

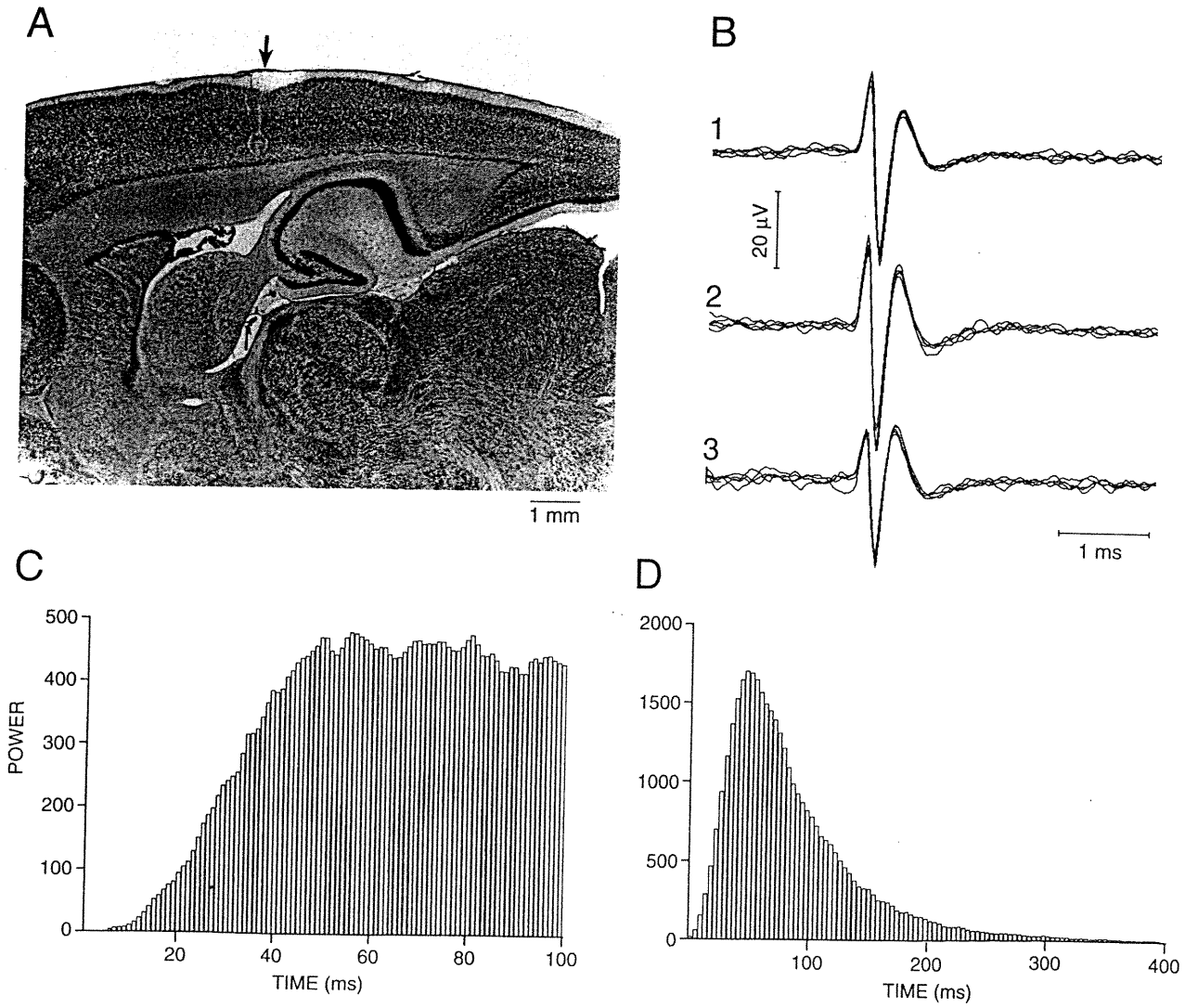

Fig. 1. Representative data from a single-unit recording in the somatosensory cortex. (A) Photomicrograph of a section (40 μm) stained with Cresyl Violet showing a typical electrode track and recording site (arrow) marked by an electrolytic lesion (10 μA for 15 s, anodal current) in layer VI of the cortex. (B) The waveform of an action potential in the beginning of the experiment (B1), 1 h later, immediately prior to DFP injection (B2), and 1 h after DFP administration (B3). These waveforms illustrate the stability of the recording across time and the clear separation of the action potential from the baseline. (C) Autocorrelogram derived from the spike train for the same cell shown in B illustrating an absence of extremely short intervals, suggesting that the recording was free of any artifacts produced by noise or by the inclusion of signals from any additional cells. (D) Smoothed interval histogram showing a unimodal peak at 46 ms and illustrating the skew of the spontaneous activity in the same cell. These two histograms were smoothed by a weighted, moving average calculated using the filter $f_i = (f_{i-1} + 2f_i + f_{i+1})/4$.

could be more readily visualized and interpreted when bin-to-bin variability was reduced by using comparatively larger binwidths (200 ms/bin) to illustrate firing frequency (Fig. 4A), and by calculating a weighted, moving average $[f_i = (f_{i-2} + 2f_{i-1} + 3f_i + 2f_{i+1} + f_{i+2})/9]$ of the data from these larger bins (Fig. 4B). This filter was used for all cells described hereinafter, ensuring comparable treatment of each. These smoothing operations also allowed a more reliable quantification of the excitatory influence of individual pulses of ACh (Fig. 4C).

The nature of the responses to single pulses of ACh varied across cells. Some cells showed either a monophasic excitatory (E) or inhibitory (I) response to ACh. Other cells had biphasic responses involving both E and I components (E–I, I–E, or E–E), or triphasic responses composed of different combinations of the E and I components (E–E–I, I–E–I, or I–I–E). The largest category of the ACh-sensitive cells showed some form of monophasic excitatory response to ACh, usually a rapid excitation that quickly reached a peak before returning to prestimulus levels of activity within 7.4 ± 5.7 s (Fig. 5A). A somewhat less common response was a slow rise in excitation followed by an even slower decay, often lasting more than 1 min before returning to

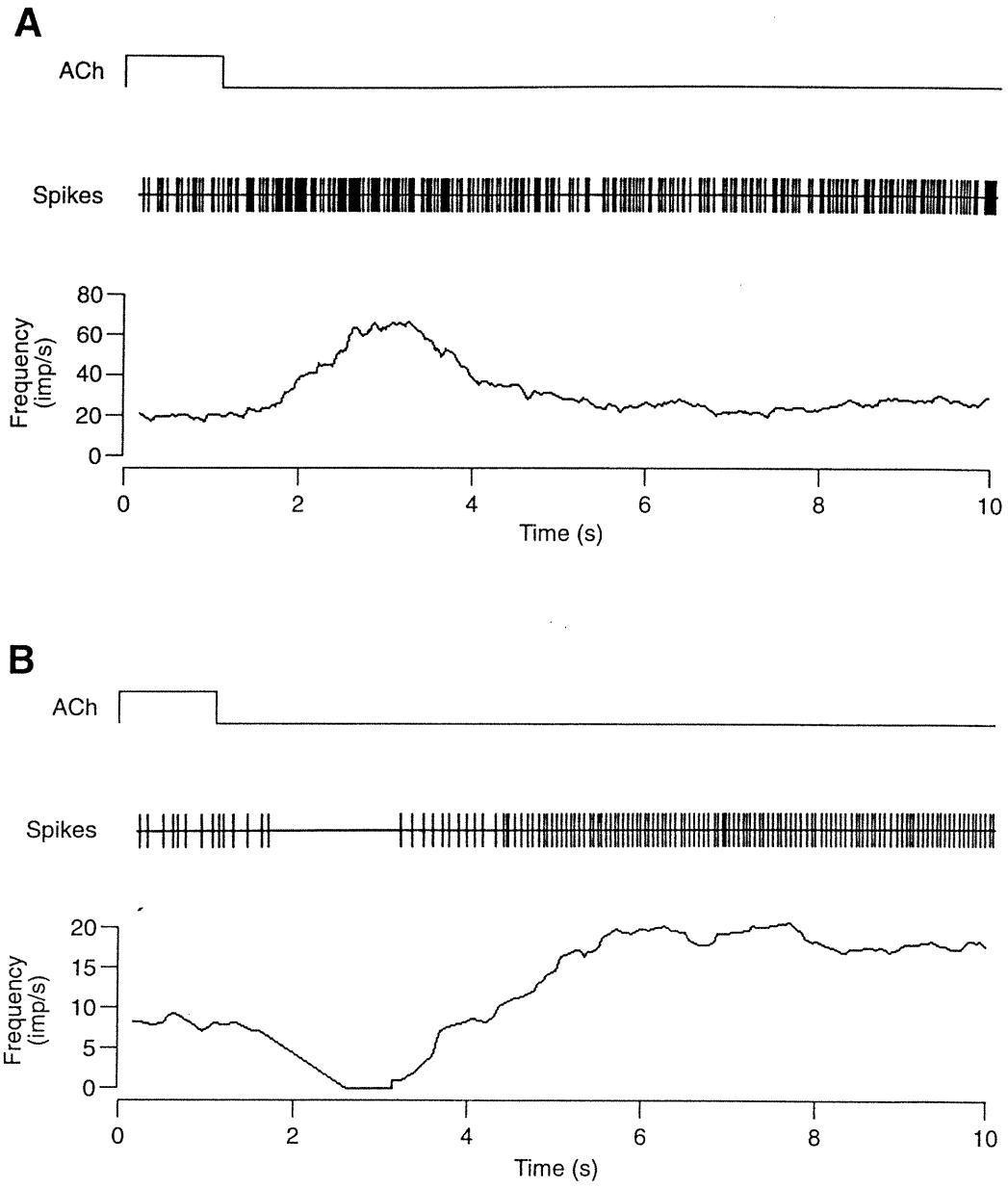

Fig. 2. Spike train and rate meter displays of the temporal relationship between the administration of a single iontophoretic pulse of ACh and neuronal firing in a cell with a monophasic excitatory response (A) and in a cell with a biphasic, inhibitory-excitatory (I–E) response to ACh (B). Both cells were recorded in urethane-anesthetized rats. A 90-nA ejection current and a − 1-nA retention current were used during iontophoresis. The depth of recordings were 1.6 mm and 1.55 mm, respectively. The influence of the iontophoretically applied ACh on neuronal activity is reflected both in the raster displays of action potentials and in the corresponding rate meter record (200 ms/bin). Temporal shifts in the rate meter record, for example in B, where the cell stops firing completely but the record shows a downward slope instead of a frequency of zero, are caused by the use of a 0.5-s integration period to smooth the data and to illustrate the average frequency of neuronal activity across time.

initial levels of activity (Fig. 5B). Two cells showed monophasic inhibitory responses. One was a rapid, initial decline followed quickly by a return to pre-stimulus activity levels (Fig. 5C) and the other was a more slowly developing inhibition followed by a more gradual return to pre-stimulus activity levels (Fig. 5D).

Other cells displayed one of several different forms of biphasic or triphasic responses to iontophoretic pulses of ACh (Fig. 6). One form of biphasic response was a rapid, initial excitation followed by another gradually developing, longer-lasting excitatory phase (Fig. 6A). Another form of biphasic response was a rapid excitatory phase followed by a gradually developing, longer-lasting inhibitory phase (Fig. 6B). A third response type was a rapid inhibitory phase followed by a rapidly developing but longer-lasting excitatory phase (Fig. 6C).

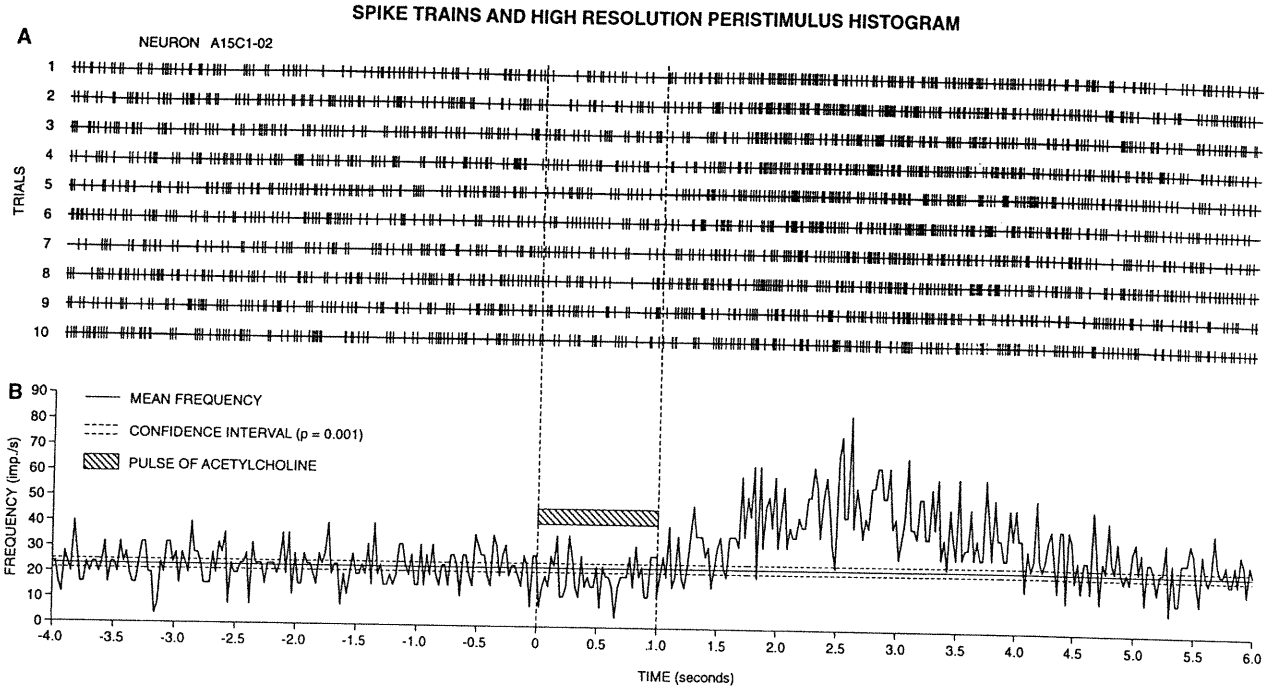

Fig. 3. (A) Raster displays of the individual responses to a series of 10 consecutive pulses of ACh in a representative cell. The onset and offset of the iontophoretic delivery of ACh are illustrated as vertical dotted lines. Note the change in neuronal activity in response to each pulse of ACh. (B) Peristimulus histogram (50 ms/bin) showing the sum of activity produced by the 10 responses to ACh shown in A. Although the number of spikes per bin is highly variable, there is an identifiable decrease and subsequent increase in response frequency following ACh administration.

Though relatively uncommon, some cells showed triphasic or still more complex responses to ACh, where these responses appeared to consist of different combinations of fast and slow excitatory and inhibitory phases (Fig. 6D–F).

Comparison of the response to acetylcholine in awake and urethane-anesthetized rats

The responses to ACh in urethane-anesthetized rats ($n = 37$ cells) and in awake rats ($n = 24$ cells) were compared on several measures. First, the number of cells assigned to the several characteristic response types (e.g., E–I, I–E, etc.) were compared and no significant differences in their distributions were found according to both the Mann–Whitney ($P > 0.25$) and the Kolmogorav–Smirnov ($P > 0.8$) tests (Table 1).

Second, there were no significant differences in the frequency of ongoing impulse activity between cells in the awake group (mean $f = 16.11 \pm 2.82$ imp./s; S.E.M.) and cells in the urethane-anesthetized group (mean $f = 10.84 \pm 1.22$ imp./s; S.E.M.), nor were there differences in the average frequency of impulse activity during the response to ACh in the two groups (mean $f = 21.47 \pm 4.05$ imp./s; S.E.M.; mean $f = 18.97 \pm 2.05$ imp./s; S.E.M., respectively), as assessed using *t*-tests. Third, the maximum frequency reached during excitatory responses did not differ between groups, as determined using the *t*-test.

Although the urethane-anesthetized and awake groups did not differ on the above measures, both the latency and the duration of the excitatory component of the response to ACh were significantly shorter in the awake rats as compared to the urethane-anesthetized rats. The mean latency for cells in the awake rats to respond to the ACh pulse was 1.3 ± 0.2 s; (mean ± S.E.M.), compared to 2.0 ± 0.2 s; (mean ± S.E.M.) in the urethane-anesthetized rats (*t*-test, $P < 0.01$). The mean duration of the excitatory component of the response to ACh for cells in the awake rats was 5.3 ± 0.6 s; (mean ± S.E.M.), compared to 9.2 ± 1.6 s; (mean ± S.E.M.) in the urethane-anesthetized rats (*t*-test; $P < 0.03$). Because these seemed to be relatively minor differences, we combined the data from the two groups for all subsequent analyses.

Detailed analysis of combined data from the urethane-anesthetized and awake rats

Table 2 summarizes certain characteristics of each response category including the number of cells, the depth of recording, the average spontaneous activity, the average firing frequency of the excitatory component of the response to ACh, the duration of that excitatory component, the delay after ACh application until maximum neuronal

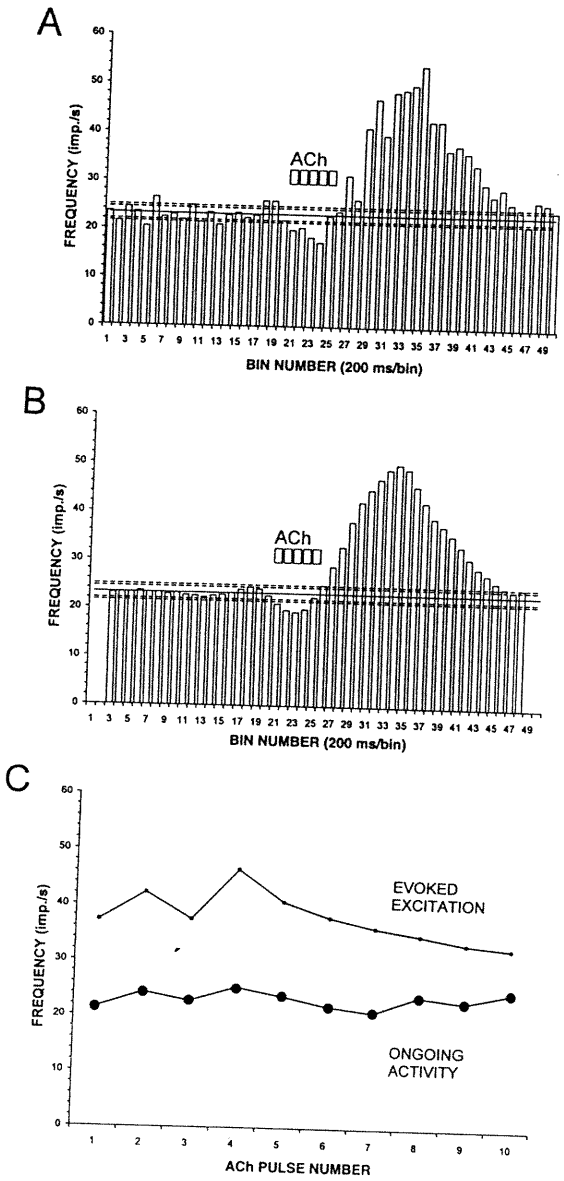

Fig. 4.

Fig. 4. (A) Peristimulus histogram of the data shown in Fig. 3 illustrated using 200-ms bin widths. Superimposed are two pairs of lines indicating threshold values centered around the mean frequency of ongoing activity. The first threshold delineates a 0.001 confidence interval, and second delineates values greater than 1 S.D. from the average prestimulus activity. Note that the appearance of the inhibitory and subsequent excitatory response to ACh is preserved in the peristimulus histogram despite the reduced variability achieved through the use of relatively large bin widths (200 ms/bin); the exact beginning and end of the inhibitory and excitatory components of the response to ACh, and the minimum and maximum levels of activity during these components, are clearly identifiable. (B) Smoothed peristimulus histogram showing the weighted, moving average frequency of the data shown in A (see text for details of this five-point smoothing procedure). The beginning and end of the inhibitory and excitatory components of the response to ACh are more readily distinguishable on this smoothed histogram; those bins that exceeded the second threshold (1 S.D. from mean prestimulus activity) were defined as belonging to either the inhibitory or excitatory component of the response. The minimum and maximum frequencies and their moments of occurrence during the inhibitory and excitatory phases are also readily determined within ± 200 ms. (C) Rate of average evoked activity during the excitatory component of the response to ACh (small black circles) and average ongoing prestimulus activity (large black circles) across the same 10 pulses of ACh depicted in the peristimulus histograms in A and B.

response was reached, the maximum firing frequency during the response to ACh, the delay after ACh application until the maximum inhibition of neuronal activity was reached, and the lowest firing frequency achieved during the inhibitory component of the response to ACh.

Many of the cells with an E–I response to ACh were recorded somewhat more superficially (1.2 ± 0.4 mm depth) than cells with I–E–I responses or E–E responses (1.5 ± 0.2 mm depth). Most neurons had ongoing activity rates of 10.6–12.3 imp./s, but those neurons with an E–E–I response tended to discharge at a relatively lower rate (8.8 ± 0.6 imp./s). The cells with the highest rate of ongoing activity were those with two adjacent inhibitory components (I–I–E) and those showing only inhibition (I) in their response to ACh. The mean frequency of discharge during the excitatory components of the various responses to ACh was lowest in those cells with an E–E–I response to ACh. Monophasic excitatory responses to ACh averaged 17.0 ± 14.6 imp./s. Triphasic, I–E–I responses to ACh averaged 25.3 ± 18.3 imp./s. The peak of the excitatory component was the smallest in neurons with E–E–I responses to ACh (19.3 ± 4.3 imp./s) whereas the largest peak responses were observed in cells with E–E responses. Finally, the latency to the peak was shortest in cells with E–E–I responses (2.4 ± 1.1 s) and longest in cells with I–E responses (5.2 ± 2.4 s). The duration of the excitatory components varied considerably with the shortest mean duration for cells with E–I responses (3.9 ± 2.1 s), and the longest mean duration for cells with I–E responses (17.4 ± 14.5 s).

Once cells were classified by their response type, principally a subjective analysis based on the appearance of the characteristic response to ACh over several individual responses, we examined several objective measures that might also distinguish our response classes. For each cell we calculated the ratio of the peak frequency during the excitatory response to the average prestimulus spike frequency and the ratio of the minimum inhibitory response to the average prestimulus spike frequency. These calculations provided the indices of excitation and inhibition shown in Table 3. With *t*-tests we determined which classes were significantly different from one another. Table 3 shows those differences that were significant and suggests that there are objective differences among the classes identified by their characteristic response.

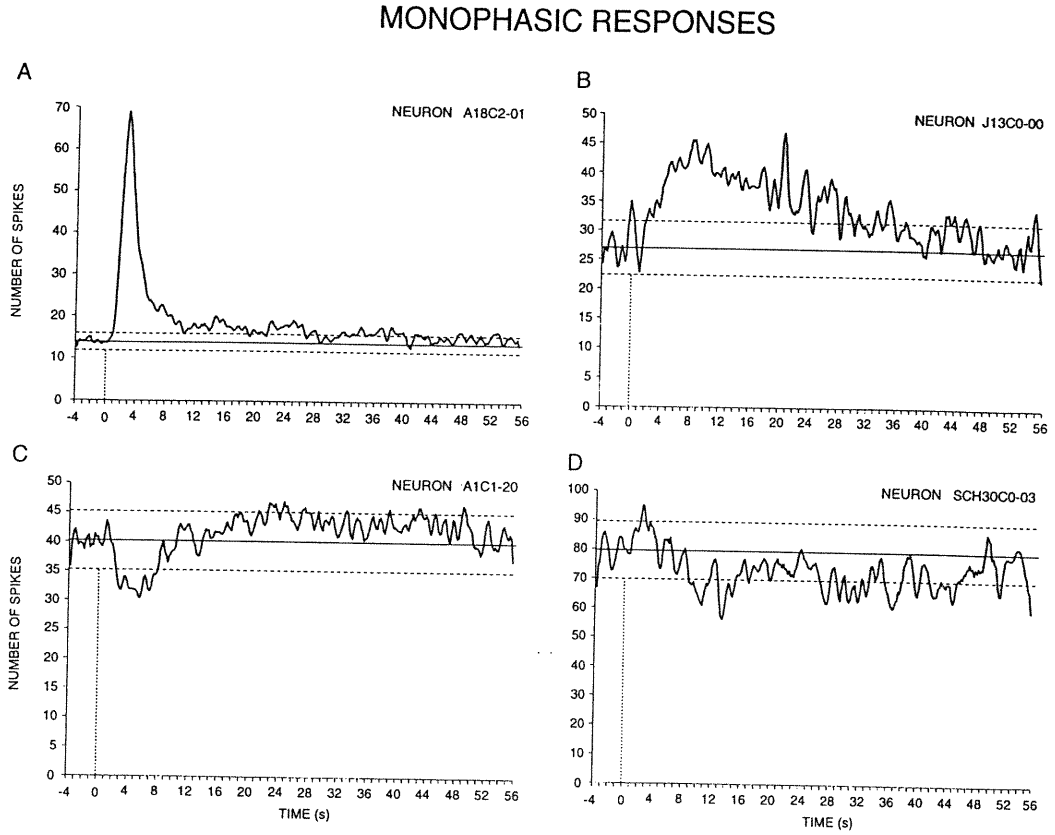

Fig. 5. Smoothed (five-point moving average) peristimulus time histograms illustrating characteristic responses of cells with monophasic responses to iontophoretic pulses of ACh. Among the different types of monophasic responses observed were rapidly developing, short-lasting excitatory responses (A), slowly developing, long-lasting excitatory responses (B), rapidly developing, short-lasting inhibitory responses (C), and slowly developing, long-lasting inhibitory responses to ACh (D). Superimposed on each histogram are the thresholds (1 S.D. from mean prestimulus activity) used to define the inhibitory and excitatory components of the response to ACh; components of the response where the frequency bins exceeded the upper threshold were defined as excitatory, and components of the response where the frequency bins fell below the lower threshold were defined as inhibitory. The cells shown in A–C were recorded in urethane-anesthetized rats and the cell shown in D was recorded in an awake rat. The recording depth, and the iontophoretic ejection and retention currents used for the cells shown in A–D were 2.2 mm, 90 nA, and − 1 nA; 1.2 mm, 90 nA, and − 1 nA; 1.3 mm, 92 nA, and 0 nA; and 0.6 mm, 85 nA, and − 7 nA, respectively. The duration of the current pulse for the cells shown in A–C was 1 s, and 2 s for the cell shown in D. The interval between successive ACh pulses was 2 min for the cell shown in A, and 1 min for the cells shown in B–D. In all cases, the response to ACh was similar in terms of its basic shape across ACh presentations. See text for details of smoothing procedure.

Analysis of relationship between neuronal activity and state of the animal

To determine whether there was a relationship between the behavioral state of the animal and the neuronal response to ACh, we first searched the data to find changes in the cortical EEG traces and EMG traces that seemed to be related to changes in the simultaneously recorded rasters of action potentials. A representative cell from an awake, undrugged rat is shown in Fig. 7. This analysis showed no consistent relationship between the variations in responsiveness to ACh and changes in either EEG or EMG. Similarly, ongoing spontaneous spike activity appeared to be unrelated to arousal as reflected in EEG or EMG activity.

Next we tried a statistical approach. Because discrete variables such as spike frequency are not directly comparable to continuous variables such as EEG and EMG activity, we first sought trials where the frequency of neuronal activity differed significantly from the other trials. Next, the power spectra of the EEG and EMG activity during the prestimulus and post-stimulus intervals of EEG and EMG activity were calculated using a fast Fourier transformation resulting in power spectra (100 bin resolution) for each of the 16 trials shown in Fig. 7. These power spectra were grouped by cluster analysis. Although a number of significant changes were found, there was no significant correlation between the trials in which the EEG or EMG had changed and those in which the neuronal responsiveness, or rates of ongoing activity changed (please see Appendix for further details). These results indicate that variation in neuronal responsiveness to ACh can occur without changes in the behavioral state of the animal.

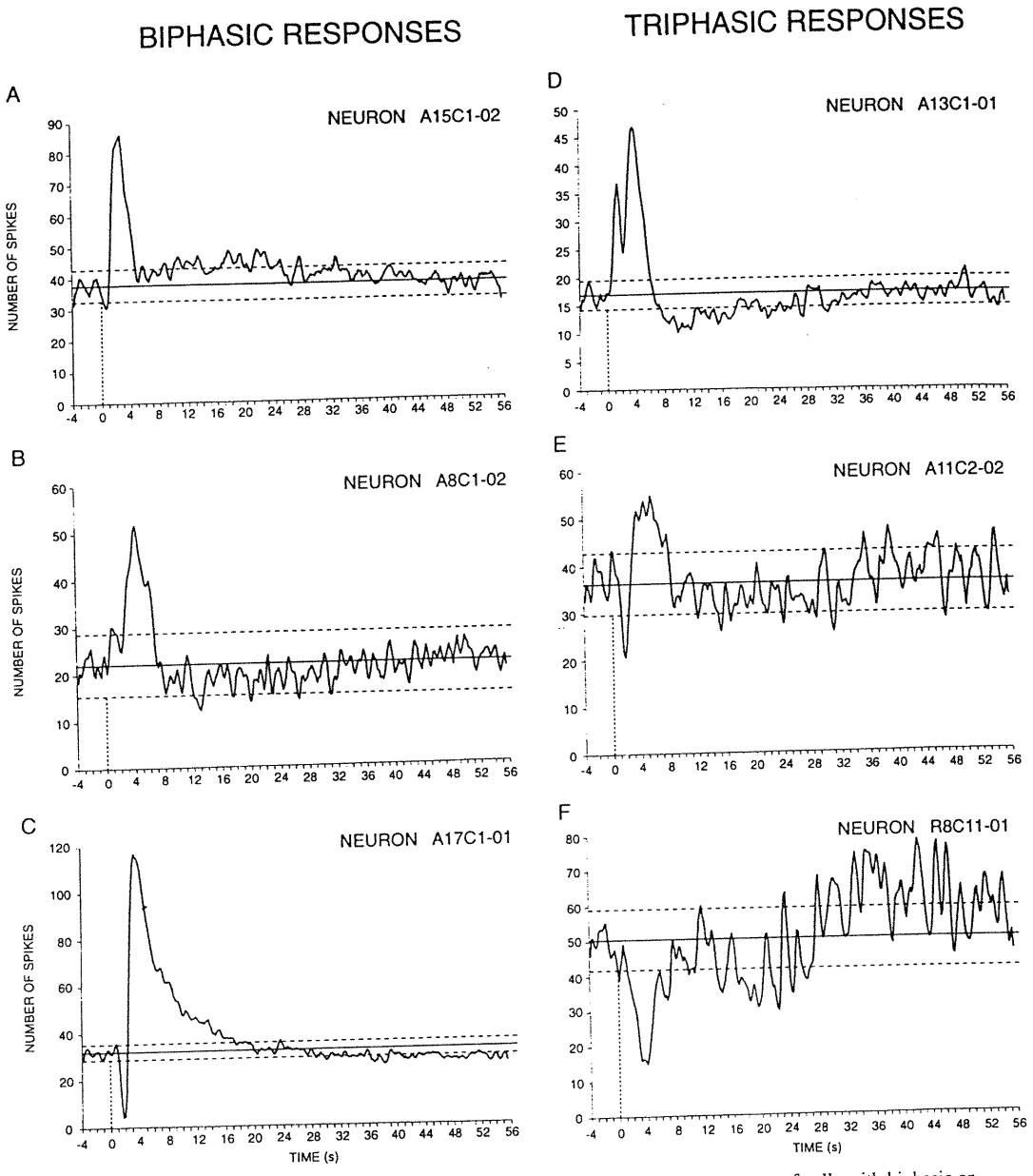

Fig. 6. Smoothed peristimulus time histograms illustrating the characteristic responses of cells with biphasic or triphasic responses to ACh. Biphasic response patterns included combinations of both short- and long-lasting excitatory responses (A), short-lasting excitatory and long-lasting inhibitory responses (B), and short-lasting inhibitory followed by short-lasting excitatory responses. Triphasic response patterns (D–F) included still more complex combinations of short- and long-lasting excitatory and inhibitory components. The cells shown in A–E were recorded in urethane-anesthetized rats and the cell shown in F was recorded in an awake rat. The recording depth and the iontophoretic ejection and retention currents used for the cells shown in A–F were 0.4 mm, 90 nA, and −1 nA; 1.8 mm, 90 nA, and −1 nA; 0.9 mm, 90 nA, and −1 nA; 1.5 mm, 90 nA, and −1 nA; 1.5 mm, 90 nA, and −1 nA; and 0.4 mm, 92 nA, and −1 nA, respectively. The interval between successive ACh pulses was 2 min for the cells shown in A–E and 1 min for the cell shown in F. All histograms were treated with the same five-point-moving average applied to previous figures.

Changes in neuronal responsiveness across repeated pulses of acetylcholine

To follow temporal changes in the magnitudes of the individual responses to ACh pulses and to compare these changes among cells, we expressed all responses for a cell as a percentage of the response to the first pulse of ACh. When plotted as a function of the pulse number, these ratios underwent large fluctuations across time. Although the patterns of changing responsiveness differed among cells, they could be grouped into three broad classes on the basis of the initial trend. The first class consisted of cells with an initially increasing responsiveness, cells in the second class were characterized by an initially decreasing

Table 1. Distribution of cells showing different types of responses to iontophoretically-administered acetylcholine in urethane-anesthetized and awake rats

Response type	Number of cells recorded in urethane-anesthetized rats	Number of cells recorded in awake rats
E–E	3 (8.1%)	1 (4.2%)
E	11 (29.7%)	7 (29.2%)
E–E–I	2 (5.4%)	1 (4.2%)
E–I	11 (29.7%)	6 (25.0%)
I–E	7 (18.9%)	2 (8.3%)
I–E–I	3 (8.1%)	4 (16.7%)
I–I–E	0 (0%)	1 (4.2%)
I	0 (0%)	2 (8.3%)
Total *n*	37 (100%)	24 (100%)

responsiveness. The third class consisted of cells with a relative absence of change in the response to repeated, single pulses of ACh.

The direction of the initial change in responsiveness was related to the form of the response to a pulse of ACh (Fig. 8). For example, in those cells having a large and rapidly rising excitatory phase (Fig. 8A), the response to successive pulses of ACh tended to increase relative to the size of the first response (Fig. 8B). The change in excitability, however, was transient and the response magnitude returned towards its initial levels within 1 h. In contrast, for cells where the inhibitory phase was relatively large and rapid (Fig. 8G), the magnitude of the response to successive pulses of ACh tended to decrease before returning to levels near the original size (Fig. 8H). In cases where excitatory and inhibitory components were roughly equivalent (Fig. 8C, E), the response magnitude showed little or no fluctuation (Fig. 8D, F).

During the first hour of the experiment, some cells went through several cycles of sensitivity to ACh. There was an inverse relationship between the duration of the excitatory response to ACh and the period of these oscillations. For example, the responsiveness of a cell with a prolonged excitatory response to ACh (Fig. 9A) showed a gradual decline in ACh-evoked response amplitude that did not recover during the first hour (Fig. 9B), but cells with excitatory phases of progressively shorter duration (Fig. 9C, E, G) showed increasingly more rapid periodicity in their responsiveness to ACh (Fig. 9D, F, H). In most of these cases, this evolution was characterized by cycles of progressively smaller amplitude, regressing towards the initial response magnitude.

A similar pattern occurred in cells showing an initial increase in responsiveness to successive pulses of ACh (Fig. 10). Cells having shorter duration excitatory components (Fig. 10A) showed higher frequencies of oscillation in responsiveness to successive ACh pulses (Fig. 10B) while cells

Table 2. Summary statistics describing the number of cells, depth, and mean frequency observed in spontaneous activity and in the excitatory and inhibitory components of the response to acetylcholine

Response type	*n*	Recording depth (mm)	Average ongoing activity (imp./s)	Average *f* of excitatory response (imp./s)	Excitatory response maximum *f* (imp./s)	Inhibitory response minimum *f* (imp./s)
E–E	4	1.1–2.2*	0.4–28.6*	3.6–44.5*	5.2–63.9*	–
		–	7.77†	18.72†	30.72†	–
		1.5 ± 0.6‡	11.2 ± 12.2‡	21.4 ± 17.2‡	32.6 ± 24.1‡	–
E	18	0.5–1.8	0.92–31.2	4.2–59.0	6.3–104.8	–
		–	8.25	12.21	16.97	–
		1.3 ± 0.3	10.6 ± 9.0	17.0 ± 14.6	27.1 ± 25.9	–
E–E–I	3	0.9–1.8	8.3–9.5	12.9–17.7	15.6–23.9	2.4–6.0*
		–	8.69	14.82	18.50	5.17†
		1.4 ± 0.5	8.8 ± 0.6	15.1 ± 2.4	19.3 ± 4.3	4.5 ± 1.9‡
E–I	17	0.6–1.8	3.3–33.5	7.5–52.3	8.8–66.9	1.0–20.8
		–	11.28	16.76	21.86	6.42
		1.2 ± 0.4	12.3 ± 8.6	20.3 ± 12.3	24.8 ± 15.0	8.6 ± 7.4
I–E	9	0.6–1.7	1.6–27.1	7.3–35.8	10.5–51.1	0.1–7.1
		–	8.14	19.47	28.03	1.00
		1.3 ± 0.3	10.8 ± 8.1	19.6 ± 9.5	28.2 ± 13.0	2.6 ± 3.1
I–E–I	7	1.2–1.6	5.6–44.6	8.0–64.2	8.5–78.7	–
		–	17.11	23.17	27.22	–
		1.5 ± 0.2	17.8 ± 13.0	25.3 ± 18.3	30.3 ± 22.5	–
I–I–E	1	–	–	–	–	–
		–	–	–	–	–
		0.4	25.3	–	–	–
I	2	1.4–1.5	14.6–50.6	–	–	–
		–	–	–	–	–
		1.45	32.6	–	–	–

*, range; †, median; ‡, mean ± S.D.; *f*, frequency; dashes indicate cases where values were not determined because the $n = 1$, precluding calculation of ranges or medians, or cases where the variable in question did not apply to a given category.

Table 3. Indices of excitation or inhibition for identified response classes and their statistical comparisons

	Response type	n	Index		t-test statistics comparing mean index values across response types				
			Excitation	Inhibition	2	3	4	5	6
1	E–E	4	2.23–11.86* 4.01† 5.52 ± 4.33‡	–	$P > 0.025$	$P > 0.05$	$P < 0.05$¶	$P > 0.10$	$P < 0.05$¶
2	E	13	1.50–39.43 2.40 5.53 ± 10.25	–	–	$P > 0.80$	$P > 0.15$	$P > 0.95$	$P < 0.05$¶
3	E–E–I	3	1.89–2.53 2.13 2.18 ± 0.32	0.28–0.63 0.63 0.51 ± 0.20	–	–	$P > 0.40$	$P > 0.30$	$P > 0.05$
4	E–I	14	1.27–4.12 1.87 2.16 ± 0.86	0.23–0.85 0.57 0.57 ± 0.18	–	$P > 0.80$	–	$P > 0.05$	$P > 0.35$
5	I–E	8	1.44–2.36 2.78 2.79 ± 0.87	0.01–0.43 0.18 0.24 ± 0.21	–	$P > 0.05$	$P < 0.01$¶	–	$P < 0.05$¶
6	(I–E)–II–(E–I)	7	1.44–2.36 1.64 1.74 ± 0.34	0.22–0.57* 0.40 ± 0.25‡ 0.56–0.88* 0.72 ± 0.22‡	–	$P > 0.20$ $P > 0.55$	$P > 0.25$ $P > 0.25$	$P > 0.20$ $P < 0.05$¶	–

*, range; †, median; ‡, mean ± S.D.; dashes indicate cases where no values were determined. For the I–E–I response category (6), two indices of inhibition and two sets of P values are given; the first for the initial inhibitory component of the response, and the second for the second inhibitory component of the response. ¶Significant values from t-tests comparing mean excitation and inhibition indices.

having longer duration excitatory components (Fig. 10C) showed lower oscillation frequencies (Fig. 10D).

Effects of diisopropylfluorophosphate administration

Between 1 and 2 h after the experiment began, when the magnitude of the responses to ACh had stabilized, DFP was administered as the periodic pulses of ACh were continued. The mean rate of ongoing activity in the 14 cells studied after systemic DFP treatment increased by 23.4% (to 123.4% ± 43.8%) of the pretreatment rate. The average frequency in response to iontophoretic administration of ACh, however, increased by only 9.7% to (109.7% ± 36.9%) after DFP. Similarly, the maximal firing frequency in response to ACh increased by only 10.2% (to 110.2% ± 27.6%) of the pre-DFP level.

Based on previous work in awake, freely moving rats,[58] we know that DFP reaches its peak effectiveness between 15 and 30 min after injection, a time when AChE inhibition reaches a stable level that lasts for at least 3 h (Fig. 11). As a consequence, the concentration of ACh in the brain starts rising, reaching a peak within 2 h and remaining at a high level. Over this same period of time, but following a control injection of saline, the concentration of ACh in the extracellular space declines slightly, probably because the animal becomes more accustomed to the conditions of the experiment.

When the responses to ACh were compared before and after DFP, as shown in the raster displays of action potentials of a typical cell depicted in Fig. 12, the neuronal response to iontophoretic administration of ACh began to vary again from pulse to pulse, much as they had before DFP treatment. Notably, these raster displays also show the somewhat surprising finding that the magnitude of the responses to pulses of ACh was increased very little (+ 10.2%) by DFP even though the acetylcholinesterase inhibition increased extracellular ACh concentrations by more than 350%.

Proceeding with the analysis, we calculated the ratio of response magnitude for each pulse to the magnitude of the first pulse so as to be able to follow the changes in responsiveness across the 2–5 h required to complete the study. These changes as a function of time are shown in Fig. 13. During the 1–3 h period following DFP injection, when we assumed that extracellular ACh concentrations had shifted to the new elevated but steady level induced by DFP, the responses to the ACh pulses underwent a series of changes that mimicked the fluctuations observed at the beginning of the experiment. Figure 13 shows three examples of this behavior which are typical of the nine cases in which we were able to hold the cell for the 1–3 h following DFP injection required to complete the experiment. Figure 13A shows a neuron that rapidly became less responsive to repeated ACh pulses during the first part of the experiment, with the gradual return in responsiveness towards the baseline level. Then, the inhibition of AChE caused by systemic injection of DFP, one hour later, resulted in a reoccurrence of the transient

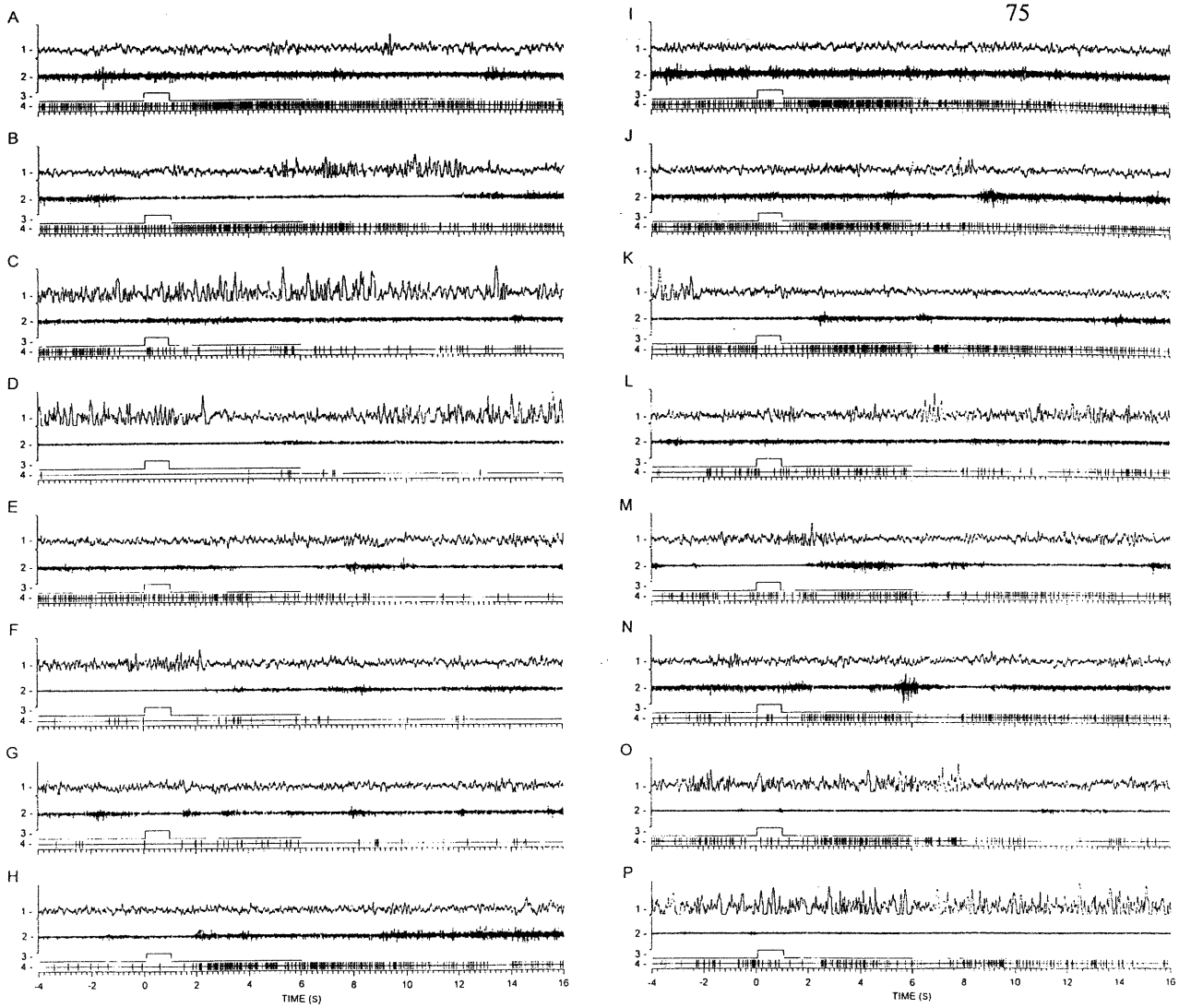

Fig. 7. Traces showing EEG (1) and EMG (2) and raster displays of neuronal spike activity (4) in relation to individual ACh pulses (3) on each of 16 consecutive trials (A–P) in an awake, undrugged rat. Note the transient but dramatic increase in spike activity following the ACh pulse in A and B, followed by several weak responses to ACh in C–G, the return of a more robust response to ACh in H–K, and another series of weaker responses to ACh in L–P. Changes in EEG and EMG activity do not appear to be consistently related to the onset or offset of the ACh pulse or to changes in ongoing spontaneous spike activity during the 2 s preceding ACh delivery or to ACh-evoked spike activity during the 16-s period following the onset of the ACh pulse (note time-scale). The temporal evolution of the amplitude of the first excitatory component and the changes in the mean frequency of the ongoing activity are shown in Fig. 9F. The average of 20 responses of this neuron to 1-s pulses of ACh is shown in Fig. 9E.

Fig. 8. Relationship between the main component of the characteristic response to ACh in individual neurons, as shown in the smoothed (five-point filter) peristimulus histograms on the left side of the figure, and the direction and magnitude of the overall change in responsiveness to repeated pulses of ACh, as shown in the smoothed (three-point filter) line graphs of the spike frequency across ACh pulses on the right. All values are expressed as a percentage of the size of the first excitatory component of the response to the first ACh pulse. Cells with a large, rapidly rising excitatory phase in their characteristic response (A) showed a transient increase (reflected in the dotted line showing the linear regression) in the evoked responses to ACh (filled circles) over time. The spontaneous activity (open diamonds, also expressed as a percentage of the size of the first excitatory component of the response to the first ACh pulse) showed parallel fluctuations in activity (B). Cells with small, relatively equal excitatory and inhibitory components in their characteristic response (C) tended to show little or no overall change in the evoked response to ACh over time (D). Cells with a large, rapidly developing inhibitory phase in their characteristic response (E) tended to show a transient decrease in the evoked response to ACh (F). Cells with the largest rapidly developing inhibitory phases (G) tended to show the greatest transient decreases in evoked response to ACh (H). C and D were from a cell recorded in an awake rat, whereas the others were from cells recorded in urethane-anesthetized rats. The recording depth and the iontophoretic ejection and retention currents used for the cells shown in A–B, C–D, E–F, and G–H were 1.4 mm, 90 nA, and − 1 nA; 1.2 mm, 50 nA, and 0 nA; 1.7 mm, 90 nA, and − 1 nA; and 1.5 mm, 90 nA, and − 1 nA, respectively. The duration of the current pulse was 1 s for all cells shown except in C and D, where the duration of the current pulse was 0.5 s. The interval between successive current pulses was 2 min for the cells shown in A–B and G–H, and 1 min for the cells shown in C–D and E–F.

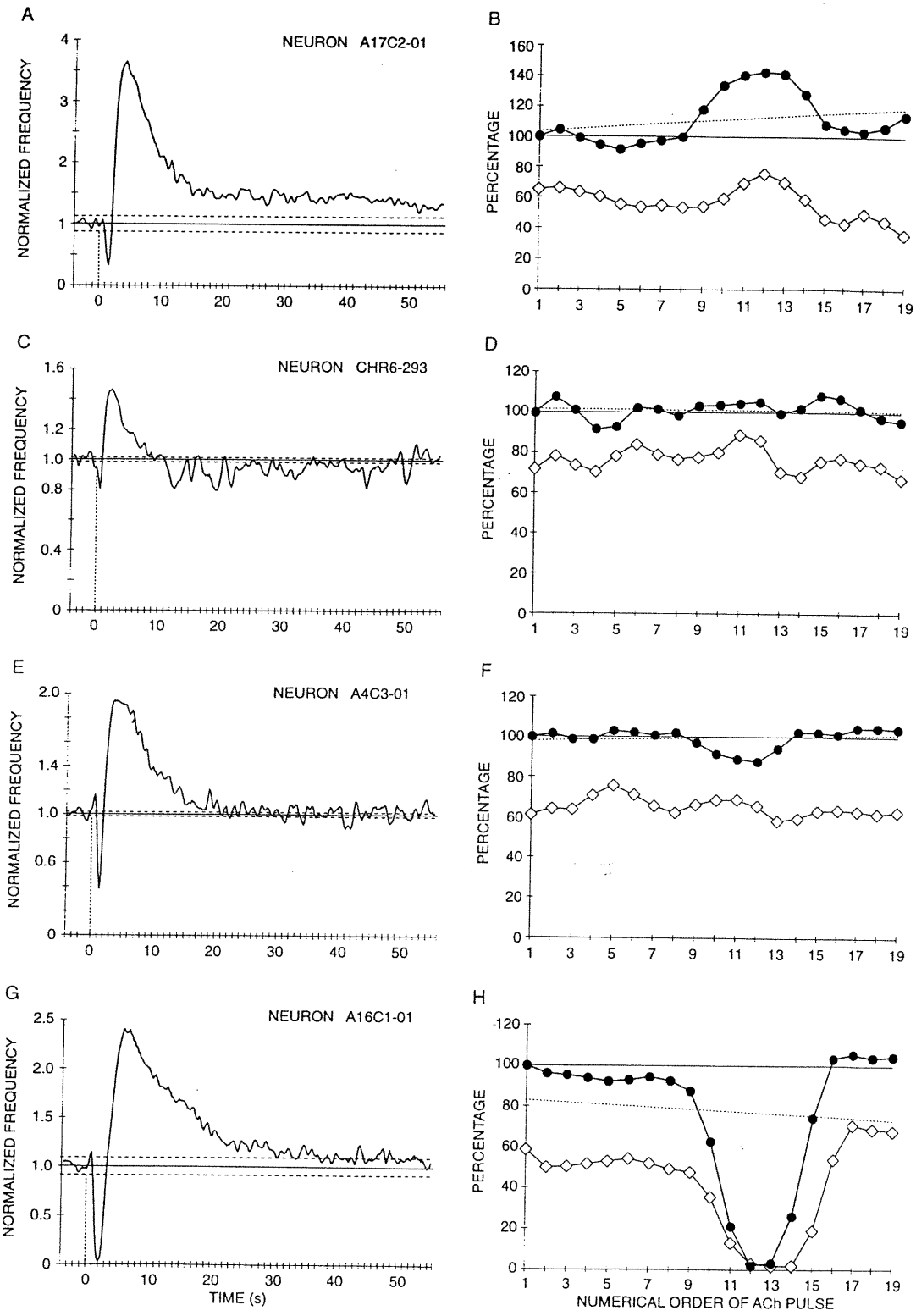
A
NEURON A17C2-01
NORMALIZED FREQUENCY
B
PERCENTAGE
C
NEURON CHR6-293
NORMALIZED FREQUENCY
D
PERCENTAGE
E
NEURON A4C3-01
NORMALIZED FREQUENCY
F
PERCENTAGE
G
NEURON A16C1-01
NORMALIZED FREQUENCY
TIME (s)
H
PERCENTAGE
NUMERICAL ORDER OF ACh PULSE

Fig. 8.

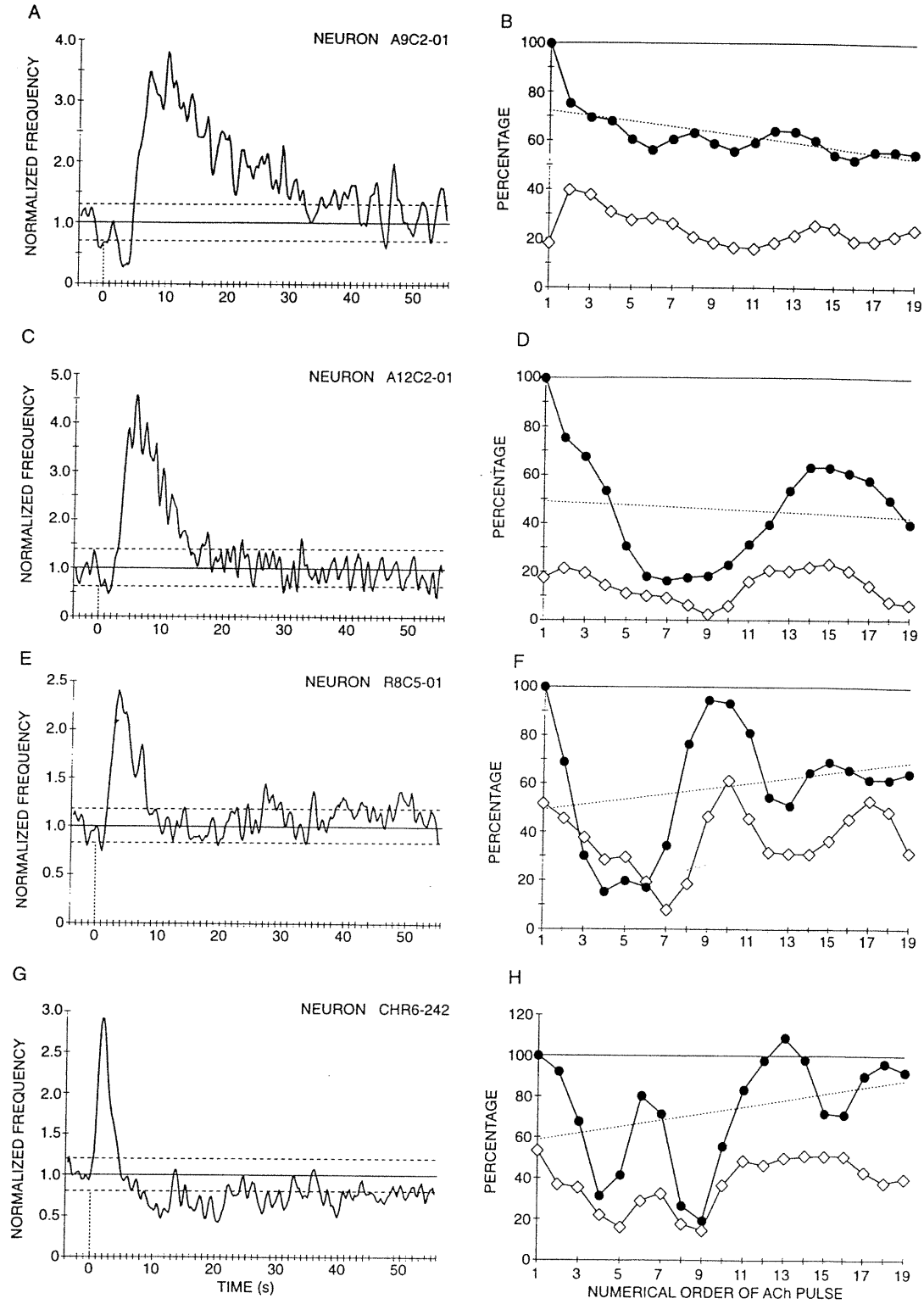
A
NEURON A9C2-01
NORMALIZED FREQUENCY
B
PERCENTAGE
C
NEURON A12C2-01
NORMALIZED FREQUENCY
D
PERCENTAGE
E
NEURON R8C5-01
NORMALIZED FREQUENCY
F
PERCENTAGE
G
NEURON CHR6-242
NORMALIZED FREQUENCY
TIME (s)
H
PERCENTAGE
NUMERICAL ORDER OF ACh PULSE

Fig. 9.

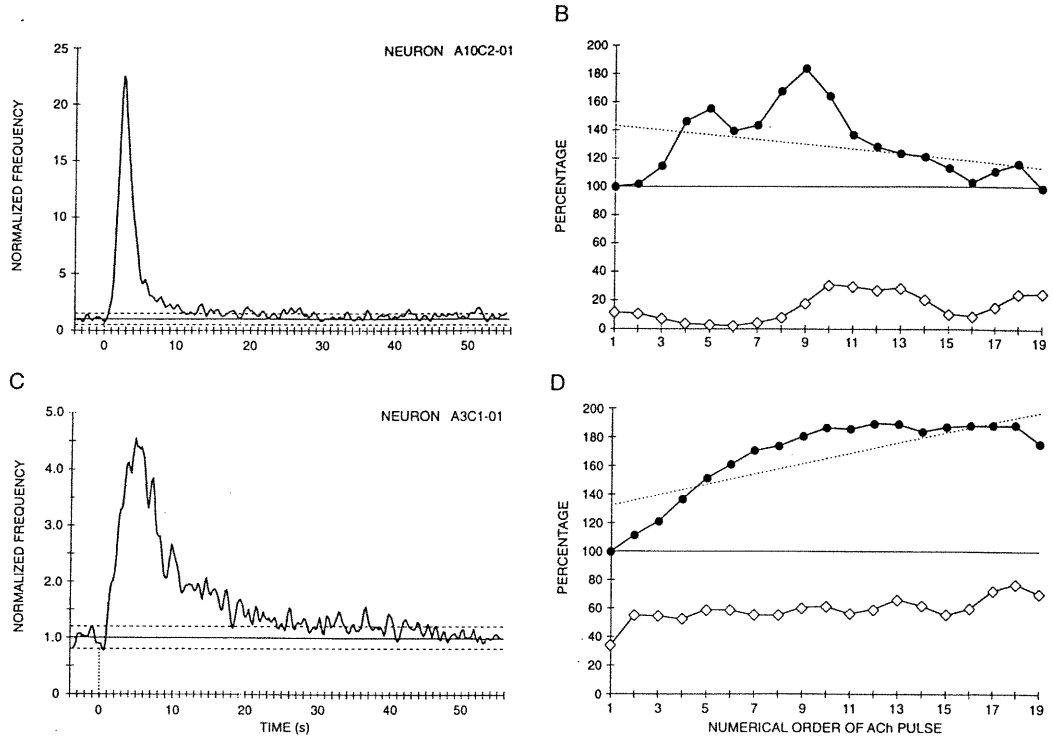

Fig. 10. Relationship between the duration of the excitatory component of the characteristic response and the frequency of oscillation in responsiveness to successive pulses of ACh for neurons showing an overall increase in responsiveness to ACh. Cells with excitatory components of relatively short duration (A) tended to show relatively rapid oscillations in ACh-evoked responses (filled circles) and in spontaneous activity (open diamonds: B). In contrast, cells with excitatory components of relatively long duration (C) tended to show slower oscillations in ACh-evoked and spontaneous activity (D). All cells shown were recorded in urethane-anesthetized rats. The depth of recording and the iontophoretic ejection and retention currents used for the cells shown in A–B and C–D were 0.4 mm, 90 nA, and − 1 nA; and 1.8 mm, 65 nA, and − 1 nA, respectively. The duration of the current pulses was 1 s in both cases. The interval between successive pulses of current was 100 s in the cell shown in A and B and 60 s in the cell shown in C and D (five-point smoothing on left and three-point smoothing on right). All values on the right are expressed as a percentage of the first excitatory component of the response to the first ACh pulse.

decrease in responsiveness observed prior to DFP treatment. Figure 13B and C shows similar examples of the reappearance of the transient decrease in responsiveness observed during the first part of the experiment following DFP administration.

The oscillations in responsiveness to repeated pulses of ACh observed before and after treatment with DFP are analysed in Fig. 14. On the first panel (Fig. 14A) are plotted the autocorrelograms from the 60 ACh pulses (at 1 min intervals) given to two cells one from a DFP-injected animal (circles) and one from a control (squares). Strong positive correlations are seen initially for both cells as the oscillatory pattern develops. However, after the injection of either saline or DFP (arrowhead) only the cell from the DFP-injected animal shows strong positive

Fig. 9. Relationship between the duration of the excitatory component of the characteristic response and the frequency of oscillation in responsiveness to successive pulses of ACh for neurons showing an overall decrease in responsiveness to ACh. Cells with excitatory components of progressively shorter durations (A, C, E, and G, respectively) showed oscillations in responsiveness to pulses of ACh that occurred at correspondingly shorter intervals (B, D, F, and H, respectively). Notice that both the oscillations in the evoked response to repeated pulses of ACh (filled circles) and the oscillations in ongoing activity (open diamonds) are of increasingly smaller amplitude, returning towards the initial level of responsiveness over time. This return is reflected by the linear regression (dotted line) of the data for the evoked response to ACh. The cells shown in A–B and C–D were recorded in urethane-anesthetized rats, whereas the cells shown in E–F and G–H were recorded in awake rats. The recording depth and the iontophoretic ejection and retention currents used for the cells shown in A–B, C–D, E–F, and G–H were 1.3 mm, 90 nA, and − 1 nA; 1.5 mm, 90 nA, and − 1 nA; 1.6 mm, 92 nA, and − 1 nA; and 1.1 mm, 36 nA, and 0 nA, respectively. The duration of the current pulse was 1 s in all cases except for the cell shown in G and H, where it was 0.5 s. The interval between successive current pulses was 2 min for the cells shown in A–B and C–D, and 1 min for the cells shown in E–F and G–H (five-point smoothing on left and three-point smoothing on right). All values on the right are expressed as a percentage of the first excitatory component of the response to the first ACh pulse.

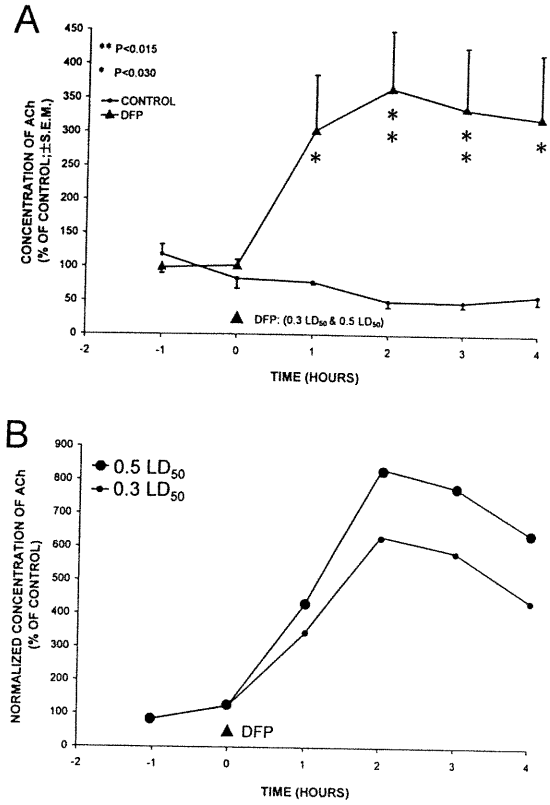

Fig. 11. Microdialysis fibers (molecular cut-off: 10,000 mol. wt) were prepared to leave 12 mm of exposed membrane in the cerebral cortex after having passed through a small craniotomy. When perfused with artificial Ringer solution (without AChE inhibitors) at a rate of 2 μl/min the fibers yielded aliquots that could be assayed by high-performance liquid chromatography for ACh content every hour. Recovery of ACh was 38% in a 10 nM solution at room temperature. (A) Normalized concentration of ACh in the brain as measured with microdialysis 2 h before and 4 h following the systemic injection of DFP at 0.3 DL_{50} and 0.5 DL_{50} and saline in awake, freely moving rats. Following injection of saline, the concentration of ACh declined, perhaps as the animals habituated to the cage (small dots). In contrast, following injection of DFP (triangles) the concentration of ACh started rising and reached the maximum 2 h following the injection. (B) The dynamics of the concentration of ACh as expressed in percent of the level of ACh following injection of saline. The curves show the ACh level following injection of DFP at 0.3 LD_{50} and 0.5 LD_{50} (data from experiments of Testylier and Dykes[58]).

correlations as the oscillations start again. The second panel (Fig. 14B) presents the averaged (± S.E.M.) autocorrelograms for two sets of cells in which ACh pulses were given at 1-min intervals. One group was three I–E cells and the other was three E–I cells. Even though they differed in their dynamics and thus had different autocorrelograms (two asterisks indicate points where a *t*-test showed differences significant at $P < 0.05$), nevertheless in each group the positive correlation reappears as the oscillations reappear.

In Fig. 14C, three other cells in which ACh pulses were given at 30-s intervals and in which the return to the original baseline was incomplete due to the shorter duration of the time before DFP, the signal after DFP was less evident in the autocorrelograms. Nevertheless, the DFP treatment produced an effect significantly different ($P < 0.05$) from three cells subjected to the same experimental paradigm but without the DFP injections.

DISCUSSION

We examined the responses of rat somatosensory cortical neurons to the repeated iontophoretic administration of ACh before and after systemic treatment with the irreversible AChE inhibitor DFP. Our results document the behavior of single neurons in a complex and dynamic system in which responses to single pulses of ACh vary among individual neurons and become modified over time. Several different temporal patterns were observed in response to single pulses of ACh. Excitatory and inhibitory responses occurred alone or in combination, resulting in neuronal responses to a single pulse of ACh that were often quite complex. Additionally, the excitatory and inhibitory phases of those responses were either very rapid or developed more gradually, suggesting two apparently distinct reactions to ACh. With repetition, the amplitude of the responses changed in a cyclic manner. After describing the evolution of the responsiveness to repeated pulses of ACh, we administered systemic injections of DFP. This second experimental condition produced transient changes in responsiveness similar to those observed at the beginning of the experiment; in each condition, these transient oscillations gradually regressed towards the original response magnitude.

Neuronal responses to iontophoretic acetylcholine administration

The most common response to iontophoretic administration of a pulse of ACh was a relatively rapid, monophasic excitation followed by a more gradual return to prestimulus levels of activity. A second type of excitatory monophasic response was characterized by a more gradual increase in activity followed by an even more gradual return to pre-stimulus activity levels (Fig. 5A, B). The distinctive differences between the slow and rapid time-courses of these responses was consistent enough to suggest that there are two broad classes of responses to ACh; one that is rapid in onset and stops within seconds after the ACh pulse is terminated, and a second that provokes changes in the cell lasting much longer than the period during which ACh is bound to the cell membrane. These excitatory monophasic responses are similar to those described by others.[4,37,41,43] Lamour *et al.*[43] noted that neurons located in different cortical layers produced responses to ACh having different time-courses. More superficially located cells produced a

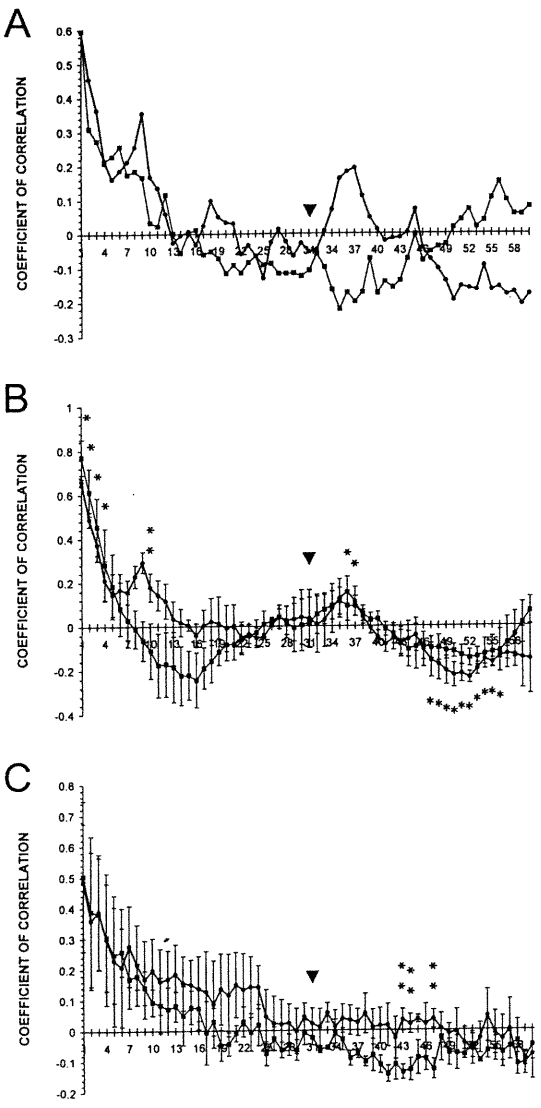

Fig. 14. (A) Sequential correlation coefficients for autocorrelograms of the mean frequency of response across 60 pulses (horizontal axis) of iontophoretically administered ACh in an inhibitory–excitatory (I–E) cell from an anesthetized, DFP-treated rat (circles) and from an I–E cell in an anesthetized, saline-treated control rat (squares). Both cells show significant correlations during early ACh pulses corresponding to the oscillations in responsiveness to repeated ACh pulses observed in these cells prior to saline or DFP treatment. Both cells also show a trend towards zero correlation across the first 30 ACh pulses, reflecting the tendency to return to the original level of responsiveness. Following DFP or saline injection (triangle), however, the two cells show distinctly different patterns of autocorrelation; note that the cell from the DFP-treated rat shows positive correlations on pulses 36–37, whereas the cell from the saline-treated rat shows negative correlations on pulses 34–38. The positive correlations seen in the autocorrelogram for the DFP cell correspond to the reoccurrence of the oscillatory pattern of responsiveness to ACh seen following DFP injection, whereas the negative correlations seen in the cell of the saline-injected animal reflect a new, negative relationship between earlier responses and responses following saline injection. (B) Autocorrelograms for two groups of cells recorded from DFP-treated rats showing the coefficients of correlation on the mean ($\pm$ S.E.M.) frequency of response across 60 pulses (horizontal axis) of iontophoretically administered ACh. The groups were created because the pattern of temporal oscillations differed between them. The first group (circles) contains three cells receiving ACh pulses at 2 min intervals and shows the re-emergence of correlation at 7–11 pulses of ACh and the second group (squares) contains three cells receiving ACh pulses at 2-min intervals and shows a negative correlation around 9–22 pulses of ACh. Prior to DFP injection, there was a significant difference between groups ($^{**}P < 0.05$) on pulse 10, where one group but not the other shows an increase in its correlation coefficient. Following DFP injection (arrow), while both groups show an increase in positive correlation soon after drug treatment, the correlation coefficients were significantly greater than zero ($^{*}P < 0.05$) for both groups on pulses 36–37. (C) Autocorrelograms for two other groups of three cells each. One group was recorded from DFP-treated rats (circles) and the other was three control animals (squares). In these animals 60 pulses (horizontal axis) of iontophoretically administered ACh were delivered at 60-s intervals. Prior to DFP treatment, the correlation coefficient values for the two groups did not differ significantly. However, following DFP injection (arrow), the correlation coefficients for the DFP-treated group were significantly greater than for the untreated group ($^{**}P < 0.05$) on pulses 43–44 and on pulse 47.

swings in the amplitude observed before DFP supports our suggestion that these variations are not due to diffusion from a point source but rather are adjustments within the cortical network that bring the excitability of the network back towards its original set-point. When this elevation of ACh caused by less than normal levels of AChE is added to the even higher concentrations produced by the ACh pulses, it seems remarkable that the responses to pulsatile ACh increased only 10% above the responses observed before DFP. Neuronal responsiveness appears to have adapted to abnormally high levels of ambient extracellular ACh.

Others experiments also suggest that the cholinergic system is highly adaptive when ACh levels are raised through AChE inhibition. For example, although extracellular ACh levels increase dramatically following AChE inhibition by DFP,[58] animals quickly develop behavioral tolerance to these levels.[15,57,60] Our results suggest that underlying this behavioral tolerance may be a rapid, homeostatic adjustment in the responsiveness to ACh in cortical neurons. We doubt that our results are an artifact attributable to the non-synaptic delivery of ACh since distribution of ACh by diffusion in the extracellular space is probably the principle mechanism of ACh delivery within the cortex.[11]

Modeling the dynamics of the response of cortical neurons to acetylcholine before and after diisopropylfluorophosphate

The delivery of equal amounts of ACh with repeated, equal current pulses is not a simple task. Between periods of ACh delivery, the open end of the pipette allows diffusive loss of ACh. This is normally countered by the retaining current which

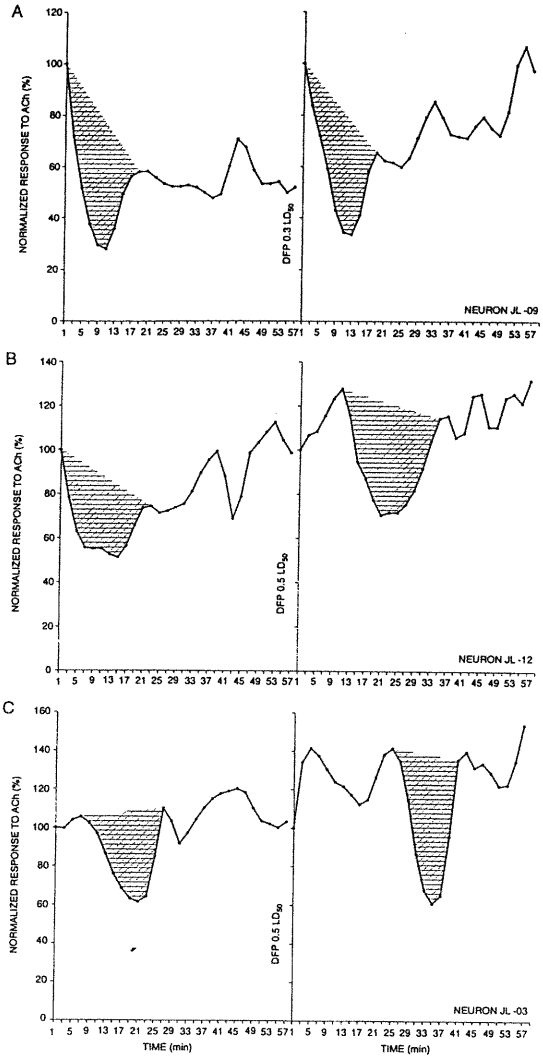

Fig. 13. Smoothed curves showing the effects of DFP on the responsiveness of single neurons to repeated pulses of ACh (three-point filter). The pattern of oscillation occurring in the evoked response to repeated pulses of ACh before systemic DFP treatment (left) reoccurs with remarkable consistency following DFP injection (right) in each of the three cells. The first major oscillations before and after DFP treatment have been highlighted (crosshatching) to illustrate this consistency. Note that in the cells shown in B and C, the appearance of these oscillations following DFP treatment occurs following a longer latency compared to their appearance after the onset of ACh administration alone in the beginning of the experiment. This delay presumably reflects the time required for a gradual increase in extracellular ACh levels associated with the gradual diffusion of DFP into the brain following systemic injection.

excites local inhibitory interneurons more rapidly than pyramidal cells and the observed suppression of activity is the result of the excitation of these unseen cells. McCormick and Prince[48] marshalled arguments for this explanation in slices of rat hippocampus; they suggested that the time-course of ACh effects are very rapid on GABAergic interneurons and that their excitation inhibits the cell being recorded before that neuron begins to respond to the direct excitatory effects of ACh. They argued that the excitatory effects developed more slowly in pyramidal cells but eventually took over and dominated the cell's behavior (see also Ref. 34).

One can generalize this model to explain other, more complex neuronal responses to ACh. For example, inhibitory interneurons more distant from the pipette might depolarize more slowly or some classes of inhibitory interneurons may have receptors or membrane properties causing them to be excited more slowly or for a longer duration. With appropriate combinations of synaptic relations, distances from the iontophoretic pipette and ACh receptor properties, one can build mechanistic explanations for the biphasic and triphasic responses we observed. The diversity of these responses suggests that the cells we recorded from are located at different points within a complex cortical circuitry where the effects of ACh can vary considerably.

Changes in neuronal responsiveness across repeated acetylcholine administrations

The rapid evolution of the response size with successive pulses of ACh is consistent with the suggestion that many cortical neurons undergo some form of habituation to elevated levels of ACh.[33,35,36,38] Whether an equilibrium in extracellular ACh in the area around the pipette is reached after a few pulses or after many pulses, the dramatic swings in the excitability of many neurons cannot be explained simply by changes in the concentration gradient. It is evident that the neurons being studied were elements within a cortical network and that the diffusion of ACh through the extracellular space must be affecting nearby cells in addition to the recorded neuron[47] and that the observed responses also reflect the contribution of other unseen members of the cortical network. We interpret the oscillatory evolution of the response magnitude as evidence that the sensitivity of the neurons in this network to ACh changed while one of them was being studied. It seems likely that this series of damped oscillations returning towards a new stable state is an adaptation of the system to elevated levels of ACh. Most of the cells seemed to have adapted within 1 h to the increase in extracellular ACh produced by the repeated iontophoretic pulses of ACh.

The influence of diisopropylfluorophosphate on the neuronal response to acetylcholine

The systemic administration of DFP must have raised extracellular ACh to levels that were comparable to those used in previous experiments.[58] This must have been a generalized, gradual elevation in extracellular ACh levels throughout the cortex rather than the localized, rapid increase that occurs with microiontophoretic delivery. That pulses of ACh again produced the same pattern of oscillatory

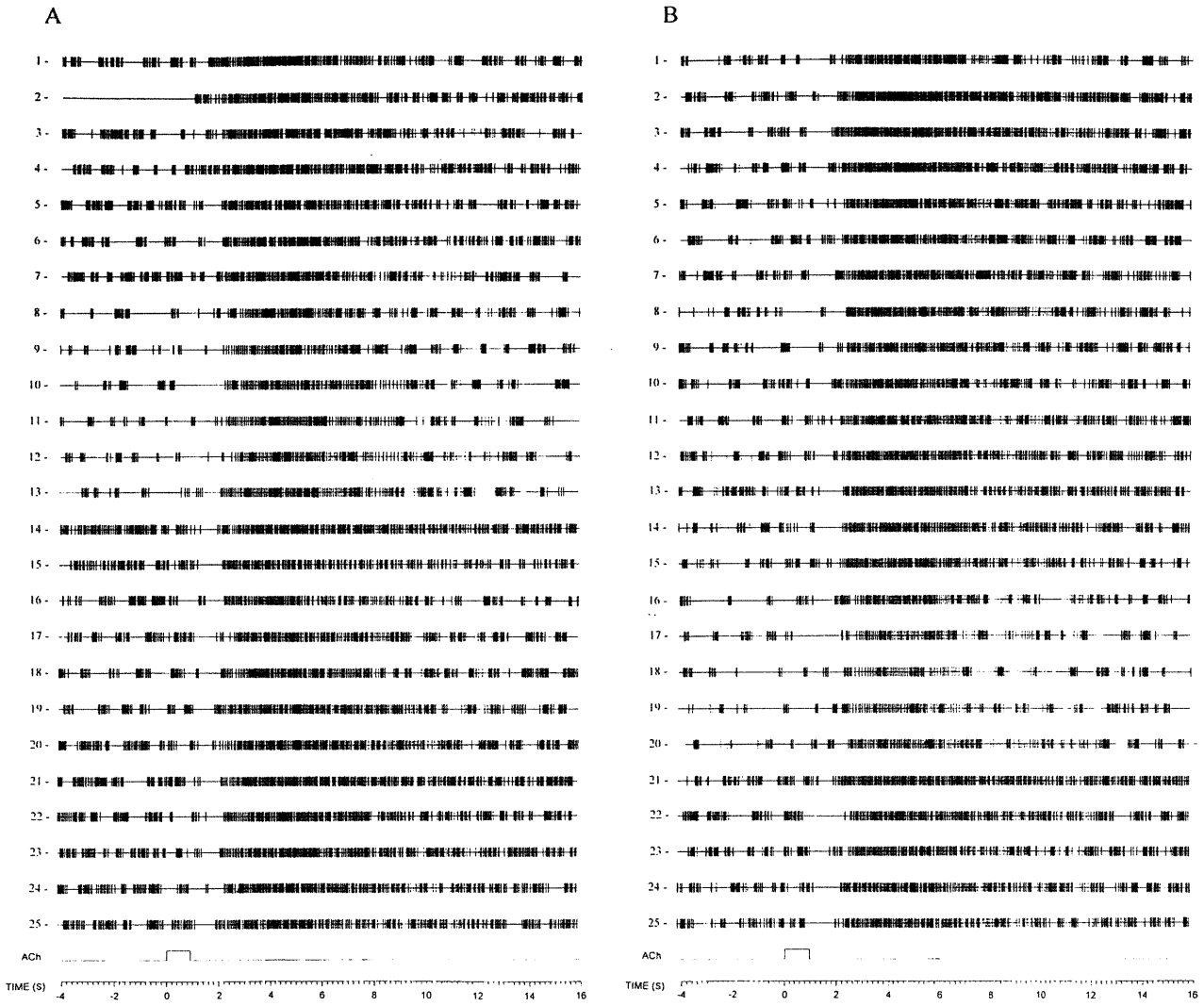

Fig. 12. Raster displays of the responses to 25 successive pulses of ACh before (A) and after DFP injection (B). Note the variation in response across pulses of ACh, including a transient decrease in ongoing and evoked activity during pulses 9–13, and subsequent return of the responses towards the initial magnitude prior to DFP injection. After DFP injection, the fluctuation in response to ACh reappears, as seen in the transient inhibition and subsequent return of the response during pulses 16–20. Note that the fluctuation in response to ACh appears to follow a similar time-course before and after DFP injection.

less robust but sustained response lasting for the duration of the ACh application, whereas more deeply located cells produced large, rapid, transient responses that fell quickly to a lower rate of discharge outlasting the duration of the ACh administration by several seconds. These two response patterns have been reported in rat somatosensory cortex[44] but because the pulses of ACh in our experiments lasted only 1 s, it is difficult to relate our response classes directly to the classes observed by Lamour *et al.*,[43,44] although it would be surprising if such a correlation does not exist.

A small number of cells exhibited monophasic inhibitory responses, with either a rapidly developing decrease in activity followed by a gradual return to prestimulus activity levels, or a more gradual decline and subsequent recovery. The pure inhibition of cortical neurons by ACh has been reported occasionally;[42,47,54] the small percentage of cells in our sample showing monophasic inhibitory responses consistent with the relative rarity of such examples in the literature.

The majority of the cells exhibited biphasic or triphasic responses to ACh, apparently consisting of some combination of the excitatory and inhibitory components observed in the monophasic cells. For example, many cells showed a rapid initial excitation followed by a more gradually developing and longer-lasting inhibition. Other biphasic responses were characterized by a rapidly developing, brief inhibitory phase followed by more slowly developing and longer-lasting excitation. The commonly accepted explanation for these initially inhibitory, biphasic responses is that the administered ACh

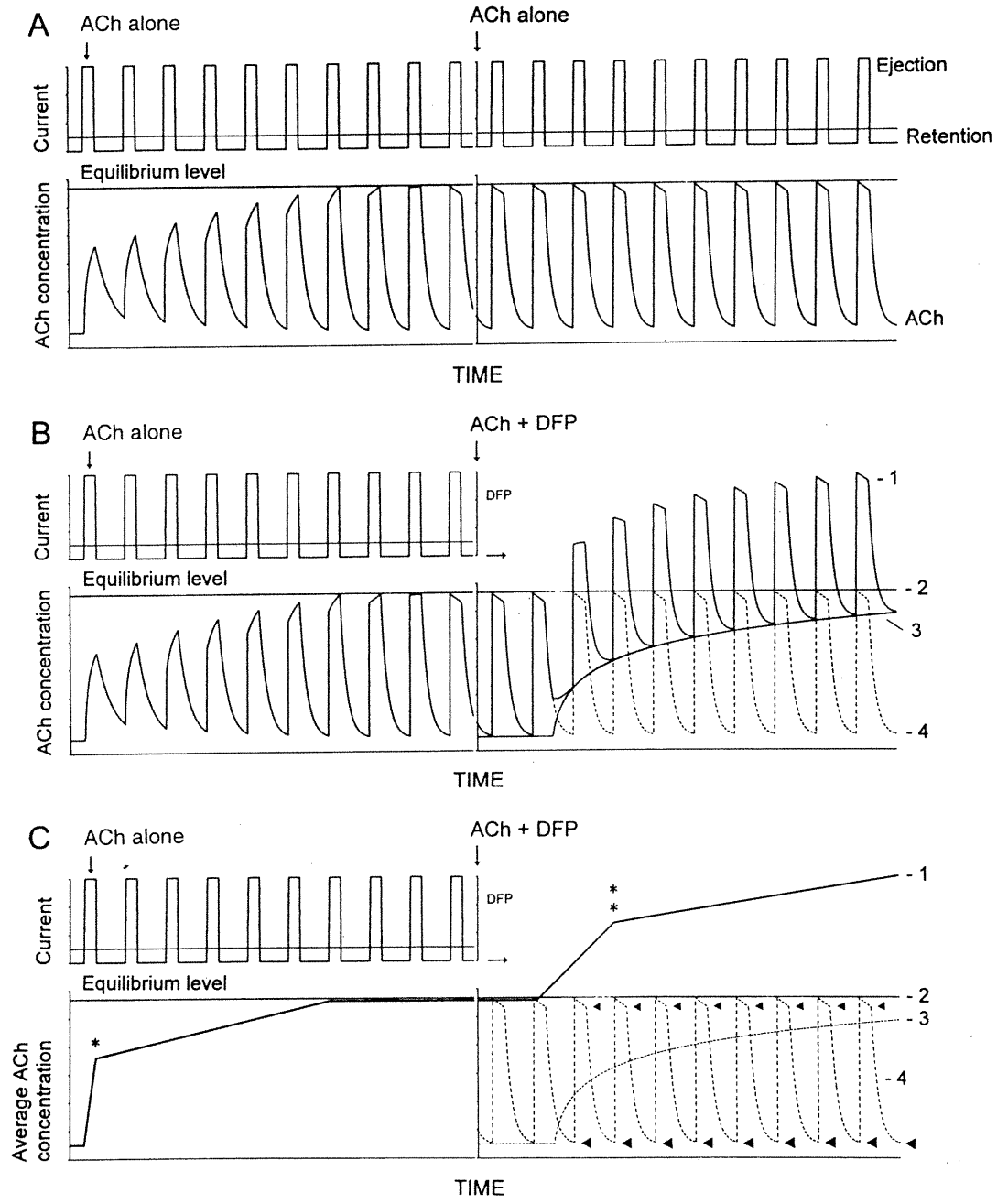

Fig. 15.

encourages the movement of ACh into the pipette. Thus, at the moment of the first current pulse, the liquid at the boundary between the pipette opening and the extracellular space contains some unknown, diluted concentration of ACh. This concentration is dependent upon the size of the pipette opening, the time since the last current pulse and the original concentration of ACh. As a result, a concentration gradient exists along the length of the pipette such that the initial current pulse ejects much less ACh than pulses delivered later in the series. With each successive pulse there will be a gradual increase in ejected ACh concentration until the ACh gradient is displaced from inside the pipette.

Also, physical factors of the cortical structure will influence the development of a steady-state extracellular concentration and influence the consequences of repeated pulsatile ejections of ACh. These factors include the size of the extracellular space, the coefficient of diffusion, the density of ACh receptors on nearby cells, and the rate of ACh degradation. Together, these factors will determine the rate at which ACh will dissipate from the vicinity of the pipette opening following each

current pulse and will influence the time-course of ACh effects on nearby and more distant cells. Nevertheless, after several current pulses, the system should come into an equilibrium. At this point, the concentration of ACh leaving the pipette with each equally spaced pulse should be constant.

Although it is impossible to describe each of these events and their time-courses precisely, nevertheless they are likely to summate temporally to establish the overall equilibrium. We hypothesize that the initial changes in the amplitude of the neuronal responses observed following the first ACh pulses may reflect the development of this dynamic equilibrium, but, once an equilibrium in the extracellular concentration of ACh is reached, the changes in response magnitude reflect adjustments in responsiveness to ACh of one or more neurons participating in the cortical network of which the recorded neuron is part.

Figure 15 represents our view of the processes occurring around the tip of the micropipette delivering ACh into the extracellular space. The horizontal axis represents time and the pulses of ACh are equally spaced. The figure shows that multiple pulses of ejection current before and after systemic injection of DFP provokes a generalized increase of ACh concentration. The upper frame of Fig. 15A represents the amount and the polarity of periodic current pulses. The thin horizontal line shows the reference (zero potential applied to the solution within the iontophoretic micropipette). When the line is below the reference, the retention current prevents the outflow of ACh. The values above the reference represent the ejection current forcing the molecules of ACh to leave the pipette. The ejection current is an order of magnitude higher than the retention current. Contrary to the illustration, the time between pulses was about two orders of magnitude greater than the pulse duration. The lower frame in Fig. 15A represents the amount of ACh delivered by each pulse of current. Based on the experiments and the calculations previously performed by others[1,52,53] we presume that the amount of ACh released from the tip of the pipette grows logaritmically in the presence of the ejection current and declines exponentially when that current is replaced with the retention current. When the gradient of ACh is finally driven from the pipette all subsequent pulses should deliver equal amounts of ACh.

Removal of extracellular ACh depends primarily on its degradation by AChE. The kinetics of this process in the extracellular space are unknown, but there is evidence of that large stores of this enzyme in an inactive form exist in the cortex.[20] Furthermore, a number of different mechanisms exist and can be brought into play rapidly to change the availability of enzymes and receptors on cell surfaces.[9,18]

Fig. 15. A representation of the authors' view of the processes occurring around the tip of the micropipette delivering ACh locally to the nearby recording electrode in the extracellular space of the cortex. Multiple pulses of ejection current before and after systemic injection of DFP provoke a general increase of ACh concentration accross the whole region. The upper frame of A represents the amount and the polarity of current as well as the time-course of its appearance. The thin horizontal line shows the reference (zero potential applied to the solution within the iontophoretic micropipette). The values below the reference represent the retention current, preventing the outflow of molecules of ACh from the pipette, while the values above the reference represent the ejection current forcing the molecules of ACh to leave the pipette. The values of the retention and ejection current schematically represent the fact that the ejection current is an order of magnitude higher then the retention one. The diagram does not show the fact that the duration of an application of retention current is about two orders of magnitude longer than the duration of the ejection current. The lower frame on A represents the amount of ACh delivered by respective pulses of ejection current into the extracellular space surrounding the recorded neuron. Based on the experiments and the calculations previously peformed by others[1,52,53] we presume that the amount of ACh released from the tip of the pipette grows logarithmically in the presence of the ejection current and declines exponentially when that current is replaced with the retention current. With sequential pulses of ejection current the concentration inside the tip of the pipette increases, so the basal level for the logarithmic function representing the release of the drug increases. This is shown with an increased kurtosis of the curves in their ascending parts. Upon the activation of local degradative mechanisms, the kurtoses of the curves representing the fall of ACh concentration on the onset of retention current also increases with the number of pulses. These growing levels of inactivation of ACh are also shown schematically with a change in direction of the slopes of curves representing the peak concentrations of ACh. Once the balance in delivery and degradation of the drug has been achieved (about the seventh pulse in the diagram; this is an assumption), the sequential pulses of ejection current deliver equal amounts of ACh and removal is modeled at an equilibrium as well. B shows the situation when the equilibrium in delivery and removal of ACh has already been established before the systemic injection of DFP. The increase in the extracellular level of ACh caused by DFP also follows a logarithmic curve (this is shown with solid line 3). Those levels of ACh concentration are systemically superimposed on the local pulses of ACh delivered iontophoretically (dotted line 4) and produces a gradient of a complex nature represented by solid line 1. The second set of 10 pulses of current are indicated by horizontally-oriented arrows. C represents the general sequence of gradients in ACh concentration in extracellular space surrounding the tip of the micropipette when DFP is systemically injected a reasonable period of time after the beginning of the treatment with ACh pulses. The process described by this diagram may be generalized to treatments with other drugs delivered microiontophoretically in situations when they are supplemented with a systemic treatment of similar nature. The asterisks indicate the moments when the shifts in ACh concentration are maximal (one asterisk indicates the first large gradient caused by iontophoresis; two asterisks indicate the second large gradient caused by systemic injection of DFP). These moments, in our opinion correspond to the time of initiation of oscillations in the strength of responses of neurons to ACh pulses.

Table 4. Significance levels from *t*-tests on all possible pair-wise comparisons of ongoing prestimulus activity and activity evoked by acetylcholine administration during 16 sequential trials (A–P) in a single cell recorded from an awake, undrugged rat

–	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	–	> 0.05	> 0.65	< 0.01*	> 0.60	< 0.01*	< 0.01*	< 0.01*	> 0.70	< 0.01*	> 0.15	< 0.01*	> 0.25	< 0.01*	> 0.35	> 0.20
B	< 0.02*	–	> 0.05	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.05	< 0.01*	> 0.60	> 0.05	> 0.65	< 0.05*	> 0.30	> 0.90
C	< 0.01*	< 0.01*	–	< 0.01*	> 0.90	< 0.01*	< 0.01*	< 0.01*	> 0.50	> 0.05	> 0.15	< 0.01*	> 0.20	< 0.01*	> 0.30	> 0.15
D	< 0.01*	< 0.01*	< 0.01*	–	< 0.01*	< 0.05*	> 0.05	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*
E	< 0.01*	< 0.01*	< 0.01*	< 0.01*	–	< 0.01*	< 0.01*	< 0.01*	> 0.30	< 0.01*	< 0.05*	< 0.01*	> 0.10	< 0.01*	> 0.10	> 0.10
F	< 0.01*	< 0.01*	> 0.05	> 0.15	< 0.01*	–	> 0.95	> 0.50	< 0.01*	< 0.01*	< 0.01*	< 0.02*	< 0.02*	< 0.05*	< 0.01*	< 0.01*
G	< 0.01*	< 0.01*	> 0.50	< 0.05*	< 0.01*	> 0.35	–	> 0.55	< 0.01*	< 0.01*	< 0.01*	< 0.05*	< 0.01*	< 0.05*	< 0.01*	< 0.01*
H	> 0.35	> 0.05	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	–	< 0.01*	< 0.01*	< 0.01*	> 0.05	< 0.01*	> 0.05	< 0.01*	< 0.01*
I	> 0.85	< 0.02*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.30	–	< 0.01*	> 0.20	< 0.01*	> 0.35	< 0.01*	> 0.50	> 0.25
J	> 0.15	> 0.25	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.55	> 0.15	–	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*
K	> 0.80	< 0.05*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.55	> 0.70	> 0.25	–	< 0.05*	> 0.95	< 0.01*	> 0.60	> 0.80
L	< 0.01*	< 0.01*	< 0.05*	< 0.01*	> 0.40	< 0.01*	< 0.02*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	–	> 0.05	> 0.65	< 0.02*	> 0.15
M	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.25	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.05	–	< 0.05*	> 0.70	> 0.80
N	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.02*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.30	–	< 0.01*	> 0.05
O	< 0.01*	> 0.20	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.02*	< 0.01*	< 0.05*	< 0.01*	< 0.01*	< 0.02*	> 0.05	–	> 0.55
P	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.02*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	> 0.15	> 0.55	> 0.20	–

*Significant *P* values from individual *t*-tests.
Horizontal rows (A–P), ongoing activity; vertical rows (A–P), evoked activity.

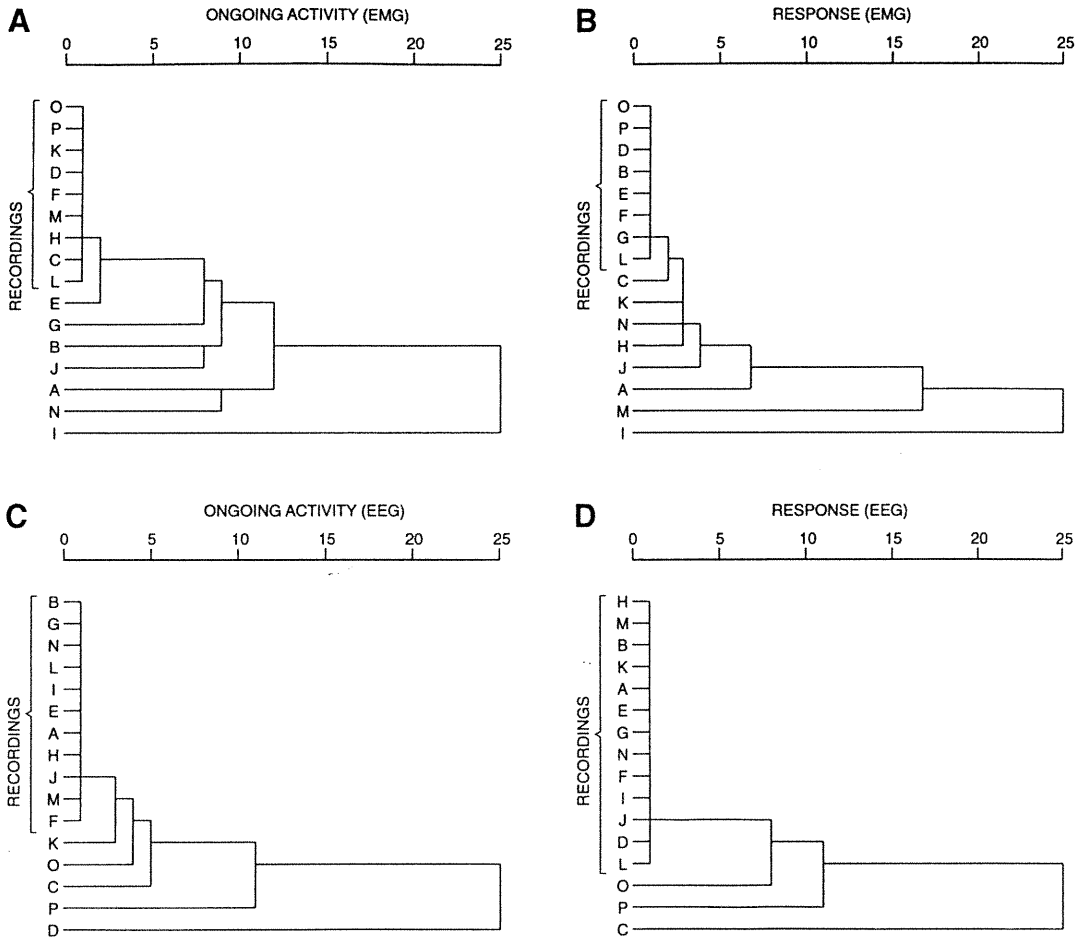

Fig. 16. Dendrograms based on cluster analyses of fast Fourier transforms of ongoing EMG activity during prestimulus periods (A) and post-stimulus, ACh-evoked response periods (B), and of ongoing EEG activity during prestimulus periods (C) and during post-stimulus, ACh-evoked response periods (D) across 16 consecutive ACh pulses (A–P) in a single neuron recorded from an awake, undrugged rat (from Fig. 7). EMG and EEG traces were transformed into power spectra (100 bin resolution) using fast Fourier transformation, divided into groups, and clustered using SPSS cluster analysis. The resulting clusters contain individual ACh pulse periods with power spectra that are similar to each other; members belonging to the same cluster differ from each other by not more than 4%. The scale at the top of each dendrogram shows the normalized distance (0–25 units) between clusters. Clusters with the greatest number of members are bracketed on the left-hand side of each figure.

Thus, the increasing rate of fall of ACh concentrations in the lower part in Fig. 15A is attributable to the activation of these mechanisms. The interplay of these factors will lead to a new equilibrium that we observed within 1 h of the beginning of the experiment. Figure 15B shows our hypothesis about the changes brought about by the systemic injection of DFP. It is assumed that the equilibrium in delivery and removal of ACh has already been established before the systemic injection of DFP. The increase in the extracellular level of ACh caused by DFP follows a logarithmic curve (line 3 in Fig. 15B). That level must add to the concentration produced locally by ACh pulses (dotted line 4) to the concentrations shown as the solid line 1 in Fig. 15B. It is evident that the pulsatile release of ACh produces extracellular concentrations that do not oscillate like the behavior observed in the neuronal responses. It is also evident that the effect of DFP must be to raise the transient ACh concentration to some new level. Since the net result of each of these perturbations of the cortical network was a very small change in the average rate of spontaneous activity of the recorded neurons and no net change of the averaged responses to pulses of ACh, we conclude that there were significant adapative processes at work in the cortex which led to a type of homeostasis of the effects of either transient or sustained increases in extracellular ACh.

Possible mechanisms underlying adaptation in the neuronal response to acetylcholine

Our observations suggest that the cholinergic system is modulated by homeostatic, dynamic mechanisms that make relatively rapid adjustments to perturbations in the concentration of extracellular ACh. A number of different cellular mechanisms exist that could

Table 5. Relationship between neuronal impulse activity (spikes), electroencephalographic, and electromyographic activity during periods of time corresponding to ongoing and acetylcholine-evoked activity across 16 trials of acetylcholine administration

	Ongoing			Evoked				
	Spikes	EEG	EMG	Spikes	EEG	EMG	(Number of pairs)	PAIRS
1	N	Y	N	N	Y	N	(20)	AF AG AL AN BN EH EJ EN FI FJ FN GI GJ GM GN IL IN JL JM JN
2	Y	Y	N	N	Y	N	(10)	AB AE AM BI BM EI GH HN IM LN
3	Y	N	N	N	N	N	(9)	AC AO AP BC CF CI CJ IO IP
4	N	N	N	N	Y	N	(8)	AD DI DJ DK DN EK KN GK
5	N	Y	N	Y	Y	N	(8)	AH AJ BH BJ HI HJ IJ MN
6	Y	N	Y	N	N	N	(7)	CK CM CO CP KO KP MO
7	N	Y	N	N	Y	Y	(6)	BE BF BG EF EG GL
8	N	N	Y	N	N	N	(5)	CD CH CL HO HP
9	N	N	Y	N	N	Y	(5)	FO FP LO DO DP
10	N	N	Y	N	Y	N	(4)	DH DM FK KL
11	Y	N	N	N	N	Y	(4)	BP EO EP LP
12	N	N	N	N	N	N	(3)	CN JO JP
13	N	N	N	N	N	Y	(2)	GO GP
14	N	N	N	N	Y	Y	(2)	BD DE
15	N	N	N	Y	N	N	(2)	CG NO
16	N	Y	Y	N	Y	N	(2)	FM HM
17	Y	N	N	Y	Y	N	(2)	AK IK
18	Y	Y	N	Y	Y	N	(2)	AI EM
19	Y	Y	Y	N	Y	N	(2)	FH HL
20	N	N	N	Y	Y	N	(1)	JK
21	N	N	Y	N	Y	Y	(1)	DL
22	N	N	Y	Y	N	N	(1)	CF
23	N	N	Y	Y	Y	N	(1)	HK
24	N	N	Y	Y	Y	Y	(1)	DF
25	N	Y	N	Y	Y	Y	(1)	EL
26	N	Y	Y	N	Y	Y	(1)	FL
27	Y	N	N	N	Y	N	(1)	BK
28	Y	N	N	N	Y	Y	(1)	DG
29	Y	N	N	Y	N	N	(1)	NP
30	Y	N	N	Y	N	Y	(1)	BO
31	Y	N	Y	N	Y	N	(1)	KM
32	Y	N	Y	Y	N	N	(1)	MP
33	Y	N	Y	Y	N	Y	(1)	OP
34	Y	Y	N	N	Y	Y	(1)	BL
35	Y	Y	N	Y	Y	Y	(1)	FG
36	Y	Y	Y	Y	Y	N	(1)	LM
37–64	All other combinations (0)							

Ongoing refers to the 4-s period prior to a given ACh pulse; Evoked refers to the 6-s period beginning at the moment of the ACh pulse.

Y (Yes) indicates that two periods of activity (ongoing or evoked) associated with two different trials, each containing a single ACh pulse (A–P) were statistically similar (based on *t*-tests for spike activity and on cluster analyses for EEG and EMG activity) to each other with respect to the parameter indicated (spikes, EEG, or EMG).

N (No) indicates that the parameter indicated was statistically different. PAIRS refers to the specific pairs of trials that meet the classification definition for each of 64 possible combinations of Y and N on the six parameters measured.

account for the initial decrease and subsequent return of responsiveness to iontophoretically administered ACh. For example, repeated administration of ACh pulses may induce a form of desensitization of membrane-bound receptors or a decrease in the release of endogenous ACh by inhibiting the muscarinic presynaptic autoreceptors located on cholinergic nerve terminals.[56] These mechanisms could contribute both to the observed development of tolerence to the ACh pulses as well as to administration of DFP. The eventual recovery in responsiveness to ACh observed in many cells may result from a delayed desensitization of inhibitory presynaptic autoreceptors.[56]

During continuous exposure to cholinergic agonists, muscarinic receptors undergo various forms of desensitization, all characterized by a decrease in muscarinic receptor activity despite increasing levels of ACh. Short-term exposure to cholinergic agonists, on the order of seconds to minutes, causes the uncoupling of the muscarinic receptor from its G-protein, resulting in a decrease in responsivity to ACh.[26] A second process, initiated shortly after agonist-induced G-protein uncoupling at the muscarinic receptor, is the sequestration of receptors in intracellular compartments where they become inaccessible to hydrophilic ligands.[45] The role of muscarinic receptor sequestration in the development of desensitization to ACh, however, is somewhat controversial.[6,24,45,55,62] Nevertheless, Yu *et al.*[63] suggested that sequestration might play a role in the resensitization of β_2-adrenergic receptors following short-term exposure to cholinergic agonists, by showing that the pharmacological blockade of sequestration reduced functional resensitization. Finally, long-term exposure to cholinergic agonists, on the order of minutes to hours, results in the down-regulation of cholinergic function by decreasing the number of cholinergic receptors available on the cell membrane. Such down-regulation can result from decreases in transcription of receptor mRNA, alterations in mRNA stability, or by degradation of receptor proteins.[45]

Implications for the pharmacological treatment of Alzheimer's disease

Treatment for Alzheimer's disease and other manipulations targeted at changing the extracellular concentrations of ACh must take into account the complex, dynamic equilibrium that apparently occurs in the neocortex. It seems quite likely that relatively rapid adjustments of degradative processes, or the masking or reduction of binding sites for ACh could minimize the effectiveness of any treatment that attempts to raise or decrease the tonic extracellular level of ACh. It is also likely that different elements of the cortical tissue will react differently to changes in the ambient level of ACh, thereby establishing a new, dynamic equilibrium in response to elevations or reductions in ambient ACh levels. Our data suggest that increasing ACh levels results in adjustments that bring the excitability of the system back towards its original level. Consequently, efforts to develop AChE inhibitors with longer half-lives than those currently available[25] may ultimately be misguided. Nevertheless, it has been suggested that AChE inhibition may produce significant improvement in cognitive function in AD patients possessing a certain genetic make-up associated with relatively greater preservation of cholinergic function compared to patients with different genetic profiles.[51]

The effectiveness of increasing levels of ACh released into the dynamic equilibrium of the cholinergic system seems to be transient; when the system is perturbed, either by increased levels of ACh administration or by reduced levels of ACh degradation, the system returns to its original level of excitability through complex adjustments presumably involving several cellular elements in the network, leaving the level of neuronal excitability relatively unchanged. These adjustments appear to return cholinergic function to levels that are comparable to pre-treatment levels, even when the ambient level of ACh has been increased dramatically. If similar adjustments occur in response to other pharmacological manipulations designed to enhance the effectiveness of ACh in disease states, the long-term effects of treatment may be considerably limited. Such reflections are consistent with the growing body of evidence that suggests that the basal forebrain is a dynamic system that plays an active role in the creation of the transient brain states associated with attentional and learning processes.[13]

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APPENDIX

To determine statistically whether variations in ongoing (prestimulus) and in ACh-evoked spike activity were unrelated to EEG and EMG, we dealt first with the discrete data (spike frequency) by comparing (with a *t*-test) the mean frequency of ongoing activity during a given prestimulus interval in a trial to each of the remaining trials. Next we repeated this procedure for mean frequency of ACh-evoked spike activity. Table 4 shows the results of all possible pair-wise comparisons on both of these measures (ongoing and evoked activity). The large number of significant comparisons (64%) suggests that both spontaneous and ACh-evoked activity varied from trial to trial, an assessment consistent with that based on a visual analyses of these data as shown in Fig. 7.

Next we subjected the prestimulus and post-stimulus intervals of EEG and EMG activity to fast fourier transformations resulting in power spectra for each interval on each trial. The power spectra were subjected to four separate cluster analyses (pre- and post-stimulus interval analyses for both EEG and EMG data). The resulting dendrograms illustrate the extent of similarity or relatedness between pairs of EEG or EMG traces on each of the 16 trials (Fig. 16). Based on the results of these cluster analyses, all possible pairs of EEG and EMG data were categorized either as having pair members that were the same (Yes) or not the same (No). Thus, just as both ongoing prestimulus activity and ACh-evoked activity were each objectively compared across trials (using *t*-tests), EEG and EMG activity during each of the same 16 trials were compared using cluster

analyses of fast Fourier transforms of these data. Finally, results from the *t*-tests on the spike data and results from the cluster analyses on the EEG and EMG data were combined (see Table 5). The data in Table 5 illustrate the inter-relationships between neuronal activity (spikes), EEG activity, and EMG activity both during the prestimulus interval and during the post-stimulus interval across each of the 16 successive trials originally shown in Fig. 7.

As shown in Table 5, the largest category of pairs of trials (row 1: 20 pairs) had the following characteristics: spike activity differed between members of each pair during both pre- and post-stimulus intervals, EEG activity did not differ between pairs during either interval, while EMG activity differed between members of each pair during both intervals. Thus, for those pairs listed (row 1), spontaneous spike activity varied from one member of each trial pair to the other, ACh administration had a different effect on spike activity for one member of each trial pair than it did on the other, and EMG activity differed from one member of each trial pair to the other, but EEG activity was similar for both members of each trial pair both during pre-and post-stimulus intervals. We concluded that the changes we observed in neural activity are not correlated with EMG and EEG activity.

GENERAL DISCUSSION

THE CEREBRAL CORTEX AS A SITE OF SYNAPTIC PLASTICITY

Evidence arguing that plasticity occurs within the cerebral cortex following repeated pairings of two innocuous sensory stimuli has been provided by sensory preconditioning experiments in adult animals. In contrast with classical conditioning, sensory preconditioning does not involve reinforcement by pain or a reward and, in the paradigms that require some form of sensory deprivation (for example whisker trimming), damage is not afflicted on the peripheral nervous system. Diamond et al. (1993, 1994) showed that trimming all but two adjacent whiskers in rats leads to a form of pairing that enhances the responses of neurons within a barrel serving one of the spared whiskers to stimulation of the other. This change appeared in the non-granular layers before it did in layer IV and affected the portion of the neuronal responses that occured later than 10 ms after the onset of whisker deflection. Since thalamocortical projections end primarily in layer IV where they activate neurons at latencies shorter than 10 ms, the authors argued that the emergence of plasticity in subcortical areas would oppose their findings.

In another case, repeatedly pairing vibratory, temporally non-coincident deflections of two non-adjacent whiskers, S1 (stimulated first) and S2 (stimulated second), was shown to increase the responsiveness of single-units located in the barrel serving S2 to the deflection of S1 (Delacour et al. 1987, 1990). This enhancement was manipulated by local microiontophoresis of pharmacological agents known to affect associative learning (Maalouf et al. 1998a). The fact that with microiontophoresis, the delivery of substances was limited to a few cells surrounding the recording site favored a cortical locus of plasticity. Finally, in experiments by Wang et al. (1994), repeated synchronous stimulation of digits 2, 3 and 4 was applied, in alternation, either to the distal or medial (or proximal in some cases) phalanges in awake monkeys. In other terms, inputs to distal segments, across fingers, always were simultaneous, as were those to the more proximal ones, but stimulations of distal and proximal segments never were temporally coincident. They

followed each other at interstimulus intervals of 200-300 ms. This procedure led to the fusion of receptive fields in the somatosensory cortex from phalanges simultaneously stimulated across different fingers and, in contrast to untrained monkeys, many units in trained animals had multiple-digit receptive fields. In addition, the cortical representations of distal and proximal segments became more sharply segregated, with the emergence of a zone that responded to the hand dorsum between them. None of these effects were obvious in the lateral division of ventral posterior nucleus of the thalamus (VPL), thus arguing again in favor of a cortical locus of plasticity.

This evidence, arguing for the CCx as a primary site of plasticity, is reinforced by two additional studies that do not use associative learning. Fox (1994) showed that trimming all but one vibrissa enlarged the cortical receptive field of the spared vibrissa to include neurons in adjacent barrels. This change could be prevented by placing lesions in the barrel serving the spared vibrissa. If changes in the cortical representation of the spared vibrissa were passively transmitted from subcortical relay nuclei, they would still take place as inputs would not pass through the spared whisker's barrel. In a study of habituation, decreased deoxyglucose uptake following increased whisker stimulation was reported only at the cortical level (Welker et al. 1992).

The results presented in the preceding paragraphs must however be interpreted with caution. First, although the observations of Diamond et al. (1993) would argue against plasticity in the principal trigeminal nucleus and the ventral posteromedial thalamus (VPM) or at thalamocortical synapses from the VPM to layer IV, they cannot exclude possible changes in synapses formed by the VPM with neurons in other layers (for example Vb) or by axons of neurons in subcortical regions such as the posterior thalamic nucleus that relay signals from the periphery at latencies longer than 10 ms (Agmon et al. 1993, 1995; Armstrong-James and Fox 1987; Armstrong-James et al. 1992; Ebner and Armstrong-James 1990; Keller 1994). A different

interpretation of the findings of Fox (1994) would be that the lesion eliminates a cortical source of excitation which is necessary for the emergence of plasticity in another brain area. The same argument can be applied in the case of Maalouf et al. (1998a) to the action of the pharmacological agents that might inhibit or enhance plasticity outside the CCx. Finally, Wang et al. (1994) did not look for possible changes in the spinal cord, brainstem or other thalamic nuclei. Although the presence of plasticity in the dorsal column nuclei (DCN) would be highly unlikely with its absence in the VPL, changes might still occur in other sections of the thalamus that relay information to the primary somatosensory cortex. Such a possibility was illustrated by Weinberger and colleagues through pupillary conditioning experiments (reviewed in Weinberger and Diamond 1987). Most cells in the magnocellular medial geniculate nucleus developed significant changes in response to an acoustic conditioned stimulus whereas none in the medial geniculate nucleus (the lemniscal line) did. So, in summary, despite some criticisms, several arguments favor the CCx as a primary site of synaptic plasticity in sensory conditioning.

MECHANISMS OF ASSOCIATIVE LEARNING

The Hebbian hypothesis

In the whisker pairing paradigm used by Maalouf et al. (1998a,b), S2 consistently follows S1, thus satisfying the contingency condition set by Hebb's covariance model. Contiguity is ensured by a 300 ms interstimulus interval that is much shorter than the inter-trial interval which lasted from 5 to 11 s. To understand, however, how this protocol enhances the responses of neurons in the barrel serving S2 to the deflection of S1, two issues need to be clarified. First, since trace conditioning was used, a procedure wherein the two stimuli never overlapped, how a simultaneous increase in both pre- and postsynaptic activity might take place is not obvious.

Second, a connection between the neurons serving S1 and those serving S2 must be demonstrated.

In anesthetized animals, an extracellularly recorded response to the deflection of a whisker consists of a single excitatory component (Simons 1983, 1985; Simons et al. 1992) although an immediately following inhibitory phase has been described intracellularly (Carvell and Simons 1988). In contrast, in the awake animal, imaging techniques that rely on voltage sensitive dyes have revealed that deflection of a vibrissa produces a more complex cortical response consisting of an alternation of excitatory and inhibitory components, starting with excitation and lasting longer than the stimulation period (Kleinfeld and Delaney 1996). Maalouf et al. (1998b) also showed that somatosensory evoked potentials (SEP) in barrel cortex of awake rats consists of alternating positive and negative components lasting at least 500 ms following stimulus onset. When recording from the center of the cortical region activated by the stimulated vibrissa, it is generally accepted that the first two components of the response reflect mainly the depolarization and repolarization of neurons directly activated by thalamocortical projections (see Martin 1991; Speckman and Elger 1993). The origins of the late components are, however, less clearly understood. One possibility is that they result from interactions of neurons in barrel cortex with those in a multitude of cortical and subcortical regions, including the cholinergic nuclei, and that these interactions represent the storage of the stimulus representation in working memory.

Baddeley (1995) defined working memory as a form of temporary storage necessary for the performance of cognitive tasks such as learning. Underlying it are probably several re-entrant circuits (Edelman 1993) that are active only when the animal is awake. The first one involves the prefrontal cortex. Neural activity is transmitted from the primary sensory areas to the posterior parietal cortex where it is projected to the dorsolateral prefrontal cortex which can re-entrantly feed neural signals back to the posterior parietal cortex. According to Desmedt and Tomberg

(1995), a functional binding is achieved by the appearance of transient and synchronous 40 Hz oscillations in both regions; this enhanced electrical activity in the dorsolateral prefrontal cortex would thus reflect the activation of somatic representations in working memory. The second circuit is based on cortico-hippocampal interactions. Following activation of the primary sensory areas by peripheral sensory stimulation, information is relayed, through the association and entorhinal cortices, to the hippocampus from where it travels back to the primary sensory regions (Fair 1992). Eichenbaum et al. (1996) argued that the parahippocampal gyri are sufficient to prolong the neural representation of external stimuli within the cerebral cortex. The hippocampus itself might be performing more complicated tasks such as the detection of regularities in the environment between two stimuli (Myers et al. 1996). A third circuit involves the re-entrant exchange of signals related to the sensory message between the thalamus and the CCx, as reflected by the appearance of synchronized, oscillatory activity around 40 Hz. Corticothalamic projections form feedback loops (Staiger et al. 1996) that have been suggested to underlie the conscious processing of sensory information and the recognition of global stimulus properties (Paré and Llinas 1995; Singer 1995; Woolf 1996). Although we do not wish to attribute our observations specifically to any of these hypothesized mechanisms, it is evident that several such processes are available to account for the maintenance of a trace of the first stimulus in the CCx.

When a whisker is deflected, the response pattern in the center of its cortical representation, described above, occurs in an attenuated form in areas of barrel cortex serving nearby vibrissae. The responses are usually less obvious and less consistent, as was shown by Kleinfeld and Delaney (1996) through optical techniques in conjunction with voltage-sensitive dyes and by Maalouf et al. (1998b) using SEPs, although the latter could still be measured as specific changes in the EEG resulting from sensory stimulation. The EEG is generated by the flow of synaptic currents through the extracellular space (Schroeder et al.1995; Speckmann and Elger

1993). The contribution of action potentials is believed to be minor because their short duration does not allow them to summate like the slower synaptic potentials (Martin 1991). Cortical SEPs consist of several components that reflect the summation of excitatory and inhibitory synaptic potentials from thousands of neurons surrounding the recording electrode, thereby indirectly providing information similar to that obtained by intracellular recordings. Several components are also influenced by events in subcortical and distant cortical regions (Pineda 1995; Sannita 1995). According to the current litterature, cortical SEPs are primarily generated by synaptic potentials in pyramidal neurons for several reasons (Birbaumer et al. 1995; Desmedt and Tomberg 1995; Schroeder et al.1995; Speckmann and Elger 1993). First, they represent around 70% of the neuronal population in the cerebral cortex (Nieuwenhuys 1994). They have larger cells bodies and more extensive dendritic arborizations. Particularly important is the apical dendritic system which integrates information from a multitude of subcortical and cortical regions and, in association with the highly elaborated axonal forward and feed back projections, may couple the electrical activities of several pyramidal neurons together (Keller 1994; Nieuwenhuys 1994). Synchronized activity leads to a higher probability of summation of synaptic potentials and is a crucial factor in the emergence of SEPs (Martin 1991). Based on all these observations, the spread of the activation from neurons in the center of the stimulated vibrissa's cortical representation is believed to be mediated by the horizontal projections of large pyramidal neurons, mainly in layer III and, to a lesser extent, in layer V (Donoghue 1995; Keller 1994; Nieuwenhyuis 1994). Pyramidal cells integrate information from all parts of their cortical columns as well as afferents originating in various regions of the brain. Through their widespread horizontal axonal projections and their contribution to reciprocal cortico-cortical connections, they are well-placed to mediate the transmission of inputs between cortical columns.

Biochemical mechanisms

The effects of whisker pairing can be modulated by local, microiontophoretic application of pharmacological agents that act upon NMDAr and NO synthase (Maalouf et al. 1998a). At the molecular level, the covariance hypothesis translates into the properties of the NMDA receptor (NMDAr; see for example Bliss and Collingridge 1993; Madison et al. 1991; Roberson et al. 1996). Activation of NMDAr requires two conditions: 1) interaction with an agonist ; 2) depolarization of the membrane on which the receptor is located. The simultaneous occurrence of these two factors removes the magnesium ion which, when the cytoplasmic membrane is at its resting potential, blocks the cation channel associated with the receptor. With the information presented in the previous sections in mind, the following Hebbian mechanism can be proposed to explain whisker pairing. The neurons located in the barrel serving S2 represent the postsynaptic element. Stimulation of S1 activates afferent fibers, releasing glutamate that subsequently interacts with NMDA receptors located on the postsynaptic membrane. Since the interstimulus interval is below 500 ms, S2 is presented when the activity of the recorded neurons is still clearly increased by stimulation of S1, leading to a stronger depolarization of the postsynaptic membrane. Thus, whisker pairing provides the conditions required for the opening of NMDA channels. A cascade of second messenger events is subsequently initiated, eventually leading to long-term potentiation (LTP) of synapses between neurons activated by the first stimulus and those activated by the second one. The ability of these horizontal connections to be potentiated by NMDA-dependant mechanisms (Glazewski and Fox 1996; Hess et al. 1996) would therefore support the finding that plasticity induced by whisker pairing involves NMDA receptors (Maalouf et al., 1998a). Many pyramidal cells also display a bursting pattern of firing (Agmon and Connors 1992; Connors and Gutnick 1990; McCormick et al. 1993; Webster et al. 1997), a process which has also been repeatedly associated with the induction of LTP.

Cristicisms of the Hebbian hypothesis

Although the Hebbian hypothesis might explain increased responses following pairing, it cannot account for the fact that certain neurons exhibit decreased or unchanged responses to S1. Unchanged responses to S1 could, on one hand, represent a failure of the animal to learn. On the other, the amount of time required by these neurons to exhibit plastic changes might be longer than the recording time. At the same time, the lack of a detectable change in spike frequency does not exclude modifications in subthreshold postsynaptic potentials. Another possibility, however, is that some types of neurons in the primary somatosensory cortex do not undergo plasticity. There are theoretical reasons to believe that unchangeable neurons may be needed to preserve a fixed representation of the basic properties of a stimulus. Indeed, Maalouf et al. (in preparation) identified a group of cells in layer III of barrel cortex that receive monosynaptic inputs from the thalamus, do not display plasticity following whisker pairing and share electrophysiological properties compatible with those of inhibitory interneurons (see below, page 106).

A failure of the animal to learn the relationship between S1 and S2 may also take the form of decreased responses to S1, as habituation develops during the course of the experiment due to factors like a lack of motivation or distraction by different stimuli. From a different perspective, decreased responses to S1 could also reflect a necessary effect of conditioning for two possible reasons. First, depression of the responses of certain classes of neurons, inhibitory interneurons for example, can lead to potentiation of the responses of different types of neurons. Second, this depression might act as a feedback control system that provides stability to cortical neural networks. Testing the adequacy of the Hebbian hypothesis as a mechanism for whisker pairing would require precise control of postsynaptic activity, for example with direct juxtacellular current application as was done by Fregnac, Shulz and colleagues in visual cortex (Fregnac et al. 1992, 1994; Shulz and Fregnac 1992) or by Cruikshank and Weinberger (1996) in auditory cortex. A possible experiment would then be to perform whisker pairing while

depolarizing or hyperpolarizing a neuron in the barrel serving S2. The use of a third whisker that is not a part of any conditioning would also be necessary to determine whether the changes in the responses to S1 following pairing are specific to the paired whisker or extend to others as part of a generalized increase in postsynaptic excitability.

THE ROLE OF ACETYLCHOLINE IN ASSOCIATIVE LEARNING

The conclusions reached in the previous paragraphs might be interpreted to mean that all the conditions necessary for plasticity to occur following associative learning are met in the CCx. This is not the case. Neural systems such as those in the CCx are characterized by a high level of stability conferred by inhibitory interneurons. Numerous studies have demonstrated that the emergence of plasticity requires counteracting inhibition, either by directly reducing it or by generally increased neuronal excitability. Both states can be accomplished by activation of cortical projections from the NBM. Some of these projecting fibers are indeed GABAergic and end on inhibitory interneurons in the CCx (Freund and Gulyas 1991). Approximately one third are cholinergic. ACh might facilitate the emergence of plasticity in the CCx in two ways: 1) through enhancement of working memory by increasing attention and arousal, thus lengthening the time during which pre-synaptic activity is increased and 2) through a direct effect on plasticity by facilitating the opening of post-synaptic NMDA channels and increasing the intracellular Ca^{2+} level to provoke induction of LTP.

Recent work combining neuropsychology and electrophysiology indicate that the NBM may play an important role in attention, a function that is significantly impaired at early stages of AD (Muff et al. 1993; Sarter and Bruno 1997; Voytko 1995). At the same time, the presence of ACh has been shown to be essential for re-entrant circuits. Granon et al. (1995) showed that injection of

cholinergic antagonists in the prefrontal cortex impairs working memory in delayed matching and non-matching to sample tasks. ACh has been proposed to shift hippocampal circuits towards a state appropriate for acquiring new inputs and preventing stored information from interfering with learning by selectively depressing transmission in intrinsic fibers while not affecting responses evoked by afferent fiber stimulation (Hasselmo 1995; Hasselmo and Bower 1993). Meck (1996) proposed a similar function for the interaction between the NBM and the prefrontal and temporal cortices and interestingly, Inglis and Fibiger (1995) found that ACh release in the prefrontal cortex and the hippocampus is increased during sensory stimulation. Moreover, ACh is one of several neuromodulators gating the transition from sleep to arousal through suppression of the low-frequency, high-amplitude oscillations generated by feedforward and feedback networks in reticular nucleus-thalamocortical circuitry during quiescent (non-REM) sleep and facilitation of fast spontaneous rythms (McCormick 1993; McCormick and Bal 1995; Steriade et al. 1993). This is supported at the cellular level by the fact that cholinergic antagonists have an inhibitory effect on the late components of sensory evoked potentials (Meador 1995; Sannita 1995).

The modulatory properties of ACh in the CNS are mediated, at least in part, by its interaction with muscarinic receptors. ACh can decrease neuronal adaptation and lengthen the duration of action potentials in pyramidal cells by decreasing several potassium (K^+) outward currents, including the voltage-dependent non-inactivating I_M, the fast-inactivating I_A and the calcium (Ca^{2+})-activated K^+ current responsible for slow after-hyperpolarization (Bernardo and Prince 1982; McCormick et al. 1993; Madison et al. 1987), and activating a voltage dependent cation non-selective inward current (Haj-Dahmane and Andrade 1996). It has also been shown to reduce inhibition by enhancing K^+ outward currents in GABAergic interneurons (see McCormick

1993). This effect is surely enhanced by the activation of GABAergic afferents from the NBM, leading to a depression of cortical inhibitory interneurons on which they end.

These same mechanisms that decrease neuronal adaptation and increase spike duration can also facilitate long term potentiation (LTP), a process believed by many to underlie memory storage. Since, under the influence of ACh, action potentials last longer, the probability and duration of opening of NMDA channels and probably voltage-dependant Ca^{2+} channels are increased, leading in turn to the entry of larger amounts of Ca^{2+} into the post-synaptic cell (Brocher et al. 1992; Segal 1992). This rise in cytoplasmic Ca^{2+} may also be achieved by muscarinic potentiation of phophoinositol turnover, resulting in the synthesis of inositol triphosphate which subsequently enhances the release of Ca^{2+} from intracellular stores (Jones 1993). The rise in intracellular Ca^{2+} would ultimately lead to the activation of the cascade of second-messenger events leading to synaptic plasticity. Consistent with this model are the findings of Rasmusson and Dykes (1988), Webster et al. (1991), Verdier (1999) and Tremblay et al. (1990a,b), who demonstrated that pairing electrical activation of the basal forebrain with hindpaw stimulation in anaesthetized rats leads to a long-term enhancement (over an hour) of cortical responsiveness to the tactile stimulus and that this enhancement requires the activation of NMDA receptors. In addition, the muscarinic antagonist atropine is sufficient to block the changes in cortical neuronal responsiveness induced by whisker pairing, an NMDA-dependent form of plasticity (Maalouf et al. 1998b).

IMPLICATIONS FOR ALZHEIMER'S DISEASE

The cerebral cortex and the basal forebrain cholinergic nuclei display characteristic

histopathologic lesions associated with extensive cell loss in AD. The severity of learning and memory impairment has been correlated with the extent of cortical and NBM degeneration (Albert 1996; Esiri 1989; Hof and Morrisson 1994; Levey 1996; Poirier et al. 1995). Interestingly, neurofibrillary tangles form primarily in the perikaryon of large pyramidal neurons located mainly in layers III and V and involved in cortico-cortical circuits (Esiri 1989; Hof and Morrison 1994). In addition, pyramidal cells, the origin of horizontal connections which have been hypothesized to be potentiated by sensory preconditioning, are specifically affected in other dementing disorders such as Down Syndrome and Pick's disease. Finally, Arendt et al. (1998) showed that neurofibrillary tangles preferentially affect areas of the brain that have retained their capacity of plastic remodeling, as assessed by the intensity of dendritic remodelling.

In view of these findings, it is understandable why treatments that aimed to restore cholinergic function only have been generally unsuccessful. To be efficient, treatments must first palliate both neocortical and cholinergic deficits. Second, they must do so in ways that respect the appropriate times and places of the enhanced neuronal processes. Consequently, it is understandable why traditional treatments that enhance cholinergic levels continuously have had a limited use (Testylier et al. 1999). AD is also characterized by degeneration in many other areas such as the hippocampus and by deficits in several neurochemical systems including noradrenaline and serotonin (Hof and Morrison 1994; Cambier et al. 1993). In this context, it would obviously be advantageous to have an experimental learning paradigm that selectively induces ACh-dependent plasticity within the cerebral cortex. Such a model could ultimately be whisker pairing and would help distinguish, if any, the specific consequences of cortical and NBM cholinergic nuclei degeneration on cognitive functions in AD, thereby facilitating the task of finding more effective treatments.

GENERAL CONCLUSION

Data supporting the hypothesis that the CCx is a primary site of ACh-dependent synaptic plasticity in associative learning have been presented in this thesis. The main findings are that sensory preconditioning of two non-adjacent vibrissae induces local changes in the responses of cortical neurons to peripheral stimuli through the activation of NMDA receptors and the inhibtion of NO synthase and that these changes require the presence of ACh during conditioning. With additional analyses, two interesting groups of neurons were isolated by their electrophysiological properties (Maalouf et al., in preparation). The first one included cells that never displayed synaptic plasticity and was hypothesized to represent a class of inhibitory interneurons. In contrast, in the second group where electrophysiological properties were believed to reflect those of pyramidal cells, plasticity did occur in some neurons.

The hypotheses presented in the discussion assume that the CCx is the center of the learning process. Weinberger (1998) proposed a similar concept, where inputs from the thalamic nuclei and the NBM converge onto the auditory cortex to produce long-term changes in neuronal responses to sensory stimuli. Although such models can be justified by the extent of cortical development in humans and the catastrophic consequences of its destruction or aberrant development on cognitive functions, they represent only a few possible interpretations of these data. One reason in particular is that the CCx and the NBM are not required for several forms of learning. Thus, much work will undoubtedly be required to understand the mechanisms of learning and memory.

Future experiments under consideration will take two parallel directions. On one side, the current findings need to be correlated with behavior, which will require the use of a complete sensory preconditioning paradigm. In other terms, whisker pairing will be followed by classical or instrumental conditioning, consisting of pairing the principal whisker (S2) with either a nociceptive stimulus (for instance an electric shock) or a reward (usually food or juice). On the other side, to reach a deeper understanding of the cortical circuits underlying synaptic plasticity

in the cortex, a more detailed knowledge of the anatomy and physiology of sensory systems is mandatory. Such a purpose may be achieved by histologic and morphologic studies (see for example Gabbott and Bacon 1996a,b) and completed by electrophysiological studies that look at changes in neuronal responsiveness throughout the CCx (see Paper 2 for the initial attempts). A new technique currently being tested involves silicone probes with muliple recording sites spread along the depth of the cortex (see Castro-Alamanco 1999). We therefore hope that results from a single experiment will reveal changes in each cortical layer and the order in which they occur.

The projects proposed in the previous paragraph represent only a few possibilities among many in a rather young but broad field of research. Our understanding of the mechanisms is undoubtedly incomplete and much work will be required to provide the insights needed to treat cognitive disorders in complex diseases such as AD or Down's syndrome.

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