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Postlethwaite, M., A. Constanti, and V. Libri. Muscarinic agonist–induced burst firing in immature rat olfactory cortex neurons in vitro. J. Neurophysiol. 79: 2003–2012, 1998. Age-related changes in pre-/postsynaptic muscarinic (mACHR) and metabotropic-glutamate (mGluR) responsiveness were studied in slices of olfactory cortex from both immature [postnatal day 16–22 (P16–P22)] and adult (≥P40) rats, using a conventional intracellular recording technique. In adult neurons, bath application of the mACHR agonist oxotremorine-M (OXO-M; 10 μM), or the selective mGluR agonist 1-aminoocyclopentane-1S-3R-dicarboxylic acid (1S,3R-ACPD; 10 μM) evoked sustained membrane depolarizations, increases in input resistance, intense repetitive firing, and the appearance of a slow poststimulus afterdepolarizing potential (sADP). Excitatory postsynaptic potentials (EPSPs) evoked by local electrical stimulation of association fiber terminals were also depressed. In contrast, in neurons from immature slices, the 10 μM OXO-M–induced membrane depolarization was followed by the appearance of spontaneous rhythmic epileptic activity, which was voltage independent and reversible on drug wash out. Epileptiform bursts were abolished or reduced by coapplication of tetrodotoxin (1 μM), atropine (1 μM), pirenzepine (100–200 nM), the N-methyl-d-aspartate (NMDA) receptor antagonist dl-amino-5-phosphonovaleric acid (dl-APV; 100 μM), the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5–20 μM), the anesthetic-sedative barbiturate pentobarbital (100 μM), or by raising the extracellular Mg2+ concentration, whereas a clear facilitatory effect was exhibited by the selective γ-aminobutyric acid-A (GABA A) receptor blocker (–)-bicuculline methiodide (10 μM). The epileptogenic effects induced by OXO-M were indistinguishable from those produced by 4-aminopyridine (4-AP; 100–200 μM), although these latter actions were unaffected by atropine. In slices from immature animals, electrical stimulation of layer III association fibers in the presence of 10 μM OXO-M was accompanied by a dramatic prolongation of evoked depolarizing postsynaptic potentials (PSPs), with the appearance of recurrent superimposed spike discharges. This effect was readily reversed on wash out of OXO-M. No comparable age-dependent differences were observed in the nature or time course of 1S,3R-ACPD–evoked pre- (or postsynaptic responses, even in immature cells where muscarinic epileptiform activity had previously been demonstrated. We suggest that the overall susceptibility toward muscarinic-induced epileptiform discharge in immature olfactory cortical neurons may depend on the functional integrity of presynaptic inhibitory mACHRs; additional contributing mechanisms were also considered.

INTRODUCTION

The mammalian piriform (primary olfactory) cortex is particularly prone to generation of epileptiform activity in a variety of chemoconvulsant models or in response to electrical stimulation of afferent fiber inputs (Hoffman and Haberly 1989, 1991; Pelletier and Carlen 1996; Piredda and Gale 1985); it is also a major center in the genesis and maintenance of kindled seizures (for review see Löschner and Ebert 1996). In vitro, paroxysmal depolarizing shifts (PDSs) (closely resembling the epileptiform events evoked by kindling in vivo) have been recorded in this neuronal region following tonic and pharmacological manipulation of olfactory cortex slices (Galvan et al. 1982; Hoffman and Haberly 1991; Libri et al. 1996b). For example, both spontaneous and electrically evoked PDSs can be observed following slice superfusion with the convulsant agent 4-aminopyridine (4-AP) (Galvan et al. 1982; Libri et al. 1996b), or by an enhancement of N-methyl-d-aspartate (NMDA)–dependent excitatory postsynaptic potentials (EPSPs) following reduction in extracellular Mg2+ concentration (Hoffman and Haberly 1989; Libri et al. 1996b). Independently of their mode of generation, PDSs can be greatly reduced by excitatory amino acid (EAA) receptor antagonists of both NMDA (Libri et al. 1996b) and non-NMDA subtypes (Pelletier and Carlen 1996), indicating a major role for these receptors in the generation and maintenance of epileptiform activity in the mature piriform cortex. Muscarinic acetylcholine receptors (mACHRs) may also play an important role in epileptogenesis in this brain region. Thus bilateral epileptic seizures have been reported following unilateral microinjection of the cholinergic agonist carbachol into the piriform cortex that were abolished by local application of the muscarinic antagonist atropine (Piredda and Gale 1985). Also, under certain conditions, such as down-regulation of γ-aminobutyric acid-B (GABA B)-receptor–mediated inhibitory synaptical processes (Malcangio et al. 1995), activation of adult olfactory cortex mACHRs may initiate atypical spontaneous cortical PDSs (Libri et al. 1996a).

In both humans and rodents, there are age-related changes in brain mACHR density, increasing rapidly after birth, reaching adult levels after ~30 days of development (Tice et al. 1996), then gradually declining with age (Blake et al. 1991). Age-dependent changes in mACHR-stimulated protein kinase C (PKC) activity and phosphoinositide (PI) turnover have also been reported both in the cerebral cortex (Tan and Costa 1995) and hippocampus (Tandon et al. 1991). In view of the well-recognized susceptibility of immature CNS to hyperexcitability and epileptogenesis (for references, see Johnston 1996; Moshè and Cornblath 1993), we investigated whether such developmental alterations may have functional consequences on the overall degree of neuronal excitability.
We previously reported that activation of mACHRs in adult olfactory cortical slices, apart from inducing a resistance increase and slow sustained depolarization of neuronal cell membranes (Constanti et al. 1993; Libri et al. 1994), also mediates a decrease in amplitude of evoked synaptic potentials, via activation of presynaptic inhibitory receptors (Williams and Constanti 1988). We therefore examined the electrophysiological properties and pre- and postsynaptic muscarinic responsiveness of piriform cortical neurons from rats at different ages, using a conventional intracellular recording technique in vitro. In addition, because metabotropic-glutamate receptor (mGluR) agonists may induce muscarinic-like excitatory postsynaptic and inhibitory presynaptic responses in several regions of the mammalian brain including the mature olfactory cortex (Constanti and Libri 1992; Constanti et al. 1993; Guérineau et al. 1995; Libri et al. 1996b, 1997; Womble and Moises 1994), we also investigated the cellular responses elicited by mGluR activation in olfactory cortical slices from immature rats, for comparison.

**METHODS**

Wistar rats of either sex were grouped according to their postnatal age (day of birth, P0) and conventionally classified as immature if they were between the postnatal days 16–22 (P16–P22) and adult (≥P40). Animals were decapitated after anesthesia with halothane and the brain rapidly removed. Transverse slices of olfactory cortex (~450 μm thick) were then prepared as previously detailed (Constanti et al. 1993) and stored in oxygenated Krebs solution at 32°C for 30 min before transferring to the recording chamber, where they were superfused continuously at ~10 ml/min with prewarmed oxygenated Krebs solution at 29–30°C. The composition of the Krebs solution was (in mM) 118 NaCl, 3 KCl, 1.5 CaCl2, 25 NaHCO3, 1 MgCl2, 6H2O, and 11 d-glucose (bubbled with 95% O2-5% CO2, pH 7.4). Conventional intracellular current-clamp recordings were made from the periamygdaloid area of the slices within the olfactory cell layers II–III, using glass microelectrodes filled with 2 M potassium acetate (tip resistances 50–70 MΩ) connected to an Axoclamp II sample-and-hold preamplifier. Orthodromic stimulation was delivered through a bipolar nichrome stimulating electrode (50 μm diam, insulated except at the tip) placed in cortical layer III, a few millimeters away from the recording electrode, to activate local association fiber terminals, projecting to layer II–III neurons. Excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively) were evoked in response to stimuli of increasing intensities (15–50 V) and constant duration (200 μs). Stimulus strength was routinely adjusted to provide a synaptic response just subthreshold for the initiation of orthodromic action potentials. Synaptic potentials recorded in control solution were averaged over five consecutive stimuli delivered at 0.033 Hz. The total response duration of synaptically evoked depolarizing postsynaptic potentials (PSPs) was measured from the stimulus artifact to return to baseline in control solution and during mACHR activation. The rise time or maximum amplitude of synaptically evoked epileptiform events [in oxotremorine-M (OXO-M) or 4-AP] could not be measured accurately due to superimposed repetitive firing activity. Most recordings remained stable for periods of 1–4 h. Current and voltage signals were recorded on a storage oscilloscope and a Gould RS 3200 ink-jet chart recorder.

In the present study, two types of spontaneous epileptiform activity were observed after mACHR activation (see Development of OXO-M–induced burst discharges in immature neurons): 1) sustained (ictalike) epileptiform discharges [termed paroxysmal depolarizing shifts (PDSs)], which were characterized by their large ampli-

tude (~15 mV), and long duration (~10 s) with multiple action potentials superimposed on a prolonged depolarizing potential and 2) spontaneous interictal discharges of varying peak amplitude (range 5–35 mV from baseline) occasionally occurred between the ictal-like discharges, consisting of brief bursts (~2 s) composed of multiple single spikes. In any given experiment, measurement of mean PDSs amplitude, duration, and spontaneous frequency (1/interburst interval) over a standard epoch time of 3 min, were made from digitized signals and chart records, respectively; these values were then used to calculate global averages ±SE over the total number of recorded cells. The duration of a PDS was measured from the time at which the voltage signal initially deflected (and remained above) the baseline, until the point of return to the preburst membrane potential; an arbitrary burst detection threshold was set at twice the peak level of the baseline noise. Interburst intervals were measured as the times between the onset of individual bursts, and burst amplitudes were taken from the baseline to the peak of the prolonged depolarizing envelope. As a measure of the synchronicity of spontaneous PDS potentials in any experiment, the coefficient of variation ([SD/mean] × 100%) of burst duration (CVb) and interburst intervals (CVi) were also measured as described by Bracci et al. (1996). These values were then used for calculation of global averages (±SE) over the total number of cells. All measurement were performed before, during, and after bath-application of pharmacological agents so that each neuron served as its own control. Data are expressed as means ± SE. Where differences between data groups were expressed as percentage (%) change versus control, statistical differences were assessed by a nonparametric Wilcoxon signed-rank test. When appropriate, statistical significance of the differences between data group means was evaluated by a standard Student’s t-test.

Drugs used in this study included oxotremorine-M iodide (OXO-M) (Semat, St. Albans, Herts, UK); 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), dl-aminophosphonic acid (dl-APV), 6-cyano-7-nitroquinoxaline (CNQX) (all from Tocris Cookson, Bristol, UK); tetrodotoxin (TTX), atropine sulphate, pirenzepine dihydrochloride (–)bicuculline methiodide, and pentobarbitone Na (all from Sigma, Poole, Dorset, UK). Drugs were routinely prepared in Krebs solution and bath-applied by superfusion (bath-exchange time ~30 s). 1S,3R-ACPD and dl-APV were predissolved in 1:1 equivalent of 100 mM sodium hydroxide (NaOH) solution, whereas CNQX was predissolved in dimethylsulphoxide (DMSO). They were frozen in 10-mM aliquots and subsequently diluted in Krebs solution immediately before use. Final bath concentrations of DMSO (up to 0.5%) or NaOH (up to 0.1%) had no deleterious effects on neuronal membrane properties or muscarinic/metabotropic responsiveness.

**RESULTS**

**Intrinsic membrane properties**

The present study is based on stable (2–4 h) intracellular recordings made from a total of 53 olfactory cortical neurons obtained from young (P16–P22; n = 44) and adult (≥P40; n = 9) rat brain slices. The electrophysiological properties and firing behavior of adult neurons recorded in these experiments were similar to those we have previously reported (Constanti et al. 1993; Libri et al. 1994), and were comparable with those reported for pyramidal cells in other cortical areas (Connors and Gutnick 1990). No statistically significant differences (P > 0.05; t-tests) were observed between immature or adult rat olfactory slices in the mean resting membrane potential (~82 ± 0.5 mV, mean ± SE), input resistance (35 ± 1.8 vs. 28 ± 1.5 MΩ), or evoked action-potential amplitude (112 ± 0.8 vs. 111 ± 1.3 mV).
of recorded neurons; however, a slow $I_{h}$-like inward rectification was seen on hyperpolarizing stimulation (−2 nA; 160 ms) in 7 of 44 immature neurons, which was not observed in adult cells ($n = 9$) (cf. Sciancalepore and Constanti 1995). When a prolonged depolarizing stimulus (1.6 s, +2 nA) was applied to the recorded neurons, a poststimulus slow afterhyperpolarization (sAHP, measured at −70 mV resting potential) of similar amplitude was observed in both immature (10.6 ± 0.7 mV) and adult cells (11.0 ± 0.6 mV).

Effects of postsynaptic mAChR/mGluR activation on neuronal excitability

As previously reported (Constanti and Libri 1992; Constanti et al. 1993; Libri et al. 1994, 1997), bath-application of a standard dose of the mAChR agonist OXO-M (10 µM; 2 min) or the selective mGluR agonist 1S,3R-ACPD (10 µM, 2 min) evoked persistent postsynaptic excitatory effects, in both immature and adult olfactory neurons. These consisted of a prolonged membrane depolarization, a small increase in input resistance, an intense neuronal discharge (~10-Hz frequency), and the appearance of a prominent slow poststimulus afterdepolarization (sADP). However, no obvious age-dependent differences were observed in the depolarization amplitude, input resistance change, or response profile of muscarinic or metabotropic-glutamate agonist-induced postsynaptic responses (Table 1).

Development of OXO-M–induced burst discharges in immature neurons

In control solution, bursts of action potentials were never evident in immature or adult neurons, either spontaneously or in response to depolarizing pulses. However, within the first 4 min of superfusion of immature olfactory slices with 10 µM OXO-M (and after the membrane depolarization was offset by applying maintained hyperpolarizing current), ~40% of neurons ($n = 16/44$) progressively developed a pronounced pattern of spontaneous synchronous activity, consisting of rhythmic, large-amplitude (mean 13.1 ± 1.6 mV) bursts of action potentials and oscillations (ictal events) lasting for ~10 s (mean 9.5 ± 1.2 s), often separated by prolonged membrane afterhyperpolarizations and occurring at a frequency of between 0.5 and 7 bursts/min (mean interburst interval, 23.4 ± 3.7 s; Fig. 1A). The average values for $C_{V o}$ and $C_{V i}$ were 31 ± 6% and 34 ± 5%, respectively, indicating an irregular burst duration and occurrence. Ictal discharges were usually followed by an ~30-s period (mean, 32.6 ± 6.3 s), during which the cell was either quiescent ($n = 11$), or spontaneous interburst spike discharges (interictal events; 5–50 mV amplitude; ~2 s duration) occasionally occurred ($n = 5$; see Fig. 2). No apparent relationship was observed between membrane potential (measured in current-clamp mode between −65 and −105 mV, $n = 4$, or in voltage-clamp mode between −70 and −90 mV; $n = 3$) and the frequency or duration of OXO-M–induced seizure discharges, suggesting they were independent of intrinsic membrane properties (Fig. 1, B and C). In view of the intense repetitive firing or occasional ‘‘depolarization block’’ observed at the peak of the OXO-M depolarization, no bursting activity could be reliably measured in immature cells without prior repolarization of the cell; however, it was clear that underlying bursts were still occurring at the more depolarized level albeit at a reduced amplitude. Because of the ongoing epileptiform activity, no poststimulus sADP could be accurately measured in the same neurons during mAChR activation. These effects remained relatively stable as long as OXO-M was present (~2 h) and were reversible on wash out (~5–10 min). In contrast, no such bursting activity was ever recorded from adult olfactory cortical neurons in the presence of OXO-M (up to 20 µM; $n = 9$).

As previously reported for adult cells (Libri et al. 1994), only immature olfactory cortical neurons encountered in deep layer III were capable of generating a prolonged neuronal excitation in response to mAChR activation (i.e., sustained membrane depolarization) and of these recorded neurons, 16 of 20 cells developed the above-described pattern of spontaneous epileptiform discharges. In the remaining four cells, rhythmic bursts induced by OXO-M appeared either as low amplitude (8.3 ± 0.9 mV), long duration (mean 66.9 ± 6.1 s), ictal-like spike discharges (frequency ~0.5 bursts/min, $n = 3$), or as a periodic increase in baseline noise ($n = 1$). On the contrary, cells identified electrophysiologically as superficial pyramidal neurons ($n = 10$) (Constanti et al. 1993; Libri et al. 1994) showed little or no sensitivity to mAChR activation and generated a somewhat weaker pattern of spontaneous activity, consisting of ictal bursts of low amplitude (mean 7.6 ± 1.6 mV), long duration (mean 34.8 ± 8.3 s), and slow frequency (0.3–1 bursts/min), with isolated spike discharges during the inactive period. Interestingly, although mGluR activation produced similar depolarizing effects to those of OXO-M, spontaneous epileptiform potentials were never observed during superfusion of immature neurons with 1S,3R-ACPD (10–50 µM; $n = 6$), even in cells where bursts had previously been demonstrated in the presence of muscarinic agonist; this suggested that mAChR sites were primarily involved in their generation.

Pharmacology of spontaneous burst discharges

After 5 min in OXO-M, the frequency of spontaneous discharges had usually stabilized, and neurons (deep or superficial type; $n = 30$) were recorded for a further 15–20 min before application of a drug; cells in which OXO-M failed to generate strong excitatory effects and any type of spontaneous epileptiform activity ($n = 14$) were regularly discarded. Established OXO-M–induced epileptiform bursts (both ictal and interictal events) completely disappeared on adding 1 µM TTX to bathing medium ($n = 3$; Fig. 2A) or by raising the extracellular Mg$^{2+}$ concentration to 5 mM (5 times normal; $n = 3$; Fig. 2B), suggesting they were synaptically mediated. Bursts were also regularly abolished after addition of atropine (1 µM; $n = 3$; Fig. 2C) or the $M_1$-receptor antagonist pirenzepine (100–200 mM; $n = 3$; not shown) (Williams and Constanti 1988), confirming their muscarinic nature. In the presence of the selective NMDA receptor antagonist d-APV (100 µM, $n = 3$), ictal bursts disappeared and were replaced by spontaneous interictal discharges, whose frequency progressively increased (mean frequency, 54 ± 9 spikes/min; Fig. 3A). A similar effect was produced in the presence of the non-NMDA receptor
antagonist CNQX, at a concentration (5 μM; n = 2) known to be effective in blocking non-NMDA responses without interfering with NMDA receptors (Collingridge and Lester 1989; Monaghan et al. 1989) (Fig. 3B). In contrast, synchronous activity (both ictal and interictal events) could no longer be detected during bath application of 20 μM CNQX (n = 2; Fig. 3C). Ictal bursts were also reversibly reduced in amplitude (3.0 ± 1.0 mV, significantly different from control; P < 0.01, t-test) but not duration (13.7 ± 4.1 s), and interictal bursts were abolished, during superfusion with the anesthetic-sedative/GABAA receptor modulator pentobarbitalone (100 μM; n = 3; Fig. 4A) (Cullen and Martin 1984; Schulz and MacDonald 1981). In the four neurons where the OXO-M–induced rhythmic activity was not fully developed, application of the selective GABAA receptor blocker (−)-bicuculline methiodide (10 μM, n = 4) caused full amplitude spontaneous bursts to appear (Fig. 4B1); subsequent exposure to atropine (1 μM; n = 3) reduced the epileptiform discharges to a pattern of spontaneous synchronous activity (consisting of repetitive depolarizations with overriding action potentials; Fig. 4B2), which were similar to those elicited by (−)-bicuculline alone (10 μM; n = 3;
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FIG. 3. A: muscarinic-induced epileptiform events require activation of N-methyl-D-aspartate (NMDA) receptors. Application of DL-APV (100 μM) during OXO-M–induced activity, led to block of recurring bursts and the appearance of spontaneous interburst discharges (period indicated by * was recorded at 2 times chart speed). A normal pattern of bursting activity returned rapidly after wash out of APV. Initial membrane potential, −83 mV (P22 neuron). B and C: block of non-NMDA receptors can also interfere with epileptiform activity induced by OXO-M. In B (P22 neuron; membrane potential, −83 mV), addition of a low dose of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 μM) during established bursting led to a reduction in incidence of ictal bursts, and an increase in frequency of interictal discharges. In C (P21 neuron; membrane potential, −80 mV), rhythmic bursts were completely blocked by adding 20 μM CNQX (affecting both non-NMDA and NMDA receptors). In both cells, normal bursting eventually reappeared on wash out of CNQX (40 min). Calibration bars apply to all traces.

Effects of presynaptic mACHR/mGluR activation on evoked synaptic potentials

The muscarinic and metabotropic-glutamate modulation of synaptic transmission in both immature and adult rat olfactory cortex slices was also compared. In adult slices, control (n = 9), focal stimulation (10–30 V; 0.2 ms) of the local association fiber terminals in layer III produced a characteristic EPSP/IPSP sequence in recorded neurons, consisting of a short latency (glutamate-mediated) EPSP (mean 18.0 ± 1.8 mV peak amplitude) with a threshold for triggering an action potential at around −65 mV, followed by an early GABA_A receptor–mediated fast (depolarizing) IPSP and a late GABA_B receptor–mediated slow (hyperpolarizing) IPSP (Libri et al. 1996a; Malcangio et al. 1995) (Fig. 5); the control mean duration of the total depolarizing PSP was 98.8 ± 9.6 ms (range 60–160 ms). No repetitive spike discharge superimposed on the depolarizing PSP was ever evoked on electrical stimulation, even after stimulus not shown). This suggests that OXO-M and bicuculline could act synergistically to induce spontaneous burst firing.

FIG. 4. A: OXO-M–induced burst discharges are reversibly reduced in amplitude (but not abolished) after application of the anesthetic-sedative barbiturate pentobarbitone (100 μM). Initial membrane potential, −86 mV (P22 neuron). B1: blockade of γ-aminobutyric acid-A (GABA_A) receptors can reveal muscarinic spontaneous discharges. In this neuron (P20; membrane potential, −77 mV), initial application of 10 μM OXO-M induced only small amplitude interictal discharges. B2: coapplication of bicuculline (10 μM) then revealed full-amplitude spontaneous bursts that were subsequently blocked by atropine (1 μM; bicuculline present throughout B1 and B2).
FIG. 5. Postsynaptic potentials (PSPs) recorded intracellularly in adult or immature olfactory cortical neurons in response to subthreshold orthodromic stimuli (5–30 V; 0.2 ms) delivered to local association fiber terminals. The PSP normally consisted of an early excitatory postsynaptic potential (EPSP) and fast (depolarizing) inhibitory postsynaptic potential (IPSP), followed by a late slow (hyperpolarizing) IPSP. Top: recordings made from an adult neuron (P36) in control solution and after bath application (2 min) of 10 μM OXO-M or 1S,3R-ACPD, respectively; note both agonists markedly reduced the PSP amplitude. Bottom: similar experiments carried out on an immature neuron (P19). Bottom, a: whereas 1S,3R-ACPD strongly depressed the PSP amplitude (as in the adult), OXO-M now induced a pronounced prolongation of the depolarizing PSP, with numerous superimposed spike discharges. Bottom, b: in a different neuron maintained at a resting potential of −90 mV by current injection (set from −84 mV to avoid orthodromic spiking), OXO-M induced a similar prolongation of the evoked PSP, which now showed characteristic irregular oscillations in membrane potential. Cell membrane potentials are indicated on the left; a 30-min wash out period was allowed between each drug superfusion.

Significant age-dependent differences were also observed in the muscarinic (but not metabotropic-glutamate) modulation of evoked synaptic potentials. As expected from previous reports (Libri et al. 1996b, 1997), in adult neurons the peak PSP amplitude evoked at −80 mV membrane potential was significantly reduced in the presence of 10 μM OXO-M (70.5 ± 5.6%; P < 0.01; n = 6) or 10 μM 1S,3R-ACPD (42.3 ± 11.5%; n = 5; both corrected for change in membrane potential; Wilcoxon signed-rank tests; Fig. 5, top). No desensitization of the synaptic muscarinic or metabotropic-glutamate–mediated effects was observed during application periods up to 30 min, and evoked PSPs returned to their control amplitudes on wash out of either agonist (5–10 and 20–30 min, respectively). On the contrary, in immature animal slices, OXO-M induced a pronounced prolongation (≥10 s) of electrically evoked PSPs that now exhibited recurrent superimposed spike discharges and potential oscillations (Fig. 5, bottom, a). A similar characteristic prolongation was also observed in cells that were previously hyperpolarized to a more negative membrane potential (−90 mV; n = 3) by steady current injection, to avoid orthodromic activation of repetitive action potentials (Fig. 5, bottom, b). This showed that intrinsic voltage-dependent postsynaptic conductances activated by cell firing were unlikely to be involved in sustaining the prolonged synaptic potential. These effects were readily reversed on wash out of OXO-M (5–10 min). 1S,3R-ACPD (10 μM) on the other hand, consistently caused a reversible inhibition of stimulus-evoked synaptic responses (50.7 ± 7.1%; P < 0.01; Wilcoxon test; n = 6), which was not significantly different from that observed in adult slices (P > 0.05; t-test); PSP superimposed spike discharges were thus never observed during mGluR activation (Fig. 5).

Comparison with effects of 4-AP

The convulsant drug 4-AP (100 μM, n = 2 or 200 μM, n = 4) induced both spontaneous and stimulus-evoked bursting activity that was indistinguishable from the cellular responses produced in immature rat olfactory cortex by 10
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\[ \text{FIG. 6. } \]

A: exposure of an immature olfactory neuron (P22) to the convulsant drug 4-aminopyridine (4-AP; 100 \( \mu \)M) induces a pattern of spontaneous epileptiform activity similar to that induced by muscarinic receptor activation, that is unaffected by coapplication of 5 \( \mu \)M atropine (cf. Fig. 2). B: evoked synaptic potentials recorded in the same neuron in control solution and in the presence of 100 \( \mu \)M 4-AP (10 min); note the dramatic prolongation of the depolarizing PSP, with superimposed repetitive spikes comparable with those induced by OXO-M (cf. Fig. 5).

\( \mu \)M OXO-M (Fig. 6), and also resembled the epileptiform discharges previously seen in adult guinea pig olfactory cortical neurons treated either with 4-AP or a low extracellular Mg\(^{2+}\) concentration (Libri et al. 1996b). The minimal effective concentration of 4-AP required to induce such activity was 10 \( \mu \)M (\( n = 3 \)). Interestingly, during continuous superfusion with 10 \( \mu \)M 4-AP, application of 10 \( \mu \)M OXO-M produced a further increase in burst frequency (\( n = 2 \)), suggesting additive effects. However, in the presence of atropine (1–5 \( \mu \)M), the epileptogenic effects induced by 10 \( \mu \)M OXO-M alone were invariably abolished, whereas those of 200 \( \mu \)M 4-AP were still present (\( n = 3 \)), indicating that they were not directly due to muscarinic receptor activation.

DISCUSSION

Muscarinic (but not metabotropic)-agonist–induced burst firing

The principal result of the present study is that in olfactory cortex slices prepared from immature rats (P16–P22), superfusion with the mAChR agonist OXO-M (but not the mGluR-selective agonist 1S,3R-ACPD), induced a progressive development of both spontaneous rhythmic and electrically evoked epileptiform activity that was not seen in olfactory slices prepared from adult rats (\( \geq P40 \)). Despite the contrasting ability of OXO-M and 1S,3R-ACPD to induce epileptiform bursts, muscarinic and metabotropic-glutamate agonist–evoked depolarization responses recorded in immature neurons were apparently indistinguishable from those elicited in adult rat neurons in the present study, or those previously reported in guinea pig olfactory cortex cells (Constanti et al. 1993; Libri et al. 1996b, 1997). Such epileptiform bursts were thought to be driven by a polysynaptic network, because they were completely abolished on adding TTX to the bathing medium or by raising the extracellular Mg\(^{2+}\) concentration. In addition, the duration and frequency of the bursts were independent of membrane potential, suggesting that they were generated by neuronal elements remote to the recorded cell soma. Bursting activity was also suppressed by atropine (thereby confirming the involvement of mAChRs) and significantly shortened by the selective NMDA receptor antagonist CNQX, suggesting that both subclasses of ionotropic glutamate receptors were necessary for its expression in the immature rat olfactory cortex. Furthermore, a similar protective action against muscarinic agonist-mediated epileptogenesis was exhibited by the anesthetic-sedative barbiturate pentobarbitalone (most likely by augmentation of GABA\(_{A}\) receptor–mediated inhibition and possible suppression of glutamate-mediated excitation (Cullen and Martin 1984; Schulz and MacDonald 1981)), whereas the selective GABA\(_{A}\) receptor antagonist (–)-bicuculline methiodide exhibited an epileptogenic facilitatory effect. These results suggest that, in addition to a direct excitatory postsynaptic action of OXO-M (e.g., sustained membrane depolarization, increase in input resistance, and inhibition of firing accommodation), release of endogenous neurotransmitter(s) was required for the occurrence of rhythmic bursts. A similar induction of epileptiform activity has been previously shown to be produced by carbachol in adult hippocampal neurons, in the presence of ionotropic glutamate and GABA\(_{A}\) receptor blockers (Bianchi and Wong 1994), and more recently in immature hippocampal slices (Psarropoulou and Dallaire 1998). Also, cholinergically triggered seizures and burst firing have been reported in many areas of the adult brain, including the cerebral cortex (Cruickshank et al. 1994; MacVicar and Tse 1989; McCormick and Prince 1986). On the contrary, carbachol has been reported to raise the threshold for picrotoxin-induced epileptiform firing in the developing rat neocortex (Sutor and Hablitz 1989). To our knowledge, the present study represents the first report of such a profound difference between the muscarinic responsiveness of adult and immature animal brain slices including the appearance of mAChR agonist-induced epileptiform activity in an area of the brain in which rhythmic bursts of action potentials are never normally recorded in adult slices, either spontaneously or in response to mAChR activation.

Age-related difference in presynaptic muscarinic effectiveness: functional significance?

The present results have also revealed that in contrast to the postsynaptic mAChR agonist–induced neuronal excitation (which is already apparently an early stage of development), the presynaptic mAChR agonist–mediated inhibition...
of synaptic transmission in the rat olfactory cortex may be lacking during the first few weeks of postnatal life. This result is consistent with previous studies showing that muscarinic agonist–induced depression of field EPSPs is significantly reduced in hippocampal slices of neonatal animals, as compared with adults (Milburn and Prince 1993). On the contrary, no obvious age-dependent differences were observed in the mGluR agonist–induced inhibition of synaptic transmission. Thus, although there may be developmental changes in the density and/or efficacy of presynaptic inhibitory mACHRs, the metabotropic-glutamatergic system in the young rat olfactory cortex seems to operate in a manner similar to that observed in the adult. Indeed, muscarinic cholinergic receptor levels in the brain are generally known to increase from birth to around P30, when they reach adult density (Tice et al. 1996). Also, a delayed development of functional presynaptic mAChRs has been suggested to occur during the first few weeks of life (Milburn and Prince 1993; Vaknin and Teyler 1991). What could be the functional significance of such a lack of presynaptic muscarinic inhibitory mechanisms? Developing neurons are known to require an excitatory drive to allow neuritic outgrowth and development of cell-to-cell connections (Holliday and Spitzer 1990; Kater et al. 1988). During prenatal and early postnatal stages of ontogenesis, neuronal proliferation is thought to be important for functional synaptic formation (for references see Sutor and Luhmann 1995); the absence or a delayed development of presynaptic inhibitory mAChRs may therefore be relevant for these effects. On the other hand, the lack of inhibitory control over excitation would be expected to increase seizure vulnerability and facilitate the appearance of seizure-like activity in immature animals (Sutor and Luhmann 1995). Other mechanisms that could also be involved in the enhanced susceptibility to epileptic discharge at early postnatal stages, e.g., a predominance of EAA-receptor–mediated cortical excitation (Insel et al. 1990; Tamaru et al. 1991) and a delayed maturation of GABAergic synaptic transmission (Agmon and O’Dowd 1992; Sutor and Luhmann 1995), cannot presently be excluded.

mACHR-mediated prolongation of evoked PSPs in immature neurons

In control medium, evoked depolarizing PSPs recorded in immature olfactory neurons displayed a duration of up to 200 ms (in contrast to ~100 ms in mature neurons) and occasionally (35% of recorded cells) showed several superimposed spike potentials. Similar synaptic responses have been recorded in other brain areas during the first three postnatal weeks (Burgard and Hablitz 1993; Kim et al. 1995). Depolarizing PSPs in young olfactory neurons were also followed by slow IPSPs of smaller amplitude than those recorded in adult cells at the same membrane potential, suggesting a deficiency of either presynaptic GABA release or postsynaptic GABA-receptor mechanisms at this stage of postnatal development. In the presence of OXO-M, evoked PSPs in immature neurons underwent a dramatic increase in duration (with the appearance of superimposed repetitive spike discharges and potential oscillations), rather than a pronounced decrease in amplitude as seen in adult neurons (Williams and Constanti 1988). It is unlikely that this effect could be attributed to the rather small (~20%) accompanying increase in membrane input resistance, because this was similar in both immature and adult cells (Table 1). Furthermore, the involvement of potential-dependent postsynaptic conductances (activated by cell firing) in sustaining the prolonged synaptic response could also be excluded, because a comparable effect was observed in immature cells in the absence of orthodromic repetitive action potentials. Unfortunately, no specific agonists and antagonists yet exist that would allow a clear dissection of the above observed pre- and postsynaptic muscarinic effects.

Although activation of mGluRs or mAChRs in adult olfactory neurons produced similar postsynaptic excitations and presynaptic inhibitions, as previously reported (Libri et al. 1997) in immature cells, 1S,3R-ACPD evoked a typical prolonged neuronal excitation, but spontaneous epileptiform burst discharges could never be detected. In addition, activation of presynaptic mGluRs always resulted in a depression (rather than prolongation) in synaptic transmission, and no PSP-superimposed spike discharges could be recorded. It is worth noting that mAChR (M₁) and mGluR (group I) subtypes believed to be involved in mediating similar postsynaptic excitatory responses in the olfactory cortex are both coupled to an increase in PI hydrolysis (Constanti et al. 1993; Libri et al. 1997); by contrast, the mAChR and mGluR subtypes mediating the suppression of olfactory cortical synaptic transmission are thought to be PI-coupled M₁ mAChR receptor and two distinct group II and III mGluR receptors (negatively linked to cAMP formation) (Libri et al. 1997; Williams and Constanti 1988). It is conceivable that in the immature olfactory cortex, pre- and postsynaptic mAChRs develop at different rates; the apparent decrease in efficacy of presynaptic inhibitory mAChRs may then reflect a low density or uncoupling of specific PI-linked G-proteins, as proposed for other neurotransmitter systems (Gaiaresa et al. 1995).

### Table 1. Comparison of muscarinic and metabotropic response properties of immature and adult rat olfactory cortex neurons

<table>
<thead>
<tr>
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<th>Immature (P16–P22)</th>
<th>Adult (≥P40)</th>
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<tbody>
<tr>
<td>OXO-M depolarization, mV</td>
<td>11.8 ± 0.8 (20)</td>
<td>11.4 ± 1.6 (9)</td>
</tr>
<tr>
<td>%Change in input resistance in OXO-M</td>
<td>19.6 ± 2.4 (20)</td>
<td>11.8 ± 2.6 (9)</td>
</tr>
<tr>
<td>1S,3R-ACPD depolarization, mV</td>
<td>9.0 ± 1.1 (6)</td>
<td>8.7 ± 1.3 (4)</td>
</tr>
<tr>
<td>%Change in input resistance in 1S,3R-ACPD</td>
<td>14.5 ± 2.9 (6)</td>
<td>19.7 ± 2.6 (4)</td>
</tr>
<tr>
<td>sADP amplitude in OXO-M, mV</td>
<td>n.m.</td>
<td>11.6 ± 1.2 (4)</td>
</tr>
<tr>
<td>sADP amplitude in 1S,3R-ACPD, mV</td>
<td>8.0 ± 0.8 (6)</td>
<td>6.7 ± 1.3 (4)</td>
</tr>
</tbody>
</table>

Data are means ± SE with number of neurons in parentheses. Depolarizations to oxotremorine-M (OXO-M, 10 μM, 2 min) or 1-aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD, 50 μM; 2 min) were measured from ~70 mV membrane potential. Changes in input resistance and slow afterdepolarization (sADP) amplitude were calculated after correcting for agonist change in membrane potential. sADPs were evoked by a standard 1.6 s, ±2 nA current pulse. Corresponding mean values from immature and adult neurons were not significantly different from each other (P > 0.05, r-tests). P16–P22, postnatal day 16–22; n.m., not measurable due to induced bursting activity (see text).
Comparison with the effects of 4-AP

The novel prolongation of the evoked depolarizing PSP seen in immature neurons in the presence of OXO-M, was comparable with that produced by 4-AP in the present experiments, or in adult guinea pig olfactory cortex slices (Galvan et al. 1982; Libri et al. 1996b). Muscarinic-induced burst discharges were also indistinguishable from those produced by this convulsant agent. We therefore considered the possibility that 4-AP and OXO-M might bind to the same receptor site and thereby inactivate the same presynaptic potassium conductance. In a cardiac preparation, atropine completely blocked the electrophysiological effects of 4-AP (Urquhart and Broadley 1991). However, in our experiments, the effects of OXO-M could be invariably abolished by coapplication of atropine (1 μM), whereas those of 4-AP were unaffected, suggesting that a common receptor mechanism of action for OXO-M and 4-AP was unlikely.

Mechanism of spontaneous burst generation

Based on our observations, the mechanism by which OXO-M promotes epileptiform activity in the immature olfactory cortex seems to require activation of mAChRs as well as NMDA and non-NMDA receptors, suggesting that the phenomenon is multifactorial. It is also not possible at present to conclude on the precise site of origin and mechanism of spread of the epileptiform bursts; however, we suggest that it may initially involve selective excitatory effects on a distinct population of neurons located in deeper cortical layers (Constanti et al. 1993; Libri et al. 1994), which are then propagated to adjacent cells within the piriform cortical network via intrinsic association fibers (Haberly 1990; Haberly and Bower 1984). It is noteworthy that in the present experiments, only deep layer III olfactory cortical neurons were capable of generating fully developed postsynaptic burst discharges in response to mACHr activation, whereas cells electrophysiologically identified as superficial pyramidal neurons (Constanti et al. 1993; Libri et al. 1994) generated a much weaker pattern of epileptiform activity, on pharmacological stimulation. This could reflect the existence of relatively stronger associative synaptic connections between deep neuronal populations in these slice preparations, compared with those between deep and superficial cells (Forti et al. 1997). Also, we cannot exclude the possibility that epileptiform activity was synaptically driven by deep cells of the subjacent endopiriform nucleus (proposed layer IV of the piriform cortex) that are known to possess spontaneous bursting properties (Hoffman and Haberly 1991; Pelletier and Carlen 1996).

Several lines of investigation now suggest that neonates and infants are more prone to epileptic seizures than adults (Freeman 1982; Holden et al. 1982; Mellits et al. 1982); in addition, certain types of epilepsy are seen only during specific developmental periods (Kellaway et al. 1979). Likewise, experimental studies of various models of epilepsy have indicated that critical periods of seizure susceptibility also exist in animals (for references see Johnston 1996; Moshè and Cornblath 1993). Our in vitro findings would therefore emphasize the importance of mAChRs in epilepsygenesis, particularly at early stages of postnatal development; they would also be consistent with the reported occurrence of absence seizures in epileptic children, following olfactory stimulation (Komárek 1994). We propose that the absence, delayed maturation, or change in function of presynaptic mAChRs during the first few weeks of life could contribute to or account for this enhanced susceptibility toward epileptiform discharge generation. Whether this preclinical profile can be usefully exploited in humans, however, remains to be seen.

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