DEVELOPMENTAL CHANGES IN PRESYNAPTIC MUSCARINIC MODULATION OF EXCITATORY AND INHIBITORY NEUROTRANSMISSION IN RAT PIRIFORM CORTEX IN VITRO: RELEVANCE TO EPILEPTIFORM BURSTING SUSCEPTIBILITY

B. J. WHALLEY* AND A. CONSTANTI
Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK

Abstract—Suppression of depolarizing postsynaptic potentials and isolated GABA-A receptor-mediated fast inhibitory postsynaptic potentials by the muscarinic acetylcholine receptor agonist, oxotremorine-M (10 μM), was investigated in adult and immature (P14-P30) rat piriform cortical (PC) slices using intracellular recording. Depolarizing postsynaptic potentials evoked by layers II–III stimuli underwent concentration-dependent inhibition in oxotremorine-M that was most likely presynaptic and M2 muscarinic acetylcholine receptor-mediated in immature, but M1-mediated in adult (P40-P80) slices; percentage inhibition was smaller in immature than in adult piriform cortex. In contrast, compared with adults, layer Ia-evoked depolarizing postsynaptic potentials in immature piriform cortex slices in oxotremorine-M, showed a prolonged multiphasic depolarization with superimposed fast transients and spikes, and an increased ‘all-or-nothing’ character. Isolated N-methyl-o-aspartate receptor-mediated layer Ia depolarizing postsynaptic potentials (although significantly larger in immature slices) were however, unaffected by oxotremorine-M, but blocked by DL-2-amino-5-phosphonovaleric acid. Fast inhibitory postsynaptic potentials evoked by layer Ib or layers II–III-fiber stimulation in immature slices were significantly smaller than in adults, despite similar estimated mean reversal potentials (~69 and ~70 mV respectively). In oxotremorine-M, only layer Ib-fast inhibitory postsynaptic potentials were suppressed; suppression was again most likely presynaptic M2-mediated in immature slices, but M1-mediated in adults. The degree of fast inhibitory postsynaptic potential suppression was however, greater in immature than in adult piriform cortex. Our results demonstrate some important physiological and pharmacological differences between excitatory and inhibitory synaptic systems in adult and immature piriform cortex that could contribute toward the increased susceptibility of this region to muscarinic agonist-induced epileptiform activity in immature brain slices. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: piriform cortex, oxotremorine-M, presynaptic muscarinic modulation, depolarizing postsynaptic potentials, fast inhibitory postsynaptic potentials, development.

Epilepsy is a serious, chronic neurological disorder affecting ~1% of the world’s population (Laidlaw et al., 1988), that manifests as recurrent spontaneous convulsions and/or a loss of consciousness, caused by the appearance of abnormal electrical seizure discharges in the brain. Seizures are characterized by episodic high frequency firing by a population of cortical neurones, typically resulting from excitatory and inhibitory synaptic imbalances, that begin, and may stay, in a specific area and/or spread to other brain regions (Ebert et al., 1995). A brain area particularly prone to epileptogenesis is the piriform (primary olfactory) cortex (PC) (Loscher and Ebert, 1996), a phylogenetically old structure located rostrally on each side of the cerebral cortex, that assimilates odor-encoded information into learning and memory (Hasselmo et al., 1992). The PC consists of three principal layers (LI–III) and the underlying endopiriform nucleus (layer IV) (Tseng and Haberly, 1989a) (Fig. 1). Layer Ia (Lia) contains lateral olfactory tract (LOT) afferents synapsing with apical dendrites of superficial (LI) and deep (LIII) pyramidal neurones, while layer Ib (Lib) contains inhibitory interneurones (Satou et al., 1983b) and association fibers (arising from basal dendrites of superficial pyramidal neurones) connecting to the pre-frontal cortex, amygdala, entorhinal and perirhinal cortices (Luskin and Price, 1983). Layers II–III (LII–III) contain intrinsic fibers (arising from basal dendrites of deep pyramidal cells), that pass rostrally or caudally, to provide excitatory feedback or feedforward modulatory stimuli (Luskin and Price, 1983). The learning and memory functions of the PC are subject to muscarinic cholinergic modulation (Saar et al., 2001) and require intrinsic oscillatory networks (Chabaud et al., 1999), a characteristic known to predispose this and other brain regions to epileptogenesis (Liljenstrom and Hasselmo, 1995; Loscher and Ebert, 1996).

We recently described the developmental properties of epileptiform seizure activity recorded in immature PC (LII–III) brain slice neurones in vitro, during exposure to the muscarinic acetylcholine receptor (mACHR) agonist, oxotremorine-M (OXO-M) (Whalley et al., 2005; Postlethwaite et al., 2006)
waite et al., 1998), the age-dependent nature of which was of particular interest, since epilepsy is one of the most common childhood neurological disorders (Gardiner, 1999). Previously (Whalley et al., 2005), we investigated some age-related postsynaptic mechanisms that could be contributing to the increased seizure susceptibility of the immature PC. Here, we examined whether developmentally-dependent changes in local excitatory and/or fast (GABA type A receptor (GABA<sub>A</sub>)-mediated) inhibitory neurotransmission were also occurring in the PC, further predisposing this area toward seizure generation. In addition, we compared the sensitivity of depolarizing postsynaptic potentials (DPSPs) and fast inhibitory postsynaptic potentials (fIPSPs) to muscarinic modulation in adult and immature PC slices, to reveal possible age- and/or layer-dependent effects. We now describe a number of novel physiological and pharmacological differences between the excitatory and inhibitory synaptic systems of adult and immature PC that could, in conjunction with the age-dependent postsynaptic neuronal changes and muscarinic responsiveness already described (Whalley et al., 2005), further contribute toward the induction of muscarinic epileptiform bursting in this brain area.

**EXPERIMENTAL PROCEDURES**

**Preparation and maintenance of slices**

All experiments were carried out in accordance with Home Office regulations (Animals (Scientific Procedures) Act 1986) and conformed to international guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering. Rostrocaudal PC brain slices (~450 μm thick) were prepared from male and female adult (P40 to P80) and immature (P14 to P30) Sprague–Dawley rats, as previously described (Whalley et al., 2005). Slices were transferred to the recording chamber and continuously superfused (~10 ml min<sup>−1</sup>) with modified Kreb’s solution (mM): NaCl 118; KCl 3; NaHCO<sub>3</sub> 25; MgCl<sub>2</sub>6H<sub>2</sub>O 1; CaCl<sub>2</sub> 2.5 and d-glucose 11 at 32 °C. All solutions were continuously carbogenated with 95% O<sub>2</sub>:5% CO<sub>2</sub> (pH 7.4).

**Electrophysiological techniques**

Intracellular recordings (1–4 h duration) were made with glass microelectrodes filled with 4 M potassium acetate (pH 7.4; adjusted with glacial acetic acid; tip resistances 40 – 80 MΩ), connected to an Axoclamp 2A pre-amplifier. For immature neurones, a 2 M potassium acetate filling solution gave more stable recordings. Voltage and current signals were monitored on an oscilloscope, a Gould RS3200 chart recorder (LDS, Test and Measurement Ltd., Royston, UK) and captured via a Digidata 1200 interface (Axon Instruments, Foster City, CA, USA) to a PC using pCLAMP6.0.1 software (Axon Instruments). Data were collected from cells identified as deep pyramidal neurones or interneurones by their similar electrophysiological responses to intracellularly applied current pulses in control conditions (Libri et al., 1994); data were pooled for the purpose of the present analysis. Cells showing electrophysiological properties characteristic of superficial pyramidal neurones (Libri et al., 1994) were rejected. Recordings were routinely made from neurones situated in LII–III of the central PC region (Fig. 1).

**Preparation and application of drugs**

All drugs were obtained from Sigma-Aldrich (UK) except where indicated. These included AFDX-116, atropine sulfate, (–)-bicuculline methiodide, methoctramine tetrahydrochloride, pirenzepine dihydrobromide, α-amino-5-phosphonovaleric acid (α-APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), CGP-52432 and telenzepine dihydrobromide. OXO-M was obtained from Tozis Cookson (Bristol, UK). All other reagents were obtained from BDH (AnalaR grade). Water-soluble drugs were dissolved in Kreb’s solution and bath-applied by superfusion. AFDX-116 and CNQX were pre-dissolved in dimethylsulphoxide (DMSO); the final DMSO bath concentration was <0.01% (control experiments revealed no deleterious effects of DMSO on cell properties at this concentration; n=3). α-APV was dissolved in the minimum necessary volume of sodium hydroxide (1 M) and further diluted with distilled water.

Fig. 1. Schematic illustration of the transverse PC brain slice preparation used in the present study, showing rostrocaudal divisions (anterior, central and posterior), laminar structure (layers I, II and III), and the locations of stimulating and recording electrodes. Unless otherwise indicated in text, recordings were routinely made from neurones situated in LII–III, in the central region of the PC.
Stock drug solutions were stored at 4 °C or −20 °C, as appropriate and thawed immediately before addition to the bathing solution for the desired bath concentration. Measurements were taken before, during and (if possible) after drug application, so each neurone served as its own control.

Recording of synaptic events

Synaptic responses were elicited via bipolar stimulating electrodes, each composed of two nichrome wires (25 μm diameter; 15 μm inner core diameter, 10 μm insulated thickness) attached to a Digitimer DS2 stimulus generator (Digitimer Ltd., Welwyn Garden, UK; stimuli: 5–20 V, 0.2 ms). One electrode was placed near the pial surface in PC Llia for evoking excitatory afferent LOT DPSPs or Llib for evoking inhibitory fIPSPs respectively; the other was placed within LIII–II, to allow independent stimulation of either intrinsic excitatory (LIII–II) fibers or LIII–II inhibitory interneurones respectively (Hasselmo and Bower, 1992) (Fig. 1). Llia was clearly distinguishable from Llib by its greater opacity, particularly in its upper, myelinated area; this also facilitated accurate placement of the second electrode into the less opaque LIII–II (Price, 1973). Subthreshold or suprathreshold ‘mixed’ DPSPs (composed of an initial, fast glutamate-mediated excitatory postsynaptic potential (EPSP) (Lester et al., 1988) and subsequent fast depolarizing and slow hyperpolarizing IPSP components, mediated by GABAA and GABAB receptors (GABAAR, GABABR) activation respectively (Tseng and Haberly, 1988)) were recorded at a standard reference membrane potential of −90 mV (i.e. −5 mV more negative than normal resting potential, maintained by intracellular current injection). This increased DPSP amplitude (for more accurate measurement) and also avoided triggering spikes (particularly with N-methyl-D-aspartate (NMDA) EPSPs in immature neurones; see below). Stimulus strengths were adjusted to elicit DPSPs large enough to remain measurable after muscarinic suppression (see Results), while also remaining small enough to elicit a subthreshold response of <40% peak subthreshold amplitude (greater amplitude DPSPs may produce postsynaptic effects that adversely affect results (Saar et al., 1999), and also, lower stimuli evoke predominantly monosynaptic responses (Hasselmo and Bower, 1990)). Stimulus strengths producing small, subthreshold DPSPs of identical amplitude for both LOT afferent and intrinsic fiber stimulation were used (each stimulus repeated five times at 30 s interval). DPSP amplitudes were measured as the difference between resting membrane potential and the peak of the response; the rate of rise was calculated from stimulus application to DPSP peak. Total DPSP duration was measured from stimulus artifact to return to baseline, whereas half-widths were measured at one-half of the peak amplitudes. Repeats were performed in control, in the presence of drug and after washout (minimum 40 min) where possible. Postsynaptic depolarizing effects of OXO-M were corrected for by intracellular negative current injection, returning the membrane potential to −90 mV before evoking DPSPs. The amplitude and time course of synaptically-evoked epileptiform events in OXO-M could not be measured accurately, due to superimposed repetitive firing and multiphasic potential transients, and their tendency to trigger full-blown bursting activity (Postlethwaite et al., 1998).

All synaptic recordings shown are composite averages of at least three consecutive traces, unless otherwise indicated. Each experimental sequence illustrated was repeated at least three times in different cells.

GABAAR-mediated fIPSPs were pharmacologically isolated by selectively blocking excitatory (glutamatergic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate- and NMDA-receptor mediated) and slow inhibitory (GABAAR-mediated) transmission using 20 μM CNQX, 100 μM d-APV and 1 μM CGP-52432 respectively, followed by depolarization of the cell to −40 mV (to avoid spiking and membrane oscillations) for 3–6 min by positive current injection, where fIPSP recordings were made. fIPSPs were evoked by direct focal electrical stimulation of inhibitory interneurones in either Llib or LIII–II; fIPSP reversal potentials were estimated by plotting peak fIPSP amplitude vs. membrane potential and extrapolating the best fit linear regression line to the zero voltage intercept. Linear regression and calculation of 95% confidence intervals for slope and intercept estimates were carried out using a least squares method (Microcal Origin; Microcal Software, Northampton, MA, USA). OXO-M-induced membrane potential changes were compensated for by injected current adjustment through the recording microelectrode. To isolate N-methyl-D-aspartate receptor (NMDAR)-mediated slow EPSPs, neurones were recorded in Kreb’s medium containing 20 μM CNQX, 10 μM bicuculline and 1 μM CGP-52432. NMDAR EPSPs were then evoked by Llia stimulation (set initially at suprathreshold level in control) at −90 and −80 mV membrane potentials; slow EPSPs remained stable at these potentials for the duration of the recording (~1 h).

Paired-pulse facilitation (PPF)

Assessment of possible presynaptic effects of OXO-M on excitatory and inhibitory neurotransmission was made using a standard paired-pulse protocol. Thus, presynaptic inhibition of transmission, would be expected to increase early PPF of evoked DPSPs or fIPSPs (Hasselmo and Bower, 1992). A conditioning stimulus was applied through a stimulating electrode followed by an identical stimulus at an interstimulus interval (ISI) of 25–200 ms (25 ms increments), and the effect of time period variation upon second evoked potential amplitude observed from plots of paired-pulse ratio (second pulse amplitude (P2)/first pulse amplitude (P1)) vs. ISI. Only short ISIs, yielding a facilitation of the second DPSP (PPF) were used and any significant differences between data sets were calculated at the point of maximal facilitation (ISI=25 ms). To avoid summation effects, P1 post-stimulus depolarization was subtracted from P2 DPSP amplitude.

Data analysis and statistical treatment of results

Data values are expressed as means±S.E.M. Statistical significance between means was determined using a two-tailed Student’s paired t-test unless data are presented as % changes between two different populations whereupon a Mann-Whitney U test was used.

RESULTS

DPSPs elicited in adult or immature PC neurones are layer-specific

Recordings of sub- and suprathreshold DPSPs were made from 65 adult and 136 immature PC neurones following Llia or LIII–II stimulation. In control adult PC slices, notable differences were seen between Llia (Fig. 2A) and LIII–II (Fig. 3A) -evoked DPSPs. Mean Llia suprathreshold DPSP duration (167±10 ms) was significantly longer than that calculated for LIII–II DPSPs (107±12 ms) (mean=56±7% greater; P<0.01), due to the presence of a prolonged depolarizing phase (Fig. 2A (ii)). Additionally, in ~15% of recordings, Llia DPSPs exhibited an ‘all-or-nothing’ character (i.e. even very small stimuli evoked a ‘maximal’ DPSP response), such that subthreshold DPSPs with a gradually increasing DPSP amplitude, could not be evoked.

As in adult PC, control suprathreshold Llia DPSPs recorded in immature slices were significantly longer (Fig. 2B (ii)) compared with LIII–II-evoked DPSPs (Fig. 3B (ii); 279±21 ms vs. 112±9 ms, mean duration=149±9%
greater; \( P<0.01 \) (Fig. 2). Immature suprathreshold LIa DPSPs also showed a significantly longer duration than the equivalent adult DPSPs (279 ± 21 ms vs. 167 ± 10 ms, 66 ± 9% greater; \( P<0.01 \)). Additionally, a larger proportion (32%) of recorded immature LIa DPSPs were found to be of the ‘all-or-nothing’ type compared with adult cells, which may be relevant for epileptiform bursting.

**OXO-M selectively enhances and prolongs LIa fiber DPSPs in both adult and immature PC slices**

In 10 \( \mu \)M OXO-M (20 min application), adult subthreshold LIa DPSPs appeared unchanged (Fig. 2A (i)); however, suprathreshold LIa DPSPs were significantly prolonged (Fig. 2A (ii)) (mean duration = 253 ± 13 ms; mean increase in DPSP duration in OXO-M vs. control = 51 ± 11%; \( P<0.01 \)). Additionally, in ∼32% of recordings, an early superimposed action potential (following the initial spike) was observed. To our knowledge, this is the first report of synaptic potential prolongation, induced by mAChR activation in adult PC slices. In a previous study (Postlethwaite et al., 1998), a dramatic prolongation of DPSPs (with superimposed firing), was observed following ‘generalized’ LII–III stimulation in immature PC slices in OXO-M. Here, using more discrete LIa stimulation, subthreshold immature LIa DPSPs recorded in 10 \( \mu \)M OXO-M demonstrated either a single, more prolonged depolarizing phase (∼84%) or a long multiphasic DPSP response (∼16%; Fig. 2B(i)), often with superimposed repetitive spike firing. Corresponding suprathreshold immature LIa DPSPs in OXO-M also exhibited either a monophasic prolongation similar to that seen in adults (Fig. 2A (ii)) (although with a significantly longer mean time course; immature: 434 ± 42 ms; 68 ± 8% longer, \( n=110 \) vs. adult: 51 ± 11% longer, \( n=65 \); \( P<0.05 \) in OXO-M, relative to their respective controls) or a dramatic prolongation with the appearance of multiphasic epileptiform components and repetitive superimposed action potentials (Fig. 2B (ii)). The time courses of such epileptiform DPSPs could not be measured accurately, as they typically segued into a full paroxysmal depolarizing shift (PDS), making comparisons with adult responses impractical. Interestingly, the incidence of observed ‘all-or-nothing’ LIa DPSPs in control (adult: 15%; immature: 32%)
was unchanged in OXO-M (adult: 16%; immature: 32%), suggesting no obvious difference in stimulation threshold in immature vs. adult PC afferent fibers.

**OXO-M produces a dramatic inhibition of intrinsic LII–III fiber DPSPs in adult, but a weaker inhibition in immature PC slices**

In contrast to LIIa-evoked DPSPs, sub- and suprathreshold adult LII–III DPSPs were significantly inhibited in 10 μM OXO-M (Fig. 3A; mean depression of subthreshold intrinsic DPSP vs. control = 77 ± 6%; P < 0.01). The effects of OXO-M on LIIa or LII–III DPSPs were fully reversed after 40 min washout. Sub- and suprathreshold LII–III DPSPs in immature neurones were also consistently inhibited in OXO-M; however, this inhibition was significantly smaller (mean subthreshold DPSP inhibition = 34 ± 5% vs. control; P < 0.01; n = 112; Fig. 3B (i)) than that observed in the adult preparations (77 ± 6% vs. control; n = 65; Fig. 3A (i)). This finding clearly differs from that described by Postlethwaite et al. (1998) in immature cells following LII–III stimulation in OXO-M (see Discussion). Depolarization of cells to −50 mV by steady current injection, reduced the initial DPSP amplitude and revealed the early hyperpolarizing GABA<sub>AR</sub>-mediated fIPSP components of LIIa and LII–III synaptic responses in both adult and immature neurones (Figs. 2A, B (iii) and 3A, B (iii)). However, to avoid any possible contamination from overlying excitatory components and/or post-spike afterhyperpolarizations (AHPs) (Constanti and Sim, 1987), we only compared layer-specific fIPSPs after pharmacological isolation in the presence and absence of OXO-M, as described later.

Log concentration-response curves, comparing percentage suppression of immature subthreshold LII–III DPSPs by OXO-M are shown in Fig. 4A (using data from P14, P30 and also pooled data from all recorded or immature cells [P14 to P30] for comparison). The estimated ED<sub>50</sub>s and apparent maximal percentage suppressions for OXO-M were 4.1 μM (17%), 2.5 μM (75%) and 3.4 μM (34%) respectively (a similar plot for all recorded adult cells [P14 to P30; not shown] was indistinguishable from the immature P30 curve). Clearly, OXO-M was less effective in suppressing intrinsic synaptic transmission in the more immature PC, as described later.

Fig. 3. Representative sub- (i) and suprathreshold (ii) DPSPs recorded from LII–III neurones, following LII–III stimulation in control and after 30 min in 10 μM OXO-M (resting potentials held at −90 mV by current injection); (iii) shows control suprathreshold DPSPs recorded at −50 mV. (A) Adult PC slice: OXO-M clearly inhibited both sub- and suprathreshold LII–III DPSPs (A i and ii). (B) Immature (P15) slice: compared with the adult, OXO-M only slightly inhibited both sub- and suprathreshold LII–III DPSPs (B i, ii). Note the smaller amplitude fIPSPs elicited following LII–III stimulation at −50 mV (A, B ii) vs. LIIa stimulation (Fig. 2A, B ii) in both adult and immature preparations. LII–III stimulus strengths (adult PC): subthreshold: 12 V, 0.2 ms; suprathreshold: 18 V, 0.2 ms; immature PC: subthreshold: 4 V, 0.2 ms; suprathreshold: 7 V, 0.2 ms; suprathreshold (at −50 mV): 5 V, 0.2 ms. Cells were depolarized to −50 mV by injection of steady positive current. Traces shown are the averages of at least three traces.
varied inversely between the ages of P14 and P30 (Whalley et al., 2005). Interestingly, by comparison, the ED50s estimated from log concentration-response curves of postsynaptic membrane input resistance change produced by OXO-M, in adult and immature (P17 to P18) neurones (n=110053 in each case; see (Whalley et al., 2005)), were 5.5 µM and 5.4 µM respectively, indicating a relatively lower postsynaptic muscarinic sensitivity (B. J. W
and A. C. unpublished observations). In summary, OXO-M caused a greater prolongation of excitatory afferent (LIA) synaptic transmission and a smaller inhibition of intrinsic (LII–III) synaptic transmission in the immature PC than in the adult. This developmentally-based difference produced a shift in synaptic transmission dynamics in the immature preparation toward a state of greater excitability and hence a greater susceptibility toward epileptiform bursting.

**Effect of ionotropic glutamate antagonists and Mg\(^{2+}\) on LIA and LII–III DPSPs**

The pharmacology and polysynaptic nature of evoked DPSPs in adult and immature PC neurones were investigated using ionotropic glutamatergic receptor antagonists and externally applied Mg\(^{2+}\) respectively. In adult PC neurones, application of the NMDAR antagonist, dl-APV (100 \(\mu\)M; \(n=6\)) had little or no observable effect on LIA DPSP amplitude or duration in control or in the presence of OXO-M (Fig. 5A), suggesting only a small contribution of NMDA-gated channels to these synaptic responses. Likewise, adult (\(n=6\)) or immature (\(n=7\)) LII–III DPSPs in control or OXO-M were also unaffected by dl-APV (not shown), indicating no detectable NMDA-mediated component to these DPSPs. In contrast, dl-APV clearly depressed the long-duration multiphasic LIA DPSPs induced by OXO-M in immature PC neurones (\(n=7\); Fig. 5B) indicating the occurrence of NMDAR activation during the epileptiform synaptic response. Subsequent application of CNQX (20 \(\mu\)M) abolished all synaptic potentials confirming the predominant involvement of AMPA-type excitatory amino acid receptors (Fig. 5A, B).

Increasing the external Mg\(^{2+}\) concentration (from 1 mM to 2 or 4 mM; 20 min at each concentration) to reduce transmitter release, and therefore decrease the probability of intercalated cell discharge, progressively transformed the long-duration multiphasic LIA DPSP in OXO-M, to a single early EPSP component (Fig. 5D), confirming that polysynaptic activity was involved in its generation (see Discussion). A similar effect was also produced by applying a low concentration of CNQX (10 \(\mu\)M; \(n=3\)), insufficient to completely block synaptic transmission (cf. (Hoffman and Haberly, 1993)). For adult LIA DPSPs in OXO-M, raising external Mg\(^{2+}\) concentration steadily reduced the long duration DPSP, leaving a single EPSP response (Fig. 5C), thereby also confirming its polysynaptic nature.

**Isolated slow NMDAR EPSPs are unaffected by OXO-M**

Since postsynaptic mACHR activation can amplify NMDAR-induced membrane depolarizations and facilitate slow neu-
nal NMDAR-mediated EPSPs (Markram and Segal, 1990; Aramakis et al., 1999), we wondered whether the observed enhancement of immature LII–III DPSPs by OXO-M was due to potentiation of a pre-existing NMDA component. We therefore recorded isolated slow NMDAR EPSPs after LII stimulation in adult and immature slices (see Experimental Procedures) in the presence and absence of OXO-M (Fig. 6). In control adult neurons, the NMDAR EPSP amplitude increased on depolarization from −90 to −80 mV (4.7 ± 0.3 mV vs. 8.3 ± 1.2 mV, P < 0.01, n = 3), due to relief of NMDA channel block by Mg2⁺ (Mayer et al., 1984) (further depolarization triggered spike firing). EPSP duration (half-width) however, was unchanged (112 ± 7 ms vs. 117 ± 9 ms, n = 3, P > 0.5; Fig. 6A). In contrast, NMDAR EPSPs recorded in immature neurons (P16) were significantly larger than those in adult cells (16.2 ± 0.6 mV at −90 mV; P < 0.001, n = 3), and were also enhanced on depolarization, evoking multiple firing (Fig. 6B), though EPSP half-width at −90 mV (105 ± 12 ms, n = 3) was similar to adult values (P > 0.5). The NMDAR-mediated component of the ‘mixed’ LIIa DPSP therefore plays a more prominent role in immature PC neurones. Interestingly, in 10 μM OXO-M, the EPSP amplitude and duration did not change in either adult or immature neurones (Fig. 6A, B); however, all isolated EPSPs (control and in OXO-M) were abolished on adding 100 μM DL-APV, confirming their dependence on NMDAR activation.

### OXO-M induced inhibition of intrinsic fiber transmission is most likely M1-mACHR mediated in adult but M2-mediated in immature PC slices

The mACHR subtype(s) mediating the synaptic effects of OXO-M in the adult and immature preparations were investigated using subtype-selective mAChR antagonists (see Whalley et al., 2005 for a description of the postsynaptic effects of these antagonists vs. OXO-M-induced neuronal depolarization and input resistance increase).

The M1-selective mAChR antagonist, pirenzepine (100 nM, n = 7; 57-fold greater affinity M1 vs. M2) (Buckley et al., 1989) (30 min exposure), in adult slices, caused a full reversal of 10 μM OXO-M-induced inhibition of LII–III DPSPs back to control level (Fig. 7A (i)); a similar effect was produced by telenzepine (20 nM, n = 7; 60-fold greater affinity M1 vs. M2 (Hulme et al., 1990)). However, the M2-mACHR antagonist, methoctramine (300 nM, n = 7; four-fold greater affinity M2 vs. M1 (Buckley et al., 1989)) (30 min exposure) (Fig. 7B (ii)) or AFDX-116 (1 μM, n = 7; seven-fold greater affinity M2 vs. M1 (Buckley et al., 1989)) did not affect the OXO-M-induced synaptic inhibition of LII–III DPSPs in adult slices, suggesting an M1-mACHR-mediated modulation. In direct contrast, pirenzepine (100 nM, n = 9) (Fig. 7A (iii)) or telenzepine (20 nM, n = 9) did not reverse OXO-M-induced LII–III DPSP inhibition in immature slices, whereas methoctramine (300 nM, n = 10 cells) (Fig. 7B (ii)) or AFDX-116 (1 μM, n = 9) fully reversed it to control level, suggesting that, unlike the adult preparation, it was most likely M2-mediated. These results imply that a novel developmental ‘switch’ occurs between presynaptic mAChR subtypes (from M2 to M1) as the PC matures, with the M2 subtype being less effective in inhibiting intrinsic excitatory transmission. Our experiments suggest that a graded, rather than sudden developmental switch was occurring, as illustrated by the apparently linear increase in degree of OXO-M-induced DPSP suppression with increasing postnatal age (Fig. 4B). Consequently, slices from P14 to P17 animals were used thereafter to ensure a predominantly M2-mediated response, an approach also adopted for recording isolated fIPSPs (see below and Fig. 10B). To ensure that prolonged exposure to OXO-M itself was not inducing the observed mAChR subtype ‘switch,’ we conducted some experiments (n = 3 for each drug), where antagonists were added and DPSPs recorded as soon as OXO-M-induced suppression was observed (within 3 min, before bursting became estab...
lished; see (Whalley et al., 2005)). The results obtained did not differ from those reported above, where the period from exposure to OXO-M to measurement of DPSP responses (after antagonist application) was typically ~30 min.

**Possible presynaptic site of action of OXO-M on excitatory DPSPs**

In order to confirm that the observed suppressant effect of OXO-M on intrinsic fiber transmission was most likely presynaptically-mediated, and not a consequence of postsynaptic muscarinic effects (Whalley et al., 2005), we performed standard paired-pulse ratio (PPR) analyses (see Experimental Procedures). Fig. 8A, B shows plots of the averaged ratio of the second to the first DPSP amplitudes (P2/P1) at various ISI values for adult and immature slices in the presence and absence of OXO-M respectively. LII–III responses showed a significant PPF increase in OXO-M in both adult and immature preparations, relative to control (Table 1) that was slightly greater in the adult (14 ± 1%) than immature preparations (11 ± 2%; P > 0.5; not significant) compared with controls. By contrast, responses following LIIa stimulation in OXO-M for either adult (n = 44) or immature (n = 56) LII–III neurones revealed only a slight increase in mean peak PPF for both adult and immature slices that was not different from control (P > 0.5) (Table 1). In 1 μM atropine, the effect of OXO-M on peak LII–III PPF was abolished in both adult (n = 11) and immature (n = 12) slices, confirming mAChR-mediation (Table 1).

The measured rates of rise and half-widths of adult or immature subthreshold DPSPs elicited following LII–III fiber stimulation in control or OXO-M were also not significantly different (P > 0.5 in each case; Table 2), suggesting that mAChR-induced modifications in PPF were not due to changes in postsynaptic membrane properties (Stuart and Redman, 1991). Additionally, the standard deviations of all PPF mean results were not significantly affected in control or in OXO-M (P > 0.5 in each case; Table 1), for adult or immature preparations, indicating that PPF changes occurred in most neurones synapsing with the recorded cell rather than a specific subgroup (e.g. solely interneurones or solely superficial pyramidal cells) (Saar et al., 1999).

**Muscarinic suppression of fIPSPs in adult PC slices is also layer-specific and most likely M1-mediated**

We next investigated any possible developmental differences in fast, GABAA,R-mediated inhibitory synaptic trans-
mission in the PC, and its sensitivity to muscarinic modulation. Isolated fIPSPs were evoked in adult LIi–III PC neurones held at −40 mV, by stimulation of local inhibitory interneurones in LIb or LIi–III (see Experimental Procedures) and confirmed as GABA<sub>R</sub>-mediated by their abolition in bicuculline (10 μM; n=3). In control, mean peak LIb fIPSP amplitude (18±0.7 mV; n=18) was significantly larger than that of LIi–III fIPSPs (13±0.8 mV; P<0.05; n=18; Fig. 9A, C) although estimated extrapolated reversal potentials were similar (LIb=−70±2.1 mV; LIi–III=−71±1.9 mV; n=4 for each). Interestingly, in 10 μM OXO-M, LIb fIPSPs were only slightly but significantly suppressed compared with controls (mean reduction in amplitude=35±6%; P<0.05; n=18; Figs. 9A and 10B), while LIi–III fIPSPs were consistently unaffected (n=18; Fig. 9C); this effect was fully reversed on washout or by adding the M1 antagonists, pirenzepine (100 nM, n=6; Fig. 9A) or telenzepine (20 nM, n=9), or atropine (1 μM; n=4). The M2 antagonists, methoctramine (300 nM, n=9) or AFDX-116 (1 μM, n=9) however, had no effect. All mACHR antagonists tested had no effect on LIi–III fIPSPs; Fig. 9C, D). No change was observed in the mean time course of LIb fIPSPs in OXO-M (measured from stimulus point to return of membrane potential to baseline: 68±10 ms control vs. 64±8 ms (normalized vs. control to account for change in amplitude) in OXO-M, P>0.5; n=18; the postsynaptic OXO-M depolarization was compensated by intracellular negative current injection). These results correlate with those obtained from adult PC slices, where muscarinic suppression of excitatory LIi–III DPSPs was

### Table 1. Peak PPF of LIi–III DPSPs and LIb fIPSPs is increased by OXO-M

<table>
<thead>
<tr>
<th>Group</th>
<th>LII–III excitatory DPSPs</th>
<th>Lib fIPSPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult LII–III</td>
<td>Immature LII–III</td>
</tr>
<tr>
<td>Control</td>
<td>1.49±0.08 (n=51)</td>
<td>1.35±0.07 (n=18)</td>
</tr>
<tr>
<td>10 μM OXO-M</td>
<td>1.63±0.08 (n=44)</td>
<td>1.57±0.07* (n=18)</td>
</tr>
<tr>
<td>+1 μM atropine</td>
<td>1.53±0.06 (n=11)</td>
<td>1.36±0.06 (n=18)</td>
</tr>
</tbody>
</table>

Peak PPF values (ISI = 25 ms) recorded from DPSP complexes and pharmacologically isolated fIPSPs (see Experimental Procedures) in control solution, OXO-M, and OXO-M+atropine.

* Significant difference from control (P<0.05) was obtained following LII–III but not LIIa excitatory fibre stimulation and following Lib but not LIi–III interneurone stimulation in OXO-M, for both adult and immature (P14 to P17) slices (blocked by 1μM atropine). Data are means±S.E.M.
Table 2. Time course characteristics of LII-III DPSPs and Lib fIPSPs are unaffected by OXO-M

<table>
<thead>
<tr>
<th>Group</th>
<th>LII-III excitatory DPSPs</th>
<th>Lib fIPSPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult LII-III (intrinsic) (n=19)</td>
<td>Immature LII-III (intrinsic) (n=21)</td>
</tr>
<tr>
<td></td>
<td>Rate of rise (mV/ms)</td>
<td>Half-width (ms)</td>
</tr>
<tr>
<td>Control</td>
<td>0.6±0.32</td>
<td>62±14.2</td>
</tr>
<tr>
<td>+10 μM OXO-M</td>
<td>0.8±0.23</td>
<td>65±11.2†</td>
</tr>
</tbody>
</table>

Characteristics of subthreshold (~10 mV amplitude) LII–III DPSPs and Lib-evoked fIPSPs measured following the first (conditioning) pulse of a paired-pulse protocol in adult or immature PC neurones (membrane potential held at −90 mV for DPSPs or −40 mV for fIPSPs, by steady current injection) in control and in OXO-M. The rate of rise of the conditioning DPSP (or fIPSP) was calculated from stimulus application to DPSP peak, while half-width was calculated as the time interval between the two points at which the trace was at 50% of the maximal DPSP amplitude. Data are means±S.E.M.; immature preparations were P14 to P17. All mean values in OXO-M were not significantly different from respective controls (P>0.05 in each case).

† Indicates values normalized vs. adult LII–III (or Lib) control to account for amplitude reduction in OXO-M; absolute values are shown in parentheses.

also likely to be M1-mediated; however, the synaptic input layer sensitivity to OXO-M for fIPSPs was opposite to that for DPSPs in the adult PC.

Muscarinic suppression of fIPSPs in immature PC slices is more dramatic and most likely M2-mediated

For comparison, fIPSPs were also recorded from 20 LII–III PC neurones in immature (P14 to P17) PC slices. Like adult preparations, the control mean peak immature Lib fIPSPs amplitude (9±0.3 mV, n=20) was significantly larger than that of corresponding LII–III fIPSPs (5±0.4 mV, n=20, P<0.05; Fig. 9B,D), despite similar estimated reversal potentials (Lib=−69±2.1 mV; LII–III=−68±1.9 mV; n=6 for each). However, both Lib and LII–III fIPSPs' mean amplitudes, in immature PC slices, were significantly smaller than equivalent responses elicited from adults (Lib=18±0.7 mV; LII–III=13±0.8 mV; P<0.05 for both data groups vs. adult equivalents) indicating a generally weaker level of inhibitory control. Additionally, the difference between Lib and LII–III peak fIPSP amplitudes was slightly, but significantly greater in immature (45±5%) than in adult (35±6%; P<0.05) preparations.

In 10 μM OXO-M, Lib fIPSP mean amplitude was more dramatically reduced (global mean [P14 to P30; n=6 for

![Fig. 9](image_url). Representative isolated fast, GABA<sub>R</sub>-mediated IPSPs elicited by Lib (A, B) or LII–III (C, D) stimulation, recorded from adult (A, C) and immature (P15: B, D) neurones (stimuli applied: 4–6 V; 0.2 ms). (A) Lib fIPSPs in an adult neurone showing suppression by 10 μM OXO-M and its reversal by pirenzepine (100 nM). (B) Lib fIPSPs of an immature (P15) neurone showing greater suppression by OXO-M than in (A) and its reversal by AFDX-116 (1 μM). (C, D) LII–III fIPSPs in adult and immature (P15) neurones were not reduced by OXO-M and were unaffected by the muscarinic antagonists. Note that the peak amplitudes of fIPSPs elicited from immature slices were consistently smaller than those elicited in adults. The bathing medium contained 100 μM d-APV, 20 μM CNQX and 1 μM CGP-52432 to block glutamatergic and GABA<sub>R</sub>-mediated transmission respectively. Cells were held at −40 mV membrane potential during fIPSP recording.
each age] = 61±5% vs. controls, P<0.05, Fig. 9B and 10B; significantly greater than in adult slices; P<0.01) with no change in time course (56±8 ms vs. 53±5 ms control, P>0.5; normalized vs. control adult Lib amplitude), while LII–III fIPSPs remained unaffected (Fig. 9D). This suppression was fully reversed on washout or after adding atropine to the bathing medium (1 μM; n=4). However, in contrast with adult PC, the effect of OXO-M was not reversed after adding pirenzepine (100 nM; n=10) or telenzepine (20 nM, n=10), but it was in the presence of the M2 antagonists, AFDX-116 (1 μM; n=10; Fig. 9B) or methoctramine (300 nM, n=10). Log concentration-response curves showing the differential potency of OXO-M in suppressing Lib fIPSPs in adult and immature neurones are shown in Fig. 10A. Estimated ED_{50} values for OXO-M were lower in the immature (2.5 μM; n=6) than in the adult (4.0 μM; n=5) preparations, suggesting a relatively greater sensitivity of immature Lib inhibitory synaptic terminals to muscarinic suppression. Fig. 10B shows that in contrast with the increasing muscarinic suppression of intrinsic LII–III DPSPs with developmental age shown in Fig. 4B, after dividing the immature population into the same age groupings, the
degree of fIPSP suppression by OXO-M decreased progressively with decreasing age up to P30, after which it was maintained at adult (P>40) suppression level.

In summary, a relatively lower level of inhibitory synaptic control was found in the immature PC than in the adult, and moreover, it could be further reduced, differentially and more effectively, by mAChR activation. In addition, the suppression of immature Lib fIPSPs by OXO-M (like immature intrinsic fiber DPSPs), was also likely to be M2-, rather than M1-mediated.

**Possible presynaptic action of OXO-M on fIPSPs**

Finally, to obtain evidence that the muscarinic suppression of Lib fIPSPs was most likely presynaptically-mediated, a paired-pulse analysis was carried out as previously described. Adult (n=18) and immature (n=20) Lib (and LII–III) fIPSPs were evoked over a range of ISIs (25–200 ms) in control conditions, 10 μM OXO-M and OXO-M plus 1 μM atropine. In both adult and immature preparations, a clear difference was found between the degree of Lib and LII–III peak PPF (ISI=25 ms) measured in control (adult Lib: ~15% > LII–III; immature Lib: ~9% > LII–III, P<0.05 in each case; Table 1), suggesting that the release characteristics of inhibitory interneurone terminals located in PC layers Ib and II–III may be different.

In OXO-M, peak Lib PPF in both adult and immature slices, was significantly increased relative to controls (adult: 18±2%; immature: 26±2%; P<0.05 in each case; Table 1) and was abolished by 1 μM atropine, in accordance with a presynaptically-mediated muscarinic effect. Interestingly, the mean percentage increase in PPF observed in immature preparations was significantly greater than that in adults (P<0.05). In contrast, peak LII–III PPF in adult and immature slices was unaffected in OXO-M, in agreement with results obtained above, suggesting little or no presynaptic mAChR-mediated modulation of GABA release from LII–III interneuronal terminals. Like DPSPs, the time course characteristics of fIPSPs were unaltered in OXO-M, regardless of the recording conditions (P>0.5; Table 2), indicating that muscarinic PPF changes were not due to postsynaptic membrane effects (Stuart and Redman, 1991). The S.D. values calculated from PPF means in OXO-M (Table 1) were also not significantly altered (P>0.5 in each case), confirming that no neurones (other than the inhibitory GABA-ergic interneurones being stimulated), were likely to be firing (Saar et al., 1999).

**DISCUSSION**

**Layer-specific differences in DPSPs in the PC, and their developmental variation: possible significance for burst generation**

Our results revealed a number of differences between control DPSPs evoked in Lib and LII–III in both adult and immature PC, that may be important for understanding epileptiform burst generation in this brain region. Thus, our finding of some ‘all-or-nothing’ Lib DSP responses in adult PC (~15% of recordings), showing a prolonged depolarizing phase, accords with a previous report (Tseng and Haberly, 1989a) and may be relevant for interpreting new olfactory information (Macrides, 1977), as well as for epileptiform burst generation and maintenance (Hoffman and Haberly, 1989; Tseng and Haberly, 1989b). It is noteworthy that longer time course ‘all-or-nothing’ LIIa responses occurred more frequently in the immature PC (~32% of recordings), compared with adult slices, according with the higher seizure susceptibility in the immature preparation (Whalley et al., 2005; Postlethwaite et al., 1998) and the generally increased tendency of this area per se to develop epileptiform bursting (Loscher et al., 1995). This heightened state of synaptic excitability in the immature PC, may be required for driving synaptic growth (Barkai et al., 1994), increasing synaptic plasticity (Holliday and Spitzer, 1990) and epileptogenesis (Milburn and Prince, 1993).

Interestingly, each afferent LOT (LII) axon typically synapises with only a single superficial pyramidal cell, while it forms di- or polysynaptic connections with each deep pyramidal neuron (Biedenbach and Stevens, 1969), indicating a greater excitatory input and a more likely expression of epileptiform events in these neurones, than in superficial pyramidal cells, as we have observed (Postlethwaite et al., 1998) (see also: Hoffman and Haberly, 1991). LII–III stimuli excite superficial and deep pyramidal neurones as well as inhibitory interneurones within the PC (Haberly and Behan, 1983) and are considered responsible for integrating new odor information into memory and memory retrieval (Barkai and Hasselmo, 1997).

**Consequences of mAChR activation upon excitatory synaptic transmission**

We found afferent and intrinsic synaptic transmission to be differentially affected by mAChR activation, in both adult and immature PC. The novel enhancing/prolonging effect of OXO-M upon ‘mixed’ LIIa-evoked DPSPs (most likely due to preferential suppression of Lib-inhibitory neurotransmission; see below), although seen in adult PC, was most pronounced in immature slices, while mAChR-mediated inhibition of LII–III DPSPs was prominent in both preparations (although significantly diminished in the immature PC). Postlethwaite et al. (1998) recorded prolonged multiphasic DPSPs with superimposed spike firing in immature PC slices following LII–III stimulation in OXO-M, a phenomenon not observed in the present study; perhaps the larger stimulating electrode (tip size 45 μm vs. 25 μm) used in these experiments was also recruiting some Lib fibers with each stimulus.

Selective muscarinic suppression of intrinsic (but not afferent) DPSPs in adult PC superficial pyramidal neurones was first described by Hasselmo and Bower (1992), and was thought to allow the preferential input of new LOT-mediated sensory data, over existing intrinsic, ‘memory type’ inputs. However, the poor postsynaptic cholinergic sensitivity of superficial pyramidal neurones, compared with deep pyramidal neurones (Libri et al., 1994), suggests that this modulation would be less pronounced in superficial layers whereas, the greater increase in postsynaptic excitability of deep pyramidal neurones following mAChR activation, would produce a much greater ampli-
fication of the consequences of cholinergic synaptic modulation. Additionally, the broadly diffuse cholinergic innervation of the PC arising from the horizontal limb of the diagonal band of Broca (Eckenstein et al., 1988), although not clearly layer-specific (Luskin and Price, 1983), is weaker in the superficial PC layers, compared with deeper layers (Eckenstein et al., 1988; Kiss and Patel, 1992). A similar differential effect of cholinergic modulation has also been described in the visual cortex (Kimura et al., 1999), suggesting this layer-selective effect on intrinsic excitatory transmission may be vital in the process of interpreting and integrating new sensory stimuli into existing associative memory (Barkai and Hasselmo, 1997) in a number of brain systems.

The significant increase in LII–III (but not LIIa) PPF observed in DPSP paired-pulse experiments (with no effect on DPSP time course characteristics), strongly suggests that the inhibitory effect of OXO-M on excitatory synaptic transmission was presynaptically mediated (Stuart and Redman, 1991). Thus, either a relatively larger population of mACHRs exists upon intrinsic fiber terminals or these receptors are more effective in inhibiting excitatory transmission. Additionally, differences in mean peak PPF in OXO-M, between adult and immature preparations, supported the single evoked DPSP data in suggesting a larger or more effectively-coupled inhibitory mACHR population on adult intrinsic terminals.

Effects of Mg2+ and DL-APV on epileptiform LIIa DPSPs induced in OXO-M

Like the spontaneous PDSs observed in immature PC slices (Whalley et al., 2005), the long-duration epileptiform LIIa DPSPs evoked in OXO-M reported here were most likely attributable to a sustained reverberatory excitatory synaptic input (acting through recurrent cortical circuits), as suggested for other brain areas (Lee and Hablitz, 1991; McCormick and Contreras, 2001; Lau and Bi, 2005), while the progressive dropout of the multiphasic DPSP in high external Mg2+ medium would also accord with its polysynaptic character (Berry and Pentreath, 1976; Vinay et al., 1995; Ben-Ari and Gho, 1988). A relatively low concentration of Mg2+ was used (see (Pelletier and Carlen, 1996)) with the aim of isolating monosynaptic EPSPs, without dramatically reducing their amplitude. Such epileptiform activity has previously been modeled in a simple neuronal dramatic reducing their amplitude. Such epileptiform with the aim of isolating monosynaptic EPSPs, without

NMDA NR2B subunit specifically contributes to prolonged epileptiform-like events in dysplastic cortex (Moddel et al., 2005) and malformed hippocampus (Calcagnotto and Baraban, 2005). Finally, the abolition of epileptiform DPSPs by CNQX indicates that AMPA-type receptors were principally involved in their generation, although a contribution from synaptic metabotropic glutamate receptors cannot be excluded (Lee et al., 2002).

Possible significance of the observed developmental ‘switch’ in presynaptic mACHR subtype and sensitivity

Our study has revealed for the first time, a clear developmental variation in intrinsic excitatory fiber sensitivity to mACHR activation. The reduced suppression of immature LII–III DPSPs by OXO-M compared with adult equivalents has a number of implications for epileptogenesis in the immature PC, as well as for learning and memory. In adult PC, preferential muscarinic inhibition of intrinsic transmission may enhance associative memory performance (Hasselmo et al., 1992) by shifting dominance from intrinsic to afferent input (Barkai and Saar, 2001; Hasselmo and Bower, 1993; Patil and Hasselmo, 1999). However, in immature PC, presynaptic mACHR activation could further prolong afferent LIIa DPSPs (already longer in control and showing a larger proportion of ‘all-or-nothing’ events), thereby increasing afferent input strength; intrinsic fiber transmission would be less inhibited than in the adult, raising excitatory drive to pyramidal neurons from this source, and placing immature LII–III neurons in a highly excitatory state that amplifies incoming excitatory signals and permits stronger postsynaptic responses, as may be necessary for associative memory functions. Accordingly, this also predetermines the immature PC toward spontaneous epileptiform bursting (Whalley et al., 2005).

A further novel finding of our study was the suggested developmental ‘switch’ in presynaptic mACHR subtype on intrinsic excitatory fiber terminals from M2 (immature) to M1 (adult) (c.f. (Williams and Constanti, 1988; Bagetta and Constanti, 1991)), associated with a decrease in muscarinic inhibitory efficacy. Since the incidence of muscarinic bursting decreases progressively with age (Whalley et al., 2005) and the proposed shift from presynaptic M2 to M1 subtype is also gradual (up to P30), this distinct change in PC synaptic circuitry is implicated in the observed bursting phenomenon. Other studies have demonstrated similarly changing cholinergic responsiveness in other brain areas (Araki et al., 1996; Milburn and Prince, 1993; Saito et al., 1991). Only M1 and M2 mACHR subtypes were investi-
gated in the present study, since no M3 or M4 mAChR-mediated effects on neurotransmission were observable (Bagetta and Constanti, 1991; Das et al., 1992). This choice was clearly vindicated by the complete reversal of the OXO-M-induced suppression of LII–III DPSPs to control levels by the appropriate subtype-selective antagonists. Comparable developmental changes have also been reported for other receptor types e.g. presynaptic GABA_A Rs (Fritschi et al., 1994). Also, a developmental switch occurs in the Ca^{2+} permeability of AMPA receptors in immature (P<16) neocortical neurones resulting from an alteration in subunit composition (Kumar et al., 2002). To our knowledge, the present data are the first report of a functional pharmacological change in mAChR subtype in the brain, with possible implications for a specific pathology (epilepsy).

**Layer-specific and developmental differences in GABA_A_R-mediated fIPSPs**

Our experiments have also revealed clear laminar and developmental differences in isolated Lib and LII–III fIPSP amplitude in adult and immature PC (in control and in their modulation by OXO-M), which further assisted our understanding of muscarinic bursting in immature slices (Whalley et al., 2005); these differences were clearly not due to an existing or substantial developmental change in the fIPSP (Cl ) reversal potential (Rivera et al., 1999). Previous studies have established the currently accepted model for inhibitory synaptic transmission in this area (Tseng and Haberly, 1988; Satou et al., 1983a,b; Patil and Hasselmo, 1999). Thus, LOT and intrinsic-derived excitatory fibers synapse with GABA-ergic interneurones present within all three PC layers (Satou et al., 1982; Solti and Redman, 1991) which in turn inhibit pyramidal neurones, via feedforward and feedback inhibitory pathways (Barkai et al., 1994). The characteristics and distribution of PC inhibitory interneurones have been extensively studied (Satou et al., 1982, 1983a,b), while immunocytochemical studies have shown that most GABA-ergic neurones are located within layer I (~50%, activated either following LOT or intrinsic fiber stimulation), with ~5% and ~15% in layers II and III respectively (Loscher et al., 1998; Haberly et al., 1987). Accordingly, we found that adult and immature Lib PC fIPSPs were consistently larger than their LII–III equivalents, indicating greater GABA_A_R-mediated inhibition in this more superficial layer, possibly reflecting a greater number of GABA-ergic interneurones, or a greater release of GABA. As PPF is directly linked to raised presynaptic Ca^{2+} levels (Stuart and Redman, 1991), the relatively higher level of peak Lib fIPSP PPF seen in control adult or immature neurones would suggest higher terminal Ca^{2+} concentrations and thus a potentially greater release of inhibitory neurotransmitter from Lib interneurone terminals, in agreement with the larger amplitude of recorded Lib fIPSPs. Kapur et al. (1997) proposed that GABA_A_ergic inhibition in the PC was primarily generated in the apical dendrites of PC pyramidal cells and that inhibitory circuits in the apical dendritic and somatic regions were independent of one another (see Ekstrand et al. (2001)). Kapur et al. (1997) further suggested that a selective decrease in feed forward inhibition in Lib, would enhance integrative dendritic processes, while feedback inhibition in the somatic region would restrain system excitability. We have now shown that Lib fIPSPs (putatively distal apical dendritic) to be selectively modulated by mAChR activation, and that this is likely to be presynaptically-mediated, while LII–III fIPSPs (putatively somatic and/or distal basal dendritic) were unaffected, clearly in keeping with the proposal of Kapur et al. (1997). The mAChR system could thus serve an important function in selectively modulating fast inhibitory synaptic transmission and therefore dendritic integration in this brain region. Distinct subgroups of neurones mediating fast inhibitory transmission (affected by different modulators), have also been described in other brain areas (Aramakis et al., 1997; Tamas et al., 1997; Thomson et al., 1996; Traub et al., 1987; Vu and Krasne, 1992).

We have also shown that immature Lib and LII–III PC fIPSPs were consistently smaller than adult equivalents (despite similar extrapolated reversal potentials), indicating a significant delay in development of fast inhibitory transmission and therefore a greater likelihood of seizure initiation; (reduced GABA-ergic inhibition in the PC is known to lead to epileptogenesis (Kanter and Haberly, 1993; Bloms-Funke et al., 1999; Gernert et al., 2000)). Our present study has therefore confirmed and extended previous work (Forti et al., 1997) relating to the role played by fast GABA_A_R-mediated synaptic transmission in suppressing seizure generation in the PC.

**Preferential effects of mAChR activation upon inhibitory synaptic transmission, and a comparable ‘switch’ in presynaptic mAChR subtype**

Muscarinic AChR activation not only preferentially inhibited Lib fIPSPs in both adult and immature PC, but the degree of inhibition was greater in the immature preparation. Consequently, both of these properties predispose the immature system to over-excitability and potential epileptiform behavior, independent of the observed developmental variation in DPSPs. Interestingly, the muscarinic fIPSP modulation (most likely presynaptic) was again found to ‘switch’ from M2 (immature) to M1 (adult), although in this case, there was a decrease in modulatory efficiency with age, rather than an increase as with DPSPs (see above). The fact that Lib fIPSP PPF was significantly increased by OXO-M, supports a presynaptic inhibitory action. Also, the relatively greater increase in this muscarinic effect on PPF in immature slices, corroborates the fIPSP data and suggests the presence of greater numbers and/or more effectively coupled inhibitory presynaptic mAChRs on these terminals than in the adult. Although the function and mechanisms underlying these novel developmental ‘switches’ and the efficiency of different mAChR-mediated presynaptic effects remain to be determined, it may be that different classes of presynaptic voltage-gated Ca^{2+} channels are present during synaptic development and maturation (Scholz and Miller, 1995), which could largely influ-
ence the nature of presynaptic mACHR inhibition (see below).

We also found that control immature LIb fIPSPs had a similar time course to those in the adult (both unaffected by OXO-M; Table 2). Interestingly, Kapur et al. (1997) found that GABA\textsubscript{A}R-mediated LI/II fIPSPs in immature PC slices, had both fast and slow components, the latter possibly being critical for regulating the NMDAR-mediated component of the DPSP (Kapur et al., 1997). Why such a slow component was absent in our immature LIb fIPSPS however, is unclear.

Possible mechanisms of muscarinic inhibition of excitatory and inhibitory neurotransmission

The mechanism(s) coupling presynaptic M1 mACHRs on PC intrinsic fiber terminals to depression of excitatory or inhibitory transmitter release have yet to be clarified, but could conceivably involve modulation of terminal Ca\textsuperscript{2+} channels involved in transmission, via a G\textsubscript{q11}, G protein-phospholipase C (PLC)-linked pathway (Porter et al., 2002), in accordance with the well known M1-linked block of somatic (particularly N- and P/Q-type) Ca\textsuperscript{2+} currents (Perez-Rosello et al., 2005). In contrast, presynaptic M2 mACHRs (via a G(i)-protein-linked pathway), could act principally to increase a terminal K\textsuperscript{+} conductance(s), thereby indirectly limiting presynaptic depolarization and Ca\textsuperscript{2+} influx necessary for release (Kimura and Baughman, 1997; Nikbakht and Stone, 1999; Fernandez-Fernandez et al., 2001), although see (Allen and Brown, 1993). Alternatively, both MACHR receptor types could modulate transmission via non-Ca\textsuperscript{2+}, non-K\textsuperscript{+} channel-linked mechanisms (Scholz and Miller, 1992; Grillner et al., 1999). The possibility that M1 mACHR-mediated synaptic depression in the PC occurs indirectly through endocannabinoid signaling (as in the hippocampus), can also not be presently excluded (Fukudome et al., 2004).

CONCLUSIONS

In conclusion, we propose that developmentally variable, layer-specific presynaptic muscarinic modulation of excitatory synaptic transmission, together with preferential muscarinic suppression of fast GABA\textsubscript{A} inhibitory transmission in more distal dendritic regions (and a generally reduced fIPSP amplitude), contributes to the generation and maintenance of the circuitry required for epileptiform activity in immature PC brain slices. These mechanisms would effectively combine with the more pronounced neuronal input resistance increase produced by OXO-M in immature PC neurones (Whalley et al., 2005), to facilitate burst generation. Thus, OXO-M, by strongly suppressing the presynaptic release of GABA from local interneurones, essentially causes disinhibition of neuronal circuits, leading to selective overactivity of LlA excitatory synapses (themselves being relatively insensitive to presynaptic muscarinic modulation). The present results have therefore furthered our understanding of this novel brain slice model of sustained epileptiform bursting (Postlethwaite et al., 1998) and have also provided additional context in which to consider other reports describing mACHR agonist-induced epileptiform activity in the hippocampus (Gruslin et al., 1989) and other cortical areas (Potier and Psarropoulou, 2004).

Acknowledgments—B.J.W. was supported by a School of Pharmacy Studentship. We are very grateful to Mr. C. Courtice for invaluable technical support and assistance.

REFERENCES


(Accepted 22 February 2006)
(Available online 17 April 2006)