Cholinergic modulation of intrinsic fibre-evoked excitatory transmission contains a nicotinic component in immature but not adult rat piriform cortex, in vitro

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Abstract

The piriform cortex (PC) is highly prone to epileptogenesis, particularly in immature animals, where decreased muscarinic modulation of PC intrinsic fibre excitatory neurotransmission is implicated as a likely cause. However, whether higher levels of acetylcholine (ACh) release occur in immature vs. adult PC remains unclear. We investigated this using in vitro extracellular electrophysiological recording techniques. Intrinsic fibre-evoked extracellular field potentials (EFPs) were recorded from layers II to III in PC brain slices prepared from immature (P14–18) and adult (P>40) rats. Adult and immature PC EFPs were suppressed by eserine (1 μM) or neostigmine (1 μM) application, with a greater suppression in immature (∼40%) than adult (∼30%) slices. Subsequent application of atropine (1 μM) reversed EFP suppression, producing supranormal (∼12%) recovery in adult slices, suggesting that suppression was solely muscarinic ACh receptor-mediated and that some ‘basal’ cholinergic ‘tone’ was present. Conversely, atropine only partially reversed anticholinesterase effects in immature slices, suggesting the presence of additional non-muscarinic modulation. Accordingly, nicotine (50 μM) caused immature field suppression (∼30%) that was further enhanced by neostigmine, whereas it had no effect on adult EFPs. Unlike atropine, nicotine antagonists, mecamylamine and methyllycaconitine, induced immature supranormal field recovery (∼20%) following anticholinesterase-induced suppression (with no effect on adult slices), confirming that basal cholinergic ‘tone’ was also present. We suggest that nicotinic inhibitory cholinergic modulation occurs in the immature rat PC intrinsic excitatory fibre system, possibly to complement the existing, weak muscarinic modulation, and could be another important developmentally regulated system governing immature PC susceptibility towards epileptogenesis.

Keywords: Piriform cortex (PC); Acetylcholine (ACh); Extracellular field potentials (EFPs); Anticholinesterase; Muscarinic; Nicotinic

Cholinergic modulation of sensory input processing and function in the cortex is well established [15, 24]. In the piriform cortex (PC), cholinergic innervation arises solely from the nucleus of the diagonal band [8], diffusing within PC layers II–III [33] where several muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptor (AChR) subtypes are expressed [4, 10, 20, 27]. The PC’s laminar structure allows independent stimulation of afferent (lateral olfactory tract; LOT) and intracortical pathways [14, 29] to investigate cortical synaptic responses. Hasselmo et al. [14] showed that presynaptic mAChR-mediated suppression of PC layer II excitatory synaptic transmission (via inhibition of glutamate release [2]) was specific to intrinsic, but not afferent excitatory synaptic transmission, demonstrating a possible role for intrinsic cholinergic innervation in selectively suppressing excitatory synaptic transmission mediated by fibres other than those originating in the olfactory bulb (hereafter referred to as intrinsic). This correlates with PC cholinergic innervation only reaching layers II–III (containing intrinsic and rising association fibres), but not layer Ia (containing afferents) [33] and led to the suggestion that ACh shifts cortical function dynamics from influence upon intrinsic, intra- and inter-cortical activity to extracortical, afferent signals [17], enhancing atten-
mACHRs mediate the majority of excitatory and inhibitory AChe effects within the mammalian CNS, where mACHR activation can suppress inhibitory and excitatory synaptic potentials [17], as well as induce postsynaptic excitation [14]. Due to sparse postsynaptic (vs presynaptic) mACHR density in the PC [8], a predominantly presynaptic (inhibitory) role is indicated for mACHRs. Our previous work showed reduced presynaptic mACHR-mediated inhibition of intrinsic fibre excitatory transmission in immature (P14–P30) but not adult PC [29] and correlated well with increased in immature PC epileptiform susceptibility [30]. Coupling of reduced mACHR-mediated inhibition with greater endogenous PC cholinergic ‘tone’ may enable excessive (postsynaptic) excitatory drive and facilitate epileptiform activity.

Compared to mACHRs, PC nACHRs have received little attention; a previous report [19] concluded that nACHR agonists had no measurable effect on evoked PC extracellular field potentials (EFPs), despite an ample nACHR presence [27]. Moreover, heteromeric (α/β-subunit containing) and homomeric (α7) nACHRs exist in rat brain during embryonic and postnatal development, although no significant difference in nACHR distribution with age has been found [27]; thus, developmental variation in nACHR functional coupling remains unclear. Importantly, α7 nACHRs are highly Ca²⁺-permeable, relative to Na⁺ [20,27] and may allow a role in modulating synaptic transmission via presynaptic terminals [27]. Furthermore, nACHRs are important for sustaining attention to sensory inputs [16] and so could play a role in immature PC which is responsible for the assimilation of early odour-encoded information into learning and memory [31]. This work aims to determine whether immature animals display greater endogenous cholinergic ‘tone’ in the PC, and assess whether cholinergic modulation of immature PC intrinsic fibre transmission involves a functional nicotinic component.

Experiments were conducted in accordance with Home Office regulations (Animals (Scientific Procedures) Act, 1986). Transverse PC slices were prepared from male and female adult (P > 40; 200–300 g) and immature (P14–18; 100–150 g) Wistar rats [7]. Animals were anaesthetised with halothane (May & Baker, Dagenham, UK) and rapidly decapitated. The brain was removed, hemisected and trimmed before mounting on a Teflon block using cyanoacrylate adhesive (Loctite Ltd., Cheshire, UK). Approximately 300 μm thick slices were cut along the LOT axis (perpendicular to the pial surface) with a Campden Vibroslice/M (Campden Instruments, UK). Slices recovered and stabilised for 30–45 min in carboxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (aCSF) at 30°C before transfer to the recording chamber, where they were continuously superfused (~5 ml min⁻¹; 25°C) with carboxygenated aCSF. aCSF composition (mM): NaCl, 118; KCl, 3; CaCl₂, 1.5; NaHCO₃, 25; MgCl₂·6H₂O, 1 and d-glucose, 11; pH 7.4; All from BDH, UK.

Evoked EFPs were elicited via cathodal voltage stimulus to PC layers II–III (containing intrinsic fibres) via a platinum/iridium electrode (25 μm diameter; insulated except at tip) with respect to a bath-submerged annular silver indifferent electrode [22]. Anode and cathode were connected to a Digitimer DS2 stimulus generator (Digitimer Ltd., Welwyn Garden, UK; stimuli: 0–24 V, 0.2 ms duration). Despite precise stimulating electrode placement, a proportion of elicited responses may also have contained contributions from association fibre activation within PC layer Ib.

Recordings were made from PC layers II–III using glass micropipettes, broken back to a ~5–10 μm diameter tip, filled with aCSF and connected to an AC-coupled preamplifier (50 kHz sampling rate; 1000× gain). Data were captured using a Tektronix 5111A storage oscilloscope (Beaverton, Oregon, USA) and a Digidata 1200 A/D (Molecular Devices, USA) to a PC, running pClamp8 (Molecular Devices, USA) for on/off-line data analysis. Each response was recorded a minimum of five times for trace averaging. Drug-induced changes to the investigated EFP component (Fig. 1A) are expressed as mean percentage vs control (±S.E.M.; n = number of slices) and statistical differences assessed via non-parametric Wilcoxon Signed Rank tests with significance accepted at P < 0.05. Different slices from different animals were used in all pooled and compared data to remove potential animal-specific responses.

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Drugs used were obtained from Sigma–Aldrich, UK unless otherwise stated and included: atropine sulphate, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris Cookson, UK), eserine (physostigmine), mecamylamine hydrochloride (MEC), methyllycaconitine citrate hydrate (MLA), neostigmine bromide, nicotine hydrogen tartrate and tetrodotoxin (TTX; Tocris Cookson, UK). Water-soluble drugs were pre-dissolved in distilled water and stored at 4 °C; dilutions were freshly prepared in aCSF and bath-applied (bath-exchange time ∼30 s). CNQX was pre-dissolved in dimethylsulphoxide (DMSO; Sigma–Aldrich, UK); final DMSO bath concentration <0.01% (w/v) which had no deleterious effects on EFP properties when applied alone (n = 3; not shown).

PC intrinsic fibre-evoked EFPs were composed of three events: two fast initial negative-going waves (population spikes: E1–E2; ranges: 200–1000 μV and 300–700 μV, respectively), followed by a longer duration positive wave (E3; range: 250–500 μV; Fig. 1A). Superfusion with 10 μM CNQX (AMPA/kainate glutamate receptor antagonist) over the slice consistently suppressed the E3 component by 100 ± 1.4% (P < 0.01; n = 10) suggesting that it represented a field excitatory postsynaptic potential (fEPSP), and also completely eliminated the secondary spike (E2; n = 10), without affecting the E1 component, suggesting it to be an orthodromic population spike, caused by stimulated neurones exhibiting action potentials on reaching firing threshold (Fig. 1B). Subsequent superfusion of the fast Na+ channel blocker, TTX (1 μM), abolished E1 (n = 10), suggesting a non-synaptically mediated (antidromic) action potential component and/or an orthodromic fibre potential of presynaptic origin. The presence of a late, small amplitude, N-wave (E4) was also noted although not investigated in this study.

Possible endogenous cholinergic ‘tone’ was investigated by superfusing adult and immature slices with the anticholinesterases, eserine (1 μM; [17]) or neostigmine (1 μM; [14]). After ∼10 min exposure, eserine significantly depressed the adult intrinsic PC EFP (mean = −32.0 ± 3.7%, n = 3, P < 0.05; Fig. 2A). Similarly, neostigmine (∼10 min exposure) also significantly depressed the adult EFP (−27.6 ± 6.8%, n = 4, P < 0.01; not shown). The action of both eserine (Fig. 2A) and neostigmine was completely reversed by application of the mAChR antagonist, atropine (1 μM; 10 min; [24]) which also revealed recovery of the EFP response amplitude to a level above control (+12.5 ± 4.9%, n = 3, P < 0.05; Fig. 2A, and +4.5 ± 2.4%, n = 4, P < 0.01, respectively), suggestive of inhibitory cholinergic ‘tone’ prior to anticholinesterase application (Fig. 2A). The same protocol, performed on immature PC slices, also demonstrated significant EFP suppression with eserine (−38.7 ± 0.9%, n = 3, P < 0.05; Fig. 2B) and neostigmine (−39.7 ± 5.8%, n = 4, P < 0.01; not shown), which was
comparable to effects seen in adult slices. However, unlike adult EFPs, immature EFPs failed to fully recover in 1 μM atropine, which only partially reversed eserine-induced suppression, leaving a mean residual depression (−27.0 ± 1.6% vs. control; n = 3; Fig. 2A). Similarly, neostigmine-induced immature EFP suppression was partially reversed by 1 μM atropine (mean residual depression = −21.9 ± 1.8% vs control; n = 4). Fig. 2C summarises effects of eserine and neostigmine applied alone and following atropine treatment, suggesting the possible nAChR involvement in cholinergic immature EFP suppression. This was further investigated by comparison of control EFP responses with those following nicotine superfusion. After ~15 min exposure, nicotine (50 μM; [12]) significantly suppressed immature intrinsic EFPs (−29.2 ± 2.7%; n = 5, P < 0.01 vs. untreated control) that were further suppressed when co-superfused with 1 μM neostigmine, indicating an additional independent muscarinic component (−21.9 ± 4.7%; n = 5, P < 0.05 vs untreated control, P < 0.05 vs. nicotine treated; Fig. 3A). In contrast, 50 μM nicotine (~15 min) caused no adult intrinsic EFP suppression although 1 μM neostigmine co-superfusion caused substantial field suppression (~33.2 ± 5.4%; P < 0.05; n = 3), confirming that mACHRs were still responsive, despite prior nicotine exposure (Fig. 3B) (cf. Ref [15]). Finally, two nAChR blockers, mecamylamine (MEC; a non-specific nAChR antagonist) and methyllycaconitine (MLA; an α7 nAChR-specific antagonist), were used to further identify the presence of a nicotine-sensitive component in immature intrinsic EFPs. Whilst atropine did not induce full recovery of immature anticholinesterase-suppressed EFPs (Fig. 2), 1 μM MEC ([26]; Fig. 4A) or 1 μM MLA ([28]; Fig. 4B) reversed suppression to supranormal levels (+4.2 ± 2.1%, n = 5, P < 0.1 and +19.6 ± 3.2%, n = 5, P < 0.05, respectively). Although MEC was expected to produce a similar reversal to that seen with MLA, full MEC-induced nAChR antagonism may not be seen until concentrations of 10 μM ([25] and below). nAChR antagonists produced no discernable effects when applied to adult slices (not shown).

This study has demonstrated that PC intrinsic excitatory fibre transmission can be modulated by endogenous cholinergic ‘tone’. Increasing synaptic ACh using acetylcholinesterases caused intrinsically evoked EFP suppression that was reversed following cholinergic transmission blockade with the nAChR antagonist, atropine, and/or the nAChR antagonists, MEC and MLA. That these antagonists revealed supranormal EFP recovery after anticholinesterase treatment (adult, Fig. 2) or application of nAChR antagonists (immature, Fig. 4) suggests the presence of endogenous cholinergic ‘tone’ in the preparation. Our results also indicate that a greater level of cholinergic modulation may exist in immature PC (vs. adults) via greater supranormal recovery in immature animals (~20%) than adults (~12%) following cholinergic transmission blockade. This finding may be relevant to increased epileptiform bursting susceptibility in immature PC [29,30].

We also found that immature but not adult cholinergic modulation of intrinsic fibre transmission contains a nicotinic component; acetylcholinesterase-induced EFP suppression was supranormally reversed by atropine in adult slices and subsequent application of MEC or MLA had no further effect, suggesting no nAChR involvement in cholinergic modulation of adult PC intrinsic fibre transmission, despite the presence of nAChRs in adult PC [27]. This accords with our previous report using adult guinea-pig PC slices [19], where several nAChR agonists had no effect on evoked EFPs. Although immature slices were not tested in this earlier study [19], guinea-pigs are born ‘neurologically competent’ [3] such that nAChR-mediated effects may be difficult, if not impossible, to demonstrate. In the present study, acetylcholinesterase-induced EFP suppression in immature PC was not fully reversed by atropine, and subsequent nAChR antagonist application was required to facilitate full recovery (Fig. 4A and B), suggestive of an nAChR component in cholinergic modulation of immature PC intrinsic fibre transmission. Our finding that acetylcholinesterase-induced immature EFP suppression was more successfully reversed using MLA (α7 nAChR-specific antagonist; [28]) rather than MEC (nonspecific nAChR antagonist [26]; Fig. 4A and B) is likely due to a non-saturating MEC concentration (1 μM), whilst the greater MLA-induced effect suggests a homomeric α7 nAChR-mediated component. A non-saturating MEC dose was used so as to avoid N-methyl-D-aspartate-type receptor (NMDAR) blocking effects that have been reported for higher MEC doses [25].

We cannot presently conclude whether nAChR-mediated effects were predominantly pre- and/or postsynaptic. A recent report [18] supports our data, showing that nicotinic and muscarinic depression of unitary excitatory postsynaptic potentials occurs in the sensory cortex. These authors suggested a postsynaptic NMDAR-mediated effect due to abolition by the NMDAR antagonist DL-APV (or MEC). Moreover, Fisher and Dani...
Fig. 4. The actions of nAChR antagonists, mecamylamine (MEC) and methyllycaconitine (MLA) on immature intrinsic PC EFPs. Control EFPs (black) were suppressed by 1 μM eserine (dark grey) and partially recovered following 1 μM atropine (light grey) application. Subsequent addition of 1 μM MEC (grey; A) or 1 μM MLA (grey; B) produced supranormal recovery, indicating basal cholinergic ‘tone’. MEC or MLA had no effect on adult slices (not shown). (C) Summary of nicotine (50 μM) effects, applied alone or in the presence of MEC (1 μM) or MLA (1 μM) on immature PC EFPs. Ordinate shows percentage change in EFP (E3) amplitude vs. control; data represent means ± S.E.M; significance levels vs. control: *P<0.01; **P<0.05; ***P<0.1. N.B.: Adult data not plotted since no nAChR-mediated effects were observed.

[9] suggested that postsynaptic α7 nAChR-mediated inhibition of NMDAR-mediated excitatory transmission may cause displacement of cytoskeletal elements, such as α-actinin and spectrin, resulting in conformational NMDAR changes. In further support, Chen et al. [5] showed strong, eserine-induced NMDAR-mediated EPSCs suppression in prefrontal cortex via allosteric nAChR activation; here, ERK activation following nAChR-mediated Ca2+ entry was suggested, causing NMDAR modulation via a microtubule-dependent mechanism. This may be of relevance to our present data, since we have previously shown a greater NMDAR-mediated component of excitatory neurotransmission in the immature PC, which may further predispose the area towards epileptiform events [29].

When considering the potential of nAChRs to inhibit postsynaptic NMDAR-mediated events, presynaptic α7 nAChR-mediated effects upon GABA release [1] also require consideration. α7 nAChRs have been shown to facilitate GABAergic activity via interneuronal excitation in hippocampal CA1 stratum radiatum [21]. Moreover, Christophe et al. [6] described direct α7 nAChR-mediated excitation of neocortical layer II–III interneurons, making it plausible that suppression of immature PC EFPs following nAChR activation is partly dependent on modulation of inhibitory mechanisms. However, this hypothesis (together with potential nAChR-mediated facilitation of glutamatergic transmission, e.g. [11,23]) will require confirmation in further experiments in the presence of GABA_A antagonists (see [12]). Finally, the absence of nicotine effects in adult PC may be explicable if one accepts that observed nicotinic effects in immature PC are indirectly mediated by NMDAR modulation and/or direct excitation of GABA interneurons; it is likely that NMDARs contribute less to excitatory synaptic potentials in adult PC [29] and would therefore be less subject to nicotinic modulation. Moreover adult PC interneurons may be less sensitive to nAChR activation, thereby releasing less GABA to affect excitatory neurotransmission.

Concluding, this study demonstrates functional nAChR modulation of intrinsically evoked excitatory transmission in immature PC and increases our understanding of developmental changes and increased epileptiform susceptibility. In terms of functional significance, we propose that nAChR-mediated modulation may provide a compensatory inhibitory mechanism, offsetting developmentally reduced suppression of excitatory synaptic transmission in immature PC [29]. If valid, then immature PC epileptiform susceptibility may be more dependent
upon developmental differences in modulation of inhibitory (increased mAChR-mediated inhibition of GABA-ergic IPSPs), than excitatory (decreased mAChR-mediated inhibition of glutamatergic EPSPs) transmission [29]. There is considerable interplay between the co-development of rat cortical muscarinic and nicotinic AChRs during the investigated age-range [32] such that excessive nAChR activation during development significantly inhibits mAChR transmission and may reflect a physiological propensity to balance the dynamic state of cholinergic transmission in the neonatal cortex. Further work is required to determine specific functional means by which nAChRs modulate synaptic responsiveness in developing PC.

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References