Muscarinic modulation of synaptic transmission via endocannabinoid signalling in the rat midbrain periaqueductal grey

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Abbreviations

ACSF, artificial cerebrospinal fluid; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H—pyrazole-3-carboxamide; carbachol, (2-Hydroxyethyl)trimethylammonium chloride carbamate; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione disodium; DAG lipase diacylglycerol lipase; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; EPSC, excitatory postsynaptic current; GABA, gamma-aminobutyric acid; HU210, (6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9 methanol; IPSC, inhibitory postsynaptic current; MAG lipase, monoacylglycerol lipase; mAChR, muscarinic cholinergic receptor; mecamylamine hydrochloride, N,2,3,3-Tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride; mGlur, metabotropic glutamate receptor; nAChR, nicotinic cholinergic receptor; NMDA, N-Methyl-D-aspartic acid; oxotremorine-M, N,N,N-Trimethyl-4-(2-oxo-1-pyrolidinyl)-2-butyl-1-ammonium iodide; PAG, periaqueductal grey; PD-102807, 3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-(12H)-isoquino [1,2-b]pyrrolo[3,2-f][1,3]benzoxazine-1-carboxylic acid, ethyl ester; physostigmine hemisulfate, (3aS)-cis-1,2,3,3a,8a-Hexahydro-1,3a,8-trimethylpyrrolo[2,3-b]indol-5-ol methylcarbamate hemisulfate; QX-314 bromide, N-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium bromide; SR95531, 6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinobutanoic acid hydrobromide; tetrahydrolipstatin, N-Formyl-L-leucine (1S)-1-[(2S,3S)-3-hexyl-4-oxo-2-oxetanylmethyl]dodecyl ester.
Abstract

The midbrain periaqueductal grey (PAG) is involved in organizing behavioural responses to threat, stress and pain. These PAG functions are modulated by cholinergic agents. In the present study, we examined the cholinergic modulation of synaptic transmission in the PAG using whole-cell voltage-clamp recordings from rat midbrain slices. We found that the cholinergic agonist carbachol reduced the amplitude of evoked inhibitory and excitatory postsynaptic currents (IPSCs, EPSCs) in all PAG neurons and this was abolished by the muscarinic receptor antagonist atropine. Carbachol increased the paired-pulse ratio of evoked IPSCs and EPSCs, and reduced the rate, but not the amplitude of spontaneous miniature IPSCs. The carbachol inhibition of evoked IPSCs was mimicked by the acetylcholinesterase inhibitor physostigmine and was reduced by the M1 and M1/M3 muscarinic cholinergic receptor (mAChR) antagonists pirenzepine and 4-DAMP, but not by the M2 and M4 antagonists gallamine and PD-102807. The carbachol inhibition of evoked IPSCs was reduced by the cannabinoid CB₁ receptor antagonist AM251 and the DAG lipase inhibitor tetrahydrolipstatin, and was abolished in the presence of both AM251 and gallamine. The carbachol inhibition of evoked EPSCs was also reduced in the presence of both gallamine and AM251. These results indicate that M1 induced inhibition of GABAergic transmission within the PAG is mediated via endocannabinoids which are produced via the phospholipase C/DAG lipase pathway and activate presynaptic cannabinoid CB₁ receptors. Thus, presynaptic muscarinic modulation of PAG function is mediated indirectly by M1 receptor induced endocannabinoid-signalling and directly by M2 receptors.
Cannabinoids modulate a number of functions within the central nervous system such as pain (Pacher et al., 2006). The midbrain PAG, a brain region which plays a pivotal role in integrating an animal's somatomotor, autonomic and behavioural responses to threat, stress and pain (Keay and Bandler, 2001), is a major site of the analgesic and anxiolytic actions of cannabinoid receptor agonists (Finn et al., 2003; Lichtman et al., 1996; Moreira et al., 2007). It has long been recognized that various stressors reduce pain and that this stress-induced analgesia is mediated via parallel opioid dependent and independent components within the PAG (Lewis et al., 1980). Recently it has been shown that microinjection of cannabinoid CB₁ receptor antagonists into the PAG reduces the opioid-independent component of stress-induced analgesia suggesting that it is mediated by endocannabinoids (Hohmann et al., 2005).

It is becoming apparent that endogenous cannabinoids play an important modulatory role in the CNS, by acting as retrograde messengers in a number of brain regions, where they are produced postsynaptically from cell bodies and travel backwards onto presynaptic nerve terminals to activate cannabinoid CB₁ receptors and reduce neurotransmitter release. Retrograde endocannabinoid signalling is initiated by Ca²⁺ influx subsequent to depolarisation of the postsynaptic neuron, and/or by activation of G₄-coupled group I metabotropic glutamate receptors (mGluR) in numerous brain regions (for review see Chevaleyre et al., 2006). In addition, activation of G₄-coupled mAChRs has also been shown to initiate retrograde endocannabinoid signalling in the hippocampus and striatum (Fukudome et al., 2004; Kim et al., 2002; Narushima et al., 2006; Narushima et al., 2007; Ohno-Shosaku et al., 2003; Uchigashima et al., 2007).

The PAG contains a dense plexus of cholinergic nerve terminals which arise from brain structures, such as the pontine tegmentum (e.g. Woolf et al., 1990). Acetylcholine produces its physiological effects via ligand gated nicotinic cholinergic receptors (nAChRs) and a heterogeneous family of mAChRs. There are five subtypes of muscarinic GPCRs, including the M2 and M4 subtypes which couple via G₁₀-proteins and the M1, M3 and M5 subtypes which couple via G₄-proteins (Caulfield, 1993). The midbrain PAG contains a range of nAChR and mAChR subtypes (e.g. Aubert et al., 1996; Yasuda et al., 1993). Microinjection of cholinergic agonists into the PAG produces analgesia and associated behavioural actions which are mediated by both nAChRs and mAChRs (Guimaraes et al., 2000; Monassi et al., 1997). Functional studies indicate that opioids and cannabinoids produce analgesia from within the PAG by reducing the inhibitory influence of GABAergic interneurons onto output neurons which form part of an endogenous descending

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analgesic pathway, a process known as disinhibition (Fields et al., 2006). At the cellular level, opioids and cannabionoids inhibit GABAergic and glutamatergic synaptic transmission in the PAG via a presynaptic mechanism (Vaughan et al., 2000; Vaughan et al., 1997). In the present study, we examined the effects of cholinergic receptor activation on GABAergic and glutamatergic synaptic transmission in the PAG and whether these effects were mediated by the endocannabinoid system.
Materials and methods

Experiments were carried out on male and female Sprague-Dawley rats (14 - 24 days old) under a protocol approved by the Royal North Shore Hospital/University of Technology Sydney Animal Care and Ethics Committee. Animals were anaesthetised with isoflurane, decapitated and the brain rapidly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) of composition (mM): NaCl 126, KCl 2.5, NaH2PO4 1.4, MgCl2 1.2, CaCl2 2.4, glucose 11, NaHCO3 25, equilibrated with 95 % O2 and 5 % CO2. Coronal midbrain slices containing PAG were then cut (300 µm) using a vibratome (VT1000S, Leica Microsystems, Nussbloom, Germany), in ice-cold ACSF, as described previously (Vaughan et al., 2000). The slices were maintained at 34 °C in a submerged chamber containing ACSF. The slices were then individually transferred to a recording chamber and superfused continuously (1.6 – 1.8 ml.min⁻¹) with ACSF (34 °C).

PAG neurons were visualized using infra-red Dodt-tube optics on an upright microscope (Olympus BX50, Olympus, Sydney). Whole-cell voltage clamp recordings (holding potential -65 mV) were made using an Axopatch 200B (Molecular Devices, Sunnyvale, USA), with an internal solution of composition (mM): CsCl 140, HEPES 10, EGTA 0.2, MgCl2 1, MgATP 2, NaGTP 0.3 (pH 7.3, osmolarity 280 - 285 mOsm.l⁻¹). Series resistance (< 30 MΩ) was compensated by 80% and continuously monitored during experiments. Electrically evoked synaptic currents were elicited in neurons (rate 0.083 s⁻¹, stimuli: 1 - 40 V, 50 - 300 µs) via unipolar glass or bipolar tungsten stimulating electrodes (tip separation = 50 µm) placed 20 - 100 µm from the recording electrode. GABA_A-receptor mediated IPSCs were obtained in the presence of the non-NMDA receptor antagonist, CNQX (5 µM) and the glycine receptor antagonist, strychnine (5 µM). Non-NMDA mediated EPSCs were obtained in the presence of picrotoxin (100 µM) and strychnine (5 µM). In some experiments, spontaneous miniature IPSCs were obtained in the presence of CNQX (5 µM), strychnine (5 µM) and the addition of tetrodotoxin (300 nM).

IPSCs and EPSCs were filtered (2 - 5 kHz low-pass filter) and sampled (5 - 10 kHz) for on-line and later off-line analysis (Axograph X, Axograph Scientific Software, Sydney, Australia). Miniature IPSCs were sampled in 5 s epochs every 6 s for analysis and IPSCs above a preset threshold (4.5 - 5.0 standard deviations above baseline noise) were automatically detected by a sliding template algorithm, then manually checked offline. Plots of detected event frequency versus time and cumulative probability distributions of event amplitudes...
and inter-event intervals were constructed. Statistical comparisons between two groups were made using
Student's paired t-tests, and those between more than two groups were made using a one-way analysis of
variance (ANOVA) followed by post-hoc comparisons using the Newman-Keuls correction for multiple
comparisons (Prism, Graphpad Software, San Diego, USA). Differences were considered significant when P
< 0.05. Dose-response curves were constructed using a logistic function (Prism). All pooled data were
expressed as means ± SEM.

4-DAMP, mecamylamine hydrochloride, oxotremorine-M, PD-102807, physostigmine hemisulfate and
SR95531 hydrobromide were obtained from Tocris Cookson (Bristol, UK); QX-314 bromide and tetrodotoxin
(TTX) from Alomone Laboratories (Jerusalem, Israel); atropine, CNQX, gallamine triethiodide, carbachol,
pirenzepine dihydrochloride, strychnine hydrochloride and (-)-tetrahydrolipstatin (Orlistat) from Sigma
(Sydney, Australia); AM251 and HU210 from Cayman Chemical Co. (Ann Arbor, USA). Stock solutions of all
drugs were prepared in distilled water, or dimethyl sulfoxide, then diluted to working concentrations with
ACSF and applied by superfusion.
Results

Cholinergic agonists and endogenously released acetylcholine presynaptically inhibit GABAergic synaptic transmission via muscarinic receptors

In the presence of CNQX (5 μM) and strychnine (5 μM), local electrical stimulation evoked IPSCs in PAG neurons which were abolished by tetrodotoxin (0.3 μM, n = 4) and the GABA_A-receptor antagonist, SR95531 (10 μM, n = 5). When a supramaximal concentration of the non-selective cholinergic agonist, carbachol (10 μM) was superfused onto midbrain slices, it produced a reduction in the amplitude of evoked IPSCs in all PAG neurons tested, which reversed following washout (n = 16), or addition of the broad spectrum mAChR antagonist, atropine (3 μM, n = 8) (Figure 1a, b). On average, carbachol (10 μM) reduced the evoked IPSC amplitude to 53 ± 2 % of control (Figure 1d, P < 0.0001, range = 36 – 72 %, n = 24). The inhibition of evoked IPSCs produced by carbachol (10 μM) was similar in neurons within the ventrolateral, lateral and dorsal/dorsolateral PAG columns (P = 0.1, n = 9, 7, 8). In most of these recordings, paired evoked IPSCs were elicited by two stimuli of equal strength, in close succession (inter-stimulus interval = 70 ms) to examine paired pulse ratios. Carbachol (10 μM) increased the ratio of evoked IPSC_2/IPSC_1 from 1.03 ± 0.06 to 1.18 ± 0.07 (Figure 1c, d, P = 0.002, n = 23). Carbachol (10 μM) had no effect on membrane current, or the conductance of the neurons at –65 mV. These changes in paired-pulse ratio suggest that carbachol inhibits GABAergic synaptic transmission via a presynaptic locus of action.

We next examined whether endogenously released acetylcholine modulated GABAergic synaptic transmission. Superfusion of the reversible acetylcholinesterase inhibitor, physostigmine (100 μM) produced a reduction in the basal amplitude of evoked IPSCs, which reversed following washout, or addition of atropine (3 μM) (Figure 2a, b, d, P = 0.0005, n = 7). In addition, physostigmine (100 μM) increased the paired pulse ratio of evoked IPSC_2/IPSC_1 from 1.00 ± 0.18 to 1.31 ± 0.26 (Figure 2c, d, P = 0.04, n = 7). Superfusion of atropine (3 μM) alone, however, had no effect on the basal amplitude of evoked IPSCs (110 ± 5 % of control, P = 0.08, n = 6), or on the ratio of evoked IPSC_2/IPSC_1 (97 ± 3 % of control, P = 0.4, n = 6). Physostigmine and atropine had no effect on membrane current, or the conductance of the neurons at –65 mV.
To further confirm the presynaptic locus of action of the cholinergic agonists, the effects of carbachol and the selective muscarinic agonist, oxotremorine on miniature IPSCs were examined. In the presence of CNQX (5 µM), strychnine (5 µM) and TTX (300 nM), spontaneous miniature IPSCs were readily observed (Figure 3e). Carbachol (3 - 10 µM) produced a reversible reduction in the rate of miniature IPSCs in the majority of PAG neurons tested, and this was associated with a rightward shift in the cumulative probability distribution of miniature IPSC inter-event intervals (Figure 3a, d, e). The reduction in miniature IPSC rate produced by carbachol (3 - 10 µM) was reversed by the addition of atropine (3 µM) (Figure 3a, n = 5). In contrast, carbachol (3 - 10 µM) did not produce a change in the amplitude or kinetics of miniature IPSCs, or in the cumulative probability distributions of miniature IPSC amplitudes (Figure 3b, c). In the presence of carbachol (3 - 10 µM), the mean miniature IPSC rate and amplitude were 55 ± 3% (P < 0.0001) and 95 ± 4 % (P = 0.2) of control, respectively (Figure 3f, n = 16). This reduction in miniature IPSC rate produced by carbachol was concentration dependent, with an IC₅₀ of 180 nM (95% confidence interval = 70 – 460 nM) and a Hill slope of 1.0 ± 0.5 (Figure 4). The selective mAChR agonist, oxotremorine also produced a concentration dependent reduction in miniature IPSC rate, but not amplitude, with an IC₅₀ of 290 nM (95% confidence interval = 31 – 2,830 nM) and hill slope of 1.2 ± 0.8 (Figure 4). Together, these results suggest that muscarinic receptor activation presynaptically inhibits GABAergic synaptic transmission in PAG.

**Cholinergic agonists inhibit GABAergic synaptic transmission via M1/M3 mAChR subtypes**

We further examined the receptor subtypes involved in the effects of carbachol by pre-incubating slices in a range of cholinergic antagonists. In slices pre-incubated in atropine (3 µM), carbachol (10 µM) had no effect on the amplitude of evoked IPSCs (Figure 5a, g, P = 0.8, n = 5). By contrast, carbachol produced a reduction in the amplitude of evoked IPSCs in slices pre-incubated with the nAChR antagonist mecamylamine (3 µM) (P < 0.0001, n = 5), which was not significantly different to the reduction produced by carbachol alone (Figure 5b, g, P > 0.05).

We then examined the effect of a range of subtype selective mAChR antagonists. In the presence of the M2 selective antagonist gallamine (100 µM), carbachol (10 µM) produced a reduction in the amplitude of evoked IPSCs (P = 0.01, n = 7), which was not significantly different to that produced by carbachol alone (Figure 5c,
g, P > 0.05). In the presence of the M4 selective antagonist PD-102807 (500 nM), carbachol (10 µM) also produced a reduction in the amplitude of evoked IPSCs (P = 0.004, n = 5), which was not significantly different to that produced by carbachol alone (Figure 5d, g, P > 0.05). Carbachol (10 µM) also produced a reduction in the amplitude of evoked IPSCs in the presence of the M1/M3 selective antagonist, 4-DAMP (30 nM, P = 0.04, n = 5), and the M1 selective antagonist, pirenzepine (300 nM) (P = 0.03, n = 6), however this was significantly less than that produced by carbachol alone (Figure 5e, f, g, P < 0.001). These results suggest that the M1, and possibly the M3 mAChR receptors predominantly mediate the effects of muscarinic receptor activation in PAG.

**Cholinergic inhibition of GABAergic transmission is mediated via endocannabinoids**

Studies in the hippocampus and striatum have shown that M1/M3-induced suppression of synaptic transmission is mediated by retrograde endocannabinoid signalling. Recent evidence suggests that the endocannabinoid involved is 2-AG, which is produced via the phospholipase C/ DAG lipase pathway (Hashimotodani et al., 2007; Hashimotodani et al., 2005; Maejima et al., 2005; Melis et al., 2004; Newman et al., 2007; Uchigashima et al., 2007). We therefore examined whether the M1/M3 induced effects on synaptic transmission in PAG are mediated by endocannabinoids. In the presence of the cannabinoid CB1 receptor antagonist AM251 (3 µM), carbachol (10 µM) produced a reduction in the amplitude of evoked IPSCs (P = 0.04, n = 7) which was significantly less than that produced by carbachol alone (Figure 6a, c, P < 0.001). The effect of carbachol was also occluded by the non-selective cannabinoid receptor agonist, HU210 (3 uM), with carbachol having no significant effect on the amplitude of evoked IPSCs in its presence (P = 0.05, n = 8). In addition, carbachol (3 - 10 µM) did not produce a significant change in miniature IPSC rate in the presence of AM251 (3 µM) (rate = 81 ± 13 % of control, P = 0.2, n = 9). Physostigmine (100 µM) also produced a reduction in the amplitude of evoked IPSCs in the presence of AM251 (P = 0.04, n = 5), which was significantly less than that produced by physostigmine alone (P = 0.008). In the presence of the DAG lipase inhibitor, tetrahydrolipstatin (10 µM), carbachol (10 µM) produced a reduction in the amplitude of evoked IPSCs (P = 0.006, n = 6) which was also significantly less than that produced by carbachol alone (Figure 6b, c, P < 0.001).
Since the carbachol induced inhibition of evoked IPSCs was not abolished by pirenzepine, AM251 or tetrahydrolipstatin, we examined whether the residual inhibition was mediated by presynaptic M2 mAChRs. In the combined presence of gallamine (100 uM) and pirenzepine (300 nM), carbachol (10 µM) had no significant effect on the amplitude of evoked IPSCs (Figure 5g, P = 0.5, n = 6). Similarly, carbachol did not significantly affect evoked IPSC amplitude the presence of gallamine (100 uM) and either AM251 (3 µM) (P = 0.09, n = 5), or THL (10 µM) (Figure 6c, P = 0.06, n = 5).

**Cholinergic inhibition of glutamatergic transmission is partly mediated via endocannabinoids**

We finally examined whether carbachol also inhibited glutamatergic synaptic transmission in PAG via endocannabinoids. In the presence of picrotoxin (100 µM) and strychnine (5 µM), superfusion of carbachol (10 µM) produced a reduction in the amplitude of non-NMDA mediated evoked EPSCs which was reversed following addition of atropine (3 µM) (Figure 7a, e, P < 0.0001, n = 11) and was associated with an increase in the ratio of evoked EPSC2/EPSC1 (P = 0.03). Carbachol (10 µM) produced a reduction in the amplitude of evoked EPSCs in the presence of AM251 (3 µM) (P = 0.005, n = 8) which was not significantly different to that produced by carbachol alone (Figure 7b, e, P > 0.05). Similarly, carbachol (10 µM) reduced the amplitude of evoked EPSCs in the presence of gallamine (100 µM) (P = 0.04, n = 6) and this was not significantly different to that produced by carbachol alone (Figure 7c, e, P > 0.05). By contrast, carbachol (10 µM) produced a reduction in the amplitude of evoked EPSCs in the combined presence of AM251 (3 µM) and gallamine (100 µM) (P = 0.04, n = 7) which significantly less than that produced by carbachol alone (Figure 7d, e, P < 0.05).
Discussion

The present study has demonstrated that cholinergic agonists and endogenously released acetylcholine inhibit GABAergic and glutamatergic synaptic transmission within the PAG via activation of both M1 and M2 mAChRs. The M1 induced cholinergic inhibition appears to be indirectly mediated via endocannabinoids, produced via the phospholipase C/DAG lipase pathway, which activate presynaptic cannabinoid CB1 receptors and subsequently decrease GABA and glutamate release from nerve terminals. These findings suggest that cholinergic actions within the PAG are, at least partly, mediated via the endocannabinoid signalling system.

A number of observations suggested that cholinergic agonists and endogenously released acetylcholine suppressed GABAergic and glutamatergic synaptic transmission via a presynaptic muscarinic mechanism. In the present study, the non-degradable cholinergic agonist carbachol caused a reduction in the amplitude of evoked GABA\textsubscript{A} mediated IPSCs and evoked non-NMDA mediated EPSCs which was associated with an increase in their paired-pulse ratios. This carbachol induced inhibition was reversed by the muscarinic antagonist atropine, but not by the nicotinic antagonist mecamylamine. Carbachol and the muscarinic agonist oxotremorine-M also produced a concentration-dependent reduction in the frequency, but not the amplitude of spontaneous miniature IPSCs and had no effect on the membrane conductance of neurons. This muscarinic presynaptic inhibition of synaptic transmission is similar to that observed in other brain regions (Behrends and Ten Bruggencate, 1993; Bellingham and Berger, 1996; Fukudome et al., 2004; Li et al., 2004; Shen and Johnson, 2000). It is also similar to that described previously for opioids and cannabinoids within the PAG (Vaughan et al., 2000; Vaughan et al., 1997), both of which produce analgesia when injected into this brain structure (Fields et al., 2006). While atropine alone had no effect on synaptic transmission, the acetylcholinesterase inhibitor physostigmine produced an atropine sensitive reduction in evoked IPSCs. This indicates that endogenously released acetylcholine can also inhibit GABAergic synaptic transmission within the PAG and tonically modulate analgesia, however, this appears to be normally suppressed by uptake and degradation, at least in the slice preparation.

Binding and immunohistochemical studies have shown that the M1, M2, M3 and M4 mAChR subtypes are present within the midbrain (e.g. Aubert et al., 1996; Yasuda et al., 1993). In the present study, the carbachol
induced inhibition of GABAergic transmission was significantly reduced by the M1 and M1/M3 antagonists pirenzepine and 4-DAMP, but not by the M2 and M4 antagonists, gallamine and PD-102807. Furthermore, complete suppression of carbachol’s inhibitory effect was obtained in the combined presence of pirenzepine and gallamine. These results suggest that the mAChR induced inhibition of GABAergic transmission was largely mediated by the M1 receptor and possibly the M3 receptors, with a smaller M2 receptor contribution, similar to that previously observed in the hippocampus (Fukudome et al., 2004; Ohno-Shosaku et al., 2003). While gallamine also had no effect on the carbachol induced inhibition of glutamatergic transmission, M2 receptors were also likely to have a role in excitatory transmission (see below). The present findings differ to those in other brain regions where cholinergic inhibition of GABAergic and glutamatergic transmission is largely mediated exclusively by M1/M3 and M2 receptors, respectively (Bellingham and Berger, 1996; Li et al., 2004; Shen and Johnson, 2000). It should be emphasised, however, that some of the antagonists used in the present and prior studies display modest selectivity (Caulfield, 1993) and the receptor subtypes involved would need to be verified by knocking out mAChR subtypes (e.g. Fukudome et al., 2004; Ohno-Shosaku et al., 2003).

The observation that the cholinergic inhibition of GABAergic synaptic transmission was largely mediated by M1 mAChRs is interesting because, like group I mGluRs (mGluR1 and mGluR5), M1/M3/M5 mAChRs are G_{q}-coupled receptors which act via the phospholipase C/diacylglycerol cascade (Caulfield, 1993). It has previously been demonstrated that group I mGluR induced inhibition of GABAergic synaptic transmission is mediated indirectly via the endocannabinoid system in a number of brain regions (for review see Chevaleyre et al., 2006), including the PAG (Drew et al., 2008). Although studied to a lesser extent, M1/M3 mAChR activation also inhibits GABAergic synaptic transmission via an endocannabinoid dependent mechanism in the hippocampus and striatum (Fukudome et al., 2004; Narushima et al., 2006; Narushima et al., 2007; Ohno-Shosaku et al., 2003). In the present study, the carbachol induced inhibition of evoked and miniature IPSCs was reduced by the cannabinoid CB1 receptor antagonist AM251 and occluded by the pan-cannabinoid receptor agonist HU210, consistent with that previously observed in the hippocampus and striatum (Fukudome et al., 2004; Kim et al., 2002; Narushima et al., 2007; Ohno-Shosaku et al., 2003; Uchigashima et al., 2007). The carbachol induced inhibition of evoked IPSCs was abolished in the combined presence of AM251 and gallamine. In addition, the carbachol induced inhibition of evoked EPSCs was
reduced by AM251 and gallamine together, but not individually. Therefore it is likely that muscarinic inhibition of GABAergic and glutamatergic synaptic transmission within the PAG is mediated by parallel mechanisms, including indirect M1 mAChR-induced production endocannabinoids which activate presynaptic cannabinoid CB1 receptors and direct presynaptic M2 mAChR activation. These findings are similar to those previously reported for acetylcholine, glutamate and serotonin which also modulate synaptic transmission via indirect Gq-coupled endocannabinoid mechanisms and direct Gi/o-coupled presynaptic inhibition in the PAG and other brain regions (Best and Regehr, 2008; Drew et al., 2008; Fukudome et al., 2004). The present findings, however, differ to the striatum where muscarinic inhibition of GABAergic, but not glutamatergic synaptic transmission is mediated by endocannabinoids (Narushima et al., 2006).

To date, two major endocannabinoids, anandamide and 2-arachidonylglycerol, have been identified which are biosynthesised via the phospholipase D and phospholipase C/DAG lipase pathways, respectively (for review see Pacher et al., 2006). There is increasing evidence that 2-arachidonylglycerol mediates the M1/M3 mAChR and group I mGluR induced inhibition of synaptic transmission because it is abolished by phospholipase C and DAG lipase inhibitors, and by knocking out phospholipase Cβ (Hashimotodani et al., 2007; Hashimotodani et al., 2005; Maejima et al., 2005; Melis et al., 2004; Newman et al., 2007; Uchigashima et al., 2007). While we did not examine the role of phospholipase C, it was found that the carbachol induced inhibition of evoked IPSCs was reduced by the DAG lipase inhibitor tetrahydrolipstatin to similar extent as that produced by AM251. This indicates that the phospholipase C/DAG lipase pathway is involved in the M1 mAChR-induced effects within the PAG, and supports the growing evidence suggesting that 2-AG, rather than anandamide is the endocannabinoid involved in M1/M3 mAChR-induced suppression of synaptic transmission.

Cannabinoids have a pivotal role in analgesia and anxiety in the PAG (Finn et al., 2003; Lichtman et al., 1996; Moreira et al., 2007), and modulation of the endocannabinoid system represents a promising new target for the treatment of anxiety and pain, particularly within the PAG ((Hohmann et al., 2005; Kathuria et al., 2003). The functional effects of cholinergic agents within the PAG are largely mediated via the GABAergic system (Monassi et al., 1999). It is thought that cannabinoids produce analgesia within the PAG in a similar fashion to µ-opioids, by indirectly reducing GABAergic transmission onto output neurons that form part of an endogenous descending analgesic system (Fields et al., 2006). The present observation of
endocannabinoid mediated presynaptic inhibition of GABAergic transmission by cholinergic agonists and endogenous acetylcholine is consistent with the analgesic and anxiolytic mechanism of action of cannabinoids and opioids in the descending system. Therefore, the present findings suggest that cholinergic induced analgesic and other behavioural effects within the PAG are at least partly mediated by endocannabinoid-dependent mechanisms. These findings also raise the possibility that like group 1 mGluRs, endocannabinoid signalling via M1/M3 mAChRs might occur in numerous brain regions. Ultimately, this will contribute to the growing number of studies suggesting that endocannabinoid signalling may be an important global phenomenon underlying cholinergic transmission within the brain.
References


receptor subtype 1 to phospholipase C beta 4 signaling cascade in the cerebellum. *J Neurosci* **25**:6826-6835.


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Figure 1. Carbachol inhibits evoked IPSCs in PAG neurons. (A) Time course of evoked IPSC amplitude (eIPSC Ampl) during application of carbachol (10µM) and then following addition of atropine (3µM). (B) Averaged evoked IPSCs before (Pre) and during application of carbachol, then after addition of atropine. (C) Averaged evoked IPSCs in response to identical paired stimuli (inter-stimulus interval = 70 ms) for the traces in B, with IPSC1 normalized. (D) Bar chart showing the amplitude of evoked IPSC1 (eIPSC1) and the ratio of eIPSC2:1 in the presence of carbachol (10 µM), expressed as a percentage of the pre-carbachol level. In (D) ** denotes P < 0.01 and *** P < 0.001. Traces in (A - C) are from the same neuron.

Figure 2. Physostigmine inhibits evoked IPSCs in PAG neurons. (A) Time course of evoked IPSC (eIPSC) amplitude during application of physostigmine (Physostig, 100 µM) and then following addition of atropine (3µM). (B) Averaged evoked IPSCs before (Pre) and during application of physostigmine (100 µM). (C) Averaged evoked IPSCs in response to identical paired stimuli (inter-stimulus interval = 70 ms) for the traces in B, with IPSC1 normalized. (D) Bar chart showing the amplitude of evoked IPSC1 (eIPSC1) and the ratio of eIPSC2:1 in the presence of physostigmine (100 µM), expressed as a percentage of the pre-physostigmine level. In (D) * denotes P < 0.05 and *** P < 0.001. Traces in (A - C) are from the same neuron.

Figure 3. Carbachol decreases the rate, but not the amplitude of miniature IPSCs. (A) Time course of miniature IPSC (mIPSC) rate during superfusion of carbachol (3 µM) and then during addition of atropine (3 µM). (B) Averaged traces of miniature IPSCs before (Pre) and during carbachol (3 µM). Cumulative distribution plots of miniature IPSC (C) amplitude and (D) inter-event interval before and during carbachol. (E) Raw current traces of miniature IPSCs before (Pre) and during superfusion of carbachol (3 µM). (F) Bar chart of the mean rate and amplitude of miniature IPSCs in the presence of carbachol (3 – 10 µM), expressed as a percentage of the pre- carbachol level. *** denotes P < 0.001. Traces in (A - E) are from the same neuron.
Figure 4. Carbachol and Oxotremorine produce a concentration dependent reduction in miniature IPSC rate. Concentration-response curves of the inhibition of miniature IPSC (mIPSC) rate produced by carbachol and oxotremorine-M, expressed as a percentage of the pre-drug value. A logistic function was fitted to the curves to determine the IC$_{50}$. Data presented as mean ± SEM for n = 3 - 12 neurons.

Figure 5. Carbachol inhibition of evoked IPSCs is largely mediated by M1 mAChRs. Averaged evoked IPSCs are shown prior to (Pre) and during addition of carbachol (10 µM) in slices pre-incubated in (A) the broad spectrum mAChR antagonist atropine (3µM), (B) the nAChR antagonist mecamylamine (Mecamyl, 3µM), (C) the M2 antagonist gallamine (Gallam, 100 µM), (D) the M4 antagonist PD-102807 (500 nM), (E) the M1/M3 antagonist 4-DAMP (30 nM) and (F) the M1 antagonist pirenzepine (Pirenz, 300 nM). (G) Bar chart showing the percentage inhibition of evoked IPSCs produced by carbachol (10 µM) alone (Control), and in the presence of mecamylamine, atropine, gallamine, PD-102807, 4-DAMP, pirenzepine, and pirenzepine plus gallamine. In (G) *, ** and *** denotes significantly different compared to pre-carbachol value (P < 0.05, 0.01 and 0.001) and ## denotes significantly different between the control and pre-treatment groups (P < 0.01). Traces in (A - F) are from different neurons. Scale bars in (A – F) are 200 pA and 10 ms.

Figure 6. Cholinergic inhibition of evoked IPSCs is mediated partly via endocannabinoids. Averaged evoked IPSCs before (Pre) and during carbachol (10 µM) in slices pre-incubated in (A) the CB$_1$ antagonist AM251 (3 µM), or (B) the DAG lipase inhibitor tetrahydrolipstatin (THL, 10 µM). (C) Bar chart showing the percentage inhibition of evoked IPSCs produced by carbachol (10 µM) alone (Control), and in the presence of AM251 (3 µM), or THL (10 µM) alone, and combined with the M2 antagonist gallamine (Gallam, 100 µM). In (C) *, ** and *** denote significantly different from pre-carbachol values (P < 0.05, 0.01, 0.001). ## denotes significantly different between control and other pre-treatment groups (P < 0.01). Traces in (A - B) are from different neurons. Scale bars in (A - B) are 100 pA and 10 ms.

Figure 7. Cholinergic inhibition of evoked EPSCs is partly mediated via endocannabinoids. Averaged evoked EPSCs before (Pre) and during carbachol (10 µM) in (A) untreated slices (Control) and in slices pre-
incubated in (B) the CB$_1$ antagonist AM251 (3 µM), (C) the M2 antagonist gallamine (Gallam, 100 µM) and (D) both AM251 and gallamine. (E) Bar chart showing the percentage inhibition of evoked EPSCs produced by carbachol (10 µM) alone (Control), and in the presence of AM251 (3µM) and/or gallamine (100 µM). In (E) *, ** and *** denotes significantly different compared to pre-carbachol value (P < 0.05, 0.01 and 0.001) and # denotes significantly different between the control and pre-treatment groups (P < 0.05). Traces in (A - D) are from different neurons. Scale bars are (A) 200 pA and 5 ms, (B) 50 pA and 5 ms, (C) 20 pA and 5 ms and (D) 50 pA and 5 ms.
Figure 1

A

B

C

D

Carbachol  Atropine

Pre  Carbachol  +Atropine

ePSC Amp (pA)

0  10  20

Time (min)

10 ms

50 pA

20 ms

100

0

ePSC (% Pre)

ePSC1  ePSC2:1
Figure 2

A

Phosphatid Atropine

eIPSC Ampl (pA)

Time (min)

B

Pre
Phosphatid

100 pA

10 ms

C

D

eIPSC (% Pre)

eIPSC1 eIPSC2:1

20 ms

* ***
Figure 4

The graph shows the effect of different concentrations of carbachol and oxotremorine on mIPSC rate (% Pre) across various concentrations in vitro. The results indicate a dose-dependent inhibition of mIPSC rate with increasing concentrations of both compounds.
Figure 5

- **A** Atropine
- **B** Mecamy
- **C** Gallam
- **D** PD-102807
- **E** 4-DAMP
- **F** Pirenz

**G**

<table>
<thead>
<tr>
<th>Condition</th>
<th>eIPSC Ampl (% Pre)</th>
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<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>Mecamy</td>
<td>***</td>
</tr>
<tr>
<td>Atropine</td>
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<td>Pirenz</td>
<td>*</td>
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<tr>
<td>Pirenz + Gallam</td>
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### Notes
- Pre and Carbachol lines indicated.
- Statistical significance indicated by asterisks: *** p < 0.001, ** p < 0.01, * p < 0.05.
Figure 6
Figure 7

A. Control

B. AM251

C. Gallam

D. AM251 + Gallam

E. Graph showing eEPSC (% Pre) with bars for Control, AM251, Gallam, AM251 + Gallam, indicating statistical significance indicated by *** and **.