

# Muscarinic Modulation of Synaptic Transmission via Endocannabinoid Signalling in the Rat Midbrain Periaqueductal Gray

Benjamin K. Lau and Christopher W. Vaughan

*Pain Management Research Institute, Kolling Institute of Medical Research, Northern Clinical School, University of Sydney at Royal North Shore Hospital, Sydney, Australia*

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## ABSTRACT

The midbrain periaqueductal gray (PAG) is involved in organizing behavioral 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 and EPSCs, respectively) 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 it 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 receptor antagonists pirenzepine and 4-diphenylacetoxy-*N*-methylpiperidine, but not by the M2 and M4 antagonists gallamine and

PD-102807 (3,6a,11,14-tetrahydro-9-methoxy-2-methyl-(12*H*)-isoquinol[1,2-*b*]pyrrolo[3,2-*f*][1,3]benzoxazine-1-carboxylic acid, ethyl ester). The carbachol inhibition of evoked IPSCs was reduced by the cannabinoid CB<sub>1</sub> receptor antagonist AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide) and the diacylglycerol (DAG) lipase inhibitor tetrahydrolipstatin, and it was abolished in the presence of both AM251 and gallamine. The carbachol inhibition of evoked EPSCs was also reduced in the combined presence of 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 CB<sub>1</sub> receptors. Thus, presynaptic muscarinic modulation of PAG function is mediated indirectly by M1 receptor-induced endocannabinoid signaling 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 that plays a pivotal role in integrating an animal's somatomotor, autonomic, and behavioral responses to threat, stress, and pain (Keay and Bandler, 2001), is a major site of the analgesic and anxiolytic actions

of cannabinoid receptor agonists (Lichtman et al., 1996; Finn et al., 2003; 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). It has been shown that microinjection of cannabinoid CB<sub>1</sub> 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 central nervous

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**ABBREVIATIONS:** PAG, periaqueductal gray; CB, cannabinoid; mGluR, metabotropic glutamate receptor; mAChR, muscarinic cholinergic receptor; nAChR, nicotinic cholinergic receptor; ACSF, artificial cerebrospinal fluid; NMDA, *N*-methyl-D-aspartic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; IPSC, inhibitory postsynaptic current; EPSC, excitatory postsynaptic current; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; DAG, diacylglycerol; PD-102807, 3,6a,11,14-tetrahydro-9-methoxy-2-methyl-(12*H*)-isoquinol[1,2-*b*]pyrrolo[3,2-*f*][1,3]benzoxazine-1-carboxylic acid, ethyl ester; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide; QX-314 bromide, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; HU210, (6*aR*)-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9 methanol; THL, tetrahydrolipstatin; eIPSC, evoked inhibitory postsynaptic current; Gallam, gallamine.

system, by acting as retrograde messengers in a number of brain regions, where they are produced postsynaptically from cell bodies and travel backward onto presynaptic nerve terminals to activate cannabinoid CB<sub>1</sub> receptors and reduce neurotransmitter release. Retrograde endocannabinoid signaling is initiated by Ca<sup>2+</sup> influx subsequent to depolarization of the postsynaptic neuron, and/or by activation of G<sub>q</sub>-coupled group I metabotropic glutamate receptors (mGluRs) in numerous brain regions (for review, see Chevalere et al., 2006). In addition, activation of G<sub>q</sub>-coupled muscarinic cholinergic receptors (mAChRs) has also been shown to initiate retrograde endocannabinoid signaling in the hippocampus and striatum (Kim et al., 2002; Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Narushima et al., 2006, 2007; Uchigashima et al., 2007).

The PAG contains a dense plexus of cholinergic nerve terminals that 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 G protein-coupled receptors, including the M2 and M4 subtypes that couple via G<sub>i/o</sub> proteins and the M1, M3, and M5 subtypes, which couple via G<sub>q</sub> proteins (Caulfield, 1993). The midbrain PAG contains a range of nAChR and mAChR subtypes (Yasuda et al., 1993; Aubert et al., 1996). Microinjection of cholinergic agonists into the PAG produces analgesia and associated behavioral actions that are mediated by both nAChRs and mAChRs (Monassi et al., 1997; Guimarães et al., 2000). However, the precise cellular mechanisms of action are unknown. Functional studies indicate that opioids and cannabinoids produce analgesia from within the PAG by reducing the inhibitory influence of GABAergic interneurons onto output neurons that form part of an endogenous descending analgesic pathway, a process known as disinhibition (Fields et al., 2006). At the cellular level, opioids and cannabinoids inhibit GABAergic and glutamatergic synaptic transmission in the PAG via a presynaptic mechanism (Vaughan et al., 1997, 2000). 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 anesthetized with isoflurane, decapitated, and the brain rapidly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) of the following composition: 126 mM NaCl, 2.5 mM KCl, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgCl<sub>2</sub>, 2.4 mM CaCl<sub>2</sub>, 11 mM glucose, and 25 mM NaHCO<sub>3</sub>, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Coronal midbrain slices containing PAG were then cut (300 μm) using a Vibratome (VT1000S; Leica Microsystems, Nussloch, 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) with ACSF (34°C).

PAG neurons were visualized using infra-red Dodt-tube optics on an upright microscope (Olympus BX50; Olympus, Sydney, Australia). Whole-cell voltage-clamp recordings (holding potential, –65 mV) were made using an Axopatch 200B patch clamp (Molecular

Devices, Sunnyvale, CA), with an internal solution of the following composition: 140 mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 1 mM MgCl<sub>2</sub>, 2 mM MgATP, and 0.3 mM NaGTP (pH 7.3; osmolarity, 280–285 mOsm). 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<sup>–1</sup>; stimuli, 1–40 V, 50–300 μs) via unipolar glass or bipolar tungsten stimulating electrodes (tip separation, 50 μm) placed 20 to 100 μm from the recording electrode. GABA<sub>A</sub>-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 online and later offline 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 and then manually checked offline. Plots of detected event frequency versus time and cumulative probability distributions of event amplitudes and interevent 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 followed by post hoc comparisons using the Newman-Keuls correction for multiple comparisons (Prism; GraphPad Software Inc., San Diego, CA). Differences were considered significant when *P* < 0.05. Dose-response curves were constructed using a logistic function (Prism). All pooled data are expressed as means ± S.E.M.

4-DAMP, mecamylamine hydrochloride (*N*,2,3,3-tetramethylbicyclo-[2.2.1]heptan-2-amine hydrochloride), oxotremorine-M [*N*,*N*,*N*-trimethyl-4-(2-oxo-1-pyrolidinyl)-2-butyn-1-ammonium iodide], PD-102807, physostigmine hemisulfate [(3*a*S)-*cis*-1,2,3,3*a*,8,8*a*-hexahydro-1,3*a*,8-trimethylpyrrolo[2,3-*b*]indol-5-ol methylcarbamate hemisulfate], and SR95531 hydrobromide were obtained from Tocris Cookson Inc. (Bristol, UK); QX-314 bromide and tetrodotoxin were from Alomone Laboratories (Jerusalem, Israel); atropine, CNQX, gallamine triethiodide, carbachol [(2-hydroxyethyl)trimethylammonium chloride carbamate], pirenzepine dihydrochloride, strychnine hydrochloride, and (–)-tetrahydrolipstatin (Orlistat) were from Sigma (Sydney, Australia); and AM251 and HU210 were from Cayman Chemical (Ann Arbor, MI). Stock solutions of all drugs were prepared in distilled water, or dimethyl sulfoxide, and 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 that were abolished by tetrodotoxin (0.3 μM; *n* = 4) and the GABA<sub>A</sub>-receptor antagonist SR95531 (10 μM; *n* = 5). When a supra-maximal concentration of the nonselective 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 after washout (*n* = 16) or addition of the broad-spectrum mAChR antagonist atropine (3 μM; *n* = 8) (Fig. 1, A and B). On average, carbachol (10 μM) reduced the evoked IPSC amplitude to 53 ± 2% of control (*P* < 0.0001; range, 36–72%; *n* = 24) (Fig. 1D). The inhibition of evoked IPSCs produced by carbachol (10 μM)

was similar in neurons within the ventrolateral, lateral, and dorsal/dorsolateral PAG columns ( $P = 0.1$ ;  $n = 9, 7$ , and  $8$ , respectively). In most of these recordings, paired evoked IPSCs were elicited by two stimuli of equal strength, in close succession (interstimulus interval, 70 ms) to examine paired pulse ratios. Carbachol ( $10 \mu\text{M}$ ) increased the ratio of evoked  $\text{IPSC}_2/\text{IPSC}_1$  from  $1.03 \pm 0.06$  to  $1.18 \pm 0.07$  ( $P = 0.002$ ;  $n = 23$ ) (Fig. 1, C and D). Carbachol ( $10 \mu\text{M}$ ) had no effect on membrane current or the conductance of the neurons at  $-65 \text{ mV}$ . These changes in paired pulse ratio suggest that carbachol inhibits GABAergic synaptic transmission via a presynaptic locus of action.

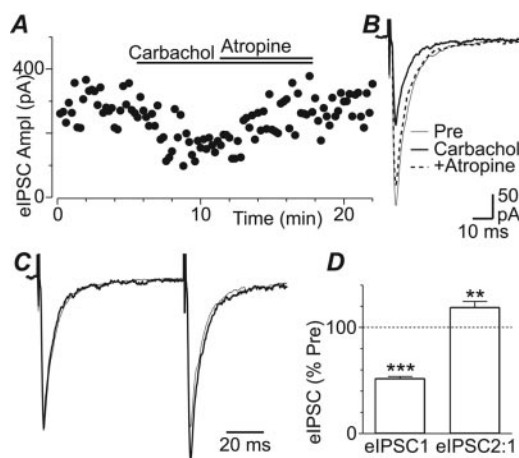
We next examined whether endogenously released acetylcholine modulated GABAergic synaptic transmission. Superfusion of the reversible acetylcholinesterase inhibitor physostigmine ( $100 \mu\text{M}$ ) produced a reduction in the basal amplitude of evoked IPSCs, which reversed after washout, or addition of atropine ( $3 \mu\text{M}$ ) ( $P = 0.0005$ ;  $n = 7$ ) (Fig. 2, A, B, and D). In addition, physostigmine ( $100 \mu\text{M}$ ) increased the paired pulse ratio of evoked  $\text{IPSC}_2/\text{IPSC}_1$  from  $1.00 \pm 0.18$  to  $1.31 \pm 0.26$  ( $P = 0.04$ ;  $n = 7$ ) (Fig. 2, C and D). Superfusion of atropine ( $3 \mu\text{M}$ ) alone, however, had no effect on the basal amplitude of evoked IPSCs ( $110 \pm 5\%$  of control;  $P = 0.08$ ;  $n = 6$ ) or on the ratio of evoked  $\text{IPSC}_2/\text{IPSC}_1$  ( $97 \pm 3\%$  of control;  $P = 0.4$ ;  $n = 6$ ). Physostigmine and atropine had no effect on membrane current or the conductance of the neurons at  $-65 \text{ mV}$ .

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 ( $5 \mu\text{M}$ ), strychnine ( $5 \mu\text{M}$ ), and tetrodotoxin ( $300 \text{ nM}$ ), spontaneous miniature IPSCs were readily observed (Fig. 3E). Carbachol ( $3\text{--}10 \mu\text{M}$ ) 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 interevent intervals (Fig.

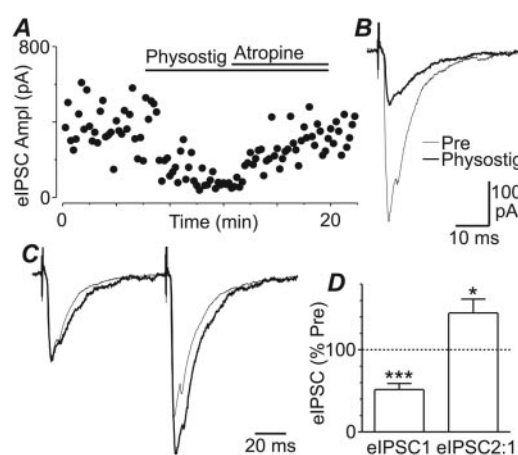
3, A, D, and E). The reduction in miniature IPSC rate produced by carbachol ( $3\text{--}10 \mu\text{M}$ ) was reversed by the addition of atropine ( $3 \mu\text{M}$ ) ( $n = 5$ ) (Fig. 3A). In contrast, carbachol ( $3\text{--}10 \mu\text{M}$ ) did not produce a change in the amplitude or kinetics of miniature IPSCs, or in the cumulative probability distributions of miniature IPSC amplitudes (Fig. 3, B and C). In the presence of carbachol ( $3\text{--}10 \mu\text{M}$ ), the mean miniature IPSC rate and amplitude were  $55 \pm 3\%$  ( $P < 0.0001$ ) and  $95 \pm 4\%$  ( $P = 0.2$ ) of control, respectively ( $n = 16$ ) (Fig. 3F). This reduction in miniature IPSC rate produced by carbachol was concentration-dependent, with an  $\text{IC}_{50}$  value of  $180 \text{ nM}$  (95% confidence interval,  $70\text{--}460 \text{ nM}$ ) and a Hill slope of  $1.0 \pm 0.5$  (Fig. 4). The selective mAChR agonist oxotremorine also produced a concentration-dependent reduction in miniature IPSC rate, but not amplitude, with an  $\text{IC}_{50}$  value of  $290 \text{ nM}$  (95% confidence interval,  $31\text{--}2830 \text{ nM}$ ) and Hill slope of  $1.2 \pm 0.8$  (Fig. 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 preincubating slices in a range of cholinergic antagonists. In slices preincubated in atropine ( $3 \mu\text{M}$ ), carbachol ( $10 \mu\text{M}$ ) had no effect on the amplitude of evoked IPSCs ( $P = 0.8$ ;  $n = 5$ ) (Fig. 5, A and G). By contrast, carbachol produced a reduction in the amplitude of evoked IPSCs in slices preincubated with the nAChR antagonist mecamylamine ( $3 \mu\text{M}$ ) ( $P < 0.0001$ ;  $n = 5$ ), which was not significantly different from the reduction produced by carbachol alone ( $P > 0.05$ ) (Fig. 5, B and G).

We then examined the effect of a range of subtype-selective mAChR antagonists. In the presence of the M2-selective antagonist gallamine ( $100 \mu\text{M}$ ), carbachol ( $10 \mu\text{M}$ ) produced a reduction in the amplitude of evoked IPSCs ( $P = 0.01$ ;  $n = 7$ ), which was not significantly different from that produced by carbachol alone ( $P > 0.05$ ) (Fig. 5, C and G). In the presence



**Fig. 1.** Carbachol inhibits evoked IPSCs in PAG neurons. A, time course of evoked IPSC (eIPSC) amplitude during application of carbachol ( $10 \mu\text{M}$ ) and then following addition of atropine ( $3 \mu\text{M}$ ). B, averaged evoked IPSCs before (Pre) and during application of carbachol, and then after addition of atropine. C, averaged evoked IPSCs in response to identical paired stimuli (interstimulus 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 ( $10 \mu\text{M}$ ), expressed as a percentage of the precarbachol level. In D, \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$ . Traces in A to C are from the same neuron.

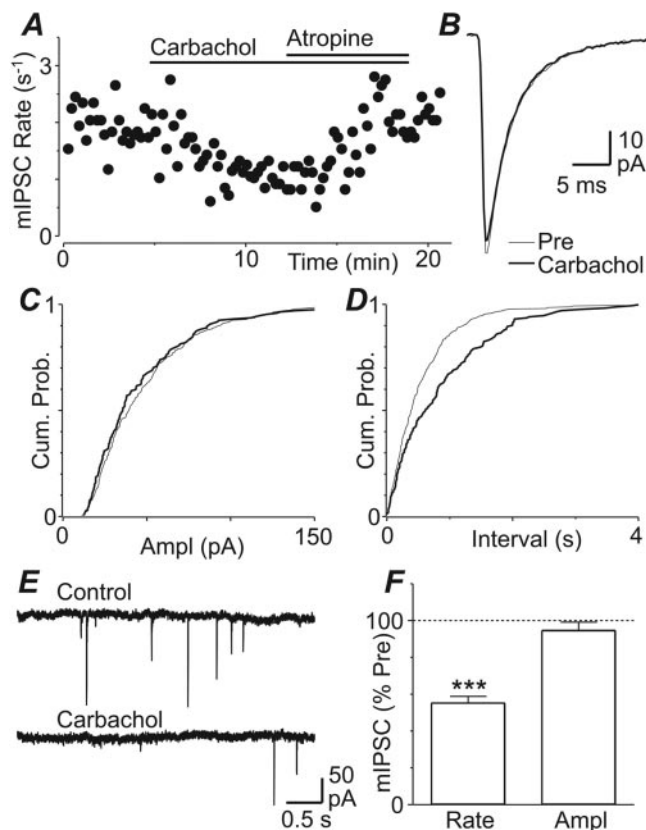


**Fig. 2.** Physostigmine inhibits evoked IPSCs in PAG neurons. A, time course of eIPSC amplitude during application of physostigmine (Physostig;  $100 \mu\text{M}$ ) and then after addition of atropine ( $3 \mu\text{M}$ ). B, averaged evoked IPSCs before (Pre) and during application of physostigmine ( $100 \mu\text{M}$ ). C, averaged evoked IPSCs in response to identical paired stimuli (interstimulus interval, 70 ms) for the traces in B, with IPSC1 normalized. D, bar chart showing the amplitude of eIPSC1 and the ratio of eIPSC2:1 in the presence of physostigmine ( $100 \mu\text{M}$ ), expressed as a percentage of the prephysostigmine level. In D, \*,  $P < 0.05$  and \*\*\*,  $P < 0.001$ . Traces in A to C are from the same neuron.

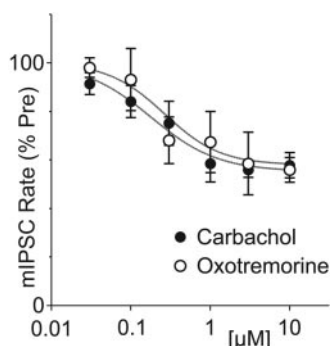
of the M4-selective antagonist PD-102807 (500 nM), carbachol (10  $\mu$ M) also produced a reduction in the amplitude of evoked IPSCs ( $P = 0.004$ ;  $n = 5$ ), which was not significantly different from that produced by carbachol alone ( $P > 0.05$ ) (Fig. 5, D and G). Carbachol (10  $\mu$ M) also 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 ( $P < 0.001$ ) (Fig. 5, E–G). These results suggest that the M1, and possibly the M3 mAChR receptors predominantly mediate the effects of muscarinic receptor activation in PAG.

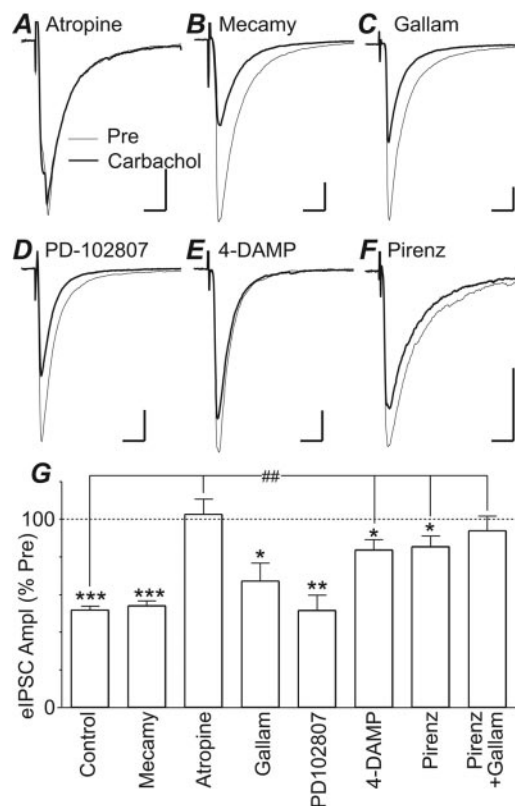
**Cholinergic Inhibition of GABAergic Transmission Is Mediated via Endocannabinoids.** Studies in the hippocampus and striatum have shown that M1/M3-induced suppression of synaptic transmission is mediated by retrograde endocannabinoid signaling. Recent evidence suggests that the endocannabinoid involved is 2-arachidonoylglycerol, which is produced via the phospholipase C/DAG lipase pathway (Melis et al., 2004; Hashimoto et al., 2005, 2007; Maejima et al., 2005; 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 CB<sub>1</sub> receptor antagonist AM251 (3  $\mu$ M), carbachol (10  $\mu$ M) produced a reduction in the amplitude of evoked IPSCs ( $P =$



**Fig. 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  $\mu$ M), and then during addition of atropine (3  $\mu$ M). B, averaged traces of miniature IPSCs before (Pre) and during carbachol (3  $\mu$ M). C, cumulative distribution plots of miniature IPSC amplitude (C) and interevent interval (D) before and during carbachol. E, raw current traces of miniature IPSCs before (Pre) and during superfusion of carbachol (3  $\mu$ M). F, bar chart of the mean rate and amplitude of miniature IPSCs in the presence of carbachol (3–10  $\mu$ M), expressed as a percentage of the precarbachol level. \*\*\*,  $P < 0.001$ . Traces in A to E are from the same neuron.



**Fig. 4.** Carbachol and oxotremorine produce a concentration-dependent reduction in miniature IPSC rate. Concentration-response curves of the inhibition of mIPSC rate produced by carbachol and oxotremorine-M, expressed as a percentage of the predrug value. A logistic function was fitted to the curves to determine the  $IC_{50}$  value. Data are presented as mean  $\pm$  S.E.M. for  $n = 3$  to 12 neurons.



**Fig. 5.** Carbachol inhibition of evoked IPSCs is largely mediated by M1 mAChRs. Averaged evoked IPSCs are shown before (Pre) and during addition of carbachol (10  $\mu$ M) in slices preincubated in the broad-spectrum mAChR antagonist atropine (3  $\mu$ M) (A), the nAChR antagonist mecamylamine (Mecamy; 3  $\mu$ M) (B), the M2 antagonist gallamine (Galam; 100  $\mu$ M) (C), the M4 antagonist PD-102807 (500 nM) (D), the M1/M3 antagonist 4-DAMP (30 nM) (E), and the M1 antagonist pirenzepine (Pirenz; 300 nM) (F). G, bar chart showing the percentage of inhibition of evoked IPSCs produced by carbachol (10  $\mu$ M) alone (Control) and in the presence of mecamylamine, atropine, gallamine, PD-102807, 4-DAMP, pirenzepine, and pirenzepine plus gallamine. In G, \*, \*\*, and \*\*\* denote a significant difference compared with precarbachol value ( $P < 0.05$ , 0.01, and 0.001, respectively) and ## denotes a significant difference between the control and pretreatment groups ( $P < 0.01$ ). Traces in A to F are from different neurons. Scale bars (A–F), 200 pA and 10 ms.

0.04;  $n = 7$ ), which was significantly less than that produced by carbachol alone ( $P < 0.001$ ) (Fig. 6, A and C). The effect of carbachol was also occluded by the nonselective cannabinoid receptor agonist HU210 (3  $\mu\text{M}$ ), 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  $\mu\text{M}$ ) did not produce a significant change in miniature IPSC rate in the presence of AM251 (3  $\mu\text{M}$ ) (rate,  $81 \pm 13\%$  of control;  $P = 0.2$ ;  $n = 9$ ). Physostigmine (100  $\mu\text{M}$ ) 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  $\mu\text{M}$ ), carbachol (10  $\mu\text{M}$ ) 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 ( $P < 0.001$ ) (Fig. 6, B and C).

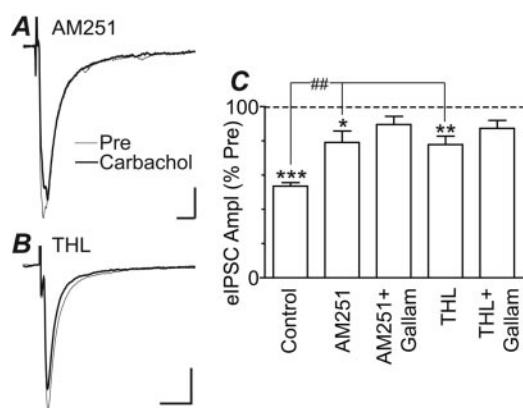
Because the carbachol-induced inhibition of evoked IPSCs was not abolished by pirenzepine, AM251, or tetrahydrolipstatin, we examined whether the residual inhibition was mediated by presynaptic M2 mAChRs. In the combined presence of gallamine (100  $\mu\text{M}$ ) and pirenzepine (300 nM), carbachol (10  $\mu\text{M}$ ) had no significant effect on the amplitude of evoked IPSCs ( $P = 0.5$ ;  $n = 6$ ) (Fig. 5G). Likewise, carbachol did not significantly affect evoked IPSC amplitude the presence of gallamine (100  $\mu\text{M}$ ) and either AM251 (3  $\mu\text{M}$ ) ( $P = 0.09$ ;  $n = 5$ ) or tetrahydrolipstatin (THL; 10  $\mu\text{M}$ ) ( $P = 0.06$ ;  $n = 5$ ) (Fig. 6C).

**Cholinergic Inhibition of Glutamatergic Transmission Is Partly Mediated via Endocannabinoids.** Finally, we examined whether carbachol also inhibited glutamatergic synaptic transmission in PAG via endocannabinoids. In the presence of picrotoxin (100  $\mu\text{M}$ ) and strychnine (5  $\mu\text{M}$ ), superfusion of carbachol (10  $\mu\text{M}$ ) produced a reduction in the amplitude of non-NMDA-mediated evoked EPSCs, which was reversed after addition of atropine (3  $\mu\text{M}$ ) ( $P < 0.0001$ ;  $n = 11$ ) (Fig. 7, A and E) and was associated with an increase

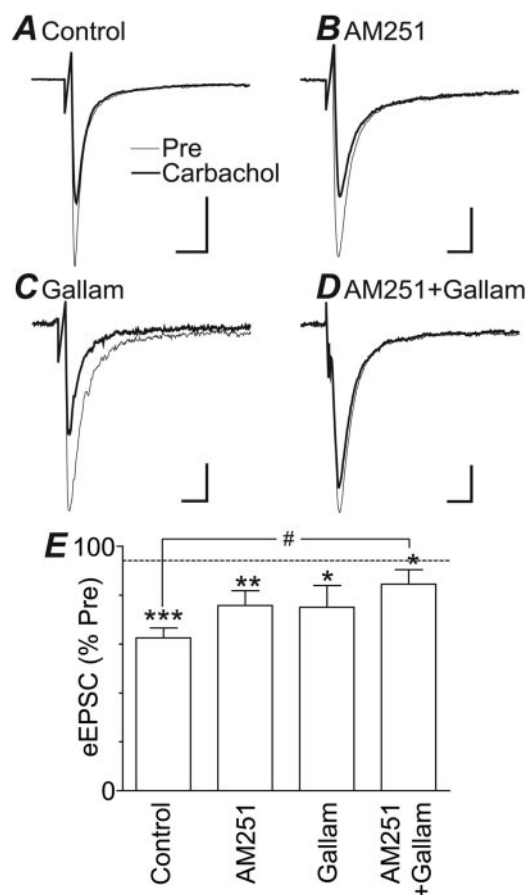
in the ratio of evoked EPSC<sub>2</sub>/EPSC<sub>1</sub> ( $P = 0.03$ ). Carbachol (10  $\mu\text{M}$ ) produced a reduction in the amplitude of evoked EPSCs in the presence of AM251 (3  $\mu\text{M}$ ) ( $P = 0.005$ ;  $n = 8$ ), which was not significantly different from that produced by carbachol alone ( $P > 0.05$ ) (Fig. 7, B and E). Likewise, carbachol (10  $\mu\text{M}$ ) reduced the amplitude of evoked EPSCs in the presence of gallamine (100  $\mu\text{M}$ ) ( $P = 0.04$ ;  $n = 6$ ), and this was not significantly different from that produced by carbachol alone ( $P > 0.05$ ) (Fig. 7, C and E). By contrast, carbachol (10  $\mu\text{M}$ ) produced a reduction in the amplitude of evoked EPSCs in the combined presence of AM251 (3  $\mu\text{M}$ ) and gallamine (100  $\mu\text{M}$ ) ( $P = 0.04$ ;  $n = 7$ ), which significantly less than that produced by carbachol alone ( $P < 0.05$ ) (Fig. 7, D and E).

## Discussion

The present study has demonstrated that cholinergic agonists and endogenously released acetylcholine inhibit GABAergic and glutamatergic synaptic transmission within



**Fig. 6.** Cholinergic inhibition of evoked IPSCs is mediated partly via endocannabinoids. Averaged evoked IPSCs before (Pre) and during carbachol (10  $\mu\text{M}$ ) in slices preincubated in the CB<sub>1</sub> antagonist AM251 (3  $\mu\text{M}$ ) (A) or the DAG lipase inhibitor THL (10  $\mu\text{M}$ ) (B). C, bar chart showing the percentage of inhibition of evoked IPSCs produced by carbachol (10  $\mu\text{M}$ ) alone (Control), and in the presence of AM251 (3  $\mu\text{M}$ ) or THL (10  $\mu\text{M}$ ) alone, and combined with the M2 antagonist Gallam (100  $\mu\text{M}$ ). In C, \*, \*\*, and \*\*\* denote a significant difference from precarbachol values ( $P < 0.05$ , 0.01, and 0.001, respectively). ## denotes significant difference between control and other pretreatment groups ( $P < 0.01$ ). Traces in A and B are from different neurons. Scale bars (A and B), 100 pA and 10 ms.



**Fig. 7.** Cholinergic inhibition of evoked EPSCs is partly mediated via endocannabinoids. Averaged evoked EPSCs before (Pre) and during carbachol (10  $\mu\text{M}$ ) in untreated slices (Control) (A) and in slices preincubated in the CB<sub>1</sub> antagonist AM251 (3  $\mu\text{M}$ ) (B), the M2 antagonist Gallam (100  $\mu\text{M}$ ) (C), and both AM251 and gallamine (100  $\mu\text{M}$ ) (D). E, bar chart showing the percentage of inhibition of evoked EPSCs produced by carbachol (10  $\mu\text{M}$ ) alone (Control) and in the presence of AM251 (3  $\mu\text{M}$ ) and/or gallamine (100  $\mu\text{M}$ ). In E, \*, \*\*, and \*\*\* denote significance difference compared with precarbachol value ( $P < 0.05$ , 0.01, and 0.001, respectively) and # denotes significant difference between the control and pretreatment groups ( $P < 0.05$ ). Traces in A to D are from different neurons. Scale bars, 200 pA and 5 ms (A), 50 pA and 5 ms (B), 20 pA and 5 ms (C), and 50 pA and 5 ms (D).

the PAG via activation of both M1 and M2 mAChRs. The M1-induced cholinergic inhibition seems to be indirectly mediated via endocannabinoids, produced via the phospholipase C/DAG lipase pathway, which activate presynaptic cannabinoid CB<sub>1</sub> 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 signaling 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 nondegradable cholinergic agonist carbachol caused a reduction in the amplitude of evoked GABA<sub>A</sub>-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; Shen and Johnson, 2000; Fukudome et al., 2004; Li et al., 2004). It is also similar in mechanism to that described previously for opioids and cannabinoids within the PAG (Vaughan et al., 1997, 2000), both of which produce analgesia when injected into this brain structure (Fields et al., 2006). Although 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 may tonically modulate analgesia; however, this seems 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 (Yasuda et al., 1993; Aubert et al., 1996). 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 the inhibitory effect of carbachol was obtained in the combined presence of pirenzepine and gallamine. These results suggest that the mAChR-induced inhibition of GABAergic transmission is largely mediated by the M1 receptor and possibly the M3 receptor, with a smaller M2 receptor contribution, similar to that observed previously in the hippocampus (Ohno-Shosaku et al., 2003; Fukudome et al., 2004). Although gallamine also had no effect on the carbachol-induced inhibition of glutamatergic transmission, M2 receptors are still likely to have a role in excitatory transmission (see below). The present findings slightly differ from those found in other brain regions in which cholinergic inhibition of GABAergic and glutamatergic transmission is largely mediated exclusively by M1/M3 and M2 receptors, respectively (Bellingham and Berger, 1996; Shen and Johnson, 2000; Li et al., 2004). It should be emphasized, however, that some of the antagonists used in the

present and prior studies display only modest selectivity (Caulfield, 1993). The specific receptor subtypes involved would need to be verified by knocking out mAChR subtypes (Ohno-Shosaku et al., 2003; Fukudome et al., 2004).

The observation that the cholinergic inhibition of GABAergic synaptic transmission is largely mediated by M1 mAChRs is interesting because, like group I mGluRs (mGluR1 and mGluR5), M1/M3/M5 mAChRs are G<sub>q</sub>-coupled receptors that 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 (Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Narushima et al., 2006, 2007). In the present study, the carbachol-induced inhibition of evoked and miniature IPSCs was reduced by the cannabinoid CB<sub>1</sub> receptor antagonist AM251 and occluded by the pan-cannabinoid receptor agonist HU210, consistent with that observed previously in the hippocampus and striatum (Kim et al., 2002; Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Narushima et al., 2007; 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 of endocannabinoids that activate presynaptic cannabinoid CB<sub>1</sub> receptors and direct presynaptic M2 mAChR activation. These findings are similar to those reported previously for acetylcholine, glutamate, and serotonin, which also modulate synaptic transmission via indirect G<sub>q</sub>-coupled endocannabinoid mechanisms and direct G<sub>i/o</sub>-coupled presynaptic inhibition in the PAG and other brain regions (Fukudome et al., 2004; Best and Regehr, 2008; Drew et al., 2008). 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 that are biosynthesized 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 (Melis et al., 2004; Hashimoto et al., 2005, 2007; Maejima et al., 2005; Newman et al., 2007; Uchigashima et al., 2007). Although 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 a 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-arachidonoylglycerol, 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 (Lichtman et al., 1996; Finn et al., 2003; 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 (Kathuria et al., 2003; Hohmann et al., 2005). 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 manner to  $\mu$ -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 behavioral 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 signaling via M1/M3 mAChRs might occur in numerous brain regions. Ultimately, this will contribute to the growing number of studies suggesting that endocannabinoid signaling may be an important global phenomenon underlying cholinergic transmission within the brain.

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**Address correspondence to:** Benjamin K. Lau, Pain Management Research Institute, Kolling Institute, Northern Clinical School, University of Sydney at Royal North Shore Hospital, St Leonards, NSW 2065, Australia. E-mail: benjaminl@med.usyd.edu.au