Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 508 (2005) 99–106

www.elsevier.com/locate/ejphar

An investigation of binding sites for paracetamol in the mouse brain and spinal cord

Lisa Godfrey[a], Amanda Morselli[a], Peter Bennion[a], Geoffrey D. Clarke[b], Susanna M.O. Hourani[a], Ian Kitchen[a,*]

[a]Pharmacology Group, School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK
[b]GlaxoSmithKline, St Georges Ave, Weybridge, Surrey KT13 ODE, UK

Received 26 November 2004; accepted 30 November 2004
Available online 5 January 2005

Abstract

Quantitative autoradiography has been used to assess whether [^{3}H]paracetamol (3 μM) binds specifically to any area of the murine brain and spinal cord and to investigate whether paracetamol (1–100 μM) competes for binding to the nociceptin opioid peptide (NOP) receptor or to the nitrobenzylthioinosine (NBTI)-sensitive adenosine transporter in the brains of mice. [^{3}H]paracetamol binding was homogenous and, although there was some indication of specific binding overall, this binding in most individual regions failed to reach statistical significance. However, thoracic segments of the spinal cord were found to have significantly higher specific binding than cervical and lumbar regions. Paracetamol did not significantly compete for binding to the NOP receptor or to the NBTI-sensitive adenosine transporter, showing that it does not mediate its effect via these sites. Although paracetamol did bind specifically to the murine brain and spinal cord, the binding was not region-specific, suggesting binding is not related to any particular neurotransmitter system.

Keywords: NBTI (nitrobenzylthioinosine); NOP (nociceptin opioid peptide); Paracetamol; Autoradiography; Adenosine uptake; Nociceptin

1. Introduction

Paracetamol was first synthesised nearly a century ago and is one of the most popular "over the counter" analgesic/antipyretics used today. However, the mechanisms by which paracetamol relieves pain has still not been elucidated. Many pathways and receptor systems have been proposed but no significant interaction between paracetamol and any pathway involved in pain transmission has yet been found.

It has been suggested that paracetamol may relieve pain through the opioid system (Sandrini et al., 2001a, b). Further, Raffa et al. (2000) have suggested that paracetamol-induced antinociception involves a "self-synergistic" interaction between spinal and supraspinal sites and, that this involves an endogenous opioid pathway. Although they showed that paracetamol at 10 μM did not bind significantly to the mu, delta or kappa opioid receptors (Raffa and Codd, 1996), others have demonstrated that paracetamol has a low (μM) affinity for [^{3}H]naloxone binding sites, suggesting that direct opioid receptor interactions may occur at high concentrations (Pini et al., 1997). An opioid related receptor (NOP) has now been characterised (Mollereau et al., 1994) and this receptor shows low affinity for naloxone (Meunier, 1997). As the endogenous peptide for the NOP receptor (nociceptin/orphanin FQ) has been shown to have a role in nociceptive pathways (Calo et al., 2000; Citterio et al., 2000), we hypothesised that it could be a target for the analgesic effects of paracetamol.

Adenosine is a ubiquitous autocoid that has also been implicated in pain pathways. Peripheral administration of adenosine has been shown to produce pronounced pro-nociceptive effects whereas centrally administered adenosine produces antinociceptive effects (Sawynok and Yaksh, 1993) and A_1 adenosine receptors have been shown to

* Corresponding author. Tel.: +44 1483 689734; fax: +44 1483 300374.
E-mail address: i.kitchen@surrey.ac.uk (I. Kitchen).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.061

modulate pain transmission at spinal level (Sawynok, 1998). The analgesic effects of adenosine are enhanced by inhibitors of uptake or metabolism (Sawynok and Liu, 2003) and thus inhibition of adenosine uptake might also be responsible for some of the analgesic effects of paracetamol. Added to this, there is a large body of evidence indicating that effects of opioids are partly mediated by adenosine, and that adenosine is released from spinal sites following opioid stimulation (Sawynok, 1998). Thus, both opioid and adenosine sites could be a target for the action of paracetamol.

Although competition binding studies have largely been used to rule out target receptors for paracetamol (Raffa and Codd, 1996), only one study has directly searched for binding sites using radiolabelled paracetamol (Courade et al., 2001). This study used [^{3}H]paracetamol at 30 nM, which is a concentration more than 1000-fold below the plasma concentration required to induce an analgesic response in laboratory animals and so it may not have been sufficient to detect specific binding (Prescott, 1996). Paracetamol readily passes the blood–brain barrier (Bannwarth et al., 1992), and there is a high CNS accumulation (Courade et al., 2001) so plasma levels are similar to levels found in the brain (Fischer et al., 1981).

The first aim of this study was to determine by quantitative autoradiography in mouse brain whether paracetamol competes for binding to NOP receptors or to the NBTI-sensitive adenosine transporter; two targets that have hitherto not been examined. The second aim was to determine by quantitative autoradiography whether [^{3}H]paracetamol, at a concentration relevant to in vivo activity (3 μM), demonstrates any specific binding in the brain and spinal cord of mice.

2. Methods

2.1. Animals

Adult (6–8 weeks) male CD1 mice (Charles River) were housed three per cage in an air conditioned unit maintained at 20–22 °C and 50–60% humidity and were allowed free access to standard rodent chow and water. Lighting was controlled on a 12-h cycle. Mice were killed by decapitation and intact whole brains and spinal cords were removed.

2.2. Autoradiographic procedures

2.2.1. NOP and NBTI competition binding

General procedures for quantitative autoradiography were performed as detailed previously (Kitchen et al., 1997; Slowe et al., 2001). Adjacent frozen coronal (20 μm) sections were cut at 300-μm intervals for the determination of [^{3}H]NBTI binding to the NBTI-sensitive adenosine transporters and [^{3}H]nociceptin binding to the NOP receptor in the presence of 0 μM, 1 μM, 10 μM and 100 μM paracetamol. The concentration of [^{3}H]NBTI (4.5 nM, 26 Ci/mmol) was approximately 10 times the value of K_d (0.46±0.14 nM; Snell et al., 2000) and was previously used by Bailey et al. (2002) to ensure all NBTI-sensitive transporters were bound. Nonspecific binding was determined using 10 μM unlabelled NBTI. The concentration of [^{3}H]nociceptin (0.4 nM, 164 Ci/mmol) was approximately 3 times the K_d (0.13 nM) as determined by Neal et al. (1999) and the nonspecific binding was determined by 1 μM unlabelled nociceptin. The sections were defrosted and then preincubated for 30 min in 50 mM Tris–HCl (pH 7.4 at 25 °C) supplemented with 2 U/ml adenosine deaminase type VIII or 0.9% NaCl for the binding of [^{3}H]NBTI or [^{3}H]nociceptin, respectively. The sections were then incubated in 50 mM Tris–HCl (pH 7.4 at 25 °C) containing either [^{3}H]NBTI (4.5 nM) or [^{3}H]nociceptin (0.4 nM) for 30 and 180 min, respectively, to determine total binding. Adjacent sections were also incubated in the presence of [^{3}H]ligand supplemented with 1 μM, 10 μM and 100 μM paracetamol to determine whether paracetamol can displace ligand binding. Nonspecific binding was determined by incubating further adjacent sections with [^{3}H]ligand with an excess of the corresponding unlabelled ligand. For the [^{3}H]NBTI binding, sections were washed three times for 10 min in ice-cold rinse buffer containing 50 mM Tris–HCl pH 7.4 at 0 °C and dipped for 30 s in reverse osmosis water at 0 °C. The sections bound with [^{3}H]nociceptin were washed for 5 min in rinse buffer containing 50 mM Tris–HCl supplemented with 3 mM $MgCl_2$ and 0.1% w/v bovine serum albumin fraction V (pH 7.4 at 0 °C). Following the wash step, sections were rapidly dried for 2 h in a cold air stream. Dehydration was continued for a further 7 days at room temperature in airtight boxes containing anhydrous calcium sulphate (Drierite). The sections were then apposed to [^{3}H]sensitive Hyperfilm together with [^{3}H]microscales in the range of 0.07–3.4 nCi/mg. After 8 and 4 weeks for [^{3}H]NBTI and [^{3}H]nociceptin binding, respectively, the film was developed using 50% Kodak D19 developer.

2.2.2. [^{3}H]Paracetamol binding

Adjacent frozen coronal, sagittal and spinal cord sections (20 μm) were cut at 300-μm intervals for the determination of total and nonspecific binding of [^{3}H]paracetamol. The sections were defrosted prior to a 15-min pre-incubation in 50 mM Tris–HCl, pH 7.4 at 25 °C and incubated for 60 min in fresh buffer supplemented with 3 μM [^{3}H]paracetamol (32 Ci/mmol). Adjacent sections were incubated in the presence of 3 μM [^{3}H]paracetamol and 3 mM unlabelled paracetamol for the determination of nonspecific binding. Sections were then washed twice for 10 min in ice-cold rinse buffer (50 mM Tris–HCl, pH 7.4 at 0 °C) and immersed for 5 s in ice-cold reverse osmosis water. Brain and spinal cord sections were then dried as above (Section 2.2.1) and apposed to [^{3}H]sensitive Hyperfilm for 4 and 5 weeks, respectively. Films were developed using 50% Kodak D19 developer.

Table 1
Quantitative autoradiography of [^{3}H]NBTI and [^{3}H]nociceptin binding and the effect of paracetamol in coronal sections of male mice

Region	% Change in [^{3}H]NBTI binding			% Change in [^{3}H]nociceptin binding		
	+Paracetamol			+Paracetamol		
	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
Olfactory bulb						
External plexiform layer	−19.1	−1.6	−8.5			
Glomeruler layer	−8.2	−8.3	0.7	−11.9	7.6	14.1
Granular layer	−15.9	−6.2	−25.9	−39.7	−40.5	−35.2
Anterior olfactory bulb				−7.6	−3.5	−7.4
Anterior olfactory nucleus	−12.7	−9.5	−6.9	−19.1	−18.2	−8.9
Cortex						
Motor						
Superficial layers	4.8	7.3	−9.6	−13.5	−17.4	−5.8
Deep layers	−5.2	0.6	−6.5	−8.5	−22.5	4.7
Prelimbic	7.2	5.2	−0.4	8.7	−20.9	−3.5
Medial orbital				9.4	−9.8	2.0
Rostral somatosensory						
Superficial layers	−11.7	1.2	−5.8	7.4	4.9	−116
Deep layers	−5.6	6.7	−3.1	−4.0	6.6	−14.4
Cingulate	−9.0	22.4	17.1			
Superficial layers				0.4	−5.4	−11.1
Deep layers				7.7	−0.0	−8.1
Caudal somatosensory						
Superficial layers	6.0	−23.6	−6.4			
Deep layers	−6.8	−20.5	−9.1			
Auditory						
Superficial layers	0.7	−20.6	−9.3	−8.6	−14.8	−7.7
Intermediate layers				6.6	−12.62	−11.8
Deep layers	−18.3	−20.7	1.8	10.5	−10.9	−10.4
Visual						
Superficial layers	−2.4	−9.8	−5.0	−10.9	−11.1	−9.6
Intermediate layers				11.5	−6.2	8.8
Deep layers	−26.8	−13.9	3.6	−7.9	−20.3	−15.6
Retrosplenial						
Superficial layers	−0.7	−14.5	−19.9	−6.8	−10.9	−1.5
Intermediate layers				4.0	0.7	10.8
Deep layers	12.8	−3.8	13.4	−14.3	−24.5	−4.9
Nucleus accumbens	1.7	16.4	4.4	−5.1	−0.8	−24.7
Caudate putamen	0.8	11.8	3.0	27.9	6.8	−19.9
Septum						
Lateral	−5.0	1.1	−3.1	8.7	5.3	−7.2
Medial	−3.9	0.9	−4.8			
Vertical limb of diagonal band	6.6	4.9	−0.9			
Triangular septal nucleus				49.4	37.6	8.7
Bed nucleus of striatal terminalis				60.6	67.3	42.3
Globus pallidus	−1.9	−20.1	−4.9	11.1	19.7	−26.2
Suprachiasmatic nucleus				−14.4	−22.4	−13.9
Thalamus	4.0	−7.8	−5.5	14.6	7.5	14.9
Amygdala	−9.7	−19.0	−9.0	−2.3	−5.5	−12.2
Hypothalamus	−4.9	−8.6	−15.4	30.2	28.8	52.6
Ventromedial nucleus	−0.4	−4.6	−5.5	0.6	−14.8	−4.9

(continued on next page)

Table 1 (*continued*)

Region	% Change in [^{3}H]NBTI binding			% Change in [^{3}H]nociceptin binding		
	+Paracetamol			+Paracetamol		
	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
Dorsal medial	2.0	−6.2	−8.6			
Medial habenula	5.8	0.3	2.2	0.1	−8.5	−0.7
Zona incerta				−9.8	−15.2	−5.4
Hippocampus				−6.4	−4.9	4.6
Stratum oriens	−2.4	−18.1	−12.3			
Stratum radiatum	−3.5	−30.5	−24.7			
Stratum molecular	8.2	−17.2	−9.1			
Dentate gyrus	−5.0	1.4	18.8	−16.7	−8.4	−3.2
Medial mammillary nucleus	10.2	6.9	4.5	−0.9	−9.3	−3.5
Medial geniculate nucleus				−27.2	−31.9	−16.8
Substantia nigra	−1.3	8.2	−8.7	−4.1	−29.6	0.9
Superficial grey layer of the superior colliculus	−10.0	−3.1	−4.0	−9.8	−2.7	4.2
Intermediate grey of superior colliculus				−29.5	−29.2	−7.9
Periaqueductal grey layer	−2.0	−6.5	−7.2	−20.4	−21.6	2.2
Interpeduncular nucleus				−14.4	−3.8	16.6
Presubiculum				−19.2	−22.5	−30.1
Pontine nucleus				−31.2	9.8	20.7
Total	−2.1	−5.7	−4.8	−2.2	−6.4	−3.0

The percent change in binding (n=5–6 ±S.E.M.) of [^{3}H]nociceptin and [^{3}H]NBTI (fmol/mg) in 20-μm coronal sections of CD1 mice. Measures were carried out at bregma coordinates described in previous studies (Bailey et al., 2002; Clarke et al., 2001). Regional determinates were made from both left and right sides of the sections 300 μm apart. Competition binding was carried out using paracetamol (0–100 μM) in a completely paired protocol, and nonspecific binding was determined by 1 μM unlabelled nociceptin and 10 μM unlabelled NBTI. Levels of binding of [^{3}H]NBTI and [^{3}H]nociceptin in the presence or absence of paracetamol were not significantly different (P>0.05, ANOVA). The percent change in binding represents the change in binding levels compared to 0 μM paracetamol with a minus sign indicating a decrease in binding.

2.3. Quantitative analysis and statistical procedures

Quantitative analysis of autoradiographic films was carried out as detailed previously (Kitchen et al., 1997) using video-based computerised densitometry. Brain and spinal cord structures were identified by reference to the mouse and rat atlases of Paxinos and Franklin (2001) and Paxinos and Watson (1986), respectively. Specific receptor binding was determined by subtraction of nonspecific binding from total binding. Specific binding was expressed in fmol/mg tissue (±S.E.M.) as determined by the [^{3}H]microscales laid down with the [^{3}H]NBTI and [^{3}H]nociceptin sections. For the brain and spinal cord sections bound with [^{3}H]paracetamol, standards were not employed due to the high level of radioactivity used to achieve binding; therefore, analysis was carried out by comparison of relative optical density (ROD). Measurements for quantitative analysis of coronal sections were taken from left and right hemispheres therefore representing a duplicate determination. All areas of the spinal cord were analysed using free hand drawing tools. Comparison of quantitative measurements of autoradiographic binding was carried out using two-way analysis of variance (ANOVA) with region and ± paracetamol as factors, for [^{3}H]NBTI and [^{3}H]nociceptin

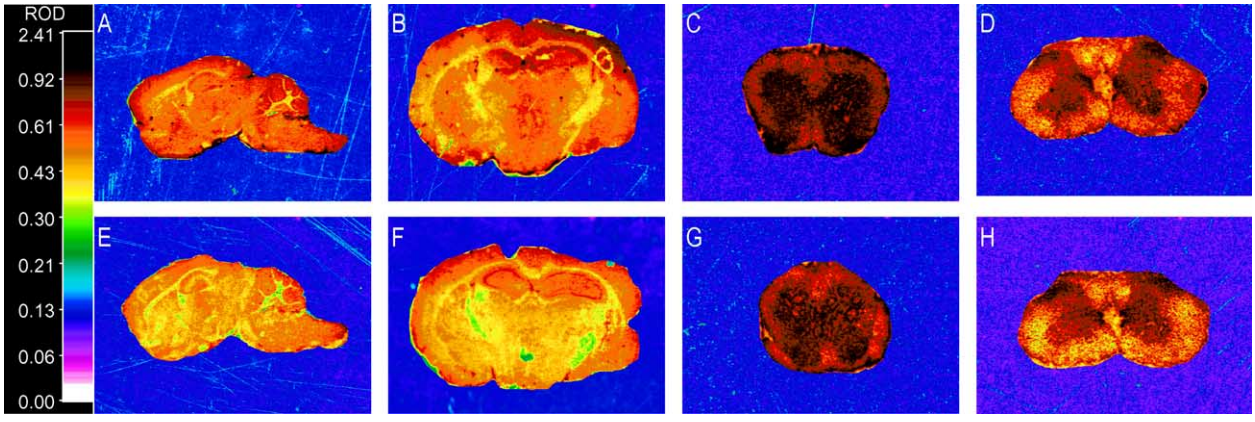

Fig. 1. Representative computer-enhanced colour autoradiograms of 20-μm sections of brain and spinal cord from CD1 mice, showing total binding of 3 μM [^{3}H]paracetamol and nonspecific binding (NSB) determined in the presence of 3 mM paracetamol. Total binding (A–D) and NSB (E–H). A and E—sagittal sections. B and F—coronal sections taken from the thalamus (Bregma −1.70 mm). C and G—coronal sections from cervical spinal cord. D and H—coronal sections from thoracic spinal cord. The colour bar shows pseudo-colour interpretation of relative density of black and white film images measured as relative optical density (ROD).

and [^{3}H]paracetamol. One-way ANOVA for comparison of total and nonspecific binding of paracetamol was performed on all spinal cord segments. Comparison of individual brain regions and spinal cord laminae was made using Fisher's LSD post hoc test.

2.4. Materials

The [^{3}H]nociceptin and unlabelled nociceptin were dissolved in 50:50 methanol/0.1 M HCl which was then further diluted 1:64 in incubation buffer [50 mM Tris–HCl, 3 mM $MgCl_2$, 0.1% w/v bovine serum albumin fraction V, bacitracin 1260 U/L and 0.2 mM ethylene glycerol-bis (2-amino ethylether)-*N*,*N*,*N*′,*N*′ tetraacetic acid (EGTA), pH 7.4 at 25 °C]. [^{3}H]NBTI and [^{3}H]paracetamol were dissolved in 50 mM Tris–HCl (pH 7.4 at 25 °C). Unlabelled NBTI and paracetamol were dissolved in 50:50 dimethyl sulfoxide (DMSO)/incubation buffer and 1:20 ethanol/incubation buffer, respectively. Both were then further diluted 1:100 with 50 mM Tris–HCl (pH 7.4 at 25 °C).

Table 2
Quantitative autoradiography of [^{3}H]paracetamol binding in coronal and sagittal sections of the brains of male mice

Region	Relative optical density (ROD)			
	Bregma/Lateral coordinates (mm)	Total	NSB	% Specific binding
Coronal				
Anterior olfactory nucleus	3.20	0.65±0.15	0.52±0.11	18.7
Cortex				
Motor	2.46	0.72±0.11	0.61±0.09	15.3
Retrosplenial agranular	1.10	0.68±0.15	0.44±0.09	35.6[a]
Nucleus accumbens	1.34	0.58±0.06	0.51±0.08	12.8
Caudate putamen	1.34	0.55±0.06	0.50±0.08	8.1
Septum	0.86			
Lateral		0.57±0.08	0.48±0.07	13.9
Medial		0.62±0.08	0.53±0.07	14.6
Vertical limb of diagonal band	0.86	0.64±0.09	0.55±0.06	14.6
Globus pallidus	−0.10	0.51±0.07	0.46±0.06	9.4
Thalamus	−1.70	0.51±0.10	0.37±0.10	27.2
Hypothalamus	−1.70	0.51±0.10	0.38±0.10	26.1
Hippocampus	−2.46			
Stratum oriens		0.63±0.12	0.43±0.10	31.3
Stratum radiatum		0.58±0.10	0.41±0.08	30.3
Stratum molecular		0.58±0.08	0.38±0.07	34.4
Amygdala	−1.70	0.56±0.11	0.43±0.11	23.0
Habenula	−1.70	0.59±0.12	0.42±0.09	28.3
Dentate gyrus	−2.46	0.46±0.08	0.38±0.08	16.6
Substantia nigra	−3.40	0.53±0.14	0.42±0.09	21.6
Periaqueductal grey	−3.40	0.47±0.10	0.38±0.08	19.9
Superficial grey layer of superior colliculus	−3.40	0.53±0.11	0.43±0.10	19.0
External cortex of inferior colliculus	−4.16	0.49±0.09	0.45±0.10	7.3
Intercollicular nucleus	−4.16	0.46±0.10	0.43±0.11	6.2
Cerebellum	−6.84	0.63±0.11	0.51±0.10	18.1
Sagittal				
Cortex				
Retrosplenial agranular	0.24	0.63±0.08	0.54±0.08	14.1
Motor	1.44	0.58±0.10	0.52±0.08	10.7
Thalamus	0.24	0.51±0.10	0.49±0.09	4.0
Hypothalamus	0.24	0.52±0.11	0.49±0.09	6.2
Nucleus accumbens	1.44	0.52±0.08	0.49±0.07	6.2
Hippocampus	1.44	0.52±0.08	0.48±0.06	19.0
External cortex of inferior colliculus	1.68	0.58±0.08	0.51±0.06	13.1
Caudate putamen	1.44	0.52±0.10	0.46±0.06	11.9
Cerebellum	1.44	0.61±0.13	0.52±0.08	15.2
Substantia nigra	1.68	0.61±0.10	0.51±0.07	15.1
Dentate gyrus	1.68	0.62±0.10	0.57±0.09	7.6
Amygdala	1.68	0.55±0.12	0.48±0.08	13.0

The mean binding in relative optical density (coronal, n=4 ±S.E.M.; sagittal n=3 ±S.E.M.) of 3 μM [^{3}H]paracetamol in 20-μm coronal and sagittal sections of CD1 mice. Nonspecific binding (NSB) was determined with unlabelled paracetamol (3 mM). Measures in the regions were carried out at the Bregma and lateral coordinates taken from the mouse atlas of Paxinos and Watson (Paxinos and Franklin, 2001). Two-way ANOVA (for the factors binding and region) showed no significant difference between regions (P>0.05), but a significant difference was observed between total and nonspecific binding (P<0.001). Post hoc analysis (Fisher's LSD test) of individual regions showed only one region where total and nonspecific binding was significantly different.

[a] P<0.05.

[^{3}H]nociceptin and [^{3}H]paracetamol were purchased from Amersham (Buckinghamshire, UK). [^{3}H]NBTI was purchased from New Life Sciences (Cambridge, UK) and unlabelled nociceptin was supplied by Bachem (Merseyside, UK). All other compounds were purchased from Sigma-Aldrich (Dorset, UK).

3. Results

3.1. Quantitative autoradiography of NBTI-sensitive adenosine transporters in the brains of mice in the absence and presence of paracetamol

Quantitative autoradiography showed a widespread distribution of NBTI-sensitive adenosine transporters in coronal sections (Table 1) throughout the fore-, mid- and hindbrain of mice with quantitative levels similar to those previously reported by us (Bailey et al., 2002).

The pattern of distribution of NBTI-sensitive adenosine transporters did not significantly alter in the presence of paracetamol. There were, however, some small quantitative differences in the presence of paracetamol. This change, however, was not concentration-dependent and two-way ANOVA did not demonstrate a significant quantitative difference overall (P>0.05) in the binding of [^{3}H]NBTI in the presence of paracetamol.

3.2. Quantitative autoradiography of the NOP receptor in the brains of mice in the absence and presence of paracetamol

Quantitative analysis revealed a wide distribution of [^{3}H]nociceptin (0.4 nM) binding (Table 1) throughout the fore-, mid- and hindbrain regions with quantitative levels similar to those previously reported by us (Clarke et al., 2001; Slowe et al., 2001). The pattern of distribution of the [^{3}H]nociceptin did not significantly alter in the presence of paracetamol. There were however some small quantitative differences. Paracetamol reduced the binding in the majority of regions but this change was not concentration-dependent. Furthermore, two-way ANOVA did not demonstrate a significant quantitative difference overall (P>0.05) in the binding of [^{3}H]nociceptin in the presence of paracetamol.

3.3. Quantitative autoradiography of the binding of [^{3}H]paracetamol in the brains of mice

3.3.1. Coronal sections

Quantitative analysis revealed a homogenous distribution of [^{3}H]paracetamol (3 μM) binding throughout the fore-, mid- and hindbrain regions (Fig. 1, Table 2). Moderate (20–35%) levels of specific binding were observed in the retrosplenial agranular cortex, thalamus and hypothalamus, hippocampus and the habenula. Low levels (<5%) of specific binding were detected in all other regions analysed including periaqueductal grey, substantia nigra, nucleus accumbens and the septum.

Two-way ANOVA (for the factors region and ± unlabelled paracetamol) showed no significant difference between regions (P>0.05) but a significant difference between total and nonspecific binding (P<0.001). Post hoc analysis (Fisher's LSD test) of individual regions showed that total and nonspecific binding was significantly different (P<0.05) only in the retrosplenial agranular cortex.

3.3.2. Sagittal sections

In sagittal sections, [^{3}H]paracetamol (3 μM) bound throughout the fore-, mid- and hindbrain with a ubiquitous

Table 3
Quantitative autoradiography of [^{3}H]paracetamol binding in the spinal cords of male mice

Region	Segments	% Specific binding
Cervical	C1 and C6	
Laminae I–III		8.32
Laminae IV–VII		8.81
Laminae VIII–IX		7.22
Laminae X		12.66
Intermediomedial cell column		3.19
White matter		−1.14
Thoracic	T1 and T3	
Laminae I–III		20.05[a]
Laminae IV–VII		15.69[a]
Laminae VIII–IX		17.78[a]
Laminae X		14.78[a]
Intermediomedial cell column		18.16[a]
White matter		18.28[a]
Lumbar	L3 and L5	
Laminae I–III		9.07
Laminae IV–VII		8.55
Laminae VIII–IX		6.82
Laminae X		9.02
Intermediomedial cell column		2.58
White matter		13.16
Sacral	S4	
Laminae I–III		2.31
Laminae IV–VII		11.23
Laminae VIII–IX		15.30
Laminae X		13.95
Intermediomedial cell column		16.98
White matter		14.04

The % specific binding (n=6 ±S.E.M.) of 3 μM [^{3}H]paracetamol in spinal cord regions of male CD1 mice. Nonspecific binding was determined with unlabelled paracetamol (3 mM). Regional determinates were made from both left and right sides of the sections. Comparison of total and nonspecific binding across all regions showed a significant difference (P<0.0001, ANOVA). Post hoc analysis (Fisher's LSD test) of individual laminae showed a significant difference between total and NSB for paracetamol in all laminae of thoracic segments, and the specific binding in thoracic laminae was significantly different from lumbar (P<0.05) and cervical segments (P<0.01) but not sacral segments (P>0.05).

[a] P<0.05.

distribution (Fig. 1, Table 2). Specific binding at levels between 10 and 20% were detected in the substantia nigra, cerebellum, and external cortex of inferior colliculus, hippocampus, retrosplenial agranular cortex, amygdala complex and the caudate. Low levels of specific binding (<10%) were observed in the dentate gyrus, hypothalamus, thalamus and nucleus accumbens. Two-way ANOVA (for the factors region and ± unlabelled paracetamol) showed no significant difference ($P>0.05$) between regions or between total and nonspecific binding.

3.4. Quantitative autoradiography of the binding of [^{3}H]paracetamol in the spinal cords of mice

Quantitative analysis showed a wide distribution of [^{3}H]paracetamol (3 μM) binding throughout the spinal cord (Fig. 1, Table 3). Specific binding was typically around 10% with the exception of thoracic regions where specific binding were around 20%. Two-way ANOVA (for the factors region and ± unlabelled paracetamol) showed a significant difference between total and nonspecific binding ($P<0.0001$). Further analysis (for factors segment and region) showed no significant difference ($P>0.05$) between regions but a significant difference ($P<0.001$) between segments. One-way ANOVA was performed on all segments and showed that paracetamol binding was significantly higher in thoracic segments when compared to lumbar ($P<0.05$) and cervical segments ($P<0.01$) but not sacral segments.

4. Discussion

4.1. Ligand binding to NBTI-sensitive adenosine transporters and NOP receptor in the presence of paracetamol

Pelissier et al. (1996) and Raffa and Codd (1996) have previously reported the lack of affinity of paracetamol for a number of receptors, uptake systems and transporters. It has been shown that paracetamol lacks affinity for nine receptor subtypes of 5-hydroxytryptamine (5-HT) (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_2, 5-HT_{2C}, 5-HT_3, 5-HT_4, and recombinant 5-HT_6, and 5-HT_7), the neuronal 5-HT re-uptake site, and several other receptors or uptake sites including, α_1, α_2, β_1 and β_2 adrenoceptors, D1 and D2 dopamine, M_2 muscarinic, NK_2 neurokinin, mu, delta and kappa opioid receptors, neuropeptide Y and sigma receptors and the noradrenaline re-uptake site (Raffa and Codd, 1996). In addition, Pelissier et al. (1996) showed no significant interaction with adenosine A_1 or A_{2A} receptors, 5-HT_{2A}, NMDA receptors, NK_1 receptors and the dopamine uptake site. However, the affinity of paracetamol for the NBTI-sensitive transporter and the NOP receptor has not previously been established. The qualitative and quantitative pattern of NBTI-binding was similar to previous studies in the mouse and rat brain (Bailey et al., 2002; Ekonomou et al., 2000; Geiger, 1987; Geiger et al., 1985; Geiger and Nagy, 1985). Paracetamol did not have a marked effect on binding, suggesting that paracetamol does not bind to the NBTI-sensitive transporter. Its analgesic effects are, therefore, unlikely to be due to interference with adenosine uptake, unlike opioid-induced analgesia which has been suggested to occur at least partially by this mechanism (Pini et al., 1997; Sandrini et al., 2001a,b).

The diffuse distribution pattern of the NOP receptor is consistent with previous data (Clarke et al., 2001; Slowe et al., 2001) and reflects the wide physiological role of this receptor system and its role in nociception. Once again, paracetamol had no significant effect on binding, suggesting that the analgesic effects of paracetamol are not mediated by an interaction with the NOP receptor.

4.2. [^{3}H]paracetamol distribution in the mouse brain

The autoradiographic study of Courade et al. (2001) using [^{3}H]paracetamol at 30 nM failed to show any specific binding to murine brains, but because this concentration is more than 1000-fold below the plasma concentration required to elicit an analgesic response in laboratory animals, our study has been carried out using a similar autoradiographic procedure but with a concentration (3 μM) more relevant to in vivo levels (Prescott, 1996). Our data confirm the results of Courade et al. (2001), showing that paracetamol binding is ubiquitous and that, although there is some indication of specific binding overall, the level of specific binding in most regions failed to reach statistical significance. Sagittal sections did not demonstrate the level of specific binding achieved in the coronal sections, which could reflect the limited discrimination of structures by sagittal sectioning.

In the spinal cord, [^{3}H]paracetamol also demonstrated specific binding, although again this binding was found to be homogenous throughout the laminae. However, specific binding was significantly higher in thoracic segments when compared to lumbar and cervical segments, suggesting that paracetamol may have a direct effect on nociception in the spinal cord, supporting the suggestions of a spinal site of action for paracetamol (Bjorkman, 1994; Pelissier et al., 1996). In relation to this, paracetamol could be binding to the recently discovered COX-1 variant (termed COX-3) which is expressed in the canine cerebral cortex and in lesser amounts in other tissues, and was selectively inhibited by paracetamol (see Chandrasekharan et al., 2002; Davies et al., 2004; Graham and Scott, 2003; Simmons, 2003). Although this COX variant does not occur in rats (Warner and Mitchell, 2004), it has been shown to be expressed in murine tissue including spleen, brain and cultured glial cells (Shaftel et al., 2003).

In conclusion, the analgesia induced by paracetamol is not mediated through the NOP receptor or the NBTI-sensitive adenosine transporter. Paracetamol does show

some specific binding in the brain and spinal cord but the binding was not region-specific, suggesting that the binding is not related to any particular neurotransmitter system.

References

Bailey, A., Matthes, H., Kieffer, B., Slowe, S., Hourani, S.M.O., Kitchen, I., 2002. Quantitative autoradiography of adenosine receptors and NBTI-sensitive adenosine transporters in the brains and spinal cords of mice deficient in the mu-opioid receptor gene. Brain Res. 943, 68–79.

Bannwarth, B., Netter, P., Lapicque, F., Gillet, P., Pere, P., Boccard, E., Royer, R.J., Gaucher, A., 1992. Plasma and cerebrospinal fluid concentrations of paracetamol after a single intravenous dose of propacetamol. Br. J. Clin. Pharmacol. 34, 79–81.

Bjorkman, R., 1994. Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. Pain 57, 259–264.

Calo, G., Guerrini, R., Rizzi, A., Salvadori, S., Regoli, D., 2000. Pharmacology of nociceptin and its receptor: a novel therapeutic target. Br. J. Pharmacol. 129, 1261–1283.

Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., Simmons, D.L., 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc. Natl. Acad. Sci. U. S. A. 99, 13926–13931.

Citterio, F., Corradini, L., Smith, R.D., Bertorelli, R., 2000. Nociceptin attenuates opioid and gamma-aminobutyric acid(B) receptor-mediated analgesia in the mouse tail-flick assay. Neurosci. Lett. 292, 83–86.

Clarke, S., Chen, Z., Hsu, M.S., Pintar, J., Hill, R., Kitchen, I., 2001. Quantitative autoradiographic mapping of the ORL1, mu-, delta- and kappa-receptors in the brains of knockout mice lacking the ORL1 receptor gene. Brain Res. 906, 13–24.

Courade, J.P., Besse, D., Delchambre, C., Hanoun, N., Hamon, M., Eschalier, A., Caussade, F., Cloarec, A., 2001. Acetaminophen distribution in the rat central nervous system. Life Sci. 69, 1455–1464.

Davies, N.M., Good, R.L., Roupe, K.A., Yanez, J.A., 2004. Cyclo-oxygenase-3: axiom, dogma, anomaly, enigma or splice error?—Not as easy as 1, 2, 3. J. Pharm. Pharm. Sci. 7, 217–226.

Ekonomou, A., Pagonopoulou, O., Angelatou, F., 2000. Age-dependent changes in adenosine A1 receptor and uptake site binding in the mouse brain: an autoradiographic study. J. Neurosci. Res. 60, 257–265.

Fischer, L.J., Green, M.D., Harman, A.W., 1981. Levels of acetaminophen and its metabolites in mouse tissues after a toxic dose. J. Pharmacol. Exp. Ther. 219, 281–286.

Geiger, J.D., 1987. Adenosine uptake and [^{3}H]nitrobenzylthioinosine binding in developing rat brain. Brain Res. 436, 265–272.

Geiger, J.D., Nagy, J.I., 1985. Localization of [^{3}H]nitrobenzylthioinosine binding sites in rat spinal cord and primary afferent neurons. Brain Res. 347, 321–327.

Geiger, J.D., LaBella, F.S., Nagy, J.I., 1985. Characterization of nitrobenzylthioinosine binding to nucleoside transport sites selective for adenosine in rat brain. J. Neurosci. 5, 735–740.

Graham, G.G., Scott, K.F., 2003. Mechanisms of action of paracetamol and related analgesics. Inflammopharmacology 11, 401–413.

Kitchen, I., Slowe, S.J., Matthes, H.W., Kieffer, B., 1997. Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. Brain Res. 778, 73–88.

Meunier, J.-C., 1997. Nociceptin/Orphanin FQ and the opioid receptor-like ORL1 receptor. Eur. J. Pharmacol. 340, 1–15.

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G., Meunier, J.C., 1994. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett. 341, 33–38.

Neal Jr., C.R., Mansour, A., Reinscheid, R., Nothacker, H.P., Civelli, O., Akil, H., Watson Jr., S.J., 1999. Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. J. Comp. Neurol. 412, 563–605.

Paxinos, G., Franklin, F.B.J., 2001. The Mouse Brain in Stereotaxic Coordinates. Academic Press, London.

Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates. Academic Press, London.

Pelissier, T., Alloui, A., Caussade, F., Dubray, C., Cloarec, A., Lavarenne, J., Eschalier, A., 1996. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine3 receptors: in vivo and in vitro evidence. J. Pharmacol. Exp. Ther. 278, 8–14.

Pini, L.A., Vitale, G., Ottani, A., Sandrini, M., 1997. Naloxone-reversible antinociception by paracetamol in the rat. J. Pharmacol. Exp. Ther. 280, 934–940.

Prescott, L.F., 1996. Paracetamol (Acetaminophen) a Critical Bibliographic Review. Taylor and Francis, London.

Raffa, R.B., Codd, E.E., 1996. Lack of binding of acetaminophen to 5-HT receptor or uptake sites (or eleven other binding/uptake assays). Life Sci. 59, L37–L40.

Raffa, R.B., Stone Jr., D.J., Tallarida, R.J., 2000. Discovery of "self-synergistic" spinal/supraspinal antinociception produced by acetaminophen (paracetamol). J. Pharmacol. Exp. Ther. 295, 291–294.

Sandrini, M., Romualdi, P., Capobianco, A., Vitale, G., Morelli, G., Pini, L.A., Candeletti, S., 2001a. The effect of paracetamol on nociception and dynorphin A levels in the rat brain. Neuropeptides 35, 110–116.

Sandrini, M., Romualdi, P., Vitale, G., Morelli, G., Capobianco, A., Pini, L.A., Candeletti, S., 2001b. The effect of a paracetamol and morphine combination on dynorphin A levels in the rat brain. Biochem. Pharmacol. 61, 1409–1416.

Sawynok, J., 1998. Adenosine receptor activation and nociception. Eur. J. Pharmacol. 347, 1–11.

Sawynok, J., Liu, X.J., 2003. Adenosine in the spinal cord and periphery: release and regulation of pain. Prog. Neurobiol. 69, 313–340.

Sawynok, J., Yaksh, T.L., 1993. Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. Pharmacol. Rev. 45, 43–85.

Shaftel, S.S., Olschowka, J.A., Hurley, S.D., Moore, A.H., O'Banion, M.K., 2003. COX-3: a splice variant of cyclooxygenase-1 in mouse neural tissue and cells. Mol. Brain Res. 119, 213–215.

Simmons, D.L., 2003. Variants of cyclooxygenase-1 and their roles in medicine. Thromb. Res. 110, 265–268.

Slowe, S.J., Clarke, S., Lena, I., Goody, R.J., Lattanzi, R., Negri, L., Simonin, F., Matthes, H.W., Filliol, D., Kieffer, B.L., Kitchen, I., 2001. Autoradiographic mapping of the opioid receptor-like 1 (ORL1) receptor in the brains of mu-, delta- or kappa-opioid receptor knockout mice. Neuroscience 106, 469–480.

Snell, B.J., Short, J.L., Drago, J., Ledent, C., Lawrence, A.J., 2000. Characterisation of central adenosine A1 receptors and adenosine transporters in mice lacking the adenosine A_{2A} receptor. Brain Res. 877, 160–169.

Warner, T.D., Mitchell, J.A., 2004. Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. FASEB J. 18, 790–804.