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SIGMA RECEPTORS AND THE IMMUNE SYSTEM:
IDENTIFICATION, LOCALIZATION AND FUNCTIONAL SIGNIFICANCE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By

Ben B. Whitlock, B.S.

*****

The Ohio State University
1997

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ABSTRACT

Sigma (σ) receptors are a class of binding sites that can interact with a variety of drugs of abuse (PCP and cocaine) and commonly prescribed drugs including antipsychotics (haloperidol) and anesthetics (pentazocine). Several subtypes (σ₁, σ₂ and σ₃) of these receptors exist in the central nervous system and in peripheral tissues. We have shown previously that σ sites are present in immune tissues and cells. In addition, we have shown that σ agonists can inhibit the proliferation of concanavalin A (ConA)-stimulated rat spleen cells via interaction with the σ₁ receptor subtype. The work presented herein grew out of two main observations and hypotheses: One, we hypothesized, based on previous studies using the σ radioligand (+)[³H]-3-PPP, that a novel subtype of σ receptor existed in immune tissues. Two, we speculated that the ability of σ agonists to inhibit the proliferation of rat spleen cells was due to direct modulation of T cell function via σ₁ sites present on T cells. Therefore, two lines of investigation arose from these observations: (1) Using (+)[³H]-3-PPP, high concentrations of a novel haloperidol-inaccessible site were found in human peripheral blood leukocytes (PBL), rat spleen and spleen cells, but were not present in rat brain. In competition binding assays the splenic sites were sensitive to all σ agonists tested with the exception of haloperidol and 1,3-di(2-tolyl)guanidine (DTG). In addition, the sites exhibited selectivity for the (-)-stereoisomers of the σ benzomorphans pentazocine and SKF 10,047 and were sensitive to the opioid antagonist naltrexone. Binding activity in spleen was destroyed by heating and phospholipase C, but not by proteases or
glycosidases. Autoradiographic studies revealed that the splenic sites were localized in a coarse punctate pattern in the marginal zones and red pulp. Haloperidol-inaccessible (+)-[\(^{3}\text{H}\)]-3-PPP binding was not present on a variety of T, B and macrophage cell lines. In addition, (+)-[\(^{3}\text{H}\)]-3-PPP binding was nonexistent in human peripheral blood mononuclear cells (PBMC), but was very high in human polymorphonuclear leukocytes (PMN). These findings suggest that the haloperidol/DTG-inaccessible, opioid-like sites are primarily located on granulocytes, and could play a role in granulocyte function or development. (2) Using (+)-[\(^{3}\text{H}\)]-pentazocine in the presence of naltrexone to block the haloperidol/DTG-inaccessible sites, we identified \(\sigma_1\) receptors in the interleukin-2 (IL2)-producing murine T cell lymphoma LBRM33. The numbers of \(\sigma_1\) sites on LBRM33 T cells were higher than those found in spleen or brain. We found that \(\sigma\) agonists could inhibit phytohemagglutinin (PHA)-stimulated production of IL2 in these cells in a manner that correlated with potency at \(\sigma_1\) sites. However, this effect was apparently due to drug-induced toxicity. We then examined whether \(\sigma_1\)-modulated inhibition of ConA-induced spleen cell proliferation was due to this toxic effect. The \(\sigma\) agonists haloperidol and (+)-pentazocine inhibited spleen cell proliferation without inducing cell death; however, only very high concentrations of haloperidol inhibited IL2 production in these cells. (+)-Pentazocine caused slight inhibition of IL2, but only at the highest concentration examined. These results indicate that while occupation of \(\sigma_1\) receptors on LBRM33 cells can lead to cell death, \(\sigma_1\) receptors on mouse spleen cells do not trigger death, but can control proliferation via a mechanism not linked to IL2.

Overall, these studies provide more information about the possible sites of action for \(\sigma\) agonists, their location on immune cells and their possible roles in immune function.
To my grandmothers, Vesta Moses and Icie Whitlock.

Their perseverance and strength has been an inspiration to all of us.
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I first want to thank Dr. Seth Wolfe for providing me with the tools and knowledge to do research and for encouraging me throughout my bouts of self-doubt.

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2. Whitlock, B.B., Y. Liu, S. Chang, P. Saini, B.K. Ha, T.W. Barrett and S.A.
Wolfe, Jr. 1996. Initial characterization and autoradiographic localization of a novel

localization of three distinct sites for sigma receptor ligands in rat spleen. *J.
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ABSTRACTS:


FIELDS OF STUDY

Major Field: Medical Microbiology and Immunology
Studies on Sigma Binding Sites in the Immune System
with Dr. Seth A. Wolfe, Jr.
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<td>A450</td>
<td>absorbance at 450 nanometers</td>
</tr>
<tr>
<td>ACK</td>
<td>ammonium chloride plus potassium lysis buffer</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>anti-μ</td>
<td>anti-immunoglobulin M</td>
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<td>ATCC</td>
<td>American Type Tissue Culture Collection</td>
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<tr>
<td>Bmax</td>
<td>maximum number of binding sites</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CHAPS</td>
<td>(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<td>Ci</td>
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<td>CNS</td>
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<td>concanavalin A</td>
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<td>C-TAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DM</td>
<td>dextromethorphan</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco's Phosphate Buffered Saline</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTG</td>
<td>1,3-di(2-tolyl)guanidine</td>
</tr>
<tr>
<td>EC50</td>
<td>effective concentration to inhibit 50%</td>
</tr>
<tr>
<td>ELISA</td>
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<tr>
<td>3-PPP</td>
<td>3-(3-hydroxyphenyl(-N-(1-propyl)piperidine</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>S.A.</td>
<td>specific activity</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCP</td>
<td>N-[1-(2-thienyl)cyclohexyl]-piperidine</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris(hydroxymethyl)-aminomethane hydrochloride</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell</td>
</tr>
</tbody>
</table>
CHAPTER 1

General Introduction

Many factors can impinge upon the health of an individual. Factors such as nutrition, sleep patterns, stress and behavior can affect immune parameters. One detrimental behavior that may influence disease-fighting ability is substance abuse. Several drugs of abuse have been shown to have adverse effects on the immune system. Drugs as diverse as alcohol, cocaine, marijuana and morphine have all been shown to have immune altering capabilities both \textit{in vitro} and \textit{in vivo} \cite{1-4}. One drug shown to have effects on immune function \textit{in vitro} is the hallucinogen phencyclidine (PCP, 'angel dust').

PCP can suppress cellular immune responses \textit{in vitro} including inhibition of proliferation of mitogen stimulated T and B cells, immunoglobulin synthesis and lytic activity of cytotoxic T cells and NK cells \cite{5, 6}. In addition, in a thorough study performed by Dornand and colleagues \cite{7} it was shown that PCP and derivatives of PCP could inhibit concanavalin A (ConA)-stimulated production of interleukin-2 (IL2) and mobilization of intracellular Ca\textsuperscript{2+} in mouse thymocytes. At the time of these initial studies it was unclear through what mechanism PCP was acting to exert these effects. PCP was known to interact with at least two receptors or binding sites in the central nervous system (CNS). One was termed the PCP receptor, later determined to be part of
the N-methyl-D-aspartate (NMDA) receptor/ion channel complex (for review see 8). The other site was a recently discovered site termed the sigma (σ) receptor (9, 10). Wolfe and colleagues (11, 12) investigated the presence of these sites in immune tissues. In radioligand binding studies utilizing [3H]N-[1-(2-thienyl) cyclohexyl]-piperidine (TCP; a potent derivative of PCP) it was found that the high affinity PCP (NMDA) receptor was not present; however, σ binding sites were present both in human peripheral blood leukocytes (PBL) and rat immune tissues (11, 12). This led to the hypothesis that PCP may exert its immunosuppressive effects through σ sites, which could be important immunomodulatory receptors on immune cells. Therefore, we undertook studies to investigate the role sigma receptors may play in the immune response by further characterizing these receptors in the immune system, as well as investigating the effect of σ agonists on immune function.

**Sigma binding sites: an historical perspective**

The existence of neuromodulatory σ receptors was first suggested by experiments carried out by Martin and co-workers (13), in which the opiate benzomorphan N-allylnormetazocine (SKF 10,047) elicited physiological effects different from other opiates. SKF 10,047 administration caused an increase in pupillary diameter and pulse rate, as well as a delirium not characteristic of other opiates (13). It was subsequently found that the (-)-stereoisomer of SKF 10,047 was a traditional, sedating opiate, while (+)-SKF 10,047 was excitatory, and its actions were not blocked by the opioid antagonist naltrexone (14). In addition, racemic SKF 10,047 was found to cause psychotomimetic effects in humans (15). Based on these findings the existence of σ opiate receptors was postulated.
Radioligand binding studies in the CNS revealed that σ binding sites were not true opioid receptors because the opiates (-)-etorphine and naloxone did not interact at these sites (9, 10), nor did any opioid peptides (16). In addition, a variety of other non-opiate drugs were demonstrated to interact at these novel binding sites, including PCP and antipsychotics such as haloperidol (9, 10). When haloperidol (better known as a dopamine antagonist) was used to label σ binding sites in guinea pig brain, it was found that the benzomorphan (+)-pentazocine (similar in structure to SKF 10,047) interacted with [3H]haloperidol-labeled sites with 10-fold higher affinity than did the dopamine antagonist spiperone (17). This strengthened the link between antipsychotics and σ binding sites. These findings led to the definition of the σ receptor as a haloperidol-sensitive, non-opioid binding site (18).

Sigma receptors were originally identified in rat spinal cord and guinea pig brain homogenates (9, 10). Anatomical studies revealed σ binding sites were widely distributed in the CNS, with concentrations in the cerebellum, brainstem and spinal cord (10, 19, 20) suggesting a role for σ sites in motor function. It became readily apparent, however, that these binding sites were also present in numerous tissues and cells outside the CNS including rat liver (21), guinea pig myenteric plexus (22) and guinea pig ileum (23). Rat endocrine tissues such as the pituitary, adrenal cortex, ovary and testis were found to contain large numbers of σ sites (24). In the immune system, human leukocytes and rat spleen were found to contain relatively high numbers of [3H]haloperidol-labeled σ sites (11, 12). In addition, σ binding sites have been found in extremely high numbers in tumors of neural and non-neural origin (25, 26). Currently, many questions remain concerning the function, signaling and molecular nature of these novel binding sites, both in the CNS and peripheral organs.
Sigma receptor heterogeneity

The initial observation that many different types of drugs have access to $\sigma$ receptors in radioligand binding assays, including phencyclidines, phenylpiperidines, benzomorphans, guanidines and butyrophenones (16), led naturally to the hypothesis that multiple subtypes of $\sigma$ sites existed in the CNS. Indeed, multiple reports have borne out this hypothesis (27-31). It appears that there are at least two subtypes that are distinct molecular entities and not differing affinity states of the same receptor (28). One subtype, $\sigma_1$, is of higher affinity (low nM) for most $\sigma$ ligands and exhibits a preference for the (+)- over the (-)-stereoisomers of the benzomorphans SKF 10,047 and pentazocine. The second subtype, $\sigma_2$, is generally of lower affinity ($10^{-8}$ - $10^{-6}$ M) for $\sigma$ ligands and shows a preference for the (-)-stereoisomers of benzomorphans (32, 33). Based on these findings, Bowen and colleagues coined the terms $\sigma_1$ and $\sigma_2$, nomenclature that has been generally accepted (34). Recently, Booth and colleagues have identified a site labeled with phenylaminotetralin compounds that binds several $\sigma$ ligands such as haloperidol and (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP), but does not interact with other $\sigma$ ligands such as (+)-pentazocine and (+)-SKF 10,047 (35). The authors have termed this site $\sigma_3$. Table 1.1 summarizes some of the known characteristics of $\sigma_1$, $\sigma_2$ and $\sigma_3$ binding sites and the accepted methods for labeling them in CNS binding studies.

$\sigma_1$ and $\sigma_2$ receptors differ in their CNS distribution. $\sigma_1$ sites are located in highest concentration in the hindbrain, midbrain and cerebellum, $\sigma_2$ sites are also high in the midbrain region, but in contrast to $\sigma_1$, are also high in the cerebral cortex (36, 37).

Receptor subtypes have also been identified outside of the CNS. Hellewell and colleagues (32) examined the heterogeneity of $\sigma$ binding sites in rat liver and kidney.
<table>
<thead>
<tr>
<th>Locations</th>
<th>σ₁</th>
<th>σ₂</th>
<th>σ₃⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discriminant Ligands</td>
<td>(+)-pentazocine high affinity</td>
<td>low affinity moderate to high affinity</td>
<td>low affinity</td>
</tr>
<tr>
<td></td>
<td>(+)-SKF 10,047 moderate to high affinity</td>
<td>very low affinity</td>
<td>low affinity</td>
</tr>
<tr>
<td></td>
<td>Dextrorphan moderate to high affinity</td>
<td>very low affinity</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td>CI,OH-PAT* unknown</td>
<td>very low affinity</td>
<td>high affinity</td>
</tr>
<tr>
<td>Nondiscriminant Ligands</td>
<td>high affinity</td>
<td>high affinity</td>
<td>moderate affinity</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>high affinity</td>
<td>high affinity</td>
<td>low affinity</td>
</tr>
<tr>
<td>DTG</td>
<td>inactive</td>
<td>inactive</td>
<td>inactive</td>
</tr>
<tr>
<td>Naloxone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stimulates Ach release; inhibits</td>
<td>inhibits contraction in ileum/myenteric plexus; elicitation of dystonia; inhibits K⁺ currents</td>
<td>stimulates tyrosine hydroxylase activity</td>
</tr>
<tr>
<td></td>
<td>carbachol-stimulated PI turnover; inhibits NMDA-stimulated [Ca²⁺] release</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second messengers</td>
<td>G-protein linked?; may cause increased [Ca²⁺]ᵢ</td>
<td>may be linked to K⁺ channels; most likely not G-protein linked</td>
<td>unknown</td>
</tr>
<tr>
<td>Structure / m.w.</td>
<td>223 aa transmembrane protein/25kDa</td>
<td>unknown / 18 - 21.5kDa</td>
<td>unknown</td>
</tr>
<tr>
<td>Immune Function</td>
<td>inhibits proliferation of rat spleen cells; may inhibit NK cells indirectly via CNS action</td>
<td>unknown</td>
<td>unknown</td>
</tr>
</tbody>
</table>

It remains to be seen if these sites are true σ sites. Many dopamine ligands interact with them, few σ ligands do. CI,OH-PAT, (+)-(trans)-1-phenyl-3-3-N,N-dimethylamino-6-chloro-7-hydroxyl-1,2,3,4-tetrahydronaphthalene. Only selected functions are shown. The information in the table was obtained from references cited in the text and the structure is based on a table originally published in (34).

Table 1.1. Characteristics of sigma binding subtypes.
where large numbers of $\sigma_1$ and $\sigma_2$ binding sites were detected by photoaffinity labeling. In addition, $\sigma_1$ and $\sigma_2$ binding sites have been found on the PC12 pheochromocytoma cell line (28) and on various tumor cell lines (38). Sigma-2 sites have also been identified in rat heart membranes (39).

For definitive pharmacological, functional and molecular studies of receptors it is critical to have specific labeling methods. Sigma-1 and $\sigma_2$ receptors can be identified by radioligand binding using different paradigms. (+)-[3H]Pentazocine (40) has been the accepted ligand for labeling $\sigma_1$ receptors, while $\sigma_2$ receptors have been labeled with the nondiscriminating ligand [3H]1,3-di(2-toly1) guanidine (DTG; 41) in the presence of a saturating concentration of a $\sigma_1$ preferring ligand such as (+)-SKF 10,047, (+)-pentazocine or dextrallorphan (34). More specific radioligands may hold promise for future detection of $\sigma_2$ (42, 43).

In general, binding studies utilizing [3H]haloperidol, performed prior to the recognition of both $\sigma_1$ and $\sigma_2$, detected $\sigma$ binding sites with a pharmacology resembling $\sigma_1$ receptors. Whereas binding studies utilizing [3H]DTG or (+)-[3H]-3-PPP may have detected $\sigma_1$ and/or $\sigma_2$ depending on the relative number of these sites in the tissue (16). For example, sigma sites labeled by [3H]haloperidol in endocrine and immune tissues exhibited a $\sigma_1$ pharmacology (11, 12). However, using [3H]DTG to label $\sigma$ sites in PC12 cells, a $\sigma_2$ pharmacology was seen because these cells have a higher number of $\sigma_2$ sites compared to $\sigma_1$ (28).

**Functional significance of sigma receptors**

Sigma receptors were originally thought to be involved in schizophrenia and other forms of human psychoses based on the observation that PCP causes a schizophrenia-like
psychosis and several antipsychotics bind σ sites (19, 44). However, σ selective drugs have shown minimal efficacy as antipsychotic drugs (45). Research on the function of σ binding sites has been hampered by the fact that most σ ligands are not selective, and also interact at other neurotransmitter receptors such as dopamine, serotonin and NMDA. In addition, selective σ antagonists have not been described. Therefore, to prove that a functional outcome is mediated through σ receptors requires correlation of the potency of a panel of σ agonists in functional studies with their potency in binding at σ receptors. In this manner, σ2 receptors have been demonstrated to be involved in elicitation of dystonia (impaired muscle tone) upon injection of σ ligands into the red nucleus of rats (46). In a like manner, the ability of σ agonists to inhibit contractions of guinea pig ileum muscle/myenteric plexus correlated highly with their binding affinity at σ2 receptors (23, 47). In neuroendocrine studies, it has been demonstrated that σ ligands can inhibit nicotine-stimulated catecholamine release and this activity correlates with drug potency at σ1 receptors in adrenal medulla (48). Other functional systems that have been shown to correlate with σ receptor binding activity include: potentiation of muscle contractions in the guinea pig vas deferens (49), inhibition of tonic K+ currents in the neuroblastoma cell line NCB-20 via σ2 receptors (31), increase in extracellular acetylcholine levels in rat frontal cortex via σ1 (50) and inhibition of carbachol-stimulated phosphoinositide turnover in brain synaptoneurosomes (51).

Recently, some compounds have shown efficacy as σ antagonists. The σ selective compounds BD1047 and BD1063 have been shown to antagonize the dystonia-eliciting effects of the σ agonists DTG and haloperidol (52). The compound Dup734 has been shown to antagonize an inhibitory effect of (+)-pentazocine on NMDA-stimulated [3H]norepinephrine release from cerebellar slices (53). However, Dup734 also has
strong serotonin antagonist properties and may not be useful as a selective antagonist (54). The effectiveness of these compounds in other systems has not been demonstrated and it is unclear if they will be clinically useful as σ antagonists.

Sigma receptors: possible second messenger systems

A hallmark of functional cellular receptors is linkage to signal transducing systems that either directly lead to new transcription/protein synthesis or intersect and modulate other signaling cascades. Evidence exists that σ receptors are linked to second messenger systems.

Sigma receptor-G-protein coupling has been investigated by examining sensitivity to agents that decrease the affinity of agonists for G-protein coupled receptors. Non-hydrolyzable analogs of GTP such as 5'-guanylylimidodiphosphate (Gpp(NH)p) are commonly used for this purpose. Exposure of membranes to these agents results in G-proteins being 'frozen' in a constant state of activation and, thus, dissociated from the receptor, which causes the receptor to remain in a lower affinity binding state for agonists, but not antagonists. Initial studies found that Gpp(NH)p decreased the affinity of (+)-[3H]-3-PPP-labeled σ receptors in rat brain (55, 56) indicating that at least some σ receptor types may be G-protein linked. Following the discovery of heterogeneous sites, it was found that binding of [3H]DTG to σ1 receptors was affected by Gpp(NH)p, whereas binding of [3H]DTG to σ2 receptors was not affected (57). In addition to sensitivity to Gpp(NH)p, Itzhak (55) showed that (+)-[3H]-3-PPP binding was sensitive to pertussis toxin (PTX). PTX catalyzes the ADP-ribosylation of the G_{i/o} subset of G-proteins, resulting in their inactivation. Further evidence for the coupling of σ1 receptors to G_{i/o}-proteins has been obtained in studies where σ ligand modulation of NMDA-induced noradrenaline release has been shown to be dependent on G_{i/o}-protein
function (58, 59). It must be noted, however, that not all studies have revealed a definitive link between $\sigma_1$ receptors and G-proteins. It appears that (+)-$[^3]H$pentazocine binding, at least in guinea pig brain, is insensitive to GTP and GTP analogs (60, 61). In addition, the affinity of haloperidol at (+)-$[^3]H$-3-PPP labeled $\sigma$ sites was unaffected by Gpp(NH)p (55) and Gpp(NH)p had no effect on $[^3]H$haloperidol binding in rat brain (Battaglia and Wolfe, unpublished). These findings led to the suggestion that (+)-pentazocine and haloperidol may be $\sigma$ antagonists; however, these drugs behave as agonists in the majority of functional studies (16).

While $\sigma_1$ receptors may be G-protein linked, little is known about second messenger systems linked to $\sigma_2$ receptors. However, $\sigma_2$ sites were originally identified on NCB-20 cells based on the ability of $\sigma$ ligands to block a tonic, outward K$^+$ current, indicating that $\sigma_2$ sites may be linked to ion channels in some manner (31). Subsequent studies demonstrated that this effect could not be abrogated by the G-protein toxins PTX and cholera toxin (CTX), lending further support to the concept that $\sigma_2$ receptors are not linked to G-proteins (62). Recent evidence suggests a link between $\sigma_2$ sites and 5-HT$_3$ receptor/ion channel function (63).

Recent studies have also examined other possible signaling events linked to $\sigma$ binding sites. Brent and colleagues (64, 65) demonstrated in rat synaptosome preparations that $\sigma$ agonists increased both [Ca$^{2+}$]$_i$ levels and baseline phosphorylation of synapsin I and dynamin (proteins involved in synaptic vesicle transport). A possible connection to calcium was also seen in human mammary adenocarcinoma cells and colon carcinoma cells, where the $\sigma$ agonist haloperidol metabolite II (reduced haloperidol) increased [Ca$^{2+}$]$_i$ (66). It was unclear in these studies which subtype(s) of $\sigma$ receptors mediated these cellular effects.
In addition, it has also been shown that, in some systems, σ agonists can modulate stimulus-induced signaling cascades. Bowen and colleagues (51) demonstrated that σ agonists could inhibit the phosphoinositide cascade generated by the muscarinic agonist carbachol. This effect correlated highly with σ₁ binding site pharmacology. Sigma agonists inhibit NMDA-stimulated enhancement of free [Ca²⁺]ᵢ and cyclic GMP in brain, effects that also appear to be σ₁ mediated (67, 68). Also, in mouse spleen cells, forskolin-stimulated cAMP formation was increased by the σ agonists (+)-3-PPP and haloperidol, but not by (+)-pentazocine (69).

These studies, taken as a whole, provide strong evidence that σ₁ and σ₂ receptors are linked to signaling systems; however, the nature of the second messengers involved is unclear. Like the functional studies outlined above, lack of selective σ ligands makes it difficult to draw definitive conclusions about which sites are linked to an observed second messenger. Therefore, as in bioassays, correlations between binding affinity and potency in signaling must be performed.

**Sigma receptors: candidate endogenous ligands**

Theoretically, receptors have not evolved to interact with synthetic drugs. Endogenous ligands must exist. Unfortunately, the identity of endogenous ligands for σ receptors has remained elusive. However, it is clear from a set of elegant studies performed by Chavkin and co-workers that endogenous ligands for σ receptors exist in the CNS (70, 71). These studies utilized an *in vitro* 'brain slice' method in which 500μm slices of rat hippocampus were subjected to depolarization with a sodium channel activator and then incubated with the σ radioligand [³H]DTG. The amount of radioligand
bound to the slices was determined and found to decrease upon depolarization. This reduction in binding was transient, and the dissociation kinetics of [3H]DTG did not change following depolarization, suggesting that the decrease in binding was not due to a conformational change in the receptor, but rather was due to a release of synaptic stores of an endogenous ligand(s), which bound σ receptors and blocked [3H]DTG binding (70, 71). Several preliminary studies partially purified peptides from brain tissue that showed potential as endogenous σ ligands (72, 73); however, none of these reports were followed up with further purification or peptide sequence information.

Several characterized peptides have been suggested as potential endogenous ligands for σ binding sites. For example, the bioactive peptide Neuropeptide Y (NPY) was reported to exhibit high affinity at σ receptors in rat brain (74), suggesting that NPY could be an endogenous σ ligand, or that σ receptors could be a form of NPY receptor. However, these findings were not reproducible (75, 76), tarnishing the possibility of NPY as an endogenous ligand. Other candidate peptides have been suggested such as calcitonin gene related peptide (77) and the NH$_2$-terminal portion of substance P (78). However, to date these peptides have not gained wide acceptance as endogenous ligands for σ sites.

Another candidate endogenous ligand was suggested by experiments conducted by Su and colleagues (79), who found that the steroid progesterone could interact with σ binding sites in guinea pig brain and spleen. The affinity of progesterone was rather low ($K_i = 376nM$ in splenic homogenates); however, the authors argued that these levels can be reached during menstruation and pregnancy (79). In addition to progesterone, other steroids such as testosterone and deoxycorticosterone also exhibit some affinity at σ sites (79, 80). Further support for progesterone as an endogenous σ ligand comes from competition binding studies carried out on rat liver membranes. In this tissue a highly significant correlation was seen between the ability of a variety of σ ligands and steroids
to block $[^3H]$progesterone and $[^3H]$haloperidol binding, suggesting that the sites labeled by the two radioligands were identical (81). Other evidence for steroid interaction at $\sigma$ sites comes from functional studies in which certain neuroactive steroids were shown to modulate NMDA-stimulated norepinephrine release in brain slices via a direct or indirect interaction with $\sigma_1$ receptors (59). These studies, combined with the recent discovery that the $\sigma_1$ site may be a steroidigenic enzyme (see below), make steroids likely candidates as endogenous binding substances.

The molecular nature of sigma receptors

Recent reports have taken a big leap in understanding the molecular structure of $\sigma$ sites. Hanner and colleagues (82) recently reported a cloned gene sequence from guinea pig liver corresponding to the $\sigma_1$ binding site. The cDNA clone codes for a 223 aa protein with a molecular weight of 25.3 kDa (82). This corresponds well to previous reports examining solubilized $\sigma_1$ sites from rat liver of 25 kDa (32) and guinea pig brain of 29 kDa molecular weight (83, 84). The predicted protein has one putative transmembrane segment near the NH$_2$-terminus and contains an endoplasmic reticulum retention signal. When expressed in yeast, the protein exhibits a classic $\sigma_1$ pharmacology, with high affinity for haloperidol and pentazocine and a preference for the (+)- over the (-)-stereoisomers of SKF 10,047 (82). mRNA transcripts were identified in many guinea pig tissues including brain, liver, ovary, placenta and spleen. This novel mammalian protein exhibits sequence homology with fungal sterol C$_8$-C$_7$ isomerase, a microsomal enzyme that shifts the C$_8$-double bond of fecosterol to position C$_7$. This represents a required step in membrane sterol synthesis in fungi. The human homologue of this gene has recently been determined by PCR-cloning using primers derived from the guinea pig sequence (85). This sequence, obtained from a human placental
choriocarcinoma cell library, was 93% identical to the guinea pig liver sequence and, when expressed in HeLa cells, bound haloperidol, DTG and (+)-3-PPP (85).

The biological significance of the cloned $\sigma_1$ binding site is not yet clear. However, a connection between $\sigma$ and microsomal enzymes has been suggested based on the observation that a significant number of $\sigma$ binding sites are present in the endoplasmic reticulum as well as the plasma membrane (36, 81), and the microsomal metabolic enzyme inhibitor proadifen (SKF 525-A) exhibits considerable affinity at $\sigma$ sites in liver (80). At this point a role for $\sigma$ agonists in mammalian sterol synthesis has not been demonstrated. However, when various steroids and steroid precursors were examined, no correlation was seen with binding affinity at $\sigma$ sites in guinea pig brain. This argues against a role for $\sigma$ binding sites in steroid synthesis (80).

Regardless of the role of $\sigma$ agonists in steroid synthesis, the implications for $\alpha_1$ binding site research are great. Given that $\sigma_1$ may be a sterol isomerase, it is difficult to interpret the data that implies that $\sigma_1$ binding sites are linked to G-proteins (57), or that $\sigma_1$ receptors can modulate other stimulus-induced pathways (51, 67, 68). However, the fact that the $\sigma_1$ binding site may be a sterol isomerase does not mean that the molecule does not have other unidentified functions, or that other signal transducing molecules could not be associated with it in some way. Indeed, recently it was suggested in solubilization and purification experiments that $\sigma_1$ sites are high molecular weight (300 - 500 kDa) entities that dissociate rapidly into smaller subunits upon purification (86).

Sigma-2 binding sites, unlike $\sigma_1$ sites, have not been genetically characterized. It is clear, however, that they are distinct molecular entities with a molecular weight of 18 - 21.5 kDa (28, 32). Although both $\sigma_1$ and $\sigma_2$ sites bind many of the same ligands, there is no data to suggest that they are structurally related. The studies observing that they
modulate different functions and may be linked to different signal transducing systems imply that they are structurally unrelated. Currently, no information is available concerning the molecular identity of σ3 binding sites.

**Sigma receptors and immune function**

Although elucidating the molecular structure, endogenous ligands and signaling mechanisms of σ sites is important, we believe that attributing functions to the σ receptor types is the first priority in understanding the role of these sites in the body. Therefore, we have concentrated on the identification and functional characterization of the σ binding sites in the immune system.

As outlined at the opening of this introduction, the original definitive connection between σ sites and the immune system came out of work performed by Wolfe and colleagues (11), who identified [3H]haloperidol-labeled σ receptors on human PBL. This was followed by the observation that σ receptors were also present in rat spleen and rat splenic mononuclear cells as well as human leukocytes (12, 87, 88). In competition binding assays the [3H]haloperidol-labeled sites on immune tissues exhibited a pharmacological profile corresponding to what is now known as the σ1 subtype (11, 12, 87, 88). In addition, (+)-[3H]pentazocine-labeled sites were identified on mouse mediastinal lymph node lymphocytes, splenic lymphocytes and thymocytes (89). In this study competition binding carried out on spleen cells showed some stereoselectivity for the (+)- over the (-)-stereoisomer of pentazocine, a characteristic of σ1 receptors (89).

Some evidence exists for the presence of σ2 receptors in the immune system. Sites labeled with [3H]DTG in rat spleen homogenates revealed a pharmacology corresponding to that of σ2 sites identified in the CNS (90). Binding experiments in our laboratory
using accepted $\sigma_2$ labeling conditions have identified saturable $[^3H]DTG$ sites on membranes of A20 cells, a B cell lymphoma, with a pharmacology corresponding to that of $\sigma_2$ receptors (Liu and Wolfe, unpublished). Specific $[^3H]DTG$ binding has also been observed on membranes of T cell lines (Liu, Whitlock and Wolfe, unpublished).

However, when identical conditions were used in autoradiographic studies to determine the location of splenic $\sigma_2$ sites, low specific binding and a non-$\sigma_2$ pharmacology was seen (91). Therefore, while $\sigma_2$ sites are most likely present in immune tissues, we cannot say on which cells they reside in vivo.

Recently, it has become apparent that $\sigma$ binding sites on immune cells are functional entities, and may influence cellular activity. The most definitive example of this is the finding by Liu and co-workers (87) that $\sigma$ agonists can suppress proliferation of rat spleen cells stimulated with the T cell mitogen ConA. This suppression is most likely mediated by interaction of agonists with $\sigma_1$ binding sites, as a good correlation ($r=0.86$) was seen between the ability of $\sigma$ agonists to suppress proliferation and binding affinity at $[^3H]$haloperidol-labeled $\sigma_1$ receptors (87). In addition, pentazocine has been shown to inhibit NK cell activity when given in vivo, an effect that favored (+)- over (-)-pentazocine (92). Other work on the effects of $\sigma$ ligands on immune function has also suggested a functional role for $\sigma$ binding sites in immune cell activity. Carr and colleagues (69,89) suggested that $\sigma$ agonists could modulate the function of splenic mononuclear cells stimulated with various mitogens. It was unclear in these studies, however, what receptor or binding site was mediating these effects, or what cell was being affected.
Hypotheses and significance

The work presented herein grew out of two main observations and hypotheses. One, we hypothesized, based on previous studies using the σ radioligand (+)-[^3H]-3-PPP, that a novel subtype of σ receptor existed in immune tissues and cells. Two, we speculated that the ability of σ agonists to inhibit ConA-induced rat spleen cell proliferation was due to direct modulation of T cell function. Through investigating these hypotheses two separate lines of investigation arose.

1. Identification of haloperidol/DTG-inaccessible sites for sigma ligands in the immune system. Previous work indicated that a large number of haloperidol-inaccessible (+)-[^3H]-3-PPP binding sites exist in rat spleen (11). Using specific labeling conditions we characterized this novel site for sigma and opioid ligands in depth, and found that it was present in large numbers in immune tissues, but not in brain. We elected to characterize these sites because they were novel, had not been previously described, and the relatively large numbers of these sites implied that they could have a role in immune function. Also, because of the relatively nonspecific nature of most σ ligands, it is critical to identify binding sites in tissues with which these drugs may interact. This site represents a possible site of drug action that must be taken into account when exploring the role of σ agonists in the immune system.

2. Sigma agonist mediated inhibition of T cell function. We followed up on previous work indicating σ1-mediated inhibition of spleen cell proliferation. We investigated the presence of σ1 receptors on T cell lines, and examined the effect of σ agonists on IL2 production. We followed this path to further characterize the σ1 receptor and its function.
on T cells. This would provide us not only with information about the role of $\sigma_1$ receptors in the immune response, but should also help to elucidate the role of these sites in cell physiology.

Research on $\sigma$ binding sites in the immune system is of potentially great significance because of the exposure of humans to compounds that interact at these sites. Commonly used drugs such as the neuroleptics haloperidol (Haldol®), chlorpromazine (Thorazine®) and thoridazine can act at these sites (16). It has been found that when patients are taken off neuroleptic therapy their immune proliferative responses rebound to above normal levels, implying a drug-mediated depressive effect on immune function may exist (93). Other widely used drugs such as the antitussive dextromethorphan (Robitussin DM®), the antihistamine chlorpheniramine, and the anesthetics/analgesics fentanyl and pentazocine (Talwin®) can interact with these sites at nanomolar concentrations (94). Importantly, abused drugs such as cocaine (95) and PCP can interact at $\sigma$ sites, and both of these drugs have immune altering capabilities (2, 7). While most of these compounds are known to elicit their CNS effects through interactions with other receptors, the widespread distribution of sigma sites in the CNS, endocrine and immune systems suggest that these drugs may also significantly affect other physiological processes through actions at $\sigma$ sites in multiple organ systems. Thus the interaction of drugs, especially drugs of abuse, with these sites could have consequences for human health.
2.1. Introduction

The present study describes a novel drug binding site for sigma and opioid ligands in immune tissues. The identification of this site grew out of our attempts to identify known sigma subtypes that exist in the CNS, peripheral organs and cells (34). At least two distinct sites for sigma ligands exist in peripheral tissues: $\sigma_1$ and $\sigma_2$. Sigma-1 designates the higher affinity binding site, which is stereoselective for (+)-optical enantiomers of pentazocine, SKF 10,047 and 3-PPP. Sigma-2 refers to a site which binds most drugs with lower affinity, and is selective for (-)-optical enantiomers of pentazocine, SKF 10,047 and 3-PPP (16, 34). Sigma-2 receptor stereoselectivity resembles that of kappa opioid receptors. However, unlike opioid receptors, sigma receptors (both $\sigma_1$ and $\sigma_2$) bind haloperidol and DTG, but do not bind the opioid antagonists naltrexone and naloxone (34). Sigma-1 and $\sigma_2$ sites have been identified in a number of peripheral tissues and cells including rat liver, kidney and heart (32, 39),
neuronal cell lines (28, 31), and a variety of tumor cell lines (38). There have been reports of further subtypes of sigma receptors (29, 35, 38, 96). Multiple types of sigma receptors can co-exist on cells (31).

In receptor autoradiography studies we have determined that σ₁ receptors are present in the T and B cell areas of rat spleen, with the highest density in the T cell regions (12, 91). Concurrently, we have shown that σ₁ binding sites modulate T cell proliferative responses (87). However, we have been unable to demonstrate comparable modulation of B cell proliferation by sigma sites (97). In addition, while we have some evidence for the existence of σ₂ receptors on immune cells (Liu, Whitlock and Wolfe, unpublished), we cannot attribute an immunomodulatory role to σ₂ sites at this time.

Carr and associates (69, 89, 98) have also reported binding sites for sigma ligands in immune tissues, and that sigma agonists modulate mitogen-stimulated lymphocyte proliferation, cytokine secretion and antibody production. Casellas, Paul and associates (90, 99) have recently expanded these findings with a variety of in vivo and in vitro binding and immune functional assays, primarily utilizing the new putative sigma receptor agonist SR 31747. However, neither of these groups demonstrated that sigma receptors were, in fact, the sites through which drugs modulated the functional activities measured.

The primary problem in these studies is the fact that most sigma agonists are not very selective, and can act at multiple sites. This list includes opioid, dopamine, serotonin, adrenergic and NMDA receptors, and dopamine and serotonin uptake sites (16). Because of these extensive cross-reactions, a key to being able to prove that compounds act at sigma receptors to modulate function is the ability to selectively label the various sigma binding sites/receptors. Also, the interpretation of any functional data is hampered by a lack of information concerning the sites of action for the σ agonists in the immune system.
We have previously reported the presence of high concentrations of sigma receptors, with a pharmacological profile consistent with what is now known as the $\sigma_1$ subtype, in human PBL, rat spleen, and isolated rat spleen cells (11, 12, 87). In these studies we identified $\sigma_1$ sites using $[\text{H}]$haloperidol, and demonstrated that (+)-3-PPP competes at these sites. In autoradiographic studies in rat spleen to determine the localization of $\sigma$ sites in immune tissues by labeling them with (+)-$[\text{H}]$-3-PPP, we found that if nonspecific binding was defined by using haloperidol to block sigma receptors there was a large amount of (+)-$[\text{H}]$-3-PPP binding to "non-sigma" sites (12). These results indicated that (+)-$[\text{H}]$-3-PPP labels an additional, haloperidol-insensitive binding site in immune tissues.

In the present study we have selectively labeled and characterized these novel binding sites with (+)-$[\text{H}]$-3-PPP in the presence of haloperidol. Using homogenate radioligand binding assays, we determined the kinetic characteristics and pharmacology of these sites. They were found to be present in extremely high concentrations in rat spleen, spleen cells and human PBL, but were not detectable in rat brain or a variety of immune cell lines. The pharmacology of these sites indicated that they were capable of binding sigma, opioid and NMDA receptor ligands. Using in vitro receptor autoradiography, we demonstrated their localization in a coarse, punctate pattern in marginal zones and red pulp of rat spleen, outside the T and B cell regions. In homogenate binding assays we showed that these novel sites were detectable in highly enriched populations of human polymorphonuclear leukocytes.
2.2. Materials and methods

2.2.1. Animals

Sprague-Dawley rats (125 - 175 g, 2.5 - 3 months old; Harlan Sprague-Dawley Inc., Indianapolis, IN) were maintained on a 12 h. light:12 h. dark cycle and received food and water ad libitum. No attempt was made to coordinate or control the estrus cycles of the animals. Animals were maintained and handled in accordance with NIH guidelines. Female rats were used for all experiments, with the exception of the autoradiography studies which also utilized some male animals.

2.2.2. Cell lines

A20, EL4.IL2 and LBRM33 clone 4A2 cell lines were obtained from the American Type Tissue Culture Collection (ATCC; Rockville, MD). A20 (mouse B cell lymphoma) and LBRM33 (mouse T cell lymphoma) cells were maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, defined grade, HyClone Laboratories, Inc., Logan UT) plus 5x10^-5 M 2-mercaptoethanol (2-ME; Bio-Rad Laboratories, Richmond, CA) and 50 μg/ml gentamicin (Gibco BRL). EL4.IL2 (mouse T cell lymphoma) cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM; Gibco BRL) supplemented with 10% horse serum (Gibco BRL) plus 5x10^-5 M 2-ME, 10mM HEPES (Gibco BRL) and 50 μg/ml gentamicin. RBL 2H3 (rat basophilic leukemia) cells were provided by Dr. Charity Fox, Ohio State. Ana-1 (mouse macrophage) cells were provided by Dr. Richard Fertel, Ohio
State. RAW 264.7 (mouse monocyte/macrophage) cells were provided by Dr. William Lafuse, Ohio State. Before binding assays, all cells were washed with Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS; Gibco BRL) and pelleted, then quick frozen with dry ice or liquid nitrogen. Cells were stored at -80°C until use in binding assays.

2.2.3. Tissue preparation

Brains and spleens were dissected from animals immediately after CO_{2} asphyxiation. Tissues were frozen using either powdered dry ice or liquid nitrogen, and stored at -80°C until use. Human peripheral blood leukocytes were obtained from the American Red Cross as leukocyte-rich buffy coats derived from normal blood donors. Five milliliters of buffy coat was added to 25ml Ca^{2+}, Mg^{2+}-free HBSS in 50ml polypropylene centrifuge tubes and mixed gently. The HBSS/buffy coat mixture was underlaid with 15ml of Histopaque (Sigma Diagnostics, St. Louis, MO) and centrifuged at 700 x g for 15 min. Leukocytes at the Histopaque-HBSS interface were collected, pooled and washed twice by centrifugation in HBSS. Viable cells were counted by trypan blue exclusion, aliquoted, pelleted by centrifugation at 200 x g for 10 min., and frozen on dry ice before being stored at -80°C. For experiments requiring single cell suspensions of splenocytes, cells from freshly dissected rat spleens were dissociated by pressing the spleens through wire mesh. Splenic leukocytes were then isolated by centrifugation over an HBSS/Histopaque step-gradient in a manner analogous to the procedure used to isolate human PBL.

Human granulocytes, obtained from the blood of healthy donors, were isolated in the laboratory of Dr. John Walters by Ficoll/Hypaque density gradient centrifugation followed by dextran sedimentation (100, 101). Residual red blood cells were lysed and
granulocytes were washed three times with Ca$^{2+}$/Mg$^{2+}$-free phosphate buffered saline prior to being resuspended in modified HBSS (1.9mM KH$_2$PO$_4$, 1.1mM Na$_2$HPO$_4$, 5mM KCl, 147mM NaCl, 5.5mM glucose, 1mM MgCl$_2$, 1mM CaCl$_2$, pH 7.3). Cells were pelleted by centrifugation at 700 x g for 15 min., frozen on dry ice and stored at -80° C until used.

Tissues to be used for autoradiography were removed as described above, embedded in M-1 embedding matrix (Shandon Lipshaw, Pittsburgh, PA), and frozen in powdered dry ice. They were sectioned (10 μm) at -20 to -16°C using a Bright cryostat (Hacker Instruments, Inc., Fairfield, NJ), thaw-mounted onto chrome alum/gelatin-subbed microscope slides, and stored desiccated at -20°C until used.

For homogenate binding assays, tissues and cell pellets were thawed and disrupted in 25 - 40 volumes ice cold 50mM Tris-HCl, pH 8.0 at 4° C, using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) for 30 sec. at 20,000 rpm. Homogenates were centrifuged at 40,000 x g for 12 min. The pellets were then resuspended in the same buffer, homogenized and centrifuged once again. Membrane pellets were then resuspended in ice-cold binding assay buffer (except where noted, 5mM Tris-HCl, pH 8.0 at room temperature), filtered through 150μm nylon mesh (Tetko, Inc., Briarcliff Manor, NY), centrifuged at 40,000 x g for 12 min. and resuspended in assay buffer. To decrease tissue loss, human PMN homogenate was not filtered with nylon mesh and was washed and resuspended in 50mM Tris-HCl for the final centrifugation instead of 5mM. PMN tissue pellets were not resuspended in 5mM Tris-HCl until just prior to assay addition. All homogenates were kept on ice until addition to binding assays.
2.2.4. Binding in membrane homogenates

A. General conditions. Except where noted in legends for individual experiments, binding assays were carried out at room temperature in 0.2 ml 5 mM Tris-HCl buffer, pH 8.0 at room temperature. Kinetic experiments were performed in 1 ml Titertubes (Bio-Rad) immersed in water baths at 0°-4°, 22° and 37° C. All other experiments were conducted at room temperature in 96-well, U-bottom microtiter plates (Corning Glass Works, Corning, NY). General binding conditions were: 1 μM (+)-3-PPP, consisting of (+)-[3H]-3-PPP (S. A. 85.2 - 107.7 Ci/mmol; New England Nuclear, Boston, MA) diluted 150- to 800-fold with nonradioactive (+)-3-PPP (and the specific activity adjusted accordingly); in the presence of 50 or 100 μM haloperidol (to block σ1, σ2 and D2 dopamine receptors); in the absence or presence of 100 μM (+)-SKF 10,047 to define nonspecific binding. Assays were incubated at room temperature for 35 min., after which bound (+)-[3H]-3-PPP was separated from free radioligand by rapid filtration and washing of membranes using a harvester (Brandel, Gaithersburg, MD) with GF/B glass fiber filters (Brandel) pretreated with 0.5% polyethylenimine (PEI, Sigma Chemical Co., St. Louis, MO) to reduce nonspecific binding (102). The retained membranes were washed for 25 sec. with approximately 20 ml 5 mM Tris-HCl, pH 8.0 per incubation well. Filters were placed in scintillation vials, and bound radioligand was quantified by liquid scintillation counting. To determine optimal binding conditions, these individual parameters were varied as noted in figure legends. All assays were performed using triplicate wells/tubes for each point.

Unless otherwise specified in figure legends, each microtiter plate well or tube contained homogenate derived from 1 mg wet weight rat spleen, 2x10⁵ human PBL or 5x10⁶ splenocytes. Protein content of the membrane preparations was determined using
a protein assay kit (Sigma Diagnostics, St. Louis, MO) based on the method of Lowry (103). In some experiments (Figs. 2.4 and 2.5) tissue amounts were titrated over a set range. In these studies tissue amounts are expressed as wet weights, not as protein levels.

Preliminary competition experiments (Fig. 2.1) were carried out in 200μl 5mM Tris-HCl buffer, pH 8.0 at room temperature, with 2.5 mg spleen/well, labeled with 5nM (+)-[3H]-3-PPP, undiluted.

**B. Saturation binding.** Membrane preparations were incubated with increasing concentrations (0.2 - 6μM for whole spleen and splenocytes, 0.2 - 2.3 μM for human PBL) of (+)-[3H]-3-PPP plus (+)-3-PPP (1:800 hot:cold for human PBL, 1:400 to 1:500 hot:cold for rat spleen) in the presence of 50μM haloperidol (to block sigma receptors), without or with 100μM (+)-SKF 10,047 to define nonspecific binding. All other conditions were as described above.

**C. Competition binding.** Except as noted for figure 2.1, binding sites were labeled under conditions comparable to those used for saturation binding. Membranes of tissue homogenates were incubated with 1μM (+)-[3H]-3-PPP plus (+)-3-PPP (1:500 hot:cold) in the presence of 50μM haloperidol, and non-radioactive competing drugs were titrated over a concentration range of 10^{-9} to 3x10^{-4} M. SKF-525A was limited by its solubility to a maximum concentration of 3x10^{-5} M. All nonradioactive drugs used in these studies were purchased from Research Biochemicals Inc., Natick, MA.
2.2.5. *Enzyme and heat treatment of splenic homogenates*

Frozen spleens were homogenized in the buffers used for subsequent treatments as follows: 50 mM Tris-HCl, pH 8.0 at 22° C for heat treatment; 50 mM Tris-HCl, pH 7.7 at 22° C for trypsin; 50mM Tris-HCl with Ca^{2+}, pH 7.5 at 22° C for proteinase K; Dulbecco's Phosphate-Buffered Saline (D-PBS; Gibco BRL) with Ca^{2+}, pH 7.3 at 4° C for phospholipase C, pronase and chymopapain; D-PBS with CaCl$_2$, pH 6.9 at 22° C for α-amylase; and D-PBS, pH 5.0 at 22° C for neuraminidase. After homogenization and centrifugation, pellets were resuspended in the appropriate buffers to yield final concentrations equivalent to 13-20 mg original wet organ weight/ml during subsequent enzyme and heat treatments. After filtration through 150 μm nylon mesh (Tetko), they were then subjected to treatments as follows: For heating, membrane suspensions were pipeted into a 15ml glass tube which was placed into a 98° C water bath. Two ml aliquots of splenic homogenates were removed and placed into an ice bath at 0 (no heat), 2.5, 5 and 10 minute intervals. Enzyme treatments were carried out for 60 - 90 min. at 37° C in the buffers listed above, with the exception of α-amylase treatment, which was carried out for one hour at 22° C. Enzymes were titrated over the following ranges: trypsin (Cat. # T8253, Sigma Diagnostics, St. Louis, MO), 100-400 μg/ml; pronase (53702, Calbiochem, San Diego, CA), 10-160μg/ml; proteinase K (LS00422, Worthington Biochemical, Freehold, NJ), 0.1-2.5 mg/ml; chymopapain (C9007, Sigma), 3-60 μg/ml; neuraminidase (LS04780, Worthington Biochemical ), 9-300 μg/ml; α-amylase (LS01013, Worthington Biochemical), 9-300 μg/ml; and phospholipase C (P7633, Sigma), 25-400 μg/ml. Post-treatment homogenates were washed by centrifugation to remove enzymes, suspended in ice-cold 5 mM Tris-HCl buffer (pH 8.0 at room temperature), and maintained on ice until addition to binding assays. Enzyme treatment conditions were per supplier’s instruction or as previously described (104, 105).
2.2.6. Detergent solubilization of splenic homogenates

Spleens were homogenized and centrifuged as outlined in Section 2.2.3. After resuspension in assay buffer, homogenates (at 100 mg original wet weight/ml) were combined with different concentrations of various detergents for membrane solubilization. The following detergents were used at the indicated concentrations: 0.2 - 0.8% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Sigma); 0.2 - 10% Triton X-100 (t-Octylphenoxypolyethoxyethanol; Sigma); 0.4 - 5% Sodium cholate (Cholic acid, sodium salt; Calbiochem); 0.2 - 5% C-TAB (Cetyltrimethylammonium bromide; Calbiochem); 0.2 - 5% α-Lecithin (L-α-phosphatidylcholine; Calbiochem); and 0.01 - 0.4% sodium dodecyl sulfate (SDS; courtesy of Dr. M. Williams, Ohio State). Homogenates were incubated with equal volumes of these agents for 1-2 h. at 4°C with frequent shaking. After incubation the tissue/detergent mixtures were centrifuged at 40,000 x g for 15 min. at 4°C, with the exception of spleen/CHAPS and spleen/Triton X-100 mixtures which were spun in an ultracentrifuge at 100,000 x g for 70 min. at 4°C. After centrifugation the supernatants were assayed for presence of solubilized binding sites in an identical manner to that outlined in Section 2.2.4. As a positive control a portion of the detergent/tissue mixture was removed before centrifugation and assayed for presence of binding sites along with the supernatants. Detergents were used at or below their critical micellization concentrations (CMC) and treatment conditions were per supplier’s instruction or as previously described (76, 106, 107).
2.2.7. Binding in slide-mounted tissue sections (108)

A. Radiolabeling of tissue sections. Slide-mounted frozen sections of rat spleen and brain were brought to room temperature and preincubated for 30 min. in ice-cold 50 mM Tris-HCl buffer, pH 8.0. They were then incubated for 30 min. in room temperature 20 mM Tris-Cl, pH 8.0, containing 2.4 nM (+)-[3H]-3-PPP (S.A., 90.1 Ci/mmol, New England Nuclear), 1 µM nonradioactive (+)-3-PPP (1:400 hot-cold ratio), and 100 µM haloperidol to block sigma receptors. Lower concentrations of Tris-HCl buffer would have been optimal for binding (Fig. 2.2), but were found to cause tissue to swell, lose morphology, and detach from slides. After a quick rinse and two 4 min. washes in ice-cold 20 mM Tris-Cl buffer, pH 8.0, sections were dipped in deionized water and rapidly dried. Adjacent serial sections were incubated in parallel in 2.4 nM (+)-[3H]-3-PPP, 1 µM nonradioactive (+)-3-PPP, 100 µM haloperidol, plus 100 µM (+)-SKF 10,047 to define nonspecific binding. To determine the pharmacology of binding, parallel incubations were also carried out in 2.4 nM (+)-[3H]-3-PPP, 1 µM nonradioactive (+)-3-PPP, 100 µM haloperidol, plus either 10 µM DTG or naltrexone at 0.1, 1, or 10 µM concentration. Two independent experiments were carried out, utilizing tissues from one male and five female animals.

B. Autoradiography. Following desiccation overnight at -20 °C, dried radiolabeled slides were brought to room temperature and apposed to Hyperfilm-3H (Amersham Life Science, Inc., Arlington Hghts., IL). To calibrate radioactivity, slide-mounted [3H]-microscale standards (Amersham) were also apposed to each film. After 12 weeks of exposure, the films were developed. The autoradiograms were then placed in an enlarger and printed. In the figures, areas of drug binding appear as light regions, corresponding to dark silver grains on the original autoradiograms. To provide
histological reference, the slide-mounted tissue sections that produced the "total" binding autoradiograms were stained with hematoxylin and eosin, photographed, and printed under identical magnification as the autoradiograms. Incubation of tissue in Tris-HCl during autoradiographic procedures caused some degradation of tissue morphology and changes in the staining properties of splenic sections, which took up less hematoxylin than sections that had not undergone autoradiographic labeling.

C. Quantification of binding. Original autoradiograms were trans-illuminated and converted to digitized images using a DAGE-MTI CCD72 camera (DAGE-MTI, Inc., Michigan City, MI) and an Everex 486 computer, running Optimas 5.2 image analysis software (Optimas Corp., Bothell, WA). Images were downloaded to storage disks. Radioactive drug binding was then quantified by image analysis using the NIH IMAGE program (version 1.58) for Macintosh computers (Ilsi with math coprocessor and RAM cache, IIci, or Performa 6115CD). Calibration to the \([^{3}H]\)microscale standards was made using either the "Rodbard" or the linear methods of the program (no significant difference in results), and drug binding was read directly from selected areas of the images.

2.2.8. Binding in spleen cell fractions (Figs. 2.17 and 2.18)

A. Histopaque fractionation. Freshly dissected rat spleens were dissociated into single cells by pressing through wire mesh into ice cold Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS (Gibco). Total splenic cells were centrifuged at 300 x g for 10 min. and resuspended in HBSS. Some of these cells were removed and frozen prior to gradient centrifugation. The remaining cells were underlaid with 10ml histopaque and centrifuged at 1000 x g for 15 min. to separate mononuclear cells from red blood cells (RBCs)/dead cells/granulocytes.
Cells at the HBSS/histopaque interface were harvested, washed and counted by trypan blue exclusion. These cells were frozen on dry ice as a pellet and stored at -80° C. Histopaque/HBSS was aspirated off the remaining RBC pellet, which was subsequently washed with HBSS, cells counted and frozen on dry ice. Prior to addition to assay all cell pellets were thawed, disrupted and washed as in Section 2.2.3. Membranes were added into binding assays at 5x10^6 cell equivalents per well.

**B. NH₄Cl-mediated lysis.** Rat spleens were dissected and dissociated into single cells as above. Total splenic cells were centrifuged at 300 x g for 10 min. and resuspended in HBSS. Some of these cells were removed and frozen prior to NH₄Cl-mediated lysis. Remaining cells were centrifuged at 300 x g for 10 min. and resuspended in 20ml ACK lysis buffer (0.15M NH₄Cl, 1M KHCO₃, 0.1mM Na₂EDTA, pH 7.4). Cells were incubated in ACK buffer for 5 min. at room temperature with occasional shaking. Cells were brought up to 40ml with HBSS and centrifuged at 300 x g for 10 min. Supernatants (which should contain RBC membranes) were decanted into 50ml centrifuge tubes and centrifuged at high speed (40,000 x g). Membrane pellets were frozen and stored at -80° C. This represents the membranes of the NH₄Cl-sensitive cells. The NH₄Cl-resistant cells (virtually 100% white blood cells (WBCs) were brought up in HBSS, washed, frozen on dry ice and stored at -80° C. All cell pellets were thawed, disrupted and washed as in Section 2.2.3. Prior to addition to binding assays, protein determinations were performed on all cell membrane homogenates to equalize each homogenate to 500µg/ml protein. Membranes were added into binding assays at 25µg protein per well.
2.2.9. Data analysis and statistics

Equilibrium dissociation constants (K_d), number of binding sites (B_max) and inhibition constants (K_i) from the saturation and competition binding experiments were determined using the nonlinear iterative curve fitting program (MacLigand) of Munson and Rodbard (109). K_i values were verified by calculation using the Cheng-Prusoff equation (110). The K_d value of (+)-[^3H]-3-PPP used for calculating the K_i's of drugs in rat spleen was 1.2μM. Correlation and Hill coefficients of Rosenthal plots were determined by the simple curve fitting program Ebd (release 2.0; Biosoft, Milltown, NJ).

Unpaired T tests were used to compare K_i values between different compounds. The results of these tests were used to construct the pharmacological profile listed in Section 23.7. This analysis was carried out using the statistical program StatView (Abacus Concepts, Inc., Berkeley, CA).

2.3. Results

2.3.1. Haloperidol-accessible (sigma) and haloperidol-inaccessible (non-sigma) binding sites for (+)-[^3H]-3-PPP are present in rat spleen.

We have previously labeled high concentrations of sigma receptors in rat spleen and human PBL using [^3H]haloperidol, and demonstrated that (+)-3-PPP competes at these sites (11, 12, 111). However, haloperidol was not a good competitor at the majority of (+)-[^3H]-3-PPP binding sites in rat splenic homogenates (Fig. 2.1). This suggested the
presence of high concentrations of additional, haloperidol-insensitive (non-sigma) binding sites for (+)-[^3]H]-3-PPP in the spleen. The prototypic sigma ligand, (+)-SKF 10,047 (13), was able to compete effectively at these splenic (+)-[^3]H]-3-PPP binding sites (Fig. 2.1). Therefore, in subsequent homogenate binding studies we labeled the haloperidol-inaccessible sites with (+)-[^3]H]-3-PPP in the presence of 50μM haloperidol, and defined nonspecific binding by the absence or presence of 100μM (+)-SKF 10,047.

2.3.2. Haloperidol-inaccessible sites bind (+)-[^3]H]-3-PPP most avidly in higher pH and lower ionic strength buffer.

When binding was carried out in Tris-HCl buffer varying from 5 to 100 mM, maximal binding occurred at lower Tris concentrations (5 - 15mM), and binding decreased as Tris-HCl increased over the range of 15 - 100mM (Fig. 2.2). Saturation binding revealed that this was due to large changes in affinity of binding (Kd), plus small variations in the number of accessible binding sites (Bmax: Fig. 2.2B). This is suggestive of competitive inhibition of binding by the Tris-HCl buffer, an effect previously documented in σ receptor binding assays (112).

When binding was carried out in buffers with differing pH, specific binding increased with rising pH (Fig. 2.3). A supraoptimal pH was not found, as these experiments were limited by the reduced solubility of the drugs at high pH. The increase in binding was due to increased binding affinity (decrease in the Kd) with increasing pH, as seen by comparing saturation binding experiments performed at pH 9.0 (Fig. 2.3B) and pH 8.0 (Fig. 2.3C). In these experiments, and the Tris-HCl experiments, a single class of binding sites was observed (Figs. 2.2B, 2.3B and 2.3C).
Figure 2.1. Haloperidol does not compete with high affinity at the majority of (+)-[^3H]-3-PPP binding sites in rat spleen, although it has been previously shown that (+)-3-PPP can compete effectively at abundant[^3H]haloperidol labeled sigma receptors in that tissue (12, 87). In contrast, (+)-SKF 10,047 does compete at these sites. This indicates the presence of haloperidol-inaccessible (+)-[^3H]-3-PPP binding sites. Binding of 5nM (+)-[^3H]-3-PPP (undiluted) was carried out as described in Section 2.2.4, and nonspecific binding was defined by a mixture of 10μM haloperidol plus 100μM (+)-SKF 10,047. Points represent means ± SEM values of triplicate determinations. Figure is representative of two independent experiments.
Figure 2.1. Haloperidol does not compete with high affinity at the majority of (+)-[^3H]-3-PPP binding sites in rat spleen.
Figure 2.2. Effect of Tris-HCl concentration on (+)-[3H]-3-PPP binding to haloperidol-inaccessible sites in rat spleen. (A): Specific binding of 1μM (+)-[3H]-3-PPP (1:400 to 1:500 ratio of radioactive:nonradioactive (+)-3-PPP), in the presence of 100μM haloperidol, in 5 - 100mM Tris-HCl, pH 8.0. (B): Rosenthal plots of saturation binding carried out at 5 (■), 25 (▲) and 100 (●) mM Tris-HCl. Saturation binding was carried out with 5mg spleen homogenate per well as described in Section 2.2.4. Points represent the mean ± SEM of three replicates. (A) is representative of three experiments, and (B) is representative of two.
Figure 2.2. Effect of Tris-HCl concentration on (+)-[^3]H]-3-PPP binding to haloperidol-inaccessible sites in rat spleen.
Figure 2.3. Effect of pH on (+)-[^3H]-3-PPP binding to haloperidol-inaccessible sites in rat spleen. (A): Specific binding of 1μM (+)-[^3H]-3-PPP ((+)-[^3H]-3-PPP, diluted 1:400 to 1:500 with nonradioactive (+)-3-PPP, and the specific activity adjusted accordingly), in the presence of 100μM haloperidol, over the pH range 7.1 - 8.3. (B) and (C): Rosenthal plots of saturation binding carried out at pH 9.0 and 8.0, respectively. Note the difference in scales of ordinate axes in (B) and (C).

Binding was carried out as described in Section 2.2.4, except 5 mg spleen homogenate was used per well, and 50mM Tris-HCl was used rather than 5mM Tris-HCl in order to provide more buffering capacity. Points represent means ± SEM values of three replicate measurements. (A) is representative of three experiments, and (B) and (C) were repeated twice.
Figure 2.3. Effect of pH on (+)-[^3H]-3-PPP binding to haloperidol-inaccessible sites in rat spleen.
Based on these findings, binding assay buffer was standardized to 5mM Tris-HCl, pH 8.0 for subsequent experiments. Although pH 9.0 would have given more avid binding, we elected to work at a lower pH because many of the compounds that we wished to use in competition binding experiments were insoluble at high pH.

2.3.3. *Haloperidol-inaccessible binding is not proportional to tissue amount in rat spleen and human PBL.*

When binding assays were performed using various amounts of spleen homogenate per well (Fig. 2.4), we found that haloperidol-inaccessible (+)-[^3H]-3-PPP binding was not proportional to the amount of tissue present. The specific binding of (+)-[^3H]-3-PPP was roughly linear up to 1-2 mg of rat spleen (Fig. 2.4). At higher tissue concentrations the binding per unit tissue decreased as the amount of tissue in the binding reaction increased, resulting in a curvilinear plot (Fig. 2.4). This was not due to depletion of (+)-[^3H]-3-PPP from the incubation mixture, as less than 10% of the radioligand was bound to the tissue even at the highest tissue amounts. The reason for this phenomenon was not determined. However, because of these findings tissue was limited to 1mg rat spleen per well in subsequent experiments.

This phenomenon was also seen when similar experiments were performed with human PBL. Specific haloperidol-inaccessible (+)-[^3H]-3-PPP binding was present in human PBL with linear binding seen up to 1 - 2x10^5 cells per well (Fig. 2.5). As with spleen, higher PBL numbers resulted in decreased amount of binding per cell. In contrast, binding in membranes derived from isolated rat spleen cells showed linear specific (+)-[^3H]-3-PPP binding up to 1x10^7 cells, the highest number tested (Fig. 2.5).
Figure 2.4. (+)-[^3H]-3-PPP binding to haloperidol-inaccessible sites in spleen is not proportional to the amount of tissue in the assay. Varying amounts of spleen membranes (equivalent to 0.2 - 5mg original wet weight per well) were incubated with (+)-[^3H]-3-PPP (diluted with nonradioactive (+)-3-PPP 1:400 - 1:500) as outlined in Section 2.2.4. Specific (+)-[^3H]-3-PPP binding is shown. Points represent the mean ± SEM of three replicates in the presence of 50μM haloperidol. Figure shown is representative of four independent experiments.
Figure 2.4. (+)-[^3]H]-3-PPP binding to haloperidol-inaccessible sites in spleen is not proportional to the amounts of tissue in the assays.
Figure 2.5. (+)-[^3H]-3-PPP binding in human peripheral blood leukocytes (PBL) (▲) is not proportional to increasing cell numbers, however binding in rat spleen cells (○) is proportional to cell number. Varying amounts of human PBL (1x10^5 - 1x10^6 cell equivalents per well) and rat spleen cells (1x10^4 - 1x10^7 cell equivalents per well) were incubated with (+)-[^3H]-3-PPP (diluted with nonradioactive (+)-3-PPP 1:300) in the presence of 50μM haloperidol as outlined in Section 2.2.4. Specific (+)-[^3H]-3-PPP binding is shown. Points represent the mean ± SEM of three replicates. Rat spleen cell curve is linear up to 1x10^7 cells. Figure shown is representative of three human PBL and four rat spleen cell experiments.
Figure 2.5. (+)-[^3]H]-3-PPP binding in human PBL is not proportional to cell number, however binding in isolated rat spleen cells is proportional to cell number.
2.3.4. Association and dissociation, a fast and a slow binding reaction, but no dissociation.

Association and dissociation of (+)-[3H]-3-PPP were carried out with rat splenic membranes at 4°, 22° and 37° C, as described in Section 2.2.4. At 22° C, an apparent steady state was reached within 15 - 20 minutes (Fig. 2.6A). At 37° C there were two components to the association reaction: a fast process reaching apparent steady-state at 15 - 20 minutes, and a subsequent very slow component that continued to increase after the initial plateau was reached (Fig. 2.6A). At 22° C, the slow binding component was not significant for the first 70 - 80 minutes of incubation (Fig. 2.6A), but could be observed with longer incubations (Fig. 2.6B, discussed below). At 4° C, the time to apparent steady-state of the fast binding event was delayed to 40 - 50 minutes. At 4° C the slow component was not observed for any incubation period (Fig. 2.6A, and data not shown), suggesting that the slow component may be due to an enzymatic or metabolic process.

The plateau after 15-20 minutes of association at 22° C (Fig. 2.6A) occurred at a low percent binding site occupancy, with less than 10% depletion of free radioligand from solution. The amount of binding at plateau was proportional to the concentration of free radioligand (data not shown).

For dissociation experiments, splenic membranes were allowed to incubate for 35 min. at 22° C with (+)-[3H]-3-PPP plus 50μM haloperidol, at which point 100μM (+)-SKF 10,047 was added to start the dissociation reaction. Although (+)-SKF 10,047 prevented the binding of (+)-[3H]-3-PPP if it was presented to the tissue simultaneously with the radioligand (this was how nonspecific binding was defined), (+)-SKF 10,047 failed to cause dissociation of (+)-[3H]-3-PPP once binding had occurred (Fig. 2.6B).
Figure 2.6. Kinetics of (+)-[³H]-3-PPP binding in rat spleen. (A) Association experiments. Washed membranes of rat spleen (1mg) were incubated with 1μM (+)-[³H]-3-PPP (1:400 to 1:500 hot:cold) in the presence of 50μM haloperidol for 0 - 80 min. at 4°, 22° and 37° C. (B) Dissociation. Tissue was incubated at 22° C with (+)-[³H]-3-PPP for 35 min. under identical conditions as above, after which 100μM (+)-SKF 10,047 was added to begin dissociation. Bound dpm differs in (A) and (B) due to differences in the specific activity of (+)-[³H]-3-PPP and tissue protein recovery in the two experiments. In both (A) and (B), nonspecific binding was determined by parallel incubations with (+)-[³H]-3-PPP and haloperidol plus 100μM (+)-SKF 10,047. Points represent the means ± SEM of three replicates. Figures are representative of four association and three dissociation experiments.
Figure 2.6. Kinetics of (+)-[^3H]-3-PPP binding in rat spleen. (A) Association. (B) Dissociation.
Moreover, there was a steady and consistent increase in specific binding over time despite the presence of 100μM (+)-SKF 10,047. The rate of this increase (Fig. 2.6B) was suggestive of the slow association process observed at 37° C (Fig. 2.6A).

The balance of this report is a description of the fast binding component, which reached apparent steady-state within 15-20 min. at 22° C. This was empirically defined by limiting all incubations to 35 minutes at room temperature.

2.3.5. *High numbers of (+)-[^3H]-3-PPP labelable, haloperidol-inaccessible binding sites are present in rat spleen and human PBL, but not in rat brain.*

Saturation binding experiments were conducted as described in Section 2.2.4. Over the concentration range of 0.2 - 6μM (+)-[^3H]-3-PPP (mixture of hot plus cold), haloperidol-inaccessible binding in rat spleen was concentration-dependent, saturable, and displayed a one-site Rosenthal plot (Fig. 2.7), with a K_d of 1.18 ± 0.08μM and an extremely high B_max of 533 ± 81 pmol/mg protein (Fig. 2.7 and Table 2.1). Haloperidol-inaccessible binding sites could not be detected in rat brain (Fig. 2.7), but were present in even greater abundance in human PBL (Fig. 2.8, Table 2.1). A comparable K_d was obtained for PBL (0.78μM), but the B_max was much larger, at 3190 ± 855 pmol/mg protein, corresponding to 4.48x10^6 ± 0.88x10^6 sites/cell.

To confirm that splenic binding sites were present on immune cells, binding assays were performed on membranes of isolated rat spleen cells. These cell populations consisted almost entirely of mononuclear cells. The affinity of binding in splenocytes was somewhat lower, but comparable to that of whole spleen homogenates (Table 2.1). A Hill coefficient of 0.98 was obtained for isolated splenocytes, consistent with the presence of a single site, and a single site fit was obtained by the iterative curve-fitting.
Figure 2.7. Saturable, (+)-[^3H]-3-PPP labeled haloperidol-inaccessible sites are present in rat spleen, but not brain. A representative sample of four saturation experiments is shown, performed as described in Section 2.2.4. Each point represents the mean ± SEM of three replicates at each ligand concentration. Inset gives the Rosenthal plot. No reasonable fit could be discerned for brain, however the data fit a single site model ($r = 0.99$) in spleen. (+)-[^3H]-3-PPP concentrations were obtained by diluting (+)-[^3H]-3-PPP 400- to 500-fold with nonradioactive (+)-3-PPP, and adjusting the specific activity accordingly.
Figure 2.7. Saturable, (+)-[^3H]-3-PPP labeled haloperidol-inaccessible sites are present in rat spleen, but not brain.
Figure 2.8. Saturable, (+)-[^3H]-3-PPP labeled haloperidol-inaccessible sites are present on human PBL. Cells (2x10^5) were incubated with increasing concentrations of (+)-[^3H]-3-PPP under conditions comparable to Fig. 2.7. Points represent the means ± SEM of three replicates at each ligand concentration. Inset gives the Rosenthal plot, indicating the fit of the data (r = 0.98) to a single site model. Figure is representative of five experiments. (+)-[^3H]-3-PPP was diluted 800-fold with nonradioactive (+)-3-PPP in order to achieve the ligand concentrations indicated.
Figure 2.8. Saturable, (+)-[^3]H]-3-PPP labeled haloperidol-inaccessible sites are present on human PBL.
Table 2.1. Haloperidol-inaccessible (+)-[3H]-3-PPP binding in rat spleen, splenocytes, brain and human PBL

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$K_d$ (µM)</th>
<th>$B_{max}$ (pmol/mg protein)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat spleen</td>
<td>1.18 ± 0.08</td>
<td>533 ± 81</td>
<td>4</td>
</tr>
<tr>
<td>Rat splenocytes</td>
<td>3.14 ± 0.60</td>
<td>458 ± 47</td>
<td>3</td>
</tr>
<tr>
<td>Human PBL</td>
<td>0.78 ± 0.14</td>
<td>3190 ± 855</td>
<td>5</td>
</tr>
<tr>
<td>Rat brain</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>4</td>
</tr>
</tbody>
</table>

$^a$ ND = specific binding was not detectable. Membrane homogenates were prepared and saturation binding carried out as described in Section 2.2. Individual experiments were analyzed using the iterative computer curve fitting program LIGAND (109). Values ($K_d$ and $B_{max}$) represent the means ± SEM of the indicated number of experiments.
program MacLIGAND. The $B_{\text{max}}$ of isolated splenocytes was comparable to that of whole spleen ($458 \pm 47$ pmol/mg protein or $7.34 \times 10^5 \pm 2.0 \times 10^5$ sites/cell; Table 2.1). The lack of enrichment of binding sites in the splenocyte population, relative to unfractionated spleen, suggests that other cells or structural elements, not recovered in the mononuclear fraction, also had binding activity.

2.3.6. Pharmacology of (+)-[^H]-3-PPP sites.

Competition binding assays were carried out as described in Section 2.2.4 using rat spleen membranes. A variety of competing compounds were tested, including drugs with high affinities for sigma receptors, compounds that bind dopamine, opioid and PCP receptors, and compounds that bind enzymes (monoamine oxidase and cytochrome P-450) reported to bind several sigma ligands (Table 2.2).

A. Stereoselectivity. Stereoselectivity of the sites was probed with pairs of optical enantiomers of SKF 10,047, pentazocine and 3-PPP. Both stereoisomers of pentazocine and SKF 10,047 competed effectively for (+)-[^H]-3-PPP labeled sites (Fig. 2.9). The (-)-stereoisomers were slightly more effective than the (+)-isomers ($P < 0.05$). This is contrary to the selectivity of the haloperidol-sensitive $\sigma_1$ site in rat spleen (12, 87, 111). Similarly, the (-)-stereoisomer of 3-PPP was in all experiments more potent than (+)-3-PPP (Table 2.2), although this difference was not statistically significant ($P = 0.22$).
B. Sites bind all sigma ligands except haloperidol and DTG. Sigma ligands fell into two closely aggregated groups. (+)- and (-)-SKF 10,047, (+)- and (-)-pentazocine, (+)- and (-)-3-PPP, dextrorphan, dextromethorphan and PCP all competed at the haloperidol-inaccessible sites (Fig. 2.10, Table 2.2). As expected, the classical sigma ligand haloperidol was ineffective (Fig. 2.10, Table 2.2). Of particular interest was the finding that DTG was also ineffective at this site (Fig. 2.10, Table 2.2). DTG is a potent σ ligand and (in the presence of blocking drug for σ1 receptors) is an acceptable ligand for labeling σ2 receptors (34).

C. Sites do not bind monoamine oxidase (MAO) or cytochrome P-450 ligands, but do bind the opiate antagonist naltrexone. The MAO inhibitors clorgyline (MAO A) and (-)-deprenyl (MAO B) showed little affinity for the binding site (Table 2.2). Proadifen (SKF 525-A), an inhibitor of microsomal drug metabolism and a ligand for cytochrome P-450 enzymes, was also ineffective. However, the most startling result was the relatively high affinity with which the opioid antagonist naltrexone competed for the haloperidol-inaccessible site (Fig. 2.10, Table 2.2).

D. Pharmacological profile. The overall rank order of potency in these competition binding assays was: (-)-SKF 10,047 ≥ naltrexone = (-)-pentazocine > (+)-pentazocine = (-)-3-PPP = (+)-SKF 10,047 ≥ (+)-3-PPP ≥ dextrorphan > dextromethorphan > PCP > clorgyline. The ligands with little or no affinity at this site, in the concentration range tested, were DTG, haloperidol, TCP, (-)-deprenyl and SKF 525-A.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Kᵢ (µM)ᵃ</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (-)-SKF 10,047</td>
<td>0.387 ± 0.060</td>
<td>3</td>
</tr>
<tr>
<td>2. Naltrexone</td>
<td>0.484 ± 0.070</td>
<td>3</td>
</tr>
<tr>
<td>3. (-)-Pentazocine</td>
<td>0.485 ± 0.014</td>
<td>3</td>
</tr>
<tr>
<td>4. (+)-Pentazocine</td>
<td>0.753 ± 0.065</td>
<td>3</td>
</tr>
<tr>
<td>5. (-)-3-PPP</td>
<td>0.783 ± 0.158</td>
<td>3</td>
</tr>
<tr>
<td>6. (+)-SKF 10,047</td>
<td>0.880 ± 0.128</td>
<td>3</td>
</tr>
<tr>
<td>7. (+)-3-PPP</td>
<td>1.022 ± 0.038</td>
<td>3</td>
</tr>
<tr>
<td>8. Dextromorphan</td>
<td>2.202 ± 0.433</td>
<td>3</td>
</tr>
<tr>
<td>9. Dextromethorphan</td>
<td>4.517 ± 0.563</td>
<td>3</td>
</tr>
<tr>
<td>10. PCP</td>
<td>33.131 ± 7.892</td>
<td>4</td>
</tr>
<tr>
<td>11. Clorgyline</td>
<td>79.461 ± 9.862</td>
<td>3</td>
</tr>
<tr>
<td>12. SKF-525A</td>
<td>&gt;15.000</td>
<td>3</td>
</tr>
<tr>
<td>13. TCP</td>
<td>&gt;150.000</td>
<td>3</td>
</tr>
<tr>
<td>14. DTG</td>
<td>&gt;150.000</td>
<td>3</td>
</tr>
<tr>
<td>15. Haloperidolᵇ</td>
<td>&gt;150.000</td>
<td>3</td>
</tr>
<tr>
<td>16. (-)-Deprenyl</td>
<td>&gt;150.000</td>
<td>3</td>
</tr>
</tbody>
</table>

⁻ Kᵢ values of drugs in competition against 1µM (+)-[^3]H]-3-PPP in the presence of 50µM haloperidol. Membrane homogenates were prepared and competition binding carried out as described in Section 2.2. Individual experiments were analyzed using the iterative computer curve fitting program LIGAND (109). Values represent the means ± SEM of the indicated number of experiments (n). Drugs are ranked from highest to lowest affinity. ᵇ Haloperidol competition experiments were carried out in the presence of 50µM DTG in lieu of haloperidol to block σ₁ and σ₂ receptors.

Table 2.2. Pharmacology of (+)-[^3]H]-3-PPP labeled haloperidol/DTG-inaccessible sites in rat spleen
Figure 2.9. Haloperidol-inaccessible (+)\(^{3}\text{H}\)-3-PPP binding sites in rat spleen exhibit slight selectivity for (-) over the (+) stereoisomers of the benzomorphans, pentazocine and SKF 10,047. Sites were labeled with 1\(\mu\text{M}\) (+)\(^{3}\text{H}\)-3-PPP (1:500 hot:cold) in the presence of 50\(\mu\text{M}\) haloperidol, in the absence or presence of 100\(\mu\text{M}\) (+)-SKF 10,047 to define nonspecific binding.
Figure 2.9. Haloperidol-inaccessible (+)-[^3]H]-3-PPP binding sites in rat spleen exhibit slight stereoselectivity for (-)- over the (+)-stereoenantiomers of pentazocine and SKF 10,047.
**Figure 2.10.** Pharmacology of (+)-[^3H]-3-PPP labeled haloperidol/DTG-inaccessible sites in rat spleen. Sites were labeled as in Fig. 2.9, with the exception of haloperidol titrations, which were carried out in the presence of 50μM DTG in lieu of a fixed amount of haloperidol. With the exception of PCP (n = 4), all competition experiments were replicated three times. Representative titrations are shown. No IC\textsubscript{50} could be determined for haloperidol, DTG, or TCP in the concentration range tested.
Figure 2.10. Pharmacology of (+)-$[^3]$H-3-PPP labeled haloperidol/DTG-inaccessible sites in rat spleen.
2.3.7. **Susceptibility of haloperidol-inaccessible (+)-[^H]-3-PPP binding sites to heat and enzyme treatment.**

Exposing tissue homogenates to near boiling temperature for 2.5 min. destroyed their ability to specifically bind (+)-[^H]-3-PPP (Fig. 2.11), suggesting that the binding entity might be a heat-denaturable protein. However, treatment of homogenates with the proteases trypsin, pronase, chymopapain and proteinase K all failed to affect binding activity (Table 2.3). Likewise, the glycosidases neuraminidase and α-amylase also failed to alter binding (Table 2.3). The only enzymatic treatment that did affect binding was phospholipase C (PLC). Treatment of splenic membranes for one hour with 25 - 100 μg/ml PLC caused significant reduction of binding in the membranes, and PLC in concentrations of 200 μg/ml or greater completely destroyed specific binding (Fig. 2.12, Table 2.3).

2.3.8. **Solubilization and isolation of (+)-[^H]-3-PPP binding sites, - no effect with a variety of detergent treatments.**

Treatment of splenic homogenates with nonionic, ionic and zwitterionic detergents failed to solubilize the haloperidol/DTG-inaccessible sites (Table 2.4). Membranes were treated with detergents, centrifuged and supernatants assayed for (+)-[^H]-3-PPP binding as outlined in Section 2.2.6. (+)-[^H]-3-PPP binding activity was not detected in any of the solubilized preparations with the exception of SDS, which solubilized a small amount of binding activity at low concentrations. However, at higher concentrations (above 0.05%) (+)-[^H]-3-PPP binding activity was destroyed in both solubilized and
Figure 2.11. Heating destroyed binding activity of haloperidol-inaccessible sites. Rat spleen membrane suspensions in 50 mM Tris-HCl buffer (pH 8.0 at 4°C) were heated to 98°C, and aliquots were removed at 0 (no heat), 2.5, 5 and 10 min. intervals and placed on ice until assayed. Specific (■) and nonspecific (○) (+)-[3H]-3-PPP binding was determined as described in Section 2.2.4. Each point represents the mean ± SEM of three replicate measurements. The figure is a representative sample of three replicate experiments.
Figure 2.11. Heating destroyed binding activity of haloperidol-inaccessible sites.
Treatment of rat splenic homogenates and binding assays were carried out as described in Sections 2.2.4 and 2.2.5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time/Conc.</th>
<th>Effect</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>heating (98°C)</td>
<td>2.5 - 10 min.</td>
<td>destroyed binding</td>
<td>3</td>
</tr>
<tr>
<td>trypsin</td>
<td>100 - 400 μg/ml</td>
<td>none</td>
<td>3</td>
</tr>
<tr>
<td>pronase</td>
<td>10 - 160 μg/ml</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>chymopapain</td>
<td>3 - 60 μg/ml</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>proteinase K</td>
<td>0.1 - 2.5 mg/ml</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>neuraminidase</td>
<td>9 - 300 μg/ml</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>α-amylase</td>
<td>9 - 300 μg/ml</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>phospholipase C</td>
<td>25 - 400 μg/ml</td>
<td>destroyed binding</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50 - 200 μg/ml)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Susceptibility of haloperidol-inaccessible splenic (+)-[3H]-3-PPP binding sites to heat and enzymes
Figure 2.12. Phospholipase C treatment destroyed binding activity of haloperidol-inaccessible sites. Various concentrations of phospholipase C were incubated with rat spleen membrane suspensions at 37°C for 1 h. in Ca^{2+}-containing PBS buffer (pH 7.3 at 4°C). Specific (■) and nonspecific (○) (+)-[^3H]-3-PPP binding was determined as described in Section 2.2.4. Each point represents the mean ± SEM of three replicates. The figure is a representative sample of four replicate experiments.
Figure 2.12. Phospholipase C treatment destroyed binding activity of haloperidol-inaccessible sites.
<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt; (n)</th>
<th>Conc. (%)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS (3)</td>
<td>0.2 - 0.8</td>
<td>none</td>
</tr>
<tr>
<td>Triton X-100 (2)</td>
<td>0.2 - 10</td>
<td>none</td>
</tr>
<tr>
<td>α-Lecithin (1)</td>
<td>0.2 - 5</td>
<td>none</td>
</tr>
<tr>
<td>Sodium cholate (1)</td>
<td>0.4 - 5</td>
<td>none</td>
</tr>
<tr>
<td>C-TAB (1)</td>
<td>0.2 - 5</td>
<td>none</td>
</tr>
<tr>
<td>SDS (3)</td>
<td>0.01 - 0.4</td>
<td>0.02-0.05% solubilized binding &gt;0.05% destroyed binding</td>
</tr>
</tbody>
</table>

<sup>a</sup> Treatment of rat splenic homogenates and binding assays were carried out as described in Sections 2.2.4 and 2.2.6.

Table 2.4. Ability of detergents to solubilize haloperidol-inaccessible splenic (+)-[3H]-3-PPP binding sites
unsolubilized splenic membranes. This exquisite sensitivity to SDS, below concentrations that gave effective solubilization, rendered SDS unacceptable as a tool for solubilization and isolation of the binding sites.

2.3.9. Haloperidol/DTG-inaccessible binding sites are undetectable in a variety of immune cell lines.

Membrane homogenates, prepared from a number of different immune cell lines, were assayed for the presence of (+)-[3H]-3-PPP haloperidol/DTG-inaccessible sites. Cell lines tested included a B cell line (A20), two T cell lines (EL4 and LBRM), a mast cell-like line (RBL) and two monocyte/macrophage cell lines (Ana-1 and RAW 264). While large amounts of specific binding were evident in the positive controls, rat and mouse spleen, little or no specific binding was seen in any cell line tested (Fig. 2.13).

2.3.10. Autoradiographic localization of splenic binding sites.

In vitro autoradiography was used to determine the anatomical localization of haloperidol/DTG-inaccessible sites in spleen. Slide-mounted frozen sections of rat spleen were incubated with 1μM (+)-[3H]-3-PPP in the presence of 100μM Haloperidol to block both σ₁ and σ₂ receptors as described in Section 2.2.7. As may be seen in figure 2.14, haloperidol-inaccessible binding sites are localized in a scattered coarse, punctate pattern in the red pulp and the marginal zones of the white pulp. They were clustered most closely in ring-like patterns in the marginal zones surrounding white pulp follicles. These sites could be blocked by (+)-SKF 10,047 (Fig. 2.15), demonstrating a pharmacology consistent with that of the binding site labeled in homogenate binding studies (Fig. 2.9).
Figure 2.13. Haloperidol-inaccessible binding sites are not present on various immune cells. Type and species of cells are as follows: A20, mouse B cell lymphoma; EL4 and LBRM33, mouse T cell lymphoma; RBL, rat basophilic leukemia; Ana-1 and RAW 264, mouse monocyte/macrophage. Binding assays were carried out on cell membranes as outlined in Section 2.2.4. Membranes were incubated with 1μM (+)-[3H]-3-PPP (1:200 hot:cold) in the presence of 50μM haloperidol, in the absence or presence of 100μM (+)-SKF 10,047 to define nonspecific binding. Bars represent means ± SEM values of triplicate determinations. Figure is representative of 2 - 3 experiments performed on each cell line.
Figure 2.13. Haloperidol-inaccessible binding sites are not present on various immune cell lines.
Figure 2.14. Haloperidol-inaccessible binding sites for (+)-[^H]-3-PPP are localized in a punctate pattern in the marginal zones and red pulp of rat spleen. (A): Hematoxylin and eosin stained, slide-mounted frozen section of rat spleen. Labeled areas are: B, B cell region of white pulp; T, T cell region of white pulp, containing the ca, central arteriole; mz, marginal zone; rp, red pulp. (B): Photographic print of autoradiogram generated by the section shown in (A). (+)-[^H]-3-PPP binding sites, labeled in the presence of 100μM haloperidol, are indicated by the white areas, corresponding to black silver grains in the original autoradiogram. See Section 2.2.7 for details of procedures. Sections shown are representative of one male and five female animals, studied in two independent experiments. Bar is equivalent to 1 mm.
Figure 2.14. Haloperidol-inaccessible binding sites for (+)-[^3H]-3-PPP are localized in a punctate pattern in the marginal zones and red pulp of rat spleen.
Figure 2.15. (+)-SKF 10,047 and naltrexone, but not DTG, compete at haloperidol-inaccessible sites in rat spleen sections. When specific binding sites were blocked with 100 μM (+)-SKF 10,047 (B), nonspecific binding was uniform over the sections. Naltrexone displaced (+)-[^3H]-3-PPP binding in a dose dependent manner (D, E and F). Radiolabeling and autoradiography were carried out as outlined in Section 2.2.7. Figure is representative of one male and five female animals, studied in two independent experiments. Bar is equivalent to 1mm.
Figure 2.15. (+)-SKF 10,047 and naltrexone, but not DTG, compete at haloperidol-inaccessible sites in rat spleen sections.
(+)-[^3H]-3-PPP binding was highly specific in marginal zone and red pulp areas (56.9 and 49.1% specific, respectively); however, little specific binding was seen in the B and T cell areas of the spleen (Table 2.5, Fig. 2.16). Because the values for total and specific binding in the marginal zones and red pulp are averages over large areas, and the binding is widely scattered, they under-represent the local concentrations of binding sites in the punctate spots. Likewise, they do not adequately represent the high specificity of the label. Since nonspecific binding was low and uniform in distribution (Fig. 2.15), while the "hot spots" were bright and highly localized (Figs. 2.14), we used the NIH IMAGE image analysis program to measure the concentration of binding sites only in those spots of the autoradiograms where binding exceeded nonspecific background levels. Binding site concentrations in these "hot spots" were 20 - 30 times higher than the number of sites seen in the T and B cell areas, and were highly specific, with 80% of the total binding representing specific binding (Table 2.5). This concentration of binding sites in punctate spots suggests that they were present in high levels on a few, scattered cells or cell clusters in the marginal zones and red pulp.

Rat brain control sections were also used in autoradiography. There were none of these sites in any area of the brain (Table 2.5). This is consistent with our findings in homogenate binding studies (Fig. 2.7).

Consistent with the pharmacology of these sites that we determined using homogenate binding assays, 10μM DTG caused only minimal blockade of binding (Figs. 2.15 and 2.16). This minimal blockade was somewhat greater than what would have been predicted given the inability of DTG to compete in homogenate binding assays (Table 2.2). Naltrexone was quite potent, and competed for binding in a dose-dependent
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Binding (fmol/mg tissue)</th>
<th>% Specific Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Nonspecific</td>
</tr>
<tr>
<td>cerebellum</td>
<td>1450 ± 110</td>
<td>1480 ± 171</td>
</tr>
<tr>
<td>cerebral cortex</td>
<td>1410 ± 260</td>
<td>1420 ± 360</td>
</tr>
<tr>
<td>hippocampus</td>
<td>1540 ± 190</td>
<td>1410 ± 190</td>
</tr>
<tr>
<td>spleen B area</td>
<td>1270 ± 190</td>
<td>1050 ± 190</td>
</tr>
<tr>
<td>spleen T area</td>
<td>1400 ± 160</td>
<td>1090 ± 210</td>
</tr>
<tr>
<td>marginal zone</td>
<td>3620 ± 430</td>
<td>1560 ± 120</td>
</tr>
<tr>
<td>red pulp</td>
<td>2850 ± 210</td>
<td>1460 ± 60</td>
</tr>
<tr>
<td>&quot;hot spots&quot;</td>
<td>7710 ± 2650</td>
<td>1510 ± 140</td>
</tr>
</tbody>
</table>

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Table 2.5. Haloperidol/DTG-inaccessible binding sites are present in 'hot spots' in the marginal zone and red pulp of rat spleen.

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*a* Values represent means ± SEM.  
*b* Specific binding was calculated for pairs of adjacent sections labeled under "total" and "nonspecific" binding conditions. Sections from four female animals were used, and three measurements were made from each anatomical region of each section. Sections were incubated in 1 μM (+)-[^H]-3-PPP in the presence of 100 μM haloperidol (in the absence and presence of 100 μM (+)-SKF 10,047 to define nonspecific binding).
Figure 2.16. Autoradiogram quantification - specific (+)-[3H]-3-PPP binding is present mainly in the marginal zone and red pulp regions of rat spleen. The pharmacology of (+)-[3H]-3-PPP binding is consistent with that of haloperidol/DTG-inaccessible sites. (+)-[3H]-3-PPP binding sites were (+)-SKF 10,047-sensitive, and naltrexone competes in a dose-dependent manner. Little specific binding is present in T and B cell areas, and naltrexone does not compete effectively in these regions. The data are from area measurements, and under-represent the highly specific binding in scattered, punctate “hot spots” (see table 2.5). Binding was measured as outlined in Section 2.2.7 and is represented as a percentage of total binding. Data are from four representative spleens, in which each region was measured in triplicate.
Figure 2.16. Autoradiogram quantification - specific (+)-[3H]-3-PPP binding is present mainly in the marginal zone and red pulp regions. The pharmacology of binding corresponds to that of haloperidol/DTG-inaccessible sites.
manner (Figs. 2.15 and 2.16). Nonspecific binding, defined with 100µM (+)-SKF 10,047 was not only low, but uniform, without anatomical localization over the spleen sections (Table 2.5, Figs. 2.15 and 2.16).

2.3.11. **Differential (+)-[^H]-3-PPP binding in spleen cell fractions.**

Rat spleens were dissected and dissociated into single cells as outlined in Section 2.2.8. When spleen cells were fractionated by density gradient separation and binding assays carried out on these fractions, we found (+)-[^H]-3-PPP binding in the mononuclear (HBSS/histopaque interface) and in the RBC/granulocyte (histopaque pellet) fraction. Levels of binding varied, however, as 3 - 4 fold more specific binding was apparent in the RBC/granulocyte fraction relative to the mononuclear cells (Fig. 2.17). In addition, (+)-[^H]-3-PPP binding was enriched in the RBC/granulocyte fraction as compared to unfractionated spleen cells (Fig. 2.17). It is important to note that the RBC/granulocyte fraction, in addition to containing 80 - 90% RBCs and 7 - 10% WBCs, also contained approximately 10% dead cells of unknown type.

The data in figure 2.17 pointed to RBCs as a possible source of this binding site in splenic homogenates. To further address this we treated rat spleen cells with 0.15 M NH₄Cl, in ACK lysis buffer, to lyse RBCs. The post-lysis RBC membranes were centrifuged at high speed and binding assays carried out on these membranes. In addition, binding assays were performed on the spleen cells resistant to ACK lysis. As with the histopaque separated cells, (+)-[^H]-3-PPP binding was seen in all cell fractions (Fig. 2.18). However, binding was lower in the RBC membranes compared to the ACK-resistant population. The ACK-resistant cells contained only 0 - 5% RBCs. In addition, (+)-[^H]-3-PPP binding was slightly enriched in the ACK-resistant spleen cells compared to total, untreated spleen cells (Fig 2.18).
Although we observed less binding in the RBC membrane homogenate, a considerable amount of binding was still apparent in this membrane population. Because of this we cannot conclude that (+)-[^3H]-3-PPP binding sites are not present on RBC membranes. However, in an experiment performed after the one shown in figure 2.18, the RBC lysis supernatant was examined under a dissecting microscope and was found to contain a relatively large number of intact nuclei and/or nucleated cells. Therefore, we may have had some contaminating cells in the RBC membrane preparation (possibly due to cells sticking to the sides of polystyrene tubes) that could have contributed to the binding seen in this homogenate.

23.12. Haloperidol-inaccessible binding sites may be primarily located on PMNs in human blood.

Haloperidol-inaccessible (+)-[^3H]-3-PPP binding assays were carried out on purified human peripheral blood mononuclear cells (PBMCs) and human polymorphonuclear leukocytes (PMNs). Cells were prepared from donor blood as outlined in Section 2.23. Various cell numbers per well were incubated with 1μM (+)-[^3H]-3-PPP in the presence of haloperidol. Human PMNs exhibited a large amount of haloperidol-inaccessible (+)-[^3H]-3-PPP binding (Fig. 2.19). This binding was seen at very low cell number (1000 dpm at 3900 cells). In addition, the pattern of binding was similar to that observed in similar experiments performed in rat spleen and human PBL (Figs. 2.4 and 2.5), in that (+)-[^3H]-3-PPP binding was not proportional to tissue at higher cell numbers. This phenomenon was not due to depletion of radioligand as less than 10% was bound even at the highest cell number. In contrast to PMNs, specific (+)-[^3H]-3-PPP binding was not apparent in PBMCs except at very low levels (Fig. 2.19).
Figure 2.17. Histopaque fractionation of rat spleens reveals (−)[3H]-3-PPP binding in all cell fractions, with a large amount of binding present in the RBC/granulocyte/dead cell fraction. Freshly dissected rat spleens were dissociated into single cells by pressing through wire mesh and cells were treated as outlined in Section 2.2.3 and 2.2.8. Total cells represent cells removed prior to gradient separation. Interface cells represent cells harvested from HBSS/histopaque interface and consists almost entirely of mononuclear cells. Pellet cells represents cells obtained from the pellet at the bottom of the histopaque layer and consists of RBCs (80%), WBCs and dead cells. Cells (includes RBCs, nucleated and dead cells) were equalized to 5x10⁶ cells per well for all cell populations prior to assay addition. (+)-[3H]-3-PPP binding assays were carried out as outlined in Section 2.2.4 with a 1:300 ratio of hot to cold (+)-3-PPP. Figure depicts specific (+)-[3H]-3-PPP binding and is representative of three similar experiments. Bars represent means ± SEM values of triplicate determinations.
Figure 2.17. Histopaque fractionation of rat spleens reveals (+)-[\textsuperscript{3}H]-3-PPP binding in all cell fractions, with a large amount of binding present in the RBC/granulocyte/dead cell fraction (pellet cells).
**Figure 2.18.** (+)-[^3H]-3-PPP binding is not enriched in ACK(NH₄Cl)-sensitive red blood cell (RBC) membranes. However, a large amount of (+)-[^3H]-3-PPP binding is seen in spleen cells resistant to NH₄Cl lysis. Freshly dissected rat spleens were dissociated into single cells by pressing through wire mesh and cells were treated as outlined in Sections 2.2.3 and 2.2.8. ‘Total cells’ represents spleen cells not treated with ACK (NH₄Cl-containing) lysis buffer and were removed and frozen prior to NH₄Cl treatment. Spleen cells were treated with ACK lysis buffer for 5 min. at room temperature. Cells were centrifuged out of lysis buffer at 300 x g. Post-lysis supernatants were poured into 50ml tubes and centrifuged at 40,000 x g for 15 min. These membranes were frozen on dry ice and represent the ‘ACK-sensitive RBCs.’ Post-lysis cells (virtually 100% WBCs) were centrifuged once again and frozen on dry ice. These cells represent the ‘ACK-resistant spleen cells.’ Membranes were thawed and protein assays (103) performed to equalize protein levels to 25μg per well. (+)-[^3H]-3-PPP binding assays were carried out as outlined in Section 2.2.4 with a 1:150 ratio of hot to cold (+)-3-PPP. Figure depicts specific (+)-[^3H]-3-PPP binding and is representative of four similar experiments. Bars represent means ± SEM values of triplicate determinations.
This cell population contained some intact WBC nuclei or cells which may have contributed to some of the binding seen in this treatment group.

Figure 2.18. (+))-[^3]H]-3-PPP binding is not enriched in ACK(NH₄Cl)-sensitive RBC membranes.
Figure 2.19. Haloperidol-inaccessible (+)-[^3H]-3-PPP binding is present at high levels in human polymorphonuclear leukocytes (PMNs), but is low in human peripheral blood mononuclear cells (PBMCs). Donor human blood was collected and separations performed in the laboratory of Dr. J. Walters as outlined in Section 2.2.3. In the figure shown PMNs and PBMCs were from the same donor. Varying amounts of PMNs (3.9x10^3 - 5x10^5 cell equivalents per well) and PBMCs (1.56x10^4 - 1x10^6 cell equivalents per well) were incubated with (+)-[^3H]-3-PPP (diluted with nonradioactive (+)-3-PPP 1:250) as listed in Section 2.2.4. Specific (+)-[^3H]-3-PPP binding is shown. Points represent the mean ± SEM of three replicates in the presence of 50μM haloperidol. PBMC curve is flat up to 1x10^6 cells. Figure shown is representative of three experiments performed on cells from different donors.
Figure 2.19. Haloperidol-inaccessible (+)-[^3]H]-3-PPP binding is present at high levels in human PMNs, but is low in human PBMCs.
2.4. Summary and discussion

We have identified and characterized a low affinity binding site for (+)-[^3H]-3-PPP in rat spleen that does not bind haloperidol or DTG, but does bind all other sigma ligands tested, and also binds the opioid antagonist, naltrexone. Sites with comparable binding affinities were also found on isolated rat splenocytes and human PBL. Competition binding experiments with DTG and (+)- and (-)-SKF 10,047 indicated that the sites on isolated splenocytes had a similar pharmacology to those of rat spleen (Whitlock and Wolfe, unpublished). Haloperidol/DTG-inaccessible sites could not be detected in rat brain.

Because a full pharmacological characterization was not performed using human PBL, we cannot definitively state that the site on PBL is identical to that in rat spleen. However, since binding assays were carried out under identical conditions, a single site was present in both tissues (Figs. 2.7 and 2.8), and the binding affinities in rat spleen and human PBL were similar (Table 2.1), it is reasonable to assume that the same sites are present in both rat and human immune tissues.

Binding was quite sensitive to pH and Tris-HCl concentration (Figs. 2.2 and 2.3). These effects were due to alteration of binding affinity (K_d), not to exposure of cryptic sites (B_max). The pH effect is similar to that reported by others using (+)-[^3H]-3-PPP to label sigma receptors in rat brain (94). Therefore, it is possible that pH may influence the charge and conformation of (+)-[^3H]-3-PPP, rather than reflecting properties of the binding sites. The detrimental effect of increasing Tris-HCl concentrations on binding affinity may reflect a low ionic strength optimum for these sites. However, Tris-HCl has been reported to inhibit binding to sigma receptors (112)
and PCP receptors (113) in a similar manner as was seen in the present experiments (Fig. 2.2). Thus, haloperidol/DTG-inaccessible sites, or their ligands, share some properties with classical sigma and PCP receptors.

An unusual feature of our assays is that binding of radioligand did not show linear proportionality to the amount of rat spleen or numbers of PBLs or PMNs, present in the binding reactions (Figs. 2.4, 2.5 and 2.19). This was not due to a depletion of radioligand from the reaction. One possible explanation is that an inhibitory endogenous substance or ligand may have been present in the tissue, and was not adequately removed during the homogenization and washing procedures. At high tissue concentration, carryover of endogenous ligand may have been sufficient to compete with the radioligand, thereby causing curvilinear tissue-binding plots (Figs. 2.4 and 2.5). This should have minimally affected the values that we obtained in saturation and competition binding assays because tissue concentrations were kept low. However, the number of binding sites (B_max values) in rat spleen and human PBL may be slight underestimates. Interestingly, this phenomenon was not seen in isolated spleen cells which are predominantly lymphocytes (Fig. 2.5). This could be due to a lack of endogenous ligands in these cell preparations, or, since it appears that granulocytes may be the primary source of these sites (Fig. 2.19), concentrations of sites may not have been high enough to reach the curvilinear, nonproportional range.

At 22°C, an initial apparent steady-state was reached within 15 - 20 minutes (Fig. 2.6A), which is comparable to that seen with (+)-[3H]-3-PPP and (+)-[3H]-SKF 10,047 at sigma sites in rat (55) and guinea pig brain (9). At 37°C, a slower binding component was evident after 20 minutes (Fig. 2.6A). The slow component of (+)-[3H]-3-PPP binding was temperature dependent, suggesting it may be the result of an enzymatic or other metabolic event.
Interestingly, the addition of excess (+)-SKF 10,047 after binding had occurred resulted in no dissociation of (+)-[3H]-3-PPP over a 90 minute period at 22° C (Fig. 2.6B), and in fact binding increased slightly in a manner suggestive of the slow binding component of the association reaction observed at 37° C (Fig. 2.6A).

Several features of these binding sites are highly unusual, and remain unexplained. The definition of a dissociation constant suggests that if the association rate is fast, and dissociation is slow, then binding must be of high affinity. Nevertheless, under our conditions the present sites are of low affinity, with fast association and no detectable dissociation. Irreversible binding should not be proportional to radioligand concentration, and should not come to apparent steady state. Yet concentration-dependent binding was seen, and this made saturation and competition binding studies possible. This may have significant biological consequences for the function of this drug binding site. Such irreversible, or very slowly reversible binding has also been observed in other drug-binding receptors (40, 114). This very unusual association and dissociation pattern raises the following issues regarding the methodology of this study:

1) An irreversible binding reaction would be expected to proceed to whichever occurred first: either full occupancy of binding sites regardless of the radioligand concentration, or depletion of radioligand from solution. Thus, saturation and competition binding assays would not be possible if this system behaved as expected for an irreversible binding system. Nevertheless, in our hands the fast binding component came to apparent steady-state within 20 min. at 22° C, at a percent binding site occupancy that was proportional to the concentration of free radioligand. We can offer no explanation for this, except to note that it suggests something other than a simple binding reaction was occurring.
From an empirical standpoint, the apparent steady state reached in 20 min. at 22° C made it possible to conduct saturation and competition assays. A similar, very slow off rate has also been reported for α₁ receptors labeled with (+)-[³H]pentazocine (40), and these too have been studied by saturation and competition binding assays. In addition, there is evidence that haloperidol binds irreversibly to sigma receptors (114) and sigma receptors have historically been studied at length using [³H]haloperidol with saturable sigma receptors being identified in various tissues (11, 17, 24, 87).

Because of the irreversible nature of this binding, affinity constants cannot be verified from association and dissociation constants. Therefore, without a better understanding of the events occurring in these binding reactions, it would be unrealistic to maintain that the dissociation and inhibition constants obtained in these experiments are accurate from a theoretical standpoint. However, we believe that the affinity constants obtained for these sites are useful representations of the behavior of these sites under conditions comparable to those used for investigation of other receptors (see above). It is important to note that only compounds that bind irreversibly, or with very slow dissociation rates, can be effective in competing with a radioligand that binds irreversibly. Therefore, the rank order of drug potency at this site does not reflect the affinity of binding of drugs whose binding to the haloperidol/DTG-inaccessible sites is rapidly reversible.

2) It has become an axiom of receptor binding that separation of bound radioligand from free may be done by filtration and washing on fiber glass filters only in the case of high affinity (nanomolar K_d) ligand-receptor systems. Filtration should not be used to harvest lower affinity binding reactions because these reactions
have faster dissociation rates, and in this case a significant amount of bound radioligand will be lost during washing (115). Thus, systems with high nanomolar or micromolar dissociation constants are usually studied using centrifugation, rather than filtration, to separate bound from free radioligand. In the present case the reason for this rule does not apply. Binding was found to be irreversible over two hours. Therefore, it is not reasonable to believe that significant dissociation and loss of ligand from receptors would occur during the 20-30 seconds of washing on a filter. Filtration over PEI-soaked glass fiber filters, which was the method employed, was appropriate in this particular case.

3) The mathematical treatment used to obtain dissociation constants and $B_{\text{max}}$ values applies to binding reactions at apparent steady-state. It may be seen in figure 2.6A that, at 37°C, association continues to increase over the entire period of these experiments. However, it may also be seen in the figure that there are two processes, a fast one that is completed within 20 min., and then a second, slow association that proceeds linearly from that point on. The slow association process appears to be occurring during "dissociation" experiments as well (Fig. 2.6B).

If we were to extend our incubation times until cessation of the slow association process, the reactions would be at apparent steady-state. However, we would then be studying two processes: a fast one, and a slow one. The present report is a study of the fast process. This is clearly completed before 35 min. at room temperature. Therefore, despite the continuation of the slow association process, the mathematical treatment is appropriate for the study of the fast process, which is, in fact, at apparent steady state at 35 min. By incubating at room temperature
and limiting incubations to 35 min., we were able to minimize the effects of the slow process to the point that a single-site binding analysis was obtained in all tissues.

Although many sigma ligands could bind these novel sites, their pharmacological profile (Table 2.2) clearly distinguished them from known sigma, PCP or dopamine receptors. The (-)-stereoisomers of the benzomorphans, as well as (-)-3-PPP, were slightly more potent than their (+)-stereoisomers, which is a trait uncharacteristic of $\sigma_1$ receptors in rat spleen (12, 111). The opiates dextromethorphan, dextrorphan and naltrexone could compete at the haloperidol/DTG-inaccessible site. The $K_J$s of dextromethorphan and dextrorphan were in the same low micromolar range as those of the benzomorphans, pentazocine and SKF 10,047. The psychoactive compound TCP, which exhibits micromolar affinity at sigma receptors in rat brain and spleen (12, 111, 116, 117, 118), had no apparent affinity at splenic haloperidol/DTG-insensitive sites. The high affinity sigma ligands haloperidol and DTG also displayed no activity at this site. Sigma receptors (both $\sigma_1$ and $\sigma_2$) are defined as haloperidol-sensitive, non-opioid sites that bind SKF 10,047 (18, 34). The haloperidol/DTG-inaccessible sites do not meet either of these criteria, as they are haloperidol-insensitive and bind the opioid antagonist naltrexone. Indeed, because of their low affinity and uncommonly large number of sites ($10^5 - 10^6$ per cell), we doubt that these drug binding sites are receptors in the classical sense.

A low affinity benzomorphan transporter, capable of binding pentazocine, has been identified in rat PBL by Marks and Medzihradsky (119). This transport system was sensitive to tricyclic antidepressants such as imipramine. In our hands imipramine did not compete for binding at haloperidol/DTG-inaccessible sites (data not shown). Therefore we do not believe these sites are the benzomorphan transporter.
Metabolic enzyme inhibitors can bind sigma receptors. These include compounds such as the monoamine oxidase inhibitors clorgyline and (-)-deprenyl, which display high affinity for (+)-[3H]-3-PPP-labeled sigma receptors in mouse brain (120). Also, the cytochrome P-450 inhibitor SKF 525A is able to compete at sigma sites in rat liver and brain (121). Since in our hands clorgyline (MAO A), (-)-deprenyl (MAO B) and SKF 525-A (cytochrome P-450) had little affinity (Table 2.2), haloperidol/DTG-insensitive sites are probably not related to cytochrome P-450 or monoamine oxidases A and B.

Dopamine receptors can bind several sigma ligands, including 3-PPP, and have been reported to be present on immune cells, but dopamine receptors are sensitive to haloperidol. Haloperidol is quite potent at D2 receptors, which have been reported on human PBL (122). Also, the D2 antagonist spiperone had little effect on the presently reported haloperidol/DTG-inaccessible sites (data not shown). D5 receptors, which bind haloperidol, have recently been reported to be present on immune cells, while D1 receptors were not found (123). D3 receptors, insensitive to 3-4 nM haloperidol, have been identified on rat splenocyte membranes using [3H]-dopamine (124). However, in CHO cells, D3 receptors have been shown to have a K_i for haloperidol of 9.8 nM (125). Therefore, CNS D3 receptors, and presumably splenocyte D3 receptors, are not haloperidol-insensitive to the extent of the presently reported haloperidol/DTG-inaccessible sites. In addition, binding of [3H]-dopamine at D3 receptors is of high affinity and reversible (124), whereas drug binding to haloperidol/DTG-inaccessible sites is of lower affinity and apparently irreversible. Therefore, we do not believe that the currently described binding site is a known type of dopamine receptor.

In addition to sensitivity to naltrexone, other aspects of the presently reported sites are similar to opioid receptors. First, the selectivity for the (-)-stereoenantiomers of the benzomorphans is consistent with opioid receptors (16). Second, the morphinan derivatives, dextromethorphan and dextrorphan, are active at these sites. While this alone
does not suggest an opioid receptor, since dextromethorphan and dextrorphan both bind predominantly to sigma receptors (126), it is consistent with the idea that the haloperidol/DTG-inaccessible sites have opioid receptor-like properties. Also, it has been shown that phenylpiperidine derivatives such as fentanyl are potent ligands at μ opioid receptors in rat brain (127). 3-PPP is a phenylpiperidine, and while it is not known as an opioid ligand, it seems possible that at higher concentrations it could have some affinity at opioid receptors or opioid receptor-like sites. Finally, PCP has micromolar affinity at opioid receptors in rat brain (128), consistent with its affinity at the haloperidol/DTG-inaccessible splenic site.

Functional opioid binding sites are present on immune cells (for review see 129, 130). Delta and κ opioid receptors have been reported (131, 132, 133). Mu receptors have been difficult to identify due to the presence of β-endorphin-specific, non-opioid binding sites in the immune system (134, 135). However, there is evidence that functional μ receptors exist on T lymphocytes. Naloxone-reversible enhancement of ConA stimulated proliferation has been reported with the μ-selective ligand, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO; 136). Recent work has confirmed that μ opioid receptor mRNA is present in human PMNs and CD4+ cells, monkey PMNs and PBMCs, and rat peritoneal macrophages (137, 138). These findings suggest that μ, δ and κ receptors are all present on immune cells.

Despite these considerations, it is unlikely that the haloperidol/DTG-inaccessible sites are classical opioid receptors. They are undetectable in whole brain homogenates known to contain μ, δ and κ receptors. Furthermore, the binding affinity of the haloperidol/DTG-inaccessible site is lower than that of opioid receptors in the CNS. Finally, the number of splenic haloperidol/DTG-inaccessible sites is approximately three orders of magnitude higher than that of traditional receptors (see B_{max} values, Table 2.1).
There is evidence pointing to the existence on immune cells of an opiate alkaloid site with little affinity for opioid peptides. Using $[^3\text{H}]$naloxone, Ovadia and colleagues (139) identified such a site on rat lymphocyte membranes. The site had a $K_d$ of 960nM and was insensitive to $\beta$-endorphin and leu-enkephalin. A similar site was found on mouse thymocytes using $[^3\text{H}]$morphine (140). This site had a $K_d$ of 50nM and exhibited slight stereoselectivity for (−)-morphine. The presently described haloperidol/DTG-inaccessible sites may be related to these low affinity opiate alkaloid binding sites.

The haloperidol/DTG-inaccessible sites are most likely not present on lymphocytes to any great extent. This is supported by several lines of evidence. First, the sites were not present in T and B cell lines (Fig 2.13). Second, the concentration per unit protein of these binding sites in splenocytes was similar to that of whole spleen (Table 2.1). If splenocytes were the predominant source of these binding sites in spleen, then isolated splenocytes would be expected to show an enrichment of binding relative to whole spleen. Since this was not the case, haloperidol/DTG-insensitive sites are probably also present on other, non-recovered cell types or structural elements of rat spleen. Third, the autoradiographic localization of binding in rat spleen confirms that these sites are probably not present in high concentrations on lymphocytes, as neither the T nor B cell zones of the white pulp showed significant labeling (Fig. 2.16 and Table 2.5). The coarse, scattered distribution of 'hot spots' in the red pulp and marginal zones suggests that these sites may be predominantly present on cells or clumps of cells such as granulocytes or subsets of macrophages. This evidence strongly supports the notion that these sites are not predominantly located on lymphocytes.

In experiments performed concurrent to the autoradiography studies, rat spleen cells (WBCs and RBCs) were fractionated using a histopaque step gradient and binding was carried out on the various cell fractions. Higher levels of (−)$[^3\text{H}]$-3-PPP binding were seen in the post-density centrifugation 'pellet' cells, than the mononuclear cells
harvested from the histopaque/HBSS interface. Importantly, the level of binding was consistently higher in the 'pellet cells' than in the 'total' or unfractionated cells indicating a relative higher number of sites per cell in this population. In histopaque gradients the 'pellet' cells consist largely of RBCs. Therefore, we examined the membranes of RBCs lysed in NH₄Cl-containing buffer for the presence of haloperidol/DTG-inaccessible sites. While some binding was seen in the NH₄Cl-sensitive membranes, it was significantly lower than the binding in the untreated or NH₄Cl-resistant spleen cells (Fig. 2.18). If RBCs were the main reservoir of these sites in spleen, a relative purification of (+)-[³H]-3-PPP sites should have been seen in the RBC membranes. These experiments suggest that neither splenic mononuclear cells nor red blood cells are the main source of haloperidol/DTG-inaccessible sites in rat spleen.

When binding was performed on membranes of human PMNs (95 - 98% pure) a large amount of (+)-[³H]-3-PPP binding was evident on these cells (Fig. 2.19). The curvilinear tissue-binding plot was very similar to rat spleen and PBL (Figs. 2.4 and 2.5). In contrast, very little binding was seen in PBMCs from the same donors. This would seem to indicate that haloperidol/DTG-inaccessible sites in human blood are primarily located on granulocytes. The 'pellet' cells, obtained in spleen cell fractionations, also contained some granulocytes, in addition to red blood cells and dead cells. Therefore, granulocytes may also be a primary source of the haloperidol/DTG-inaccessible sites in rat spleen.

The binding studies performed on human PMNs, spleen cell fractions and the labeling seen in spleen sections, strongly support the localization of these sites primarily on granulocytes. However, if this is true then it is unclear why we were able to identify these sites in splenocytes and human PBL, which were isolated on histopaque gradients and should have consisted almost entirely of mononuclear cells. While we do not have a definite answer for this, we most probably had contaminating granulocytes in our
preparations. Our human PBL were isolated in mass purifications from leukocyte-rich buffy coats obtained from the Red Cross. Our gradients may have been overloaded with blood, which can lead to a build-up of cells at the HBSS/histopaque interface, compromising purification. It is obvious from our binding experiments in purified PMNs, that, given the apparent high levels of haloperidol/DTG-inaccessible sites on these cells (1000 dpm bound in only 3900 cells), that contaminating PMNs in our cell preparations could have contributed to the (+)-[3H]-3-PPP binding seen in human PBL.

The cellular location of haloperidol/DTG-inaccessible sites in spleen is not as clear. The punctate, scattered pattern of binding in the spleen suggest these sites are not present on trabeculae or reticular fibers. The spleen is not known as a source of PMNs. However, the spleen can be slightly granulocytopoietic, especially in young animals, and senescent granulocytes are removed in the spleen (141). Therefore, PMNs could contribute to the (+)-[3H]-3-PPP binding seen in rat spleen. We can offer no direct evidence for this, however, and cannot rule out the contribution of other cell types, such as marginal zone macrophages, to the pattern of (+)-[3H]-3-PPP binding in this organ.

Stefano and Makman (142,143) have described lower affinity opioid receptors, dubbed μ3 receptors, on human monocytes and granulocytes. The present findings of naltrexone-sensitive/low affinity binding sites, the autoradiographic localization of binding sites in a punctate pattern in the red pulp of the spleen, and the presence on human PMNs are all consistent with the notion that the binding sites that we have observed may be related to these μ3 receptors. However, μ3 sites have been reported to be plentiful on RAW 264.7 cells (144), brain astrocytes and brain microglia (145). Under the conditions employed in the present study, we detected no significant specific haloperidol-insensitive binding of (+)-[3H]-3-PPP to RAW 264.7 cell membranes (Fig. 2.13) or rat brain membranes/sections (Fig. 2.7, Table 2.5). In addition, these sites are insensitive to the drug dextrorphan up to 10^{-5} M (143), while the haloperidol/
DTG-inaccessible sites are sensitive to this drug with a $K_i$ equal to 2.2$\mu$M (Table 2.2). Therefore, while we cannot rule out the possibility that the presently reported binding sites are $\mu_3$ receptors, we think that it is unlikely.

At this time, we know little regarding the molecular nature of these novel binding sites. Their susceptibility to heat (Fig. 2.11, Table 2.3) suggests that they may be composed of proteins. However, binding activity was not diminished by treatment of washed membranes with trypsin, pronase, proteinase K or chymopapain. Therefore, if these binding sites are protein in nature, they must be protected from or resistant to proteases. Neuraminidase and $\alpha$-amylase did not alter binding (Table 2.4), which indicates that carbohydrates are not crucial to the ability of the sites to function. The fact that phospholipase C did destroy binding activity in the membrane fraction (Fig. 2.12, Table 2.4) suggests that the drug binding moiety may be lipid in nature, or may require a lipid environment to function (146).

To further characterize and isolate these sites we attempted to solubilize the haloperidol/DTG-inaccessible sites with detergents commonly used in receptor studies (75, 106, 107). Surprisingly, treatment of spleen membranes with a variety of detergents with different properties did not yield solubilized sites (Table 2.4). Limited and inconsistent success was obtained with SDS; however, concentrations of SDS above 0.05% destroyed (+)-[^3H]-3-PPP binding (Table 2.4). The effective concentrations of SDS (0.02 - 0.05%) were below optimal concentrations for membrane solubilization. This strong inhibitory effect of SDS suggests that the binding site, if a protein, might consist of more than one polypeptide subunit.

It is axiomatic to receptor binding that if one increases concentrations of radioligand beyond that required to label high affinity receptors, one will always detect
additional, lower affinity drug binding sites. In the absence of a known function, such low affinity sites are usually of little interest. The presently reported binding sites are of interest because:

1) The high concentration of these binding sites prevented the selective labeling of $\sigma_1$ receptors in immune tissues by accepted techniques (see Chapter 3; 34). The contribution of these sites was apparent in a recent autoradiographic study demonstrating that certain kappa opioid ligands could displace high affinity (+)-$[^3H]$-PPP binding in spleen and liver (147). The displacement seen in this report could have been due, at least in part, to the interaction of opiates at haloperidol/DTG-inaccessible sites. Therefore, an immediate, practical result of the present investigation is our ability to slightly modify the accepted conditions (34) in order to obtain selective, highly specific labeling of $\sigma_1$ receptors in immune tissues. If haloperidol/DTG-insensitive sites are blocked with naltrexone, then (+)-$[^3H]$pentazocine ($\leq 25$ nM) is highly selective in labeling haloperidol-sensitive $\sigma_1$ receptors in spleen (Fig. 3.4). Therefore, future studies of sigma receptors in immune tissues should be conducted in the presence of blocking naltrexone.

2) The ability of these sites to bind naltrexone suggests that they may also bind other opiate compounds, such as morphine. The effects of opiates and opioid receptors on the immune system appear to be complex, and the literature on this subject is confusing and often contradictory (reviewed in 129 and 130). The presently reported binding sites may be one reason high affinity $\mu$, $\delta$ or $\kappa$ receptors have not been easy to label on immune cells using traditional opiate compounds such as naloxone, naltrexone or morphine (129). This literature should be re-evaluated in light of the present finding.
3) Because of the overlap between the pharmacology of phencyclidine (NMDA), sigma, serotonin (5-HT), dopamine and adrenergic receptors, one must be cautious about assigning drug actions at one of these receptors as the sole mechanism of the physiologic effects of a given compound. The present report introduces another such site which must be taken into consideration when dealing with this group of drugs.

In summary, we have identified, characterized, autoradiographically localized, and performed preliminary experiments probing the molecular nature of a novel drug binding site in rat spleen that is capable of binding both sigma and opioid ligands. This site was found to be present in high concentrations in both rat and human immune tissues, but was not detectable in rat CNS. The compounds that bind to these novel sites also are known to bind a multitude of receptors, including sigma, opioid, NMDA, and dopamine receptors. Although we do not believe that the haloperidol/DTG-inaccessible site is any of these entities, it must be taken into consideration when using these broad-acting compounds to probe the activities of these other receptor types. At the present time we know of no function of this binding site. It could be involved in modulatory effects of opiates on PMN function. There is some evidence for non-traditional opiate effects and binding sites on granulocytes (129, 130). In addition, these sites could be involved in some of the immunomodulatory effects of sigma agonists, as the drug concentrations that induce immune functional changes are typically in the μM range (69, 89, 99). Thus, these binding sites may have significant effects on in vivo experiments with both opiate and sigma compounds. The localization of these sites in a coarse, punctate pattern in the marginal zones and red pulp of the spleen suggests that they may be present on granulocytes or macrophage subsets. The presence of these sites on human PMNs suggest that they may play a role in granulocyte function or development.
CHAPTER 3

Identification and Functional Significance of Sigma-1 Binding Sites on T Lymphocytes

3.1. Introduction

As we indicated in the general introduction, sigma-1 binding sites are present in immune tissues and can inhibit ConA-stimulated proliferation of rat spleen cells (87). In this study we extended these findings by identifying and functionally characterizing σ₁ receptors on T cells.

Of the three σ sites (σ₁, σ₂ and σ₃), the first site to be pharmacologically identified in receptor binding studies was what is now known as the σ₁ site (9, 10, 17). Sigma-1 sites can modulate stimulus-induced functions in several tissues including: inhibition of carbachol-stimulated phosphoinositide turnover in rat brain (51), inhibition of nicotine-stimulated catecholamine-release in adrenal chromaffin cells (48), and inhibition of NMDA-stimulated intracellular calcium release in rat cortical neurons (68).

In the immune system, previous work from our laboratory found a highly significant correlation ($r^2=0.71$) between the ability of σ agonists to inhibit ConA-induced rat spleen cell proliferation and their potency at [$^3$H]haloperidol-labeled σ₁ sites in splenic
homogenates (Fig. 3.1; 87). ConA is a potent T cell mitogen; therefore, these results indicate that agonists acting at $\sigma_1$ sites can inhibit T cell function, although since this study was performed with mixed cell populations it did not prove that the $\sigma_1$ sites were on the responding T cells.

Other studies have also suggested a role for $\sigma$ agonists in modulation of T cell activity. Domand and associates (7) demonstrated that PCP, at $\mu$M concentrations, could inhibit ConA-induced proliferation and IL2 synthesis of mouse spleen cells. PCP exhibits $\mu$M affinity for $\sigma_1$ sites in the immune system (11, 12, 87). Carr and co-workers (89) demonstrated that some $\sigma$ ligands could inhibit the proliferation of ConA-stimulated mouse spleen cells and thymocytes. In addition, it was found that (+)-pentazocine could slightly inhibit IL2 and/or IL4 production in these cells, as assayed by CTLL-2 proliferation (89). Finally, Casellas and colleagues (99) observed that a novel $\sigma$ ligand, SR 31747, could also inhibit ConA-induced proliferation of mouse splenic lymphocytes. These studies are all indicative of $\sigma$ agonist-mediated inhibition of T cell function; however, none of these reports addressed the site(s) of action.

We hypothesized that functional $\sigma_1$ receptors existed on T cells and that production or release of the primary T cell cytokine IL2 was inhibited via these sites. The present study was designed to address this by using radioligand binding assays to examine IL2-producing T cell lines for the presence of $\sigma_1$ sites, then utilizing the $\sigma_1$ positive cells in functional assays to examine the effect of $\sigma$ agonists on IL2 production.

In the previous chapter we reported the identification and characterization of an alternate binding site for $\sigma$ ligands in immune tissues. This site was present in such large numbers that it precluded our use of (+)-[3H]pentazocine, the preferable $\sigma_1$ radioligand (34). In the present study we exploited the pharmacology of these novel sites to develop specific labeling conditions for $\sigma_1$ receptors: (+)-[3H]pentazocine in the presence of
naltrexone to block haloperidol/DTG-inaccessible sites. Using this method we were able to obtain highly selective labeling of sigma-1 receptors in mouse brain, spleen and a murine T cell line, LBRM33.

We found that \( \sigma \) agonists could inhibit PHA-stimulated production of IL2 in LBRM33 cells, and this inhibition correlated strongly with the previously determined drug potencies \( (K_i) \) at \( \sigma_1 \) binding sites. This effect, however, was apparently due to drug-induced toxicity as measured by trypan blue exclusion and lactate dehydrogenase (LDH) release. We then examined whether \( \sigma_1 \)-modulated inhibition of ConA-induced spleen cell proliferation was also due to this toxic effect. We tested the ability of the \( \sigma \) agonists haloperidol and (+)-pentazocine to inhibit proliferation and IL2 production in ConA-stimulated mouse spleen cells. In these assays both (+)-pentazocine and haloperidol inhibited spleen cell proliferation at 48 h without inducing cell death; however, only very high concentrations of haloperidol or (+)-pentazocine significantly inhibited IL2 production in spleen cells. These results indicate that while occupation of \( \sigma_1 \) receptors on LBRM33 cells can lead to cell death, \( \sigma_1 \) receptors on mouse spleen cells do not trigger death. Splenic \( \sigma_1 \) receptors controlled proliferation, but an effect on IL2 with the \( \sigma \) selective compound (+)-pentazocine could not be demonstrated, indicating that the effect on spleen cell proliferation was not linked to either IL2 inhibition or cell death.
Figure 3.1. The ability of σ agonists to suppress ConA-stimulated proliferation of rat spleen cells (EC₅₀) highly correlates with the ability of these drugs to bind to [³H]haloperidol-labeled σ₁ receptors (Kᵢ). ConA proliferation studies and [³H]haloperidol binding studies were performed as described elsewhere (87, 88). EC₅₀ and Kᵢ values were calculated as outlined in Section 3.2.9 and each point represents a mean of 3 - 5 experiments.

The work represented in this figure was performed by Dr. Yuhong Liu, Ph.D. and is an adaptation of a figure previously published by Dr. Liu in her dissertation and elsewhere (87, 88).
Figure 3.1. The ability of σ agonists to suppress ConA-stimulated proliferation of rat spleen cells highly correlates with the ability of these drugs to bind to $[^3$H]haloperidol-labeled σ₁ receptors (87, 88).
3.2. Materials and methods

3.2.1. Animals

Male C57Bl/6J mice (20 - 25 g, 6 - 8 weeks old; Jackson Laboratories, Bar Harbor, ME) were utilized for the majority of radioligand binding and immune function experiments. Some female Lewis and Sprague-Dawley rats (125 - 175 g, 2.5 - 3 months old; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used for preliminary characterization of (+)-[3H]pentazocine binding and establishment of labeling conditions. All animals were maintained on a 12 h. light:12 h. dark cycle and received food and water ad libitum. Animals were maintained and handled in accordance with NIH guidelines.

3.2.2. Cell lines

The Jurkat, EL4.IL2 and LBRM33 clone 4A2 cell lines were obtained from the ATCC (Rockville, MD). The murine T cell lymphoma LBRM33 and the human T cell leukemia Jurkat were maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS (defined grade, HyClone Laboratories, Inc., Logan UT ) plus 5x10^{-5} M 2-ME and 50 μg/ml gentamicin (Gibco BRL). LBRM33 cells adhered loosely to culture flasks and were scraped lightly before passage or assay. The mouse T cell lymphoma EL4.IL2 was maintained in DMEM (Gibco BRL) supplemented with 10% horse serum (Gibco BRL) plus 5x10^{-5} M 2-ME (Bio-Rad Laboratories, Richmond, CA), 10mM HEPES, 1.5mM L-glutamine and 50μg/ml gentamicin. Cells used in binding assays were washed with HBSS (Gibco BRL) and pelleted prior to quick freezing with dry ice or liquid nitrogen. Cells were stored at -80° C until used in binding assays.
3.2.3. Preparation of membrane homogenates

Brains and spleens were dissected from animals immediately after CO₂ asphyxiation. Tissues were frozen using either powdered dry ice or liquid nitrogen, and stored at -80° C until use.

For homogenate binding assays, tissues were thawed and disrupted in 25 - 40 volumes ice cold 50mM potassium phosphate, pH 8.0 at 4° C, using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, CT) for 30 sec. at 20,000 rpm. Similarly, frozen cell pellets containing 1 - 5x10⁸ cells were thawed and disrupted in 7 - 10ml 50mM potassium phosphate, pH 8.0 at 4° C. Homogenates were centrifuged at 40,000 x g for 15 min. The pellets were then resuspended in the same buffer, homogenized and centrifuged once again. Membrane pellets were then resuspended in ice-cold 50mM potassium phosphate, pH 8.0 at room temperature and centrifuged at 40,000 x g for 12 min. Prior to centrifugation, spleen and brain homogenates were filtered through 150μm nylon mesh (Tetko, Inc., Briarcliff Manor, NY). After the final centrifugation, membrane pellets were resuspended in 50mM potassium phosphate, pH 8.0 at room temperature at the desired concentration. All homogenates were kept on ice until addition to binding assays.

For preliminary binding assays utilizing rat tissues (Figs. 3.2 and 3.3), the homogenates were prepared in an identical manner to that listed above with the exception of the buffers used. Rat spleen and cerebellum were homogenized in 50mM Tris-HCl, pH 8.0 at 4° C and resuspended after the final wash in assay buffer (5mM Tris-HCl pH 8.0 at room temperature). Potassium phosphate buffer was chosen over Tris-HCl for subsequent experiments because binding carried out in potassium phosphate yielded a higher total to nonspecific binding ratio (signal-to-noise) than binding carried out in Tris-HCl.
3.2.4. Binding in membrane homogenates

A. General conditions. Except where noted in legends for individual experiments, binding assays were carried out at room temperature in 0.2ml 50mM potassium phosphate buffer, pH 8.0 at room temperature. Assays were conducted at room temperature in 96-well, U-bottom microtiter plates (Corning Glass Works, Corning, NY). General binding conditions were: 5nM (+)-[^3]H]pentazocine (S.A. 31.6 or 42.0 Ci/mmol; New England Nuclear, Boston, MA); in the presence of 100μM naltrexone (to block opioid and haloperidol-inaccessible sites); in the absence or presence of 1μM haloperidol to define nonspecific binding. Assays were incubated at room temperature for 2 or 6 h., after which bound (+)-[^3]H]pentazocine was separated from free radioligand by rapid filtration and washing of membranes using a harvester (Brandel, Gaithersburg, MD) with GF/B glass fiber filters (Brandel) pretreated with 0.5% PEI (Sigma Chemical Co., St. Louis, MO) to reduce nonspecific binding (102). The retained membranes were washed for 25 sec. with approximately 20ml per incubation well using room temperature 50mM potassium phosphate buffer, pH 8.0. Filters were placed in scintillation vials, and bound radioligand was quantified by liquid scintillation counting. All assays were performed using triplicate wells for each point.

In preliminary saturation and competition binding assays utilizing rat tissues (Figs. 3.2. and 3.3) the binding conditions were somewhat different - 5nM (+)-[^3]H]pentazocine in the absence or presence of 100μM (+)-SKF 10,047 to define nonspecific binding. These experiments were not performed in the presence of naltrexone. These changes are noted in the figure legends.

Unless noted differently in legends, each microtiter plate well contained homogenate derived from 2.5 - 5mg wet weight mouse spleen, 5mg wet weight mouse
brain, or \(2.5 \times 10^6\) LBRM33 T cells. Protein content of the membrane preparations was determined using a protein assay kit (Sigma Diagnostics) based on the method of Lowry (103). In some experiments (Figs. 3.5 and 3.6) tissue and cell amounts were titrated over a set range. In these studies tissue amounts are expressed as wet weights and cell numbers, not as protein amounts.

**B. Saturation binding.** Except as noted for figure 3.2, membrane preparations were incubated with increasing concentrations (0.5 - 98nM) of (+)-[\(^3\)H]pentazocine in the presence of 100\(\mu\)M naltrexone, without or with 1\(\mu\)M haloperidol to define nonspecific binding. All other conditions were as described above.

**C. Competition binding.** Except as noted for figure 3.3, binding sites were labeled under conditions comparable to those used for saturation binding. Membranes of tissue homogenates were incubated with 5nM (+)-[\(^3\)H]pentazocine in the presence of 100\(\mu\)M naltrexone, and non-radioactive sigma ligands were titrated over a concentration range corresponding to their known affinity at \(\sigma_1\) receptors (40, 87). All drugs used in these studies were purchased from Research Biochemicals Inc. (Natick, MA)

### 3.2.5. PHA-stimulation of LBRM33 cells

LBRM33 T cells, seeded at approximately \(2 \times 10^5\) cells/ml, were harvested in log phase of growth by lightly scraping culture flasks with a sterile cell scraper. Cells were transferred into 50ml polypropylene tubes and centrifuged at 400 \(\times\) g for 10 min. After centrifugation, cells were resuspended in 30ml Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS (Gibco BRL) and recentrifuged at 400 \(\times\) g. Cells were then resuspended in 5ml HBSS and counted using a
hemocytometer. While counting, cells were brought up to 30ml with HBSS and centrifuged a final time. The LBRM33 cells were resuspended at 1.5x10^6 cell/ml in culture media, and seeded in U-bottom 96-well plates (Corning) at 1.5x10^5 cells/well. Drugs were added to cultures in 50μl amounts at concentrations ranging from 10^-9 to 10^-3 depending on drug solubility and expected potency. Cells were pre-incubated for 2 h. at 37° C prior to addition of 5μg/ml phytohemagglutinin (PHA; cat. number L-9132, Sigma). Cultures proceeded for 24 h. at 37° C, after which time cell supernatants (100μl) were harvested from each well and IL2 levels detected by ELISA.

Experiments assessing viability of cells were carried out essentially as described above, without PHA addition. An additional 5μl of supernatant was taken from each culture well to measure LDH levels.

3.2.6. ConA-stimulation of mouse spleen cells

Freshly dissected mouse spleens were dissociated into single cells by pressing through wire mesh into Ca^{2+}/Mg^{2+}-free HBSS (Gibco BRL). Cells were transferred into 50ml polypropylene tubes and centrifuged at 400 x g for 10 min. at 4° C. Cell pellets were resuspended in HBSS and incubated on ice to allow for cellular debris to settle. Cells were transferred into 50ml tubes and underlaid with 10ml 70% Percoll (Pharmacia, Uppsala, Sweden). Density gradients were then centrifuged with no braking at 1000 x g for 15 min. at 4° C. Mononuclear cells at the HBSS/Percoll interface were harvested with transfer pipettes and brought up in HBSS and centrifuged at 400 x g for 10 min. at 4° C. After centrifugation, cells were suspended in 5ml HBSS and counted. While counting, cells were suspended in 30 - 40ml HBSS and centrifuged a final time. Cells were brought up to 2x10^6 cells/ml in RPMI-1640 (Gibco BRL), containing 5x10^{-5} M 2-ME, containing 5x10^{-5} M 2-ME,
50µg/ml gentamicin and supplemented with 10% FBS. Cells were added to flat-bottom 96-well plates at 2x10^5 cells per well. The σ agonists haloperidol and (+)-pentazocine were added to culture wells in 50µl volumes for a final concentration of 10^{-10} to 10^{-4} M. Cells were pre-incubated with drugs for 2 h. at 37°C, after which time 2.5 or 5µg/ml ConA was added to each well. Cultures proceeded at 37°C for 24 or 48 h. At the end of the incubation period, supernatants were harvested for measurement of LDH and IL2.

To measure cell proliferation, 0.5 µCi [³H]thymidine (New England Nuclear) was added to the cultures for the last 6 h. of incubation. After 6 h. the cells were harvested onto glass fiber filters (GF/B; Brandel) and filters washed with a large volume of distilled water. Radioactivity was determined in an identical manner to radioligand binding assays.

3.2.7. Interleukin-2 (IL2) measurement

Supernatants, harvested from cultures of LBRM33 or mouse spleen cells, were assayed for IL2 by ELISA. A pair of antibodies suitable for ELISA were purchased from PharMingen (San Diego, CA) and assays were carried out per supplier's instructions. Briefly, high binding ELISA plates (Corning) were coated overnight at 4°C with 1µg/ml anti-IL2 capture antibody in 0.1M NaHCO₃, pH 8.2. The following day plates were blocked with 10% FBS in phosphate buffered saline (PBS) for 2 h. prior to sample addition. Cell supernatants (100µl) were added directly to ELISA plates from culture wells and incubated either for 4 h. at room temperature or overnight at 4°C. Biotinylated anti-IL2 antibody (1µg/ml in PBS/10% FBS) was then added for 45 min., followed by 1µg/ml avidin-peroxidase (in PBS/10% FBS) for 30 min. Color was developed by the addition of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) plus
30% H$_2$O$_2$. Color was allowed to develop for 15 - 30 min. before being read at 405nm in a plate reader (Multiskan MCC; Titertek, Huntsville, AL). IL2 was quantified by comparison to a standard dilution curve of recombinant mIL2 (PharMingen).

3.2.8. Lactate dehydrogenase (LDH) measurement

Supernatants harvested from cultures of LBRM33 cells or mouse spleen cells were used to determine LDH release. LDH was measured using a commercial colorimetric assay kit (Sigma Diagnostics) modified for small volume samples. This assay is based on the reaction of 2,4-dinitrophenylhydrazine with pyruvate to form a colored product which can be measured in a spectrophotometer. The amount of pyruvate remaining is inversely proportional to the amount of LDH activity in the sample. Reactions were carried out in polypropylene titertubes (Bio-Rad) in 105μl volumes. Sodium pyruvate substrate plus 1mg/ml NADH (50μl) was incubated with 5μl sample for 30 min. at 37° C prior to addition of 50μl color reagent. Color was allowed to develop for 20 min. at room temperature, after which time 500μl 0.4N NaOH was added to each tube and 200μl transferred to flat bottom 96-well plates (Corning). Absorbance was read at 450nm in a 96-well plate reader (Titertek). A450 values were compared to a standard curve of pyruvate to determine amount of LDH released. Maximal LDH release was defined as LDH levels present in supernatants of Triton X-100 treated control cultures.

3.2.9. Data analysis and statistics

Equilibrium dissociation constants (K$_d$) and number of binding sites (B$_{max}$) from the saturation binding experiments were determined using the nonlinear iterative curve
fitting program (MacLIGAND) of Munson and Rodbard (109). Correlation and Hill coefficients of Rosenthal plots were determined by the simple curve fitting program Ebda (release 2.0; Biosoft, Milltown, NJ).

EC_{50} values for the inhibition of IL2 and release of LDH were calculated by probit transformation of the data and a least squares fit to a logarithmic-probit plot (148). Correlation of EC_{50} values with K_i values was accomplished by simple linear regression. Paired T tests were used to compare the EC_{50} values for IL2 inhibition and LDH release. Regression analysis and paired T tests were carried out using StatView (Abacus Concepts, Inc., Berkeley, CA).

3.3. Results

3.3.1. Saturable \sigma_1 receptors cannot be identified in spleen with (+)-[^3]H]pentazocine using accepted labeling conditions.

We attempted to label \sigma_1 receptors in rat spleen with the selective radioligand (+)-[^3]H]pentazocine using conditions known to label and saturate \sigma_1 receptors in other tissues (34, 40). Saturable, high affinity (+)-[^3]H]pentazocine binding sites were easily demonstrated in rat cerebellum; however, saturation was not attained in rat spleen (Fig. 3.2). Splenic (+)-[^3]H]pentazocine sites were largely insensitive to haloperidol except at high concentrations, whereas (+)-[^3]H]pentazocine sites in cerebellum were highly sensitive to haloperidol (Fig. 3.3). This showed that accepted \sigma_1 labeling conditions ((+)-[^3]H]pentazocine in the absence or presence of (+)-SKF 10,047 to define nonspecific
binding) could not be used in spleen due to the presence of overwhelming amounts of a lower affinity non-sigma binding site that binds both pentazocine and (+)-SKF 10,047, but not haloperidol (see Chapter 2; 149).

3.3.2. *Naltrexone increases the specificity of (+)-[3H]pentazocine binding in spleen.*

Characterization of the haloperidol-inaccessible sites revealed that they were sensitive to the opioid antagonist naltrexone (Fig. 2.2), while sigma receptors in the CNS exhibited little or no affinity for this compound (16). When naltrexone was competed against 5nM (+)-[3H]pentazocine in rat spleen, approximately 25% of binding could be displaced (bars 2 - 4, Fig. 3.4). Haloperidol alone (1μM) displaced approximately 50% of (+)-[3H]pentazocine (bar 6, Fig. 3.4). However, approximately 80% of naltrexone-inaccessible sites were blocked by haloperidol (bar 5 vs. bar 4, Fig. 3.4) suggesting that a highly selective and specific label could be achieved by (+)-[3H]pentazocine in the presence of naltrexone. Therefore, we elected to label σ₁ receptors in the immune system using (+)-[3H]pentazocine in the presence of naltrexone to block haloperidol-inaccessible binding sites.

The reason for the difference in haloperidol sensitivity seen between figures 3.3 and 3.4 is most likely due to the fact that the experiments utilized different assay buffers. The experiment in figure 3.4 utilized 50mM potassium phosphate buffer, which may be detrimental to high ionic strength-sensitive haloperidol-inaccessible binding sites (Fig. 2.10; 149).
**Figure 3.2.** Saturation binding with (+)-[^3]H)pentazocine under accepted σ1 binding conditions (34) in rat spleen and cerebellum. Saturation of high affinity sites is seen in cerebellum, but not in spleen. Spleen and cerebellum homogenates were prepared as in Section 3.2.3. Binding assays were performed as outlined in Section 3.2.4, with some modification. Tissues (10mg wet weight per well) were incubated in 5mM Tris-HCl (pH 8.0) assay buffer for 2 h. with 0.2 - 85 nM (+)-[^3]Hpentazocine in the absence or presence of 100μM (+)-SKF 10,047 to define nonspecific binding. Figure depicts specific binding and is representative of three experiments performed under similar conditions.
Figure 3.2. Saturation binding with (+)-[\textsuperscript{3}H]pentazocine under accepted \( \sigma_1 \) binding conditions (34) in rat spleen and cerebellum. Saturation was observed in cerebellum, but not in spleen.
Figure 3.3. Haloperidol effectively blocks for (+)-[3H]pentazocine binding sites in rat cerebellum, but does not complete effectively with (+)-[3H]pentazocine in rat spleen. Spleen and cerebellum homogenates were prepared as in Section 3.2.3. Competition binding assays were performed as outlined in Section 3.2.4, with some modification. Membranes (10mg wet weight per well) were incubated in 5mM Tris- HCl (pH 8.0) assay buffer for 2 h. with 5nM (+)-[3H]pentazocine plus 0 - 10⁻³ M haloperidol. Data are present as mean percentage ± SEM of (+)-[3H]-pentazocine binding displaced. Figure is representative of two independent experiments.
Figure 3.3. Haloperidol effectively competes for (+)-[^3]H)pentazocine binding in rat cerebellum, but does not compete with high affinity at the majority of binding sites in rat spleen.
Figure 3.4. (+)-[^3]H]Pentazocine binding in spleen is more sensitive to haloperidol, if naltrexone is present to block low affinity binding sites. Rat spleen membranes (5mg), prepared as outlined in Section 3.2.4, were incubated with 5nM (+)-[^3]H]pentazocine in 50mM potassium phosphate buffer (pH 8.0). Different treatment conditions included: bar 1 — no treatment, bars 2 - 4 — 10^{-9}, 10^{-6} or 10^{-4} M naltrexone, bar 5 — 10^{-4} M naltrexone plus 1μM haloperidol and bar 6 — 1μM haloperidol. Bars represent the mean binding ± SEM of triplicate wells. Figure is representative of two independent experiments.
Figure 3.4. (+)\([\text{H}]\)Pentazocine binding in spleen homogenates is more sensitive to haloperidol if naltrexone is present to block low affinity splenic binding sites.
3.3.3. (+)-[^3H]Pentazocine binding sites are present in mouse spleen and T cell lines.

Initial experiments revealed (+)-[^3H]pentazocine binding sites in mouse spleen and three different T cell lines (Figs. 3.5 and 3.6). Binding was proportional to tissue levels up to 8mg mouse spleen and 1x10^7 T cells per well. Levels of (+)-[^3H]pentazocine binding varied among the T cell lines tested. Binding was higher in the mouse T cell lymphomas LBRM33 and EL4.IL2 compared to the human T cell leukemia Jurkat. For the reasons outlined in Section 3.4, LBRM33 cells were chosen for subsequent radioligand binding and functional assessment experiments.

3.3.4. Sigma-1 receptors are present in mouse spleen and the murine T cell line, LBRM33.

Using adjusted \( \sigma_1 \) labeling conditions ((+)-[^3H]pentazocine in the presence of 100\( \mu \)M naltrexone), saturable sites were present in mouse brain, spleen and LBRM33 cells (Fig. 3.7A). Rosenthal plots (Fig. 3.7B) of the data revealed a single binding site. Binding affinities were comparable among all three tissues, with slightly higher \( K_d \)s in immune tissues as compared to brain (Table 3.1). Numbers of binding sites (\( B_{\text{max}} \)) were much higher in LBRM33 cells with 3990 fmol/mg protein in these cells compared to 1487 fmol/mg protein for spleen and 355 fmol/mg protein (Table 3.1) for brain. The number of sites per LBRM33 cell was calculated to be 41,192 ± 6,764.

The saturable (+)-[^3H]pentazocine sites seen in mouse spleen and LBRM33 cells were determined to be \( \sigma_1 \) receptors as seen by their pharmacology (Fig. 3.8). The \( \sigma \) agonists haloperidol, DTG and (+)- and (-)-SKF 10,047 displaced (+)-[^3H]pentazocine
Figure 3.5. Specific binding sites exist for (+)-[3H]pentazocine in mouse spleen and the binding is proportional to the amount of tissue present. Splenic membranes at 0 - 8 mg per well were incubated for 2 h. with 1nM (+)-[3H]pentazocine in the absence or presence of 1μM haloperidol to determine non-specific binding. Naltrexone (100μM) was present in all wells to block binding of (+)-[3H]pentazocine to lower affinity sigma/opioid binding sites. Points represent mean specific binding ± SEM of triplicate wells at each tissue amount. Figure is representative of four separate experiments.
Figure 3.5. Specific sites for (+)-[^3]H]pentazocine exist in mouse spleen and the binding is proportional to tissue amount.
Figure 3.6. (+)-[^H]Pentazocine binding sites exist in the mouse T cell lymphomas, LBRM33 and EL4.IL2, and the human T cell leukemia, JURKAT, at varying levels. Membranes of T cell lines were prepared in 50mM potassium phosphate (pH 8.0) as described in Section 3.2.3. Membranes were incubated for 2 h. in 5nM (+)-[^H]pentazocine in the absence or presence of 1µM haloperidol to determine non-specific binding. Naltrexone (100µM) was present in all wells to block binding of (+)-[^H]pentazocine to lower affinity sigma/opioid binding sites. Points represent mean specific binding ± SEM of triplicate wells at each cell number. Figure is representative of two independent experiments.
Figure 3.6. (+)-[^3]H]Pentazocine binding sites exist in several T cell lines.
Figure 3.7. Saturable, high affinity (+)-[^3H]pentazocine labelable sites exist in mouse spleen, mouse brain and the T cell line LBRM33. Membranes of mouse spleen (4 or 5mg), brain (5mg) and LBRM33 cells (2.5x10^6 cells) were incubated for 6 h. with increasing concentrations of (+)-[^3H]pentazocine in the absence and presence of 1μM haloperidol to define nonspecific binding. Naltrexone (100μM) was present in all wells to block binding to lower affinity sigma/opioid binding sites. Each point represents the mean ± SEM of three replicates at each radioligand concentration. Fig. 3.7A -- saturation curves. Fig. 3.7B -- Rosenthal plot of the data, the triangles in the bottom left corner correspond to mouse brain. All curves fit a single site model using the program LIGAND (109).
Figure 3.7. Saturable, high affinity sites for (+)-[^3H]pentazocine exist in mouse spleen, mouse brain and the T cell line LBRM33.
Membrane homogenates were prepared and saturation binding carried out as described in Sections 3.2.3 and 3.2.4. Individual experiments were analyzed using the iterative computer curve fitting program LIGAND (109). Values (K_d and B_max) represent the mean ± SEM of the indicated number of experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>K_d (nM)</th>
<th>B_max (fmol/mg protein)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse brain</td>
<td>6.06 ± 1.52</td>
<td>355 ± 81</td>
<td>4</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>10.14 ± 0.99</td>
<td>1487 ± 47</td>
<td>3</td>
</tr>
<tr>
<td>LBRM33 T cells</td>
<td>10.70 ± 0.25</td>
<td>3990 ± 341</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.1. (+)-[^3H]Pentazocine binding sites in mouse brain, spleen and LBRM33 cells
Figure 3.8. (+)-[3H]Pentazocine-labeled sites in mouse spleen (A) and LBRM33 T cells (B) are σ₁ receptors based on their pharmacology. Sigma ligands were titrated over a concentration range known to interact with σ₁ receptors in other tissues (40, 87). Competition binding was carried out on membranes of mouse spleen (2.5 or 4mg) and LBRM33 cells (2.5x10⁶ cells). Sites were labeled with 5nM (+)-[3H]pentazocine in the presence of 100μM naltrexone. Nonspecific binding was defined by 1μM haloperidol and data are represented as mean percentage of specific binding displaced. Standard errors were ≤ 5% of means. All drugs were examined 3 - 4 times in each tissue in independent experiments.
Figure 3.8. (+)-[^3]H]Pentazocine-labeled sites in mouse spleen (A) and LBRM33 T cells (B) exhibit $\sigma_1$ receptor pharmacology.
in mouse spleen and LBRM33 cells in a rank order identical to (+)-[^3]Hpentazocine-labeled σ₁ receptors in other tissues (40). The (+)-stereoisomer of SKF 10,047 was approximately 100 fold more potent than the (-)-stereoisomer of SKF 10,047, which is a hallmark of σ₁ receptors (16). Competition binding carried out on mouse brain membranes yielded identical results (data not shown).

3.3.5. Sigma agonists suppress IL2 production in PHA-stimulated LBRM33 T cells.

The LBRM33 cell line is capable of producing IL2 when stimulated with the mitogen PHA (150). When PHA-stimulated LBRM33 cells were incubated with σ agonists, IL2 production was suppressed (Fig. 3.9). Sigma agonists fell into two groupings in regard of potency. One group, consisting of the σ agonists haloperidol, haloperidol metabolite II (or reduced haloperidol) and (-)-butaclamol was slightly more potent than the σ agonists DTG, (+)-3-PPP and the benzomorphans, pentazocine and SKF 10,047 (Fig. 3.9, Table 3.2). There was no stereoselectivity between the (+)- and (-)-stereoisomers of pentazocine and SKF 10,047. EC₅₀ values of all sigma agonists were in the 10⁻⁵ to 10⁻⁴ M range (Table 3.2). The nonselective σ antagonist BMY 14,802 (16) and the opiate antagonist naltrexone were also tested and found to have EC₅₀s greater than 10⁻³ M. Attempts to antagonize the effects of the σ agonists with BMY 14,802 were unsuccessful (data not shown).
**Figure 3.9.** Sigma agonists suppress the production of IL2 by PHA-stimulated LBRM33 T cells. LBRM33 cells, seeded at $1.5 \times 10^5$ cells per well, were exposed to various concentrations of $\sigma$ agonists in triplicate wells for 2 h. prior to addition of 5$\mu$g/ml PHA. At 24 h. post PHA addition, supernatants were harvested and IL2 levels detected by ELISA. Each drug was examined 3 - 4 times in independent experiments. Data are presented as a mean percentage of IL2 produced in the absence of drug. Standard errors were $\leq 10\%$ of mean values.
Figure 3.9. Sigma agonists suppress the production of IL2 by PHA-stimulated LBRM33 T cells.
Table 3.2. Potencies of various sigma agonists to suppress PHA-stimulated IL2 production in LBRM33 T cells.

<table>
<thead>
<tr>
<th>Druga</th>
<th>EC50 (x10^-5 M)b</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Haloperidol metabolite II</td>
<td>2.08 ± 0.67</td>
<td>3</td>
</tr>
<tr>
<td>2. Haloperidol</td>
<td>2.79 ± 1.13</td>
<td>3</td>
</tr>
<tr>
<td>3. (-)-butaclamol</td>
<td>4.14 ± 1.37</td>
<td>3</td>
</tr>
<tr>
<td>4. (-)-Pentazocine</td>
<td>12.90 ± 1.50</td>
<td>4</td>
</tr>
<tr>
<td>5. (+)-Pentazocine</td>
<td>13.50 ± 1.90</td>
<td>3</td>
</tr>
<tr>
<td>6. DTG</td>
<td>19.40 ± 4.40</td>
<td>4</td>
</tr>
<tr>
<td>7. (+)-3-PPP</td>
<td>26.10 ± 13.00</td>
<td>3</td>
</tr>
<tr>
<td>8. (-)-SKF 10,047</td>
<td>38.40 ± 5.30</td>
<td>4</td>
</tr>
<tr>
<td>9. (+)-SKF 10,047</td>
<td>46.20 ± 4.40</td>
<td>3</td>
</tr>
</tbody>
</table>

a The σ antagonist BMY14,802 and the opioid antagonist naltrexone were also tested, and found to have EC50 values greater than 10^-3 M.
b EC50 values of drugs to inhibit IL2 production in LBRM33 cells as measured by ELISA. Cells were seeded and stimulated with 5μg/ml PHA as outlined in Section 3.2.5. Values represent the concentration at which the drug inhibited 50% of IL2 produced in the absence of drug and were calculated using a least squares fit to a logarithmic-probit analysis as described in Section 3.2.9. EC50s are given as means ± SEM of the indicated number of experiments (n). Drugs are ranked from most to least potent.
3.3.6. The ability of $\sigma$ agonists to inhibit IL2 production by LBRM33 cells correlates with the pharmacology of splenic $\sigma_1$ receptors.

When the inhibitory effect of $\sigma$ agonists on IL2 production in LBRM33 cells was correlated with our previously published pharmacology of rat splenic $\sigma_1$ receptors (87), a highly significant correlation was seen ($r^2=0.71$; Fig. 3.10). The grouping of the $\sigma$ agonists around the regression line is very similar to the correlation of $\sigma_1$ $K_i$ values versus $\sigma$ agonists ability to inhibit ConA-stimulated proliferation of rat spleen cells (Fig. 3.1; 87). A correlation of the inhibitory effect of $\sigma$ agonists on proliferation with their effects on IL2 production, also revealed a highly significant relationship ($r^2=0.82$; Fig. 3.11).

ANOVA on the mean square values of both correlations showed a statistically significant correlation between these two variables (for Fig. 3.10 $F = 16.76$; for Fig. 3.11 $F = 32.09$; both $P<0.005$). All drugs fell within or on the 95% confidence interval of the regression line.

3.3.7. Suppression of IL2 production by LBRM33 cells is due to early events (first 7 - 11 h.) in culture.

As seen in figure 3.12, inhibition of IL2 by haloperidol was due to an event that occurred early in the culture period. The late addition of drug (after 7 - 11 h.) did not lead to IL2 suppression. In this experiment haloperidol was added at various times relative to the addition of PHA. Strong inhibition was seen when haloperidol was given prior to (-4 and -2 h.) or at the same time as PHA (0 h.). However, the inhibitory effect of haloperidol began to decline when added 7 h. after PHA and completely disappeared.
Figure 3.10. Pharmacology of sigma agonist-induced suppression of IL2 in LBRM33 T cells correlates with previously published drug potency at $[^3H]$haloperidol-labeled $\sigma_1$ receptors in rat spleen (87). ANOVA on the mean square values yielded a highly significant correlation between the $K_i$ and EC$_{50}$ values ($F = 16.76; P<0.005$). Numbers correspond to drugs listed in table 3.2.
Figure 3.10. Pharmacology of $\sigma$ agonist-induced suppression of IL2 production by LBRM33 T cells correlates with drug potency at $\sigma_1$ receptors in rat spleen. Numbers correspond to drugs listed in table 3.2.
Figure 3.11. Pharmacology of sigma agonist-induced suppression of IL2 in LBRM33 T cells correlates with the ability of σ agonists to inhibit ConA-induced proliferation of rat spleen cells (previously published in 87). ANOVA on the mean square values yielded a highly significant correlation between these two variables (F = 32.09; P<0.005). Numbers correspond to drugs listed in table 3.2.
Figure 3.11. Pharmacology of \( \sigma \) agonist-induced suppression of IL2 production by LBRM33 T cells correlates with ability to inhibit ConA-induced proliferation of rat spleen cells. Numbers correspond to drugs listed in table 3.2.
Figure 3.12. Time course of σ agonist-induced suppression of IL2 production in LBRM33 T cells - inhibitory events occur during the first 11 h. of culture. LBRM cells, seeded at 1.5x10^5 cells per well, were exposed to different concentrations of haloperidol in triplicate wells for various times relative to PHA addition. PHA at 5μg/ml was added to all wells at 0 hours. Haloperidol was added 4 and 2 h. before PHA addition, at the same time as PHA addition (0 h.), and at 7, 14 and 20 h. after PHA addition. At 24 h. the supernatants in all wells were harvested and IL2 levels determined by ELISA. Points represent mean IL2 amounts ± SEM at each time point and concentration. Figure is representative of two experiments performed under identical conditions.
Figure 3.12. Time course of $\sigma$ agonist-induced suppression of IL2 production in LBRM33 T cells - inhibitory events occur during the first 11 h. of culture.
when added late in the culture period (14 and 20 h. post PHA addition). The effect was
dose related as $10^{-4}$ M haloperidol could be given later in culture (7 h. post PHA addition)
and still cause inhibition; whereas the addition of $10^{-5}$ M haloperidol at this time caused
only slight inhibition. The decrease in IL2 levels seen with no drug at 7 h. post PHA
addition was probably due to the cells in these culture wells being exposed to a higher,
possibly detrimental concentration of PHA for 7 - 20 h. Because the volumes of the
cultures were increased by the addition of haloperidol, the concentration of stimulating
PHA (initially 5 $\mu$g/ml) was reduced at the time of drug addition. Therefore, the cells
receiving drug at -4, -2 and 0 h. spent less time in the presence of the higher PHA
concentrations.

3.3.8. The $\sigma$-agonist mediated inhibition of IL2 production by LBRM33 cells correlates
with induction of cell death.

Sigma agonists inhibited IL2 production and/or release in LBRM33 cells at high
drug concentrations, $10^{-5}$ M and above. When LBRM33 cells, incubated in the presence
of the $\sigma$ agonists haloperidol and (+)-pentazocine for 24 h., were examined for viability
by trypan blue dye exclusion it was found that these drugs killed the cells at
concentrations just slightly higher than their EC$_{50}$s to suppress IL2 (Fig. 3.13). To
examine this more closely we elected to look at the release of LDH as a measure of
toxicity. These experiments were carried out in the absence of PHA, which, in addition
to its IL2-stimulating effect, causes apparent cell death in LBRM33 cells (150). Both (+)-
pentazocine and haloperidol caused a dose dependent increase in LDH in the culture
medium of LBRM33 cells exposed to drug for 24 hours (Fig. 3.14). The toxic effect of
haloperidol was more potent than that observed with (+)-pentazocine, which was
Figure 3.13. Sigma agonists induce cell death in LBRM33 T cells as measured by trypan blue dye exclusion. LBRM33 cells, seeded in 24 well plates at 1x10^5 cells per well, were exposed to haloperidol and (+)pentazocine for 24 h. After 24 h, cells were harvested by scraping, and viability determined using 0.04% trypan blue. The figure shown is representative of two experiments and each point represents the mean of two determinations. Standard errors were less than 7%.
Figure 3.13. Sigma agonists induce cell death of LBRM33 T cells as measured by trypan blue dye exclusion.
Figure 3.14. Sigma agonists induce cell death of LBRM33 T cells as measured by LDH release. LBRM33 cells, seeded at $1.5 \times 10^5$ cells per well, were incubated with haloperidol and (+)-pentazocine in triplicate wells for 24 h. Supernatants were harvested and LDH release was determined by a colorimetric assay (Sigma) described in Section 3.2.8. Maximal LDH release was determined by lysis of cells with Triton X-100 and data are presented as mean percentage of maximum LDH released. Standard errors are $\leq 5\%$ of mean values. Each drug was examined three times in separate experiments.
Figure 3.14. Sigma agonists induce cell death of LBRM33 T cells as measured by release of lactate dehydrogenase (LDH).
<table>
<thead>
<tr>
<th>Drug</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; to suppress IL2 (M)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; to release LDH (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79x10^-5 ± 1.13 (3)</td>
<td>1.17x10^-5 ± 0.24 (3)</td>
</tr>
<tr>
<td>(+)-Pentazocine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.35x10^-4 ± 0.20 (3)</td>
<td>1.73x10^-4 ± 0.10 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> EC<sub>50</sub> values of haloperidol and (+)-pentazocine to inhibit PHA-stimulated IL2 production as measured by ELISA. EC<sub>50</sub> values in the induction of LDH release as measured by a colorimetric assay (Sigma) described in Section 3.2.8. EC<sub>50</sub> values for IL2 suppression were calculated as described in table 3.2 and Section 3.2.9.. The EC<sub>50</sub> values for induction of LDH release were calculated similarly by converting the percent maximal LDH release values to a logarithmic-probit equation. EC<sub>50</sub> values are given in molar means ± SEM of the number of experiments indicated in parentheses.

<sup>b</sup> No significant difference in EC<sub>50</sub> values for haloperidol (P = 0.28, paired T test).

<sup>c</sup> No significant difference in EC<sub>50</sub> values for (+)-pentazocine (P = 0.32, paired T test)

Table 3.3. Sigma agonist suppression of IL2 in LBRM33 cells correlates with induction of cell death
consistent with effects on IL2 production. In addition, there was agreement between the
dose of haloperidol and (+)-pentazocine required to inhibit IL2 and to cause release of
LDH (Table 3.3).

3.3.9. The σ agonists haloperidol and (+)-pentazocine inhibit the proliferation of ConA-
stimulated mouse spleen cells.

To determine whether the toxic effect of haloperidol and (+)-pentazocine was
specific to the LBRM33 cell line, we examined the effect of σ agonists on mouse spleen
cells. As expected, haloperidol and (+)-pentazocine inhibited ConA-induced proliferation
of mouse spleen cells at 48 h. in a dose dependent manner (Fig. 3.15). Haloperidol was
slightly more potent than (+)pentazocine, consistent with previously observed effects on
ConA-stimulated rat spleen cell proliferation (87). However, unlike LBRM33 cells,
inhibition of proliferation in mouse spleen cells was not due to a toxic effect as measured
by release of LDH (Fig. 3.16). Importantly, it could be demonstrated by Triton X-100
lysis that LDH was present in these cells. When cells incubated at the highest drug
concentrations (10^-4 M) were lysed with Triton X-100, LDH levels significantly above
baseline were attained (data not shown). Therefore, it is likely that the toxic effect of σ
agonists on LBRM33 cells are a characteristic of the transformed cell line, and is not the
mechanism of σ1 receptor modulation of mouse and rat spleen cell proliferation (Fig.
3.15; 87).
3.3.10. Haloperidol and (+)-pentazocine have differential effects on IL2 production by ConA-stimulated mouse spleen cells.

The σ agonists (+)-pentazocine and haloperidol inhibited ConA-stimulated proliferation of mouse spleen cells (Fig. 3.15). Therefore, the effect of haloperidol and (+)-pentazocine on IL2 production in these cells was also examined. The supernatants of ConA-stimulated mouse spleen cell cultures were harvested 24 and 48 h. after addition of ConA, and assayed for IL2 by ELISA. At 24 h., haloperidol inhibited IL2 production/release at 5x10^-5 and 10^-4 M, whereas (+)-pentazocine had only a slight inhibitory effect at the highest concentration of drug examined (Fig. 3.17). LDH levels in 24 h. supernatants were not increased at the highest drug concentrations used (data not shown). At 48 h., there was much less IL2 in the culture medium, and wells incubated with (+)-pentazocine exhibited a marked enhancement of IL2 over baseline (Fig. 3.18). However, the levels of IL2 attained with higher concentrations of (+)-pentazocine did not represent an increase over the baseline IL2 amounts seen in the 24 h. cultures (Fig. 3.17). Forty-eight hour cultures treated with haloperidol showed a consistent increase in IL2 at 10^-5 M haloperidol, but a slight decrease in IL2 at higher drug concentration (Fig. 3.18). The significance of this decrease is difficult to interpret, however, given the overall reduction of IL2 present at 48 h. vs. 24 h. (Fig. 3.18 vs. Fig. 3.17). Taken as a whole, these experiments (Figs. 3.15, 3.17 and 3.18) suggest a difference in the mechanisms of action of haloperidol and (+)-pentazocine in the modulation of ConA-stimulated mouse spleen cell function.
Figure 3.15. Proliferation of ConA-stimulated mouse spleen cells is inhibited by the σ agonists haloperidol and (+)-pentazocine. Mouse spleen cells were obtained by density gradient separation from male C57Bl/6J mice as outlined in Section 3.2.6. Cells were seeded into 96-well tissue culture plates at $2 \times 10^5$ cells per well and exposed to drugs for 2 h. prior to addition of 2.5 or 5 μg/ml ConA. Cultures were incubated in a 5% CO$_2$ environment at 37° C for 48 h. $[^3]$HThymidine was added for the last 6 h. prior to harvest on to glass fiber filters. Radioactivity was determined by liquid scintillation counting. Data are expressed as mean percentage ± SEM of the $[^3]$Hthymidine incorporation in control wells. Figure is representative of 3 - 4 independent experiments.
Figure 3.15. Proliferation of ConA-stimulated mouse spleen cells is inhibited by the $\sigma$ agonists haloperidol and (+)-pentazocine.
Figure 3.16. The sigma agonists haloperidol and (+)-pentazocine do not cause release of LDH from mouse spleen cells. Spleen cells were obtained by density gradient separation from male C57Bl/6J mice as outlined in Section 3.2.6. Cells were seeded in triplicate wells at $2 \times 10^5$ cells per well and exposed to drugs for 2 h. prior to addition of 2.5 or 5 $\mu$g/ml ConA. After 48 h. supernatants were harvested and LDH release was determined by a colorimetric assay (Sigma) described in Section 3.2.8. Maximal LDH release was determined by lysis of cells with Triton X-100 and data are presented as mean percentage ± SEM of maximum LDH released. The data shown are from two separate experiments, accounting for the difference in baseline LDH levels. Each drug was examined 3 - 4 times in independent experiments.
Figure 3.16. The sigma agonists haloperidol and (+)-pentazocine do not cause release of LDH from mouse spleen cells.
Figure 3.17. Effect of σ agonists on mouse spleen cells stimulated with ConA for 24 hours. IL2 production was inhibited by haloperidol, but only slightly by (+)-pentazocine. Mouse spleen cells were obtained by density gradient separation as outlined in Section 3.2.6. Cells were seeded in 96-well tissue culture plates at $2 \times 10^5$ cells per well and exposed to drugs for 2 h. prior to addition of 2.5 μg/ml ConA. After 24 h., supernatants were harvested and IL2 levels determined by ELISA. Data are expressed as mean IL2 ± SEM of triplicate wells. Figure is representative of three independent experiments.
Figure 3.17. Effect of $\alpha$ agonists on mouse spleen cells stimulated with ConA for 24 hours. IL2 production was inhibited by haloperidol, but only slightly by (+)-pentazocine.
Figure 3.18. Effect of σ agonists on mouse spleen cells stimulated with ConA for 48 hours. IL2 production was enhanced at $10^{-5}$ M haloperidol and inhibited by higher doses of haloperidol. Levels were increased in cells exposed to (+)-pentazocine. With the exception of the incubation time, experiments were performed exactly as in figure 3.17. After 48 h supernatants were harvested and IL2 levels determined by ELISA. Data are expressed as mean IL2 ± SEM of triplicate wells. Figure is representative of five independent experiments.
Figure 3.18. Effect of σ agonists on mouse spleen cells stimulated with ConA for 48 hours. IL2 production was inhibited by high doses of haloperidol. Levels were increased in cells exposed to high concentrations of (+)-pentazocine.
3.4. Summary and discussion

Sigma-1 binding sites can inhibit the proliferation of rat spleen cells stimulated with the T cell mitogen ConA (Fig. 3.1; 87). The objective of the current study was to further examine the role σ₁ receptors have in inhibiting T cell functions. To this end we identified σ₁ sites on the T cell line LBRM33 and demonstrated that σ₁ sites on these cells inhibit IL-2 production, an effect that appeared to be due to cell death. This toxic effect may be limited to cell lines, however, because exposure of ConA-stimulated mouse spleen cells to the σ agonists haloperidol and (+)-pentazocine did not result in cell death, but still decreased the proliferative capabilities of these cells.

We first attempted to identify σ₁ receptors on T cells. Although our previous work utilized [³H]haloperidol to label splenic σ₁ sites (87), we wanted to label these receptors in immune tissues and T cell lines with the more σ₁-selective ligand (+)-[³H]pentazocine because this radioligand had become the most accepted, specific label for σ₁ receptors in the CNS (34, 40). However, using (+)-[³H]pentazocine (in the presence or absence of (+)-SKF 10,047 to define nonspecific binding) in saturation assays we could not identify saturable sites with comparable affinities to σ₁ receptors previously identified by [³H]haloperidol in immune tissues (Fig. 3.1; 11, 12, 87). This was in disagreement with a previous study performed by Carr and co-workers (89), who reported saturable sites for (+)-[³H]pentazocine in enriched murine T and B lymphocytes. The reported binding affinities of these sites were in the 300 - 400nM range. They were sensitive to κ opioid ligands and were relatively insensitive to haloperidol (89). Sigma binding sites are classified as high affinity, non-opioid and haloperidol-sensitive (34).
Therefore, it appears that the sites labeled by Carr et al. (89) were not true \( \sigma \) sites and may represent binding of \((+)-[\text{H}]\)pentazocine to opioid receptors or the haloperidol/DTG-inaccessible sites described in chapter two.

In preliminary competition binding assays carried out in rat spleen using \((+)-[\text{H}]\)pentazocine, a large portion of haloperidol inaccessible binding was seen (Fig. 3.3). This indicated that \((+)-[\text{H}]\)pentazocine was probably interacting at the previously described low affinity, high capacity haloperidol/DTG-inaccessible sites (149), thus preventing specific labeling of \( \sigma_1 \) receptors.

The opioid antagonist naltrexone exhibits a relatively high affinity at haloperidol/DTG-inaccessible sites (Chapter 2; 149), but exhibits little or no affinity at \( \sigma \) binding sites (16). Therefore, we elected to use naltrexone to occupy lower affinity sites in the tissue, thereby allowing \((+)-[\text{H}]\)pentazocine to bind to \( \sigma_1 \) sites with high specificity. This is demonstrated in figure 3.4. As can be seen in this experiment carried out on splenic homogenates, the presence of naltrexone increases the selectivity of \((+)-[\text{H}]\)pentazocine for haloperidol-sensitive sites (compare bars 1,4, 5 and 6, Fig. 3.4). These results implied that in immune tissues we could label \( \sigma_1 \) sites by using naltrexone along with \((+)-[\text{H}]\)pentazocine in the absence or presence of haloperidol to define nonspecific binding.

In this manner we identified specific \((+)-[\text{H}]\)pentazocine binding sites in mouse spleen, the murine T cell lines EL4.IL2 and LBRM33 clone 4A2, and the human T cell line Jurkat (Figs. 3.5 and 3.6). These T cell lines were chosen for their ability to produce IL2 when exposed to a variety of stimuli. For further receptor characterization and functional studies we selected the LBRM33 cell line for several reasons. One, we consistently saw higher levels of \((+)-[\text{H}]\)pentazocine binding in these cells (Fig. 3.6). Two, \((+)-[\text{H}]\)pentazocine binding sites in the EL4.IL2 cell line did not appear to be
saturable and of high affinity (data not shown). Three, the high number of Jurkat cells required for adequate levels of (+)-[^H]pentazocine binding (1x10^7 per well, Fig. 3.6) was prohibitive for performing experiments on a daily basis.

In subsequent saturation binding assays, saturable, high affinity sites were found in mouse brain, spleen and LBRM33 cells (Fig. 3.7). Binding affinities were comparable among all three tissues tested and were consistent with values previously seen for σ₁ receptors in other tissues (40, 87). Kᵓ values were slightly higher in spleen and LBRM33 cells (Table 3.1). Relative numbers of binding sites were 2.5 to 10 fold higher in the LBRM33 T cells than in either mouse brain or spleen, indicating a probable enrichment of σ₁ sites in T cells (Table 3.1). Competition binding assays carried out on mouse brain (not shown), mouse spleen (Fig. 3.8A) and LBRM33 cells (Fig. 3.8B) indicated a σ₁ pharmacology. (+)-[^H]Pentazocine-labeled sites were sensitive to haloperidol and DTG, and favored the (+)- over the (-)-stereoisomer of SKF 10,047 (Fig. 3.8). This is strong evidence that we identified σ₁ receptors in immune tissues using our current methodology.

The finding that σ₁ binding sites are enriched on T cells is not surprising in light of recent autoradiographic studies in rat spleen using the same labeling conditions outlined in the current study (91). In these studies we found that σ₁ sites were present in the lymphocyte areas of the spleen, with a relative enrichment in the T cell areas of the white pulp (91). This finding, along with the current data (Table 3.1), suggests that higher numbers of σ₁ sites reside on T cells than other immune cell types. However, it must be noted that other studies have indicated that high numbers of σ binding sites are present on a variety of tumors and tumor cell lines (25, 26, 38). In addition, Bem and colleagues (26) have shown that σ binding sites appear to be upregulated in human tumor tissue compared to normal tissue from the same individuals, suggesting overexpression.
of σ receptors in malignant cells. Therefore, the high numbers of σ₁ sites on LBRM33 T cells may be due to either the transformed nature of these cells or their T cell phenotype.

LBRM33 cells can be stimulated to produce IL2 upon exposure to the mitogen PHA. Incubation of cells with σ agonists inhibited PHA-induced production of IL2 in a dose dependent manner (Fig. 3.9). All σ ligands tested inhibited IL2 production with the exception of the putative σ antagonist BMY 14,802 (Table 3.2). BMY 14,802, while originally defined as a σ antagonist, exhibits nonspecific agonist-like properties in many functional studies (16). BMY 14,802 did not abrogate σ agonist mediated inhibition, indicating that this drug did not have σ₁ antagonistic properties in these cells (data not shown). High concentrations of σ agonists were required for IL2 inhibition, with EC₅₀ values in the 10⁻⁵ - 10⁻⁴ M range (Table 3.2). This inhibition appears to be mediated via σ₁ receptors, because when σ agonist EC₅₀ values were correlated with previously determined Kᵢ values at σ₁ receptors (87), a highly significant correlation was seen (Fig. 3.10; P<0.005). In addition, a strong relationship was seen between the inhibition of IL2 production by LBRM33 cells and our previous experiments showing σ agonist-induced inhibition of ConA-stimulated rat spleen cell proliferation (Fig. 3.11; 87). This suggests that σ agonists were acting at the same site to inhibit both of these activities.

No stereoselectivity was seen between the (+)- and (-)-isomers of SKF 10,047 and pentazocine in inhibition of IL2 production in LBRM33 cells (Table 3.2). This is contradictory to what was expected given the preference for the (+)- over the (-)-stereoisomers of these compounds at σ₁ binding sites, and suggests the existence of additional sites of action for these compounds on LBRM33 cells. The (+)- isomers of SKF 10,047 and pentazocine were slightly more potent than the (-)-isomers in inhibiting ConA-induced proliferation of rat spleen cells, however, this difference was small and did not reflect the difference in potency seen in competition binding assays (87).
weak or nonexistent stereoselectivity has been observed in other bioassays of \( \sigma \) receptor function (48, 51, 151). The lack of stereoselectivity in our assay probably does not represent interaction of (-)-pentazocine or (-)-SKF 10,047 with opioid receptors, as preincubation of LBRM33 cells with the opioid antagonist naltrexone did not abrogate the ability of these compounds to inhibit IL2 production (data not shown). Sigma-2 receptors exhibit reversed stereoselectivity for SKF 10,047 and pentazocine, favoring the (-)- over the (+)-isomers (16, 27). Therefore, the lack of stereoselectivity could represent a contribution of \( \sigma_2 \) sites in agonist-mediated inhibition of IL2 production. At present, we have no evidence for this. It should be emphasized, however, that both the (+)- and (-)-stereoisomers of these compounds fell at or within the limits of our correlation. Therefore based on our data, no other sites of action, besides \( \sigma_1 \), are predicted to modulate the inhibition of LBRM33 cell function.

The concentrations of \( \sigma \) agonists required to inhibit IL2 production in LBRM33 cells were approximately 100 - 1000 fold higher than the affinity of these compounds at \( \sigma_1 \) binding sites. This is common to \( \sigma \) receptor bioassays in many different tissues (16, 48, 87, 89, 151). The reason for this is unclear; however, some possible explanations include: 1) Over the course of the incubation time \( \sigma \) agonists may be metabolically inactivated, thereby requiring higher initial drug concentrations to maintain an effect. 2) Sigma sites may reside inside the cell as well as on the cell surface (82, 152). If biologically active \( \sigma_1 \) sites reside within cells, and cell membranes are not completely permeable to \( \sigma \) agonists, the external drug concentration required to occupy a significant number of sites would be higher. 3) Sigma-1 binding sites may require a high percentage occupancy in order to trigger a biological function. We have no evidence to
support or rule out any of these possibilities, however the recent finding that the \( \sigma_1 \) binding site may be an intracellular molecule involved in sterol synthesis favors suggestions 2 and 3 (82, 85).

While higher \( EC_{50} \) than \( K_i \) values appear to be a fairly consistent phenomenon in \( \sigma \) bioassays (16, 87, 89), concentrations required for inhibition of IL2 in LBRM33 cells were approximately 10 - 100 fold higher than needed to inhibit ConA-induced proliferation of rat spleen cells. This led us to examine potential drug toxicity in this cell line. Exposure of LBRM33 cells to the \( \sigma \) agonists haloperidol or (+)-pentazocine resulted in cell death as measured by trypan blue dye exclusion and release of LDH (Figs. 3.13 and 3.14). The effective concentrations of these drugs to inhibit IL2 production and release LDH were statistically identical (Table 3.3), which leads us to conclude that \( \sigma \) agonist suppression of IL2 in LBRM33 cells is due to induction of death in these cells. However, two caveats to this conclusion must be stated. One, we did not test all the \( \sigma \) agonists used in the IL2 assays, therefore some of these drugs could have been acting via mechanisms other than toxicity to inhibit IL2. Two, the LDH determinations were carried out on LBRM33 cells that had not been exposed to PHA. PHA, in addition to stimulating IL2 production, also causes these cells to become 'leaky' (150). This was prohibitive to cell death experiments because exposure to PHA resulted in large amounts of LDH being released into the culture supernatants. Therefore, while it appears that \( \sigma \) agonists shut down IL2 production indirectly via cell death, it is possible that in PHA-stimulated cells exposure to drug may have inhibited IL2 production via other mechanisms.

Although \( \sigma \) agonist-induced inhibition of LBRM33 cell function was mediated via cell death, the correlation of biological effect with \( \sigma_1 \) binding affinity suggests that this agonist-mediated cell death is due to interaction at \( \sigma_1 \) receptors (Fig. 3.10 and 3.11). There is precedent for this scenario. Vilner and colleagues (153), in addition to
identifying σ binding sites on a variety of neuronal and non-neuronal tumor cell lines (38), have shown that, in many of these same cell lines, certain σ agonists can cause cytotoxic effects (cell shrinkage and growth inhibition). In this study haloperidol and reduced haloperidol (metabolite II) were the most potent σ agonists in eliciting toxic effects (153). This agrees well with the present study in LBRM33 cells, where haloperidol and haloperidol metabolite II were the most potent inhibitory compounds tested (Table 3.2). However, in contrast to our findings, Vilner and associates (153, 154) found that (+)-pentazocine did not elicit a robust toxic effect in C6 glioma cells. No cell lines of immune origin were examined in this report (153). In addition, Brent and Pang (151) demonstrated that several σ ligands, including (+)-pentazocine, could inhibit the proliferation of mammary and colon carcinoma cells. Haloperidol was clearly more potent than (+)-pentazocine in inhibiting carcinoma cell proliferation (151), which agrees with our findings in this (Figs. 3.9 and 3.15) and previous studies (87). The authors speculated, based on cell appearance, that inhibition of proliferation by σ agonists could be due to induction of apoptosis in these cells (151). The apoptosis-inducing property of one σ agonist, reduced haloperidol (metabolite II), has been subsequently verified in mammary adenocarcinoma cells (66). While apoptosis is an intriguing explanation for the effects we observed in LBRM33 cells, the toxic effects of haloperidol and (+)-pentazocine were characterized by a loss of membrane integrity at 24 h., indicating that if these drugs were causing apoptosis it was being induced early in the culture period and was in the late stages by 24 h. The timing requirements for drug addition vs. inhibition of IL2 production by LBRM33 cells (Fig. 3.12) suggest that this could be the case. Our findings in LBRM33 cells, combined with the studies outlined above, provide evidence for toxic effects in some cells being mediated via σ binding sites.
Exposure of LBRM33 cells to σ agonists resulted in cell death; therefore, we elected to examine the specificity of this effect by examining murine spleen cells. We chose mouse spleen cells because σ₁ sites are prevalent in mouse spleen (Fig. 3.7; 91) and the LBRM33 T cell line is a murine cell line. As expected, ConA-induced proliferation of mouse spleen cells was inhibited by the σ agonists haloperidol and (+)-pentazocine (Fig. 3.15). This effect was not due to disruption of cell membranes as measured by LDH release (Fig. 3.16). The concentrations of haloperidol and (+)-pentazocine required to inhibit proliferation in these cells were higher than those required to inhibit rat spleen cell proliferation (87), with estimated EC₅₀s of 5x10⁻⁵ and 5x10⁻⁴ M, respectively. EC₅₀s for these compounds in inhibition of rat spleen cell proliferation were approximately 10 fold lower (87). This may represent a species-specific difference, or may be due to the higher levels of ConA used in the current study, 2.5 or 5μg/ml versus 0.5μg/ml used to stimulate rat cells (87). These levels induced near maximal proliferation, therefore possibly requiring more drug to effectively inhibit proliferation. These levels of ConA were necessary to induce adequate, measurable levels of IL2.

When the effect of haloperidol and (+)-pentazocine on IL2 production in mouse spleen cells was examined, differing effects were seen. Haloperidol caused a dose-dependent decrease in IL2 production at 24 h., whereas no effect was seen with (+)-pentazocine at this time, except for slight inhibition at the highest concentration tested, 10⁻⁴ M (Fig. 3.16). At 48 h. of incubation, baseline IL2 levels were much lower. However, (+)-pentazocine caused an increase of IL2 over baseline (Fig. 3.18). With haloperidol, a consistent spike in IL2 was seen at 10⁻⁵ M, with subsequent ablation of IL2 at 10⁻⁴ M (Fig 3.18). It appears, however, that this increase in IL2 is likely due to
decreased usage of IL2, rather than true enhancement, since baseline IL2 levels at 48 h. were low and IL2 in drug exposed cells was not increased over amounts seen at 24 h. (compare Figs. 3.17 and 3.18).

Taken together, these data suggest a difference in mechanism between haloperidol and (+)-pentazocine, with haloperidol causing a decrease in IL2 and proliferation, and (+)-pentazocine causing a decrease in proliferation, but no effect on IL2 production. However, it should be noted that the only concentrations of haloperidol that significantly decreased IL2 production (Fig. 3.17) are concentrations that resulted in total inhibition of proliferation in these cells (Fig. 3.18). Therefore, both drugs were more potent in inhibiting proliferation than in inhibiting IL2 production. This difference in potency suggests that the suppression of proliferation may be independent of effects on IL2 production.

Therefore, with the possible exception of the highest concentrations of haloperidol, the mechanism of proliferation inhibition observed in these studies is unclear. The lack of effect on IL2 production (Figs. 3.17 and 3.18) indicates that the initial mitogen-induced signaling cascade is not affected in T cells. At first look this would appear to disagree with the inhibitory effects seen in LBRM33 cells, which seemed to be due to a relatively early event in culture; however, inhibition was seen when drugs were added up to 7 h. after PHA which indicates that the initial signaling events may not have been affected in these cells. These drugs could be acting to inhibit the IL2 signaling cascade leading to proliferation, or could be affecting the cell cycle machinery in some manner. The lack of a toxic effect of σ agonists on mouse spleen cells agrees with our previous study in rat spleen cells (87). Rat spleen cells exposed to σ agonists were not permeable to trypan blue dye after 48 h. incubation and could be restimulated after removal of agonists (87). We cannot rule out, however, that in the current study σ agonists were causing apoptotic cell death, especially in light of the recent data suggesting
That σ agonists may cause apoptosis (66, 151). Regardless of the inhibitory mechanism, it is clear that σ agonists are modulating T cell activity via σ1 binding sites (Figs. 3.10, 3.11 and 3.15; 87).

T lymphocytes appear to be the predominant immune cell type that display σ1 binding sites (Fig. 3.7, Table 3.1; 91). We speculate that these sites are directly interacting with σ agonists to modulate proliferation. However, because we cannot demonstrate a specific inhibitory effect on IL2 either in T cell lines or mouse spleen cells, it is possible that these drugs are not acting directly on T cells to mediate their anti-proliferative action. At this time we cannot rule out the possibility that accessory cells could be affected by σ agonists, thereby inhibiting co-stimulatory mechanisms necessary for effective T cell proliferation. In summary, the main contributions of the experiments contained in this chapter are:

(1) This study establishes that σ1 binding sites are present on T cells, as determined by using (+)-[3H]pentazocine under σ1 labeling conditions selective for immune tissues (Figs. 3.6 - 3.8). Sigma-1 sites are present in the T cell line LBRM33 and, in addition, σ1 sites are concentrated in the T cell areas of the white pulp (91), which indicates σ1 sites are present on T cells in vivo.

(2) Sigma agonists inhibit IL2 production in LBRM33 T cells, apparently due to cell death. Inhibition of IL2/induction of cell death correlated highly with the pharmacology of σ1 binding sites (Figs. 3.10 and 3.11) suggesting an effect mediated via σ1 receptors on LBRM33 cells. The toxicity observed in these cells disagrees
with previous studies in rat spleen cells (87) and current studies in mouse spleen cells (Fig. 3.16), where no cell death, as determined by trypan blue exclusion or LDH release, was seen. This may be indicative of a differential effect on transformed cell lines versus \textit{ex vivo} cells.

(3) The \(\sigma\) agonist-induced inhibition of ConA-stimulated spleen cell proliferation is probably largely independent of IL2 production.

Therefore \(\sigma_1\) sites are present on T cells and modulate cell proliferation, although the physiological mechanism of this, and cell physiology of \(\sigma_1\) binding sites in general, remains to be determined.
CHAPTER 4

General Discussion

Since their discovery in 1976, the true nature of sigma binding sites has eluded scientists. Most studies investigating the possible role for these sites in physiology have concentrated on the CNS, where the sites were initially identified. The discovery of sigma sites outside the CNS led investigators to examine the possible function of σ sites in other areas such as muscle physiology, endocrine and immune function (for reviews see 155). In our research we have worked to understand the role of these novel, complex and heterogenous binding sites in the immune system. In this volume we focused on the identification, cellular location and function of novel haloperidol/DTG-inaccessible sites and σ₁ sites in immune tissues (see table 4.1 for summary).

Novel opioid-like haloperidol/DTG-inaccessible sites

In chapter two we outlined the identification of a novel binding site for σ ligands in rat spleen and human blood that was not present in brain. These low affinity, high capacity sites bound all σ agonists tested with the exception of the high affinity σ ligands, haloperidol and DTG. Sigma sites are defined as haloperidol-sensitive (34), thus these sites do not fit the definition of σ receptors. In addition, the haloperidol-inaccessible sites
<table>
<thead>
<tr>
<th></th>
<th>Sigma-1 sites</th>
<th>Haloperidol/DTG-inaccessible sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labeling Conditions</strong></td>
<td>(+)-[^H]-pentazocine (in the presence of naltrexone)</td>
<td>(+)-[^H]-3-PPP (in the presence of haloperidol)</td>
</tr>
<tr>
<td></td>
<td>+/- haloperidol to define nonspecific</td>
<td>+/- SKF 10,047 to define nonspecific</td>
</tr>
<tr>
<td><strong>Discriminant Ligands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>high affinity</td>
<td>inactive</td>
</tr>
<tr>
<td>DTG</td>
<td>moderate to high affinity</td>
<td>inactive</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>inactive</td>
<td>moderate affinity</td>
</tr>
<tr>
<td><strong>Nondiscriminant Ligands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-Pentazocine</td>
<td>high affinity</td>
<td>moderate affinity</td>
</tr>
<tr>
<td>(+)-SKF 10,047</td>
<td>high to moderate affinity</td>
<td>moderate affinity</td>
</tr>
<tr>
<td>PCP</td>
<td>low affinity</td>
<td>low affinity</td>
</tr>
<tr>
<td><strong>Molecular nature</strong></td>
<td>223 aa transmembrane protein/25kDa[^a]</td>
<td>unknown/sensitive to PLC and heat</td>
</tr>
<tr>
<td></td>
<td>homologous to fungal sterol isomerase</td>
<td></td>
</tr>
<tr>
<td><strong>Immune tissue locations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rodent spleen and spleen cells;</td>
<td>rat spleen and spleen cells; human PBL; rat marginal zone macrophages?; most likely on human granulocytes</td>
</tr>
<tr>
<td></td>
<td>human PBL; LBRM33 T cell line; possibly other T and B cell lines</td>
<td></td>
</tr>
<tr>
<td><strong>Immune Functions</strong></td>
<td>inhibits proliferation of ConA-stimulated rat and mouse spleen cells; may inhibit NK cells indirectly via CNS action; causes cytotoxicity in LBRM33 T cells</td>
<td>unknown - role in innate immune function?</td>
</tr>
</tbody>
</table>

[^a] This data comes from a cDNA sequence obtained from guinea pig liver, not from immune tissues. mRNA present in guinea pig spleen (82). The information in this table represents work reported in this document, previous studies (11, 12, 87, 91, 111) and unpublished data (Wolfe, Liu and Whitlock).

Table 4.1. Characteristics of sigma-1 and haloperidol/DTG-inaccessible sites in immune tissues.
bound the opioid antagonist naltrexone, favored the (-)-stereoisomers of the benzomorphans SKF 10,047 and pentazocine over their (+)-isomers and bound the morphinan derivatives dextromethorphan and dextrorphan (Table 2.2). All of these characteristics point to the possible opioid-like nature of these sites.

While these sites are not σ binding sites, the identification and description of these novel sites has significant implications for the σ receptor field. As demonstrated in our studies (Fig. 3.2), the presence of this site is prohibitive for the identification of σ₁ sites in immune tissues, and they must be blocked by saturating concentrations of naltrexone to allow specific σ labeling with [³H]benzomorphan and morphinan compounds. Also, the probable location of this site in other peripheral tissues (Wolfe and DeSouza, unpublished observation) suggests that it may interfere with σ binding assays in other tissues as well. These sites may also play some role in σ bioassays, both indirectly and directly. The high number (Table 2.1) and apparent irreversibility of binding to these sites (Fig. 2.6) may contribute indirectly to effects seen with some σ agonists because the sites may tie up compounds, thus impeding their interaction with other receptors. Interestingly, the σ agonists that exhibited no affinity at these sites, haloperidol and DTG, are the most potent agonists at inhibiting ConA-stimulated proliferation of rat spleen cells (87). In contrast, the σ agonists with a relatively high affinity for these sites, pentazocine and SKF 10,047, are less potent in ConA-assays (87). If the haloperidol/DTG-inaccessible sites were present in these cultures, they may have contributed to lower the potency of pentazocine and SKF 10,047 by effectively removing these compounds from solution. In addition, these binding sites may be functional entities, thus they may represent yet another site that must be taken into account when using these drugs. The fact that many σ agonists modulate biological responses at concentrations in the μM range (16, 48, 89, 151), levels sufficient to interact with these novel sites, suggests that the haloperidol/DTG-inaccessible sites could contribute to the effects observed in some bioassays.
The low affinity and high numbers of haloperidol/DTG-inaccessible sites in spleen, combined with the lack of haloperidol-inaccessible (+)-[^3]H)-3-PPP binding in brain (Fig. 2.7), suggests that these sites are not classical \( \mu, \delta \) or \( \kappa \) opioid receptors. However, these sites may be related to lower affinity sites for opiate alkaloid compounds present in immune tissues (139, 140, 143). In addition, these sites may have contributed to the difficulty in identifying classical opioid receptors on immune cells (129). Saturable opioid receptors have been difficult to identify on immune cells using opiate alkaloids such as [^3]H)morphine and [^3]H]naloxone (129). The presence of low affinity haloperidol/DTG-inaccessible sites may have contributed to the lack of saturability and high nonspecific binding in immune tissues.

The absence of haloperidol/DTG-inaccessible sites on T and B cell lines, the lack of binding in the white pulp of rat spleen, and the lack of binding site enrichment in isolated splenic mononuclear cells suggests this site is not present on lymphocytes. The presence of high numbers of these sites in purified human PMN preparations points to their probable existence on granulocytes (Fig. 2.19). The possibility of these sites being present on PMNs is intriguing given the evidence for nontraditional opiate mediated effects on granulocytes. Makman and colleagues (143) found that morphine and other opiate alkaloids, but not opioid peptides, could inhibit tumor necrosis factor \( \alpha \) (TNF\( \alpha \))-induced activation and chemotaxis of human granulocytes. Morphine can inhibit the release of lipid mediators in Ca\( ^{2+} \)-ionophore-stimulated granulocytes in a naloxone-irreversible manner (156). In addition, some studies have noted agonistic effects mediated by naloxone such as inhibition of \( N \)-formyl methionyl leucyl phenylalanine (FMLP)-stimulated superoxide release in human neutrophils (157). In addition, granulocytopoiesis is inhibited in bone-marrow cultures exposed to naloxone (158). Interestingly, in several descriptive case studies pentazocine has been shown to induce a marked, transient granulocytopenia in humans (159 - 161). It is tempting to speculate
that the haloperidol/DTG-inaccessible sites could be linked to some of these observations, although we currently have no evidence to support or refute this. To date we have observed only minimal modulation of respiratory burst in human PMNs (data not shown). To the best of our knowledge nothing is known about the effects of other haloperidol/DTG-inaccessible site ligands (SKF 10,047, DM, 3-PPP, etc.) on granulocyte function.

**Sigma-1 sites and T cells: Identification and functional significance**

In chapter three we used the information gained from the pharmacological characterization of the haloperidol/DTG-inaccessible sites to label \( \sigma_1 \) sites in immune tissues using the \( \sigma \) radioligand \((+)^3\text{H}\)pentazocine. Binding was carried out in the presence of naltrexone because this compound could block haloperidol/DTG-inaccessible sites and allow specific labeling of \((+)^3\text{H}\)pentazocine to \( \sigma_1 \) sites. Using this method we were able to demonstrate the existence of saturable \( \sigma_1 \) sites on mouse spleen and the T cell line LBRM33 (Fig. 3.7). Higher numbers of \( \sigma_1 \) sites were present on LBRM33 T cells as compared to mouse spleen (Table 3.1). In autoradiographic studies higher numbers of \( \sigma_1 \) sites are apparent in the T cell areas of the white pulp as compared to the other areas of the spleen (87). These findings suggest that T cells display the greatest number of \( \sigma_1 \) sites in vivo.

Sigma agonists inhibited PHA-stimulated production of IL2 by LBRM33 cells, an effect that correlated highly with the pharmacology of \( \sigma_1 \) binding sites (Fig. 3.10) and the ability of \( \sigma \) agonists to inhibit proliferation of spleen cells (Fig. 3.11), an effect that we have previously attributed to \( \sigma_1 \) sites (87). However, \( \sigma \) agonists appeared to cause cytotoxicity at identical concentrations to those that inhibited IL2 in these cells (Table 3.3). Thus, \( \sigma \) agonists appear to be acting via \( \sigma_1 \) sites to cause toxicity in LBRM33 cells.
In contrast, the σ agonists haloperidol and (+)-pentazocine inhibited the proliferation of ConA-stimulated mouse spleen cells (Fig. 3.15) without causing apparent toxicity (Fig. 3.16). In addition to affecting proliferation, haloperidol inhibited IL2 production in ConA-stimulated spleen cells. It is noteworthy, however, that the concentrations of haloperidol necessary to inhibit IL2 production were approximately 10-fold higher than the amounts necessary to inhibit proliferation. (+)-Pentazocine also had no effect on IL2. This suggests that the effect of σ agonists on proliferation is largely independent of IL2 inhibition.

Because of the strong correlation of potency with binding affinity (Fig. 3.10), the LBRM33 cell data implies that if all σ agonists tested cause cytotoxicity, they mediate toxicity via σ1 sites. A precedent exists for specific σ agonist-mediated toxicity in transformed neuronal cell lines (153) and σ agonist-mediated apoptosis in carcinoma cells (66, 151). We do not know the toxic mechanism for the actions of haloperidol and (+)-pentazocine in LBRM33 cells. The membrane perturbation evident in these cells at 24 h. suggests necrotic rather than apoptotic death; however, apoptosis could have been initiated rapidly and been in the late stages by 24 h. In addition, we cannot rule out apoptosis as a possible mechanism for the inhibition of proliferation in spleen cells.

The apparent difference in mechanism of inhibition between the T cell lymphoma and mouse spleen cells may be related to the higher numbers of σ1 sites on LBRM33 cells as compared to spleen cells, or may be due to the transformed nature of LBRM33 cells. All reports thus far that have detected potential σ mediated toxic effects on cells have been performed using immortal tumor cell lines (66, 151, 153). It is tempting to suggest, based on these findings and the apparent large numbers of σ sites on tumors and tumor lines (25, 26, 33, 38) that σ sites may possibly play a role in cancer growth regulation. It is important to note, however, that the viability determinations were performed on spleen cells stimulated with ConA, whereas the LBRM33 cells were unstimulated. Therefore,
the potential difference in mechanism may simply be due to diverse effects on stimulated vs. unstimulated cells. This would represent an interesting dichotomy and warrants further investigation.

To briefly summarize our findings, it appears that agonists acting via these sites inhibit ConA-stimulated proliferation of spleen cells in an IL2 independent manner and can cause cell death in a T cell lymphoma LBRM33. Agonists may be acting via cell surface σ1 receptors to stimulate an inhibitory signaling cascade that can modulate activation signals. However, because of the recent evidence that σ1 sites may be intracellular enzymes (82, 85), we must evaluate these results in terms of them not being mediated in a classical cell-surface fashion. The guinea pig and human σ1 sites share homology with a fungal C9-C7 sterol isomerase that is involved in the conversion of fecosterol to episterol, a required step in the formation of the membrane sterol, ergosterol (82, 85, 162). Moebius and co-workers (162) have recently shown that σ agonists can inhibit the function of this enzyme in S. cerevisiae, leading to the build-up of sterol intermediates and the inhibition of cell growth. Whether σ1 sites mediate sterol synthesis in mammalian cells is unknown; however, it seems possible given the predominance of σ1 sites in endocrine tissues (24) and the data indicating that steroids can interact with σ1 sites (79 - 81).

Importantly, Hanner and colleagues (82) showed that progesterone and testosterone could interact at σ1 sites expressed in yeast, confirming previous studies (79 - 81). Progesterone inhibits mitogen-induced T cell proliferation. Testosterone can inhibit or augment lymphocyte function depending on the hormone concentration and cell type (163). In many cases it appears that these steroids can mediate their effects via non-classical, membrane bound sites (164); therefore, it is possible that steroids could be acting via σ1 sites to mediate at least some of their effects. Interestingly, other steroids
such as corticosterone and dehydroepiandrosterone, that have been shown to modulate cellular immune activity, can interact at $\sigma$ sites with $\mu$M affinity (79, 80).

The most outstanding question that faces us, as well as the rest of the $\sigma$ receptor field, is how cellular function is modulated via $\sigma_1$ sites/enzymes. It is well accepted that altered cell membrane sterol content can effect the function of a number of transmembrane proteins including receptors (165 - 167). To the best of our knowledge the effect of altered membrane sterol content on T cell activation is not known; however, it seems conceivable that $\sigma$ agonist induced-inhibition of T cell proliferation could be mediated in this manner.

The role and significance of $\sigma$ binding sites in the immune response

Our work has helped to determine the potential role(s) $\sigma$ agonists may play in immune function. Agonists acting via $\sigma_1$ sites can inhibit proliferation of T cells, although the mechanism whereby this inhibition occurs is still unknown. We have been unable to demonstrate an effect on early activation events as seen by the lack of strong effects on IL2 production; however, it is obvious that cell growth, and presumably cell cycle progression, is being inhibited via $\sigma_1$ sites. In contrast to the effects of $\sigma$ agonists on T cells, a corresponding inhibitory function has not been evident in B cells. Haloperidol potentiated anti-\(\mu\) stimulated B cell proliferation (97); however, other $\sigma$ agonists were ineffective, suggesting that haloperidol was not acting via $\sigma$ sites to affect B cell function. At this time we have been unable to demonstrate a physiological role for $\sigma$ sites in any other immune cells tested, although the high number of haloperidol/DTG-inaccessible sites on granulocytes suggests that $\sigma$ agonists could modulate certain of their functions such as phagocytosis and chemotaxis, or their hematopoietic production.
While our studies have focused entirely on the direct exposure of immune cells to drugs, it appears that σ agonists can also indirectly affect cellular immune activity. Exposure of mice to (+)-pentazocine in vivo inhibited NK cell activity, whereas direct exposure of mouse spleen cells to (+)-pentazocine in vitro had no effect on NK-mediated cytolysis (92). In addition, in vivo administration of the σ ligand SR 31747 to BALB/c mice inhibited LPS-induced production of IL6, an effect that could be abrogated by adrenalectomy or the glucocorticoid antagonist RU 486 (168). These studies imply that σ agonists can indirectly affect immune function via CNS or endocrine mechanisms.

It seems clear from our studies and the work of others (69, 89, 92, 99, 168) that σ sites may play a role both in the innate and adaptive arms of the immune response. However, the significance of σ agonist-induced immune dysfunction is still unclear. As mentioned in the introduction, several commonly prescribed and abused drugs can interact at σ sites and may affect immune function in humans. There is a growing body of evidence suggesting that drugs of abuse can compromise the human immune system and possibly contribute to the likelihood of infection in users (169). The role of σ binding sites in this scenario warrants attention, perhaps by examining σ agonist effects in animal models of bacterial, fungal or viral infection.

**Future research on σ binding sites**

Since it appears that the haloperidol/DTG-inaccessible sites are located predominantly on granulocytes, future research on these sites will concentrate on their possible role in PMN function and/or development. Several of the ligands that bind to these sites have been shown to modulate chemotaxis, activation and development of granulocytes (143, 157, 158). In addition, convincing evidence exists for depressed PMN phagocytosis and killing in heroin addicts and in morphine/methadone-treated mice.
This depression of PMN function may be one reason why opportunistic bacterial infections are common in heroin addicts (172). This imparts a measure of importance to these sites and justifies further work to elucidate their nature.

Future studies on the role of $\sigma_1$ binding sites must involve using the newly determined genetic information to further clarify the role of these sites in T cell function. The presence of $\sigma_1$ sites in immune tissues should be confirmed by mRNA detection. From our work it is clear that $\sigma_1$ receptors play a role in inhibition of T cell proliferation and possibly in cytotoxicity. These functions of $\sigma_1$ can now be confirmed either at the level of the cell via antisense oligonucleotides or with targeted gene knockouts. In addition, the role of $\sigma_1$ sites in sterol synthesis in T cells should be examined both in terms of hormonal and membrane steroids. This information should then be used to attempt to determine the role alteration of steroid synthesis has on T cell activation.

Sigma research has been hampered by lack of specific $\sigma$ antagonists. Therefore, with the notable exception of some well-studied functional correlates, including ours (87), the role of $\sigma$ binding sites in cell and organism physiology has remained a mystery. The recent molecular description of $\sigma_1$ binding sites will lead to the use of the $\sigma_1$ gene sequence as a 'genetic antagonist' via knockout and antisense methodologies. This will undoubtedly lead to emerging functions for these enigmatic binding sites.


