SOLID-STATE NMR IN DRUG DESIGN AND DISCOVERY FOR MEMBRANE-EMBEDDED TARGETS

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Abstract | Observing drugs and ligands at their site of action in membrane proteins is now possible through the use of a development in biomolecular NMR spectroscopy known as solid-state NMR. Even large, functionally active complexes are being examined using this method, with structural details being resolved at super-high subnanometre resolution. This is supplemented by detailed dynamic and electronic information about the surrounding ligand environment, and gives surprising new insights into the way in which ligands bind, which can aid drug design.

Drug discovery for membrane targets will be a major focus for the foreseeable future, as acknowledged fully by the drug industry[1,2]. At present, more than three-quarters of all drugs act on only 5% of known membrane targets, and it is predicted that two out of three new targets in the future will be membrane proteins. This is, perhaps, not surprising, as most (~85%) of the cell signals are generated through the plasma membrane, and a membrane protein constitutes at least one component in all known metabolic and signalling pathways. Such information powerfully illustrates the potential of this class of target for further exploration and exploitation.

Even in the absence of high-resolution structural details for the target protein, of which ion channels, G-protein-coupled receptors (GPCRs), transporters and ion pumps are all examples, screening and other approaches have resulted in small-molecule therapies. The importance of having knowledge of membrane protein structure for understanding cell function has been recognized by several of the Nobel prizes that have been awarded during the past 15 years — most recently, in 2003, by that presented to McKinnon and Agre for the structural resolution of the potassium channel[3] and aquaporin (water channel)[4], respectively. Several new companies (for example, 7-TM Pharma and m-phasys) have been founded with mission statements that relate to the use and production of membrane proteins as drug targets, and ~2,000 of the new patents that are filed each year involve drugs and membrane proteins. However, the lack of essential high-resolution structural details at the molecular level, and the well-acknowledged technical difficulties that are associated with the crystallography of membrane proteins, have hampered the development of rational drug discovery for membrane targets, with computational approaches generally being used to compensate for this lack of knowledge (BOX 1).

For determining the complete structures of membrane proteins at atomic resolution, there is no substitute for crystallography, but the resolution afforded by solid-state NMR (BOX 2) of structural details at the subnanometre level for small bound molecules at their site of action is unsurpassed. In addition, intimate dynamic information and electronic details for bound ligands, as well as details of the binding site itself, can be resolved. Other applications, such as the determination of chirality or the partitioning characteristics of small molecules, are also possible, which make solid-state NMR a versatile tool for drug discovery and design (FIG. 1). Some examples for which this information has been obtained and the potential for including such information in the drug design process will be discussed in this review.

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Many structural studies of membrane proteins begin with a bioinformatics analysis and the models can then be tested through direct experimentation. This route is no less valid for the study of ligand–target interactions using solid-state NMR. Computational approaches can take the form of homology modelling that is based on a known structure, especially around a putative ligand-binding site. For example, the recently resolved high-resolution (0.35 nm) structure of retinal rhodopsin[55], which itself is a seven-transmembrane-domain G-protein-coupled receptor (GPCR), has been used to generate structural models of neurologically important GPCRs, even though these are ligand, rather than light, activated. Although the primary sequence similarity between GPCRs might be low, the major secondary structure elements could be similar, and it is the important details of ligand binding and structure, which NMR can reveal, that are functionally relevant.

Docking programs (for example, Autodock) and molecular dynamics, which are coupled with simulated annealing methods, are now regularly used even in the absence of any structural data. These methods are based on algorithms that are generated either empirically or energetically from known information to give putative high-resolution data. NMR can be used to test the results of such simulations, and NMR-generated structural information can be included in docking routines to increase their use and applicability, and to improve and refine the algorithms. In particular, electronic information about ligand–target interactions could be included more rigorously than at present, because most of the data that are used come from crystallographic studies in which such interactions might only be inferred from electron-density profiles.

Challenges of membrane proteins as targets

Although they represent a sizeable proportion (~30–40%) of the proteins that are expressed from open reading frames (ORFs) in the human genome, the number of high-resolution (<0.3 nm) complete structures of membrane proteins (~20 unique structures and ~70 structures in total) in the protein database derived from crystallographic methods does not reflect their occurrence when compared with soluble proteins (~22,000 total structures). As high-resolution information is essential for resolving potential target sites, as well as understanding the ligand–target interaction at the molecular level, the past and future development of drugs for membrane targets has and will be hampered by the lack of direct structural information.

An important factor that limits the availability of structural information for membrane proteins is the challenge of obtaining such proteins in sufficient quantity and purity for structural studies; the functionality must also be preserved. Although the copy number of putative membrane-embedded drug targets can range from exceptional cases of 30–40% of the total membrane protein (as with the gastric ATPase in peptic ulcer therapy), to a few tens or less copies per cell (as with some GPCRs), the usual situation is low copy number and, therefore, poor availability. This is a direct result of the high efficiency with which membrane processes occur. Even when they are readily available, the isolation and purification of membrane proteins for structural studies can be difficult owing to the requirement for detergents to prevent aggregation, which would cause loss of functionality.

In cases in which wild-type proteins are not readily available, recombinant technology is required to express a target of interest. Some breakthroughs in the

use of new vectors, such as baculovirus and bacterial systems (notably *Escherichia coli*) for eukaryotic proteins, have allowed the limited production of membrane proteins, but only a couple of GPCRs out of the possible 2,000 that are thought to be produced from ORFs in the human genome have been expressed to any reasonable extent, so far, for structural studies[5–7]. Recombinant technology therefore has a long way to go to fulfil the needs of structural work. Even commercial efforts (for example, m-phasys) that are aimed at GPCR-specific expression are still expending considerable efforts to satisfy the tremendous potential demand for such proteins, and successes are eagerly awaited.

Most applications of solid-state NMR in the study of membrane targets, similar to other direct methods, have so far been limited to method development with readily available wild-type membrane proteins and only a limited number of studies of recombinant proteins. TABLE 1 lists the typical amounts of target that are required for both solution-state and solid-state NMR studies of ligand–target interactions. The availability of stronger magnetic fields and novel experimental techniques are also helping to improve sensitivity for NMR methods.

Solid-state NMR in structural studies

NMR is a tool that is used to derive local (magnetic) information at the atomic level and is, therefore, limited by complexity in large molecules. There is a theoretical limit to protein molecular mass for structural determinations of ~30–40 kDa because of size (slow TUMBLING and broadening of spectral information) and multiple overlapping resonances (BOX 2). Therefore, even for a seven-transmembrane-domain protein with a molecular mass of ~40 kDa, the amount of information that is available under ideal conditions is, so far, too great for complete structure determination[8], although recent advances might extend this limit for some proteins and possibly even a membrane protein in detergent micelles[9]. When embedded in a membrane, the situation is further complicated owing to slow molecular tumbling and line broadening, even for a protein up to a molecular mass of this predicted limit[10].

However, defining ligand–protein interactions does, fortuitously, require local information and so NMR comes into its own. Indeed, it has been successfully used for a significant range of soluble, weakly binding drug–target interactions[11,12] with applicability in screening procedures, such as structure–activity relationships (SAR) by NMR[13] using solution-state NMR. There are some drawbacks to this approach and it might not be possible to gain information about the bound (motionally restricted) ligand itself. These approaches cannot be explored for membrane targets, because of the size of the target, or for tightly bound ligands (BOX 2).

Observing drugs at their membrane-embedded target directly is an important challenge that solid-state NMR is now addressing[14–17]. For the first time,

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TUMBLING
A term used to describe molecules moving freely in solution and reorienting themselves quickly with respect to any given point.

it is possible to 'peer' extensively into the ligand binding site with confidence about the information that is being gained. The challenge, therefore, is to design the solid-state NMR study so that the NMR signals from the ligand provide useful information about its location within a membrane target with sufficient detail to obtain the structure, dynamics and electronic environment, as well as information about its binding site. This detail is exactly what is required in drug design and discovery, and if target conformational changes on activation are also accessible, this could provide worthwhile additional information. As an


Box 2 | Solid-state NMR of large biomolecules

Macromolecules (molecular mass ≥30,000 kDa) do not tumble quickly enough to average out the orientationally dependent (anisotropic) spin-magnetic interactions in NMR. It can be shown that any membrane complex that contains only a few hundred (in this case, ~200) proteins (each of molecular mass >40,000 kDa) with associated lipids (each of molecular mass ~1,000 kDa) at room temperature in aqueous medium, tumbles too slowly to average out the proton dipolar couplings, and the ^{13}C or ^{15}N CHEMICAL SHIFT anisotropy. This slow motion and the rich natural abundance of large γ-protons cause significantly broadened spectra through dipolar interactions, and are the main reasons why large macromolecular complexes produce broadened NMR spectral lines (even excluding relaxation effects) and structural resolution becomes difficult. Some new high-frequency transverse relaxation-optimized spectroscopy (TROSY) NMR methods[6] combined with ^{2}H and ^{15}N labelling might extend this limit for full structural determinations for isolated membrane proteins in suitable small (radius <10 nm) detergent micelles.

Wide-line solid-state NMR has been developed to exploit the anisotropic characteristics of large complexes and to give, in particular, molecular orientation in ordered systems, such as fibres or membranes[56,57]. High-resolution solid-state NMR involves mechanically averaging the anisotropic spin interactions by rotating a sample in a rotor. Here, the rotor is set at the 'magic angle', which is 54.7° with respect to the z axis of the static magnetic field (see figure) and is the angle that is made by the cross diagonal of a unit cube. Any point on this diagonal now has identical x,y,z coordinates (see figure). Therefore, the anisotropic detail is lost. When the NMR sample is spinning (at a frequency of ω_r), the broad lines from the immobile sample begin to become continuously narrowed, with a significant improvement in the signal amplitude and sensitivity. At fast spinning rates (where $\omega_r > \nu_A$ and ν_A is the width of the anisotropically broadened NMR spectrum), the spectrum eventually collapses to an isotropic-like spectrum (see figure).

Spinning can lead to morphological changes in the sample, especially those that are induced by hydration, because at high g values (ω_r >12 kHz in the liquid state), the water of hydration can be expelled from the sample and can cause denaturation. Intermediate spinning rates ($0 < \omega_r < \nu_A$) produce a central band at the resonant frequency and a set of associated side-bands that are spaced at the spinning rate (ω_r), and this spectral form can be a useful way of obtaining orientational and structural information[21]. The resolution of the narrow NMR spectra from spinning the sample at the magic angle (magic-angle spinning (MAS)) methods does not match those of high-resolution solution-state NMR spectra, mainly because of sample disorder and slow molecular motional effects. The mechanical averaging means that many of the informative spectral features from which the structure can be deduced, such as dipolar couplings, are lost. Reintroducing the dipolar couplings requires NMR pulse methods that are designed specifically to reinstate these interactions between selected isotopically enriched sites (BOX 3).

Using either static or MAS methods, solid-state NMR can now be applied to a range of noncrystalline biological macromolecules that are of interest in drug discovery and design. Size is not a limitation (as long as sensitivity is not a problem) and often heterogenous systems with other components can be studied; these include membrane-embedded drug or hormone receptors[14–17], amyloid fibrils and other fibrous (noncrystalline and crystalline) proteins[58], natural membranes as targets for antibiotics, toxins and other perturbing agents[59,60], proteins in crystals[61] and small molecules (such as drugs) that are used to resolve uniquely the polymorphism or chirality, which is vital in drug synthesis[61] (BOX 6).
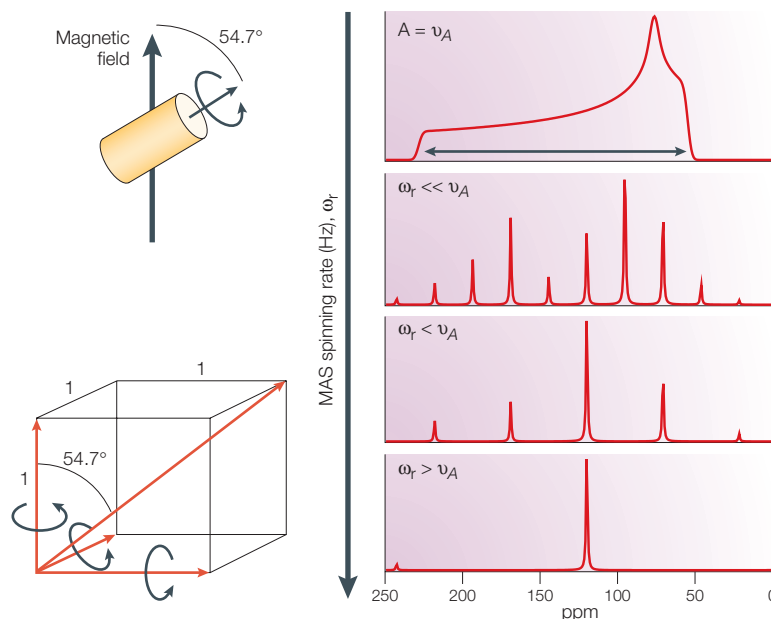
CHEMICAL SHIFT
The chemical shift of a particular nucleus is a measure of the dependence of the resonance frequency of the nucleus on its chemical environment.
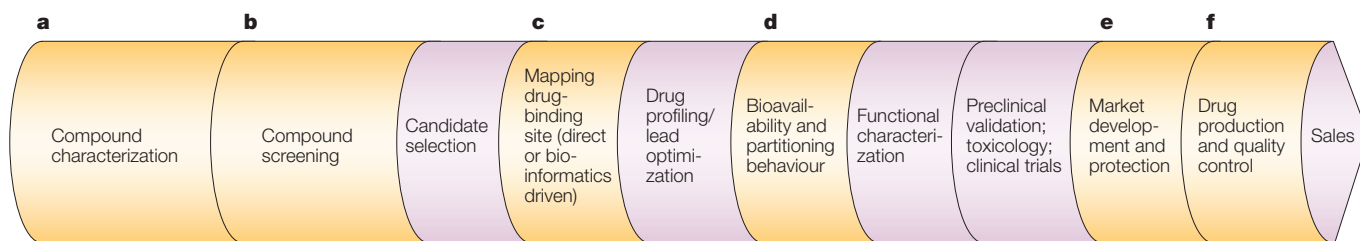
Figure 1 | **Solid-state NMR in drug discovery.** The stages at which solid-state NMR can aid in the drug discovery process are highlighted in gold. **a** | Solid-state NMR gives direct chemical bonding, chirality and compositional information in the solid (amorphous, semi- or fully crystalline) form for newly synthesized compounds[67–69]. Structural conformers, which are rapidly averaged in solution and might not be resolved, are observed directly and individually in the solid state. **b** | Selective drug-binding studies can be undertaken in the solid state for large macromolecular complexes[10,14–17]. Isotopic labelling (BOX 3), or the identification of a readily identifiable nucleus, such as ^{19}F, might be required. The nonspecific and specific binding of a drug to a target can be identified in the solid-state NMR spectrum of a heterogeneous macromolecular complex. The dissociation constant (K_d) values can also be determined using solid-state NMR[19]. Inspection of the chemical or electronic requirements that are characteristic of drugs that are found to bind to a target can help to make a more educated selection of other drugs for the target at the same binding site. **c** | Solid-state NMR spectral parameters for a drug that is bound at its target site can give detailed insight into the electronic and conformational nature and components of a binding site. **d** | The manipulation of bioavailability is important, either through solubility or partitioning to a membrane target through the bilayer, which can be distinguished using solid-state NMR[48]. **e** | Patent infringement investigations through polymorphic differences of competing drugs can be resolved, often unambiguously and more powerfully than by crystallography[67–69]. **f** | The quality control of drug synthesis is readily monitored using solid-state NMR, particularly when the chirality and enantiomeric form are important.

extension, it should be possible (although it has not been demonstrated so far) to obtain local details about multiple binding sites or more than one ligand (agonist/antagonist) on the same target. The targets that are amenable to study range from single-pass transmembrane peptide ion channels to large multi-subunit receptors. They are always embedded in a membrane (either a natural microsomal preparation or a model reconstituted membrane), with functional competence (ligand binding is just one indicator of functionality) being an essential criterion to ensure that the structural information is relevant. The following sections consider this application of solid-state NMR for detecting ligands at their site of action, resolving drug structures at their site of action, defining target binding sites, resolving ligand orientation, resolving bound drug dynamics, and assessing drug authenticity and partitioning.

Detecting ligands at their site of action
In solution-state NMR, the spectrum is dominated by narrow features that originate from isotropically, quickly tumbling molecules. Large, slowly tumbling molecules give broad signals (BOX 2) and any bound ligand within a large target will also necessarily give a broad, usually indistinguishable, signal. What is ideally required is a means to visualize only the bound drug or ligand without the complication of narrow resonances from unbound (or exchanging) ligands. Solid-state NMR can deliver this requirement using specialized pulse sequences that are able to select signals from bound ligands, whether they are exchangeable or covalently bound. This has been an important breakthrough in allowing the investigation of ligands directly at their site of action, which is a requirement for drug design and discovery.

Using isotopic labelling (BOX 3) to aid detection, ligands with both tight and weak binding behaviour can be observed using solid-state NMR. Indeed, at one extreme, even covalently bound ligands can be resolved structurally and uniquely, without any knowledge of the rest of the protein scaffold. At the other extreme, relatively weakly binding ligands (K_d values in the millimolar range) can be detected, as long as the residence time in the occluded site is long on the NMR timescale, as shown for exchanging ^{13}C-labelled sugars (where α- and β-anomers of occluded glucose were distinguished) in membrane-bound transporters[18]. As an example of a binding isotherm that has been used to characterize a weakly binding ligand by solid-state NMR, [1-^{13}C]-D-galactase has been titrated into its binding site in the lactose-transport protein (LacS) from *Streptococcus thermophilus*[19] (FIG. 2). Here, K_d can be estimated from a conventional Scatchard analysis, using the fractional ratio of the ^{13}C line height for the bound substrate. Although this kind of study has not been performed for any drug–target interaction, and the line heights are used because chemical shifts for the ligand are known, these data do indicate the potential for multiple ligand-competition studies to determine selectively recognized molecules at specific binding sites — an approach not dissimilar to determining structure–activity relationships (SAR) by solution-state NMR. This might provide a direction for future studies.

As an example of a tightly covalently bound ligand, the Schiff-base-linked (to a lysine) retinal in bacteriorhodopsin (an early example used for modelling GPCRs) and, later, rhodopsin (the present 'model') (BOX 1), is the protein–ligand complex that has been most extensively studied using solid-state

Table 1 | **Comparision of solution-state and solid-state NMR**

	Solution-state NMR	Solid-state NMR
Observation of unbound drug		
For drug observation only in solution	Yes	NS
For drug observation as solid (polymorphism determinations)	NS	Yes
Nuclei observable	^1H, ^{13}C, ^{15}N and ^{19}F	^{13}C, ^{15}N, ^{19}F, ^2H and ^{17}O
Target molecular size		
For complete structure determination	Yes (if M_r <30,000 unless TROSY can be applied)	NS (except for small membrane peptides so far)
For direct drug observation at target binding site	Yes (if M_r <40,000 unless TROSY can be applied)	No limit
For indirect (rapidly exchanging into solution) drug–target observation	No limit	NS
Sample size	10–500 μl	10 μl–1 ml
Detection levels of target–drug complex (^{13}C, ^{19}F and ^{15}N)	mM (for M_r <30,000)	>20 nmoles*
Detection levels for drug only	>1 nmole	>1 nmole*
Temperature range	Not frozen	Any
Isotopic-labelling requirements for target	None or ^{13}C, ^{15}N selectively and/or uniformly	None or selective target labelling (residues of a single type or a minimal number of types) only
Isotopic-labelling requirements for drug	Preferable for enhanced sensitivity or to aid assignments	Yes for assignment and sensitivity enhancements for ^{13}C and ^{15}N; no for rare nuclei (such as ^{19}F)
K_d range	mM to μM (for large targets); mM to nM (for small targets)	<mM (no limit)

*With proton cross-polarization to enhance sensitivity. K_d, dissociation constant; M_r, relative molecular mass; NS, not suitable; TROSY, transverse relaxation-optimized spectroscopy.

NMR. Retinal is a vitamin A derivative that is coloured from yellow to purple in proteins. Indeed, this was the first membrane protein ligand for which the method was demonstrated[20]. The orientation (*cis* or *trans* with respect to the polyene chain) of the β-ionone ring of the retinal was determined using constraints that were determined from precise (±0.03 nm) interatomic distance determinations between strategically placed ^{13}C atomic labels for identification and measurement (BOX 4). In later studies, the complete structural constraints of specifically deuterated (^2H) retinal at its binding site were resolved for a bacterial and a mammalian photoreceptor[21,22]. Most recently, solid-state NMR has been used to give high-resolution (±25 pm) distance measurements and torsion angles within protein-bound retinal, which have yielded high-resolution details for the chromophore[23].

Resolving drug structures at their site of action
The molecular structure determination of bound ligands is usually achieved by inspection of the residual electron density ($|F_c - F_o|$) after the refinement to backbone-modelled protein structures in crystallography. Recent advances in automation and informatics have aided the development of structure-based lead discovery for soluble targets to almost the level of high-throughput screening (HTS)[24]. This progress is highly

successful in the best cases, although the residual electron density is often modelled and is of lower resolution than the refined backbone structure, and there are many cases in which small-molecule structure determination is not possible. Added to this is the possible difficulty of crystallizing a drug–target complex, which has not been achieved for any membrane drug target so far, and so any alternative approach to define ligand structure is a welcome addition. Even when all of the necessary criteria have been fulfilled, the resolution and electronic details for ligand binding might not be sufficient from any structural method. Solid-state NMR can fill this gap in the best cases, and the levels of structural details are high in defining intra- and intermolecular constraints, as highlighted by the two examples described below.

Gastric proton pump. Inhibitors of the gastric proton pump, which reduce the secretion of gastric acid, have proved highly successful clinically, and continue to generate considerable revenues as a result of the widespread occurrence of gastric and duodenal ulcers, and gastro-oesophageal reflux disease (GERD). Even with *Helicobacter pylori* infestation, stomach acidity needs to be reduced for antibiotics (such as amoxicillin and clarithromycin) to be effective. The target, which is the P-type H^+/K^+-ATPase in the gastric lumen, is a large heterodimeric (α- and β-subunits) protein (with

Box 3 | **Isotopic substitution for drug-target studies by solid-state NMR**

The identification and ASSIGNMENT of NMR resonances require defined chemical labelling or spectroscopic approaches that are designed for these purposes. The NMR-visible nuclei that can be chemically introduced in studies of drug–target interactions commonly include ^{13}C (the natural abundance of which is 1.1%), ^{15}N, ^{2}H, ^{31}P and ^{19}F (almost 50% of all drugs are fluorinated with this NMR-visible naturally abundant isotope as shown in the table). ^{17}O has not found widespread application owing to its low sensitivity and the need for high NMR magnetic fields, but its use is expanding in small-molecule biological applications[62]. All of these 'spin probes' are nonperturbing and more precursors are becoming available for *de novo* synthesis, including amino acids either for use in peptide ligands or labelling proteins.

The overriding consideration in experimental design is how many sites need labelling (too many labels might be difficult to resolve) and where the labels should be placed, as illustrated in the figure for two fluorine-containing ligands. To achieve the required answer in this task, a structural modelling or simulation study is vital in labelling strategies. Distance measurements in a bound small molecule are limited to the strength of the dipolar couplings (BOX 4) and, therefore, the distance range that can be covered (typically 1–15 Å)[57]. Modelling methods can therefore help in the selection of sites for labels, either by giving a distance between the nuclei and constraining a drug structure by defining torsion angles, or by probing a binding site and defining the involvement of specific moieties in the binding process. Even defining limits can be useful, so the lack of detection of any dipolar interaction between defined sites might imply a distance that is longer than the predicted possible range for dipolar coupling.
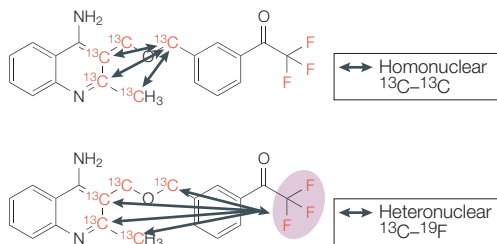

Table | Properties of commonly used and biologically relevant NMR-visible isotopes

NMR-visible isotope	Substitution	Natural abundance compared with ^{1}H	Relative sensitivity	Main NMR property exploited
^{13}C	^{12}C	1.1%	0.016	Dipolar coupling and chemical shift
^{2}H	^{1}H	0.015%	~0.001	Anisotropic quadrupolar interactions and dynamics
^{15}N	^{14}N	0.37%	~0.001	Anisotropic chemical shifts
^{19}F	Non or ^{1}H	100%	0.83	Strong dipolar couplings
^{31}P	Non	100%	0.006	Anisotropic and isotropic chemical shifts

ASSIGNMENT
The process of attributing a resonance in an NMR spectrum to a particular nucleus in a molecule.

molecular mass of ~114 kDa and ~35 kDa, respectively). One member of this ubiquitous ATPase family, the sarcoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase, has (uniquely, so far) been crystallized in two functional forms and with an inhibitor (thapsigargin) *in situ*[25]. However, no such detail is available for the proton- or sodium-pump analogues and their respective inhibitors.

In studies of porcine gastric membranes, which were prepared through the removal of peripheral proteins to enrich the proton pump to 30–35% of the total membrane protein, high-resolution structural details of some representative members of the substituted imidazole pyridines based on the Schering SCH library have been determined[26,70]. Several SCH28080 analogues were synthesized that gave useful half-maximal inhibitory concentration (IC_{50}) values for proton-pump inhibition in the micromolar range. Subsequent solid-state NMR

investigation of the ^{13}C- and/or ^{19}F-labelled analogues allowed internuclear distances in the bound ligand to be determined with high precision (±0.03 nm; BOX 4). A close-to-planar structure of all the analogues was observed (FIG. 3), with deviation from planarity owing to intramolecular flexibility in the binding site (see below). This is a little surprising, because such ligands with complete freedom around their torsion angles might be expected to take on a conformationally more constrained structure within their target site. This is the first example of a drug structure being resolved at its site of action in a membrane target, with the added benefit of being defined with structural details of such high resolution in the absence of the protein structure and in a membrane-embedded target[70].

Sodium pump. Another example of such high-resolution structure determination has been reported for ouabain, which is the parent compound of the cardiac glycoside (digitalis) family, the members of which are found in a number of plants and are usually extracted from the foxglove (*Digitalis lanata*). They are used in controlling atrial fibrillation and their action as positive inotropes also gives them a role in the management of congestive heart failure. The ouabain molecule is comprised of two moieties, a rhamnose sugar and a rigid steroid nucleus, which are linked through a single flexible ether link. As with the H^{+}/K^{+}-gastric ATPase, kidney microsomes that are enriched in the Na^{+}/K^{+}-ATPase to 35–40% of the total protein can be produced by negative purification (removing peripheral proteins in salt). Density-gradient centrifugation produces a membrane preparation that is suitable for direct NMR studies or functional assays. Here, a distance of 0.90 ± 0.05 nm was determined between the ^{13}C pairs and the ^{13}C–^{19}F nuclei that were incorporated within target-bound ouabain modified with diacetonides across the diol functions[27]. This distance restricts the two moieties to a conformation of 90° with respect to each other, with the rhamnose probably extending into solution and the steroid constrained on the protein surface (see below). All of the modified ligands showed inhibition (IC_{50} values in the nanomolar to micromolar range) of the Na^{+}/K^{+}-ATPase in the enriched kidney membranes, but they were not of the same magnitude as the potent parent compound (IC_{50} values in the nanomolar range or lower).

Defining target binding sites
Intramolecular structural details for a bound ligand alone do not give a direct insight into the binding site, although they might help in locating and understanding putative drug–target interactions. Defining the structure is therefore a useful first step, but some further information is then required. This can come from modelling that is based on other similar studies, direct information, such as NMR chemical shifts, as well as heuristic data and functional activity measurements, which often involve site-directed mutagenesis (SDM), discussions of which are beyond the scope of this review. Two types of example are provided here: the
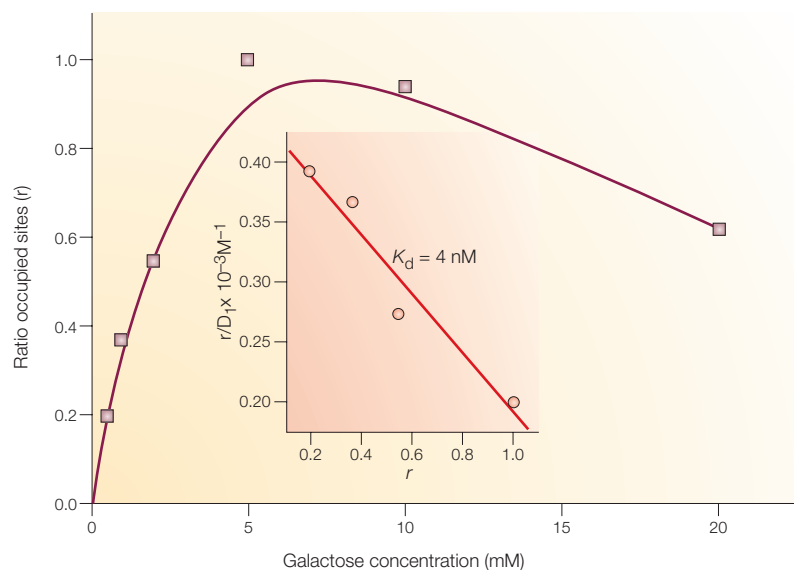
Figure 2 | **Determining K_d for ligands.** The NMR spectral line heights for a ^{13}C-labelled, weakly binding ligand (galactose here) increases as the concentration of ligand is added to a fixed amount of target protein (a sugar transporter here). The fractional ratio of bound ligand, which is specifically selected for using NMR methods, then gives a binding isotherm and, for this case, a K_d ~4 mM (REF. 19). By suppressing the NMR spectrum from isotropic, unbound ligand, and only observing bound ligand, an equilibrium K_d is determined directly.

cation pumps that were discussed in the previous section and the nicotinic acetylcholine receptor (nAChR), which is a ligand-gated ion channel.

Cation pumps. The sarcoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase has overall ~26% sequence similarity with the gastric proton pump (H^+/K^+-ATPase), but ~40% identity in the putative cation-location domain for the region in the membrane where 10 transmembrane domains assemble to form the ion-translocation pathway. This reasonable similarity allows modelling of the putative proton-translocation pathway in the H^+/K^+-ATPase based on the Ca^{2+}/Mg^{2+}-ATPase crystal structure, as well as speculation as to how the protein alters conformationally between the E1 and E2 states of the functional cycle, which are known to be kinetically similar for both homologues. With this information, and indications from SDM that Tyr925 and Glu765 — located at the luminal end of transmembrane domains 8 and 5 in the Ca^{2+}/Mg^{2+}-ATPase, respectively — might be crucial residues for ion translocation and inhibitor binding, the locality of the binding site can be tentatively defined (FIG. 3). NMR chemical shift changes that were observed for the $-O-^{13}CH_2-$ ether link of the pyridine in the bound SCH28080 analogue confirmed the possible Van der Waals contact that was identified in the model (within ~0.3–0.5 nm) of an aromatic residue to the pyridine moiety[28]. Similarly, a small chemical shift change of the resonance from the imidazole-$N(^{13}CH)_3$ indicated a charge interaction of the N^+/N^+-imidazole of bound ligand with the protein, which, together with the SDM-implied

Glu765 residue, and Glu824 domain 6, is proposed to participate in the ion binding site II as identified in the homologous Ca^{2+}/Mg^{2+}-ATPase[25].

Using a docking routine (Autodock$_{TM}$ 4.0), a more detailed description of the binding site is possible, even though this is an unconventional application for a membrane target[28]. Imposing a predetermined solid-state NMR-resolved structure on the ligand before docking increases (from ~55% for unconstrained ligand to 95% for constrained ligand) the population of the ligand at the anticipated ligand-binding site.

Similarly, the chemical shift perturbation of ^{13}C-acetonide-modified rhamnose derivatives of ouabain, when bound to the sodium pump (see above), strongly imply an electronic interaction with the essential Tyr108, which, when mutated, confers ouabain resistance on the pump in studies of the recombinant protein[27]; this is also the residue that is mutated in some warfarin-resistant rats.

Ligand-gated ion channel. Drug design for interaction with the nAChR at neuromuscular junctions is of particular interest for the treatment of many neurological diseases, including schizophrenia, Alzheimer's disease and attention-deficit/hyperactivity disorder. High-resolution atomic structures of the receptor are not available (although a structure at 4.6-Å resolution is available from electron-microscopic studies[29,30]) and, more importantly, details of the neurotransmitter-binding site are not known, even though, paradoxically, nAChR is the best understood of the cell-surface ligand-gated receptors. A good model for the human receptor is that in the *Torpedo* sp. electric organ, which can be prepared with 30–35% of the total protein being the receptor of interest. The five transmembrane subunits surround a water-filled ion-conducting channel of nAChR, which is activated by the agonist acetylcholine (a small neurotransmitter that causes 5×10^3 Na^+ per min to pass through the channel into the cell). Questions relate to channel action, not least because neurotransmitter binding occurs remotely some 6–7 nm away from the membrane surface and, therefore, the pore site[30].

Solution-state NMR studies of the neurotransmitter in solution and in the presence of the receptor do not reveal the agonist structure or provide binding-site information, as this is averaged quickly when the ligand departs the binding site (exchange rates are in the millisecond range or faster)[31]. Therefore, considerable discussion about the mechanism of acetylcholine binding was only resolved when a solid-state NMR study showed that the binding mechanism was mediated through an aromatic protein residue(s) interaction with the $-N^+(CH_3)_3$ quaternary ammonium moiety of the activator molecule, rather than a simple charge–charge interaction with an anionic residue in the binding pocket (FIG. 4). This electronic contribution to binding was reported through chemical shift perturbations of ~1.6 ppm for the ^{13}C-labelled trimethyl group of bound neurotransmitter using NMR pulse-sequence methods that were specifically designed to exclude any free

unbound ligand from detection[32,33]. Subsequently[34], the cation–π interaction has been suggested to stabilize the folding of proteins between, for example, Asp or Lys residues and aromatic residues, thereby giving stabilization energies as high as 10 kcals per mole.

The implications of this simple piece of novel information for aiding drug design that involves the nAChR are clear. For example, the potential capacity of compounds to act at neuronal nAChRs and exert beneficial effects in central nervous system disorders has prompted a search for agents with improved safety and pharmacokinetic profiles similar to those of nicotine (3-(1-methyl-2-pyrrolidinyl)pyridine). Some of these compounds are analogues of nicotine, which,

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Box 4 | Distance methods in solid-state NMR

Method development for recoupling dipolar interactions in magic-angle spinning (MAS) solid-state NMR is now a mature field[63]. These methods have been introduced because of the averaging of all the dipolar coupling interactions that occur as a result of sample spinning (BOX 2). The distances can be measured to ultra-high resolution, which is typically ±0.05 nm and often better. This is a direct result of the strong sensitivity of the magnetic dipolar coupling (b) to the distance (r). Methods are available for determining b between similar nuclei (homonuclear) and different nuclei (heteronuclear) as shown below. Explicitly, as shown in equation 1:

$$b_{IS} = \left(\frac{\mu_o}{4\pi}\right)\frac{\gamma_I\gamma_S\hbar}{r^3{}_{IS}} \tag{1}$$

where b_{IS} is the dipolar coupling between nuclear spins I and S, μ_O is the permeability of free space, γ_I and γ_S are the gyromagnetic ratios of spins I and S respectively, $\hbar$ is Planck's constant divided by 2π and r_{IS} is the distance between nuclear spins I and S.

For coupling between a ^{13}C pair, this relationship simplifies to equation 2:

$$r_{IS} = \sqrt[3]{\frac{7.59382 \times 10^3}{b_{IS}}} \tag{2}$$

Experimentally, for homonuclear recoupling (panel **a**), the recoupling is achieved under 'rotational resonance' conditions by setting the sample spinning rate (BOX 2), ω_r, to multiples of the frequency difference (ν_{AB}) between the NMR resonances ($|\nu_A - \nu_B|$); that is, $\omega_r = n\nu_{AB}$, where $n = 1, 2, 3$ and so on. During the mixing period (τ_m; panel **a**), the transfer of magnetization occurs, thereby reducing the spectral intensity (panel **b**). The decay curve (panel **c**) of the intensity reduction as a function of the mixing time (τ_m) then provides a means of quantifying the dipolar interaction (b). Typical distances of 0.7 ± 0.03 nm for ^{13}C pairs and 1.5 ± 0.05 nm for ^{19}F pairs can be measured under ideal conditions. For strong couplings at short distances the curves are oscillatory, but at larger distances they become close to exponential. Although the mixing curves (panel **c**) might seem to have considerable scatter, owing to motion or spectral noise, when computer generated curves at 0.05-nm intervals are compared with the experiment, the data can be seen to be well defined.

For heteronuclear recoupling[64], the signal amplitude is again determined, but as a function only of mixing time at any sample spinning rate that is sufficient to resolve the resonance line from the coupled nucleus. This is a less complicated method than rotational resonance, but the labelling (BOX 3) might be more complex. Many of these methods were designed and perfected for 'ideal' samples, such as solid compounds or those with little motion, and might not be directly applicable to some systems.
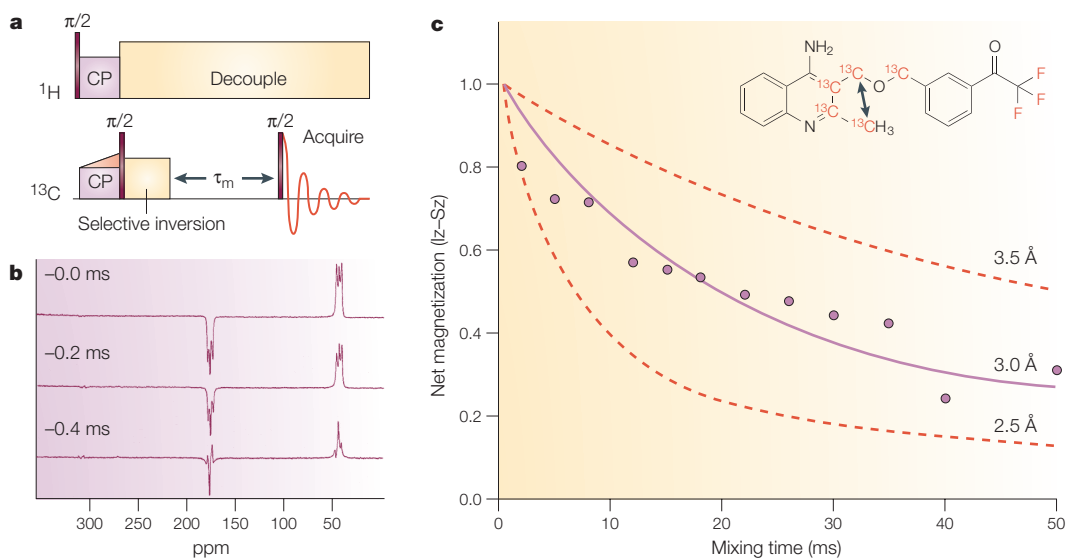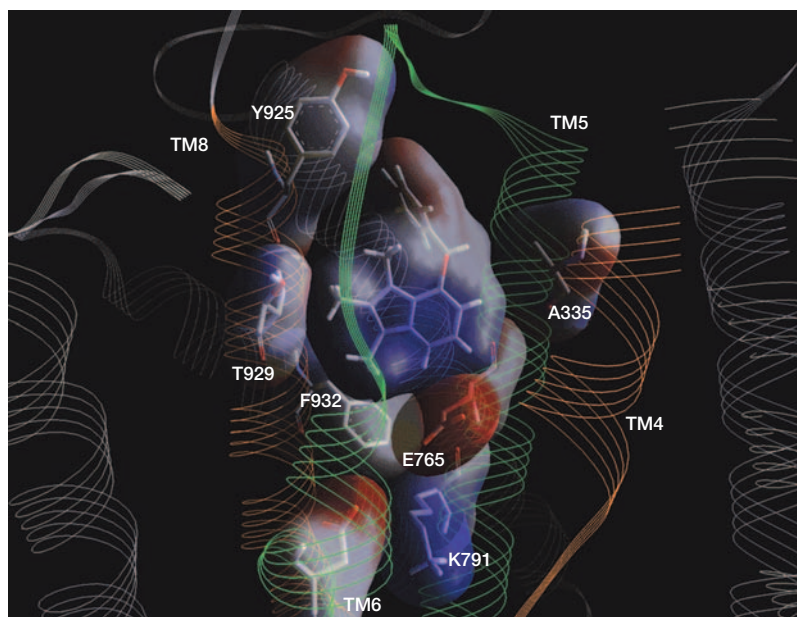
Figure 3 | **Resolving drug structures at the site of action.** The binding site of the H^+/K^+-ATPase for the substituted imidazole-pyridine class of drugs resolved using solid-state NMR in combination with site-directed mutagenesis (SDM) and bioinformatics approaches is shown. The intramolecular drug structure at the target site has been resolved to high resolution (interatomic distance to ±0.03 nm; BOX 4), as has the electronic environment (from NMR chemical shift perturbations). Essential π–π electronic interactions of Tyr926 (an essential residue identified from SDM and functional studies) with the mobile pyridine moiety, and charge–charge interactions from the constrained imidazole nucleus and Glu765 (from SDM), give insight into the requirements for drug design in this site (C. G. Kim, J. A. Watts and A. Watts, unpublished observations).

them in the design process, could result in greater hit rates in the discovery process. NMR of bound ligands, whether in membrane or soluble targets, clearly has a role to play here.

Resolving ligand orientation

Membranes are asymmetric and vectorial in their function. Drugs must find a binding site and, if this is occluded, they might have reduced efficacy. As membrane targets are uniquely orientated, any drug will have an (average) orientation with respect to the membrane. If this can be resolved, it can help in modelling the drug and its target site, because most membrane proteins have a bundle of transmembrane helices as a major structural feature.

Some forms of solid-state NMR spectra from orientated samples can be produced in which the anisotropic magnetic interactions dominate. Although most magnetic interactions are anisotropic in nature, some nuclei can be exploited more readily to show molecular anisotropy, such as ^{31}P and ^{15}N (in the chemical shift), and ^2H (in the quadrupolar interactions). Macroscopically orientated membranes are required to resolve molecular orientation details, but no knowledge of protein structure is needed, because only intramolecular information with respect to the membrane normal is resolved.

A well-studied example of ligand orientation in a membrane-embedded receptor is retinal in photosensitive proteins, such as bacteriorhodopsin and bovine rhodopsin[20,21]. These well-studied receptors (one of which is a GPCR) serve to demonstrate the methodology and resolution of measurement (±5°), and, when compared with subsequent highest-resolution crystal structures (early structures for bacteriorhodopsin[42] had errors or missing retinal density), the NMR-derived structural constraints are shown to be close to the highest-resolution (1.55 Å) wild-type protein structures[43]. In both cases, the receptor can be studied in membranes that can be stacked and orientated in the magnetic field, either when in the native purple membrane for bacteriorhodopsin or in a reconstituted membrane for rhodopsin[20,44,45].

Solid-state NMR studies of deuterated acetylcholine-$(N^+(C^2H_3)_3)$ have shown that the neurotransmitter binds to its α-subunit binding site at an angle for the long molecular axis of 42 ± 5°. This angle agrees favourably (to within 3°) with that determined from electron-microscopic structural information[29]. No drugs have yet been studied with the goal of resolving their orientation at their target site, but examples of simulations aimed at obtaining this information have been reported — any experimental information here would strengthen the simulations and help their extension into other systems.

with its -N^+CH_3 moiety (nicotine itself is 70% ionized under physiological conditions) could explain its competitive behaviour with acetylcholine for the receptor (although the K_d values of nicotine and acetylcholine are 10^{-9} and 10^{-4} M, respectively). Nicotine has now even been tentatively suggested to have therapeutic effects in schizophrenia, Alzheimer's disease, Tourette's syndrome and Parkinson's disease[35–39]. One compound (ABT-418), which progressed as far as Phase II clinical trials, has the pyridine ring of nicotine replaced by the (S)-methyl-5-isoxazole moiety, and shows cognitive-enhancing and anxiolytic-like activities in animal models, with an inhibition constant (K_i) in the nanomolar range[40]. Additionally, it is interesting to note that schizophrenics often become chain smokers[41]; the similar mechanisms of the cholinergic agonist nicotine and drugs with quaternary ammonium-like or N^+-CH_x-containing groups allow them, possibly, to compete for acetylcholine at the receptor, thereby modulating neurological activity. These searches have not acknowledged the solid-state NMR information, but clearly it could be of significant use in similar discovery processes, and would limit compound library searches and, therefore, make them more efficient.

Initially, identifying directly the electronic contributions to drug binding, which are notoriously difficult to resolve from electron-density maps of target binding sites, and then understanding and exploiting

Resolving bound drug dynamics

It is acknowledged that ligands have considerable degrees of freedom within binding sites, but ways of visualizing or quantifying this motion are scarce. Such experimental information is required if approaches,
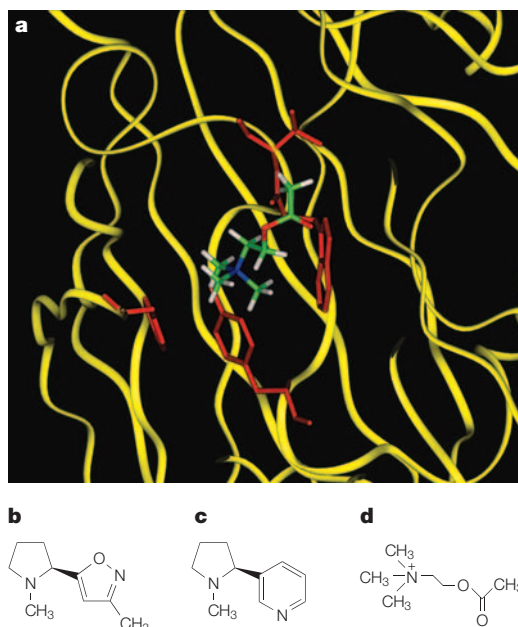
Figure 4 | **Identifying bound ligand environment.** Direct indications that a mechanism involving a cation–π interaction between agonist and receptor came from ring-current-induced chemical shifts that were observed in solid-state NMR studies of $(^{13}CH_3)_3N^+$-acetylcholine when resident at the receptor binding site, compared with an aqueous agonist. **a** | The agonist binding site is shown for the nicotinic acetylcholine receptor (1OL4.pdb chick α7 receptor subunit), which highlights (in red) the aromatic residues (Tyr91, Trp147, Thr148 and Tyr186) that line the agonist-binding pocket, within which deuterium solid-state NMR studies (BOX 3) have shown fast rotation of the agonist –$(CH_3)_3$ group[32,33]. Nicotine (3-(1-methyl-2-pyrrolidinyl)pyridine) (**b**) and ABT-418 (**c**) both modulate receptor function, and could potentially bind to the acetylcholine receptor through a cation–π interaction that is similar to that for the natural agonist acetylcholine (**d**).

such as the stochastic-tunnelling technique (STUN), are applied to screen a database of chemical compounds to the target site using atomistic force fields that include the internal rotational degrees of freedom of the ligand[46]. Solid-state NMR is sensitive to dynamics, as are other spectroscopic methods. In addition, it is not justified to assume that the rigid 'structure' of a bound ligand is representative of the whole ligand — one moiety might be highly mobile, whereas a linked moiety might be motionally restricted.

One NMR approach that can be used to gain an insight into ligand motion is labelling with the ^2H nucleus, which gives rise to NMR spectra with sensitivity to motions in the functionally important millisecond to picosecond timescale. Depending on the situation, narrow spectra can imply fast (microsecond to picosecond) motion and broad spectra can indicate slow (greater than microsecond) motion (BOX 5). This motion can also be of large amplitude (close to isotropic) or more restricted, but the spectra remain sensitive to these possibilities.

Fast motion of the ligand while at its binding site in a membrane receptor was first shown unequivocally

for an acetylcholine agonist at its target site[32]. Here, fast –$N^+(CH_3)_3$) group rotation, even at –60°C, was observed to be in the picosecond time range. Rotating these methyl groups through the π-orbital of an aromatic ring (see above) might be a contributing energetic factor in driving structural changes (ion-channel opening) in the receptor.

There are only two examples, so far, in which the differential motion of a bound ligand has been resolved. Again, these are for the proton and sodium pumps, because the chemistry (deuteration) is defined for ligand-labelling schemes. For a substituted imidazole, the pyridine moiety moves quickly around an average position in the proximity of a neighbouring aromatic residue (Phe or Tyr) when bound (FIG. 5), whereas the imidazole moiety is constrained and undergoes slow motion — it is probably constrained through electrostatic interactions with the binding site and charge–charge interactions (see above). Similarly, ouabain is constrained to the protein at its steroid nucleus, but is highly mobile in the rhamnose moiety, which is probably involved in a Tyr π-bond-sharing interaction (see above).

The significance of ligand mobility might be in generating small increments of thermally derived energy to induce conformational changes in the protein during inhibition. The ion channel in the acetylcholine receptor is 7-nm remote from the agonist-binding site, and energy is required to rotate the five helices and open the distant channel. Similarly , the ATPases are conformationally locked into inhibitory conformations by small-molecule drugs and, as yet, the mechanisms by which receptors are activated have only been resolved structurally for the Ca^{2+}/Mg^{2+}-ATPase[25] — the energetics that are required and the molecular dynamics that are involved have been little studied.

Assessing drug partitioning

The delivery of a drug to its target might be influenced significantly by its ability to partition into, and distribute between each side of, the plasma membrane. Also, translocation across the blood–brain barrier is a requirement in some cases. Certain drugs might act on the plasma membrane itself and others might need to enter the cell for (putative) intracellular target binding sites. Chemically, partition behaviour can be manipulated by increasing or reducing hydrophobicity or through fluorination. Despite its simplicity as a concept, partition behaviour is difficult to quantify. The standard filtration or separation methods for its measurement are plagued by artifacts, as seen in any literature search for the range of partition coefficients for any compound, which often vary by orders of magnitude.

Solid-state NMR can be used to visualize all molecularly distinct components, not only those that are free in solution, but also those that are bound to membranes or proteins. Exchange can be fast or slow as long as the residency time of the labelled component is long in any one environment on the NMR timescale (milliseconds; BOX 5). Deuterated small
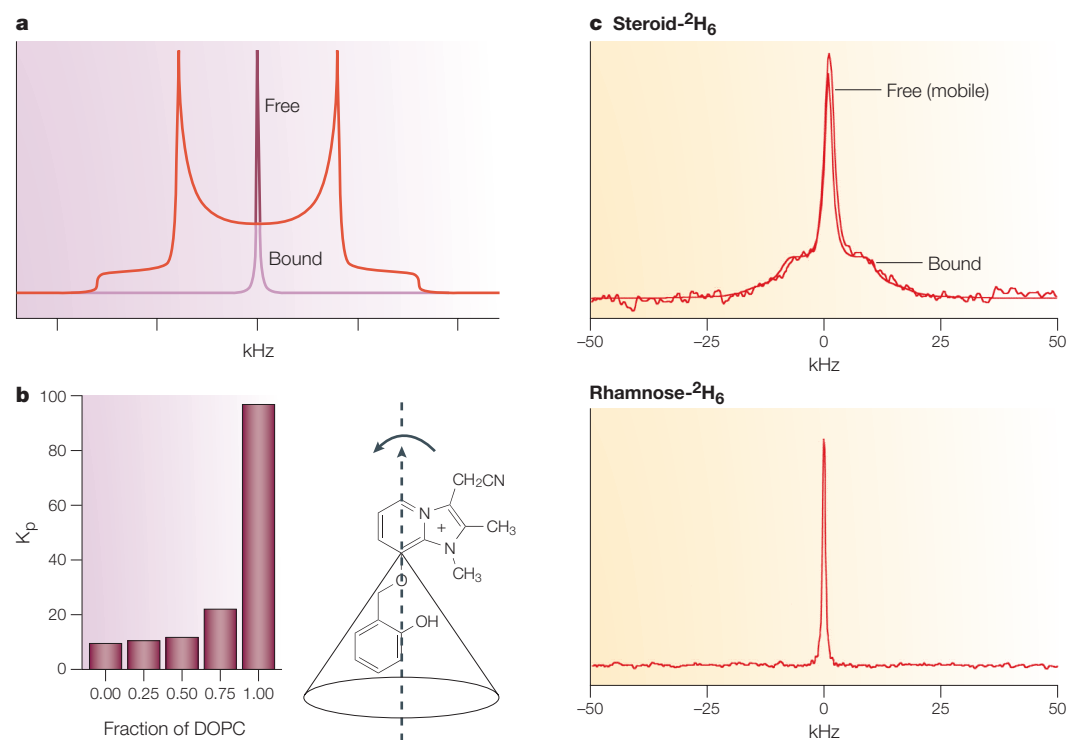
Box 5 | **Identifying the partitioning behaviour and motion of bound ligands**

Deuterium is a low-natural-abundance quadrupolar nucleus that shows high motional and order sensitivity. The maximum quadrupolar coupling constant for a $-C-^2H$ group is ~175 kHz, and is averaged through motional rate and amplitude. For a freely (isotropically) and rapidly (rate >> ~175 kHz) tumbling molecule, a narrow isotropic line is recorded (labelled as 'free' in this simulated spectrum), and for a slowly moving (rate < ~175 kHz) constrained molecule, a broadened spectrum is observed, which is typical for a small molecule that is partitioned into a membrane (panel **a**). Fast and slow motion are therefore spectroscopically defined and spectral shape can be used diagnostically for motional rate (the case of anisotropic motional broadening is also useful, but is only useful in ordered systems).

Partitioning behaviour of drugs. The deuterium wide-line NMR spectra of ligands that can partition into a membrane show well-defined spectra for each motionally distinct environment (panel **a**). This distinction only occurs if the exchange rate ($\nu_{ex} = 1/\tau_{ex}$) of a freely tumbling small molecule between the environments is slow on the quadrupolar averaging timescale, as defined above (ν_{ex} <175 kHz). As long as the experimental conditions (particularly the relaxation delays) are sufficient, then spectral integration (the measured areas, A_p and A_i, for the partitioned and isotropic spectral components, respectively) gives directly the relative concentrations of the small molecule in each phase in equilibrium. From this, a partition coefficient, K_p, can be determined as $K_p = A_p V_a / A_i V_m$, where V_a and V_m are the aqueous and membrane volumes, respectively.

As an example of how membrane composition can affect drug (a substituted imidazole pyridine is shown) partitioning behaviour, a model system of bilayers was made of varying proportions of less-fluid saturated lipids and more-fluid unsaturated lipids (dioleoyl phosphatidylcholine or DOPC; panel **b**)[48]. As the proportion of fluid lipid was increased, the partition coefficient increased, as expected. This kind of determination would be useful for any drug for which partitioning behaviour is unknown and accessibility to a target is required through the bilayer.

Differential dynamics of bound ligands. Differential motional behaviour of the same bound ligand has been reported for a substituted imidazole pyridine (as shown) bound to the H^+/K^+-ATPase and for ouabain bound to the Na^+/K^+-ATPase[48]. This implies that parts of the drug have an average position in or on its binding site, rather than a highly rigid location for the whole molecule. Deuterium substitutions in one of the two main moieties give rise to either a motionally broadened or a motionally narrowed spectrum (panel **a**), depending on which part of the molecule carries the label. In the example shown, the method is sensitive enough to differentiate unbound ligand as a narrow ('free') signal from the tightly (dissociation constant (K_d) values in the nanomolar range or smaller) 'bound' ouabain (panel **c**, upper) when the deuterium label is carried on the steroid nucleus of ouabain. By contrast, for a similar drug carrying the deuteron in the rhamnose sugar moiety, a narrow spectrum characteristic of a highly mobile component is recorded (panel **c**, lower graph). The difference in motional rates are probably 2–3 orders of magnitude.

a

Free

Bound

kHz

b

K_p

Fraction of DOPC

CH$_2$CN
CH$_3$
CH$_3$
OH

c Steroid-$^2H_6$

Free (mobile)

Bound

kHz

Rhamnose-$^2H_6$

kHz

molecules of interest are generally used, because the large degree of spectral (quadrupolar) anisotropy gives rise to a high degree of differentiation between the motionally different environments of the small molecule — even different motional modes for water (^2H_2O) can be distinguished. The applicability and success of the solid-state NMR partition method

is limited by the amounts of membrane that are available and the labelling of the small partitioning species, but it does give direct and assumption-free (about degrees of hydration, membrane amounts and so on) quantification of partition behaviour. Such approaches can be used to estimate the amount of access to a protein target for a membrane-acting drug

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Box 6 | **Resolving polymorphism in drug design and synthesis**

Polymorphic state, hydration, amorphous states or unpredictable polymorphic transitions of drugs can adversely affect their efficacy, processing and bioavailability[65–67]. In some cases, financially expensive patent issues[61] or adverse side effects have been caused through ill-characterized or controlled polymorphisms, which might be uncovered through conventional crystallography if suitable crystals can be formed. Despite their importance, the predictive or systematic preparation of polymorphic crystal forms is limited. The preparation and detection of polymorphs is, therefore, largely empirical, but remains essential in quality control.

Solid-state NMR has a powerful and definitive role in polymorph characterization, discrimination and quantification, either alone or with other methods. Detailed inter- and intramolecular conformations can be deduced at high (>0.1 nm) resolutions (BOX 4), and proton sites and hydrogen bonds, which are often lacking in crystallographic studies or averaged in solution-state NMR, can be directly determined. In a crystalline material, the number of molecules in the asymmetric unit can be determined and sensitive aspects of the electronic structure, such as the aromaticity or the groups that act as donors or acceptors for hydrogen bonds, can be deduced.

As an example of polymorph characterization, various forms of derivatives of sulphanilamides, which act as antibacterials, have been studied[68]; these are versatile in their ability to crystallize in various solid-state forms and have formed the subject of an extensive literature since their discovery in the 1930s. However, recent solid-state NMR studies have uncovered new polymorphs and hundreds of solvates. The most common polymorphs[68] are shown in panel **a**, in which clear spectral differences can be distinguished. Some forms have been variously and wrongly described by other methods, probably because of compound instability and changing hydrate form, which depend on the sample-preparative methods. The changing polymorphic behaviour (for example, with time or temperature) of solid compounds can also be assessed, as well as the rates of internal rotation of the groups relative to each other. If the motions are slow on the frequency averaging timescale, many solid-state NMR spectra are recorded, which can give an insight into the transitions that might occur, even at room temperature, and that are relevant to drug-storage requirements. The quantification of polymorphic states in the same sample is regularly a requirement and the integration of spectral lines can give accurate discrimination of the proportions of particular polymorphs; in the case shown in panel **b**, this value is 46% of polymorph I (REF. 69). Here, as in any quantification study by NMR, it is necessary to determine whether the relaxation behaviour of each polymorph is similar (as it often is), to prevent spectral suppression owing to the incomplete relaxation of any one form and, hence, erroneous quantification. Such studies demand a better description of drug polymorphs, and more consistency between the pharmaceutically and crystallographically defined nomenclature of the same drugs. In the case of sulphathiazole (panel **b**), five polymorphs were described more than 50 years ago, but these were not differentiated until they were resolved by solid-state NMR, which uncovered the irreproducibility of crystallization, a tendency to form mixed crystal forms, sample-to-sample variation and other differences that were overlooked for more than 40 years. Importantly, the commercially available materials are not as they were described originally owing to this confusion.
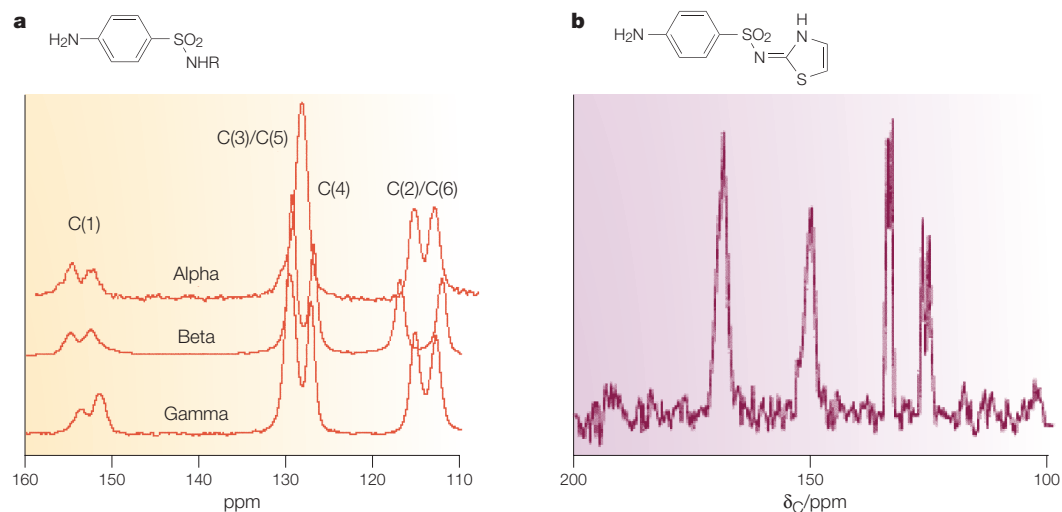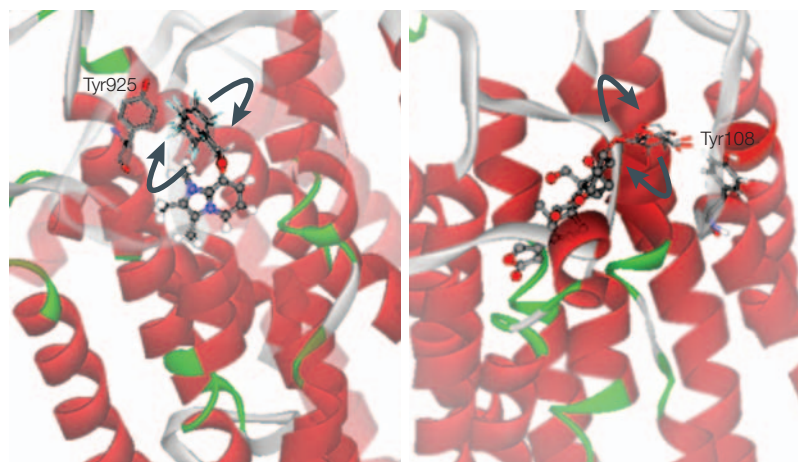

Figure 5 | **Differential dynamics of bound ligands.** Differential dynamics are detected for bound ligands in their target site in membrane proteins. One moiety is fixed relative to the protein, and the other is highly mobile both in rate and amplitude of motion. Here, the two examples for which data are available, the substituted imidazopyridine in the proton pump (**a**) and ouabain in the sodium pump (**b**), are shown. The imidazole and steroid components are the more restricted parts of the ligands, whereas the pyridine and rhamnose moieties are allowed greater degrees of freedom of motion about the average positions that are determined by dipolar recoupling methods[27] (BOX 4).

and any reduction in the concentration of the drug that is available at the target owing to nonspecific membrane partitioning can be estimated.

The approach has been applied to the simple membrane-partitioning alcohol hexanol[47], and a substituted imidazole pyridine[48] that has specific target binding. Such studies have shown a more complex situation than simple two-phase (membrane or aqueous phase) partitioning as, in the case of hexanol, some membrane-embedded alcohol was identified together with a surface-bound (third) component. The free unbound aqueous alcohol is readily distinguished, because it gives rise to a narrow spectrum and the measurements are performed under equilibrium conditions, which makes quantification possible without the need to centrifuge or filter the membrane.

Future prospects and needs

Two important pieces of information are lacking in present studies of ligand binding as described above: the spatial coordinate (intermolecular) details of

binding sites and the binding-site architecture. Both of these are also missing for some of the best-characterized soluble drug targets for a range of technical reasons. To obtain this information, labels are required in the binding site itself, and this necessitates either recombinant protein with specific labels, as shown only for bacteriorhodopsin so far[49,50], or the external introduction of non-NMR labels but specifically sited chemical modifications[19]. With labels in both the binding site and ligand, geometric triangulations that use distance measurements over short ranges (up to 1.5 nm) can reveal structural, as well as electronic (from chemical shifts) and orientational, details. With such detailed information, both electrostatic and — possibly with the aid of ^{17}O as a sensitive NMR probe — hydrogen-bonding contributions to drug–target interactions can be described[51]. This exercise in mapping depends on the successful protein production and labelling of target and ligand, and any experimentation will need to be led by a modelling and bioinformatics approach to determine which sites to label.

Some headway is already being made in describing conformational changes in targets that are induced by small-molecule binding or activation, again by light in bacteriorhodopsin[52,53]. So far, structural changes that are induced in bacteriorhodopsin have been observed using solid-state NMR — here, the same sample can, in principle, be studied in two or more forms directly in the NMR spectrometer without sample removal. Helix orientational and loop conformational changes can be detected, and some ligands bind to loop regions rather than sites in the protein for some classes of target (the loops of proteins can readily be visualized in NMR[10,54], even though they are often elusive in crystallographic structures).

In conclusion, the details of membrane target binding sites are now becoming accessible at the structural and dynamic levels. This new information can clearly help in the design and discovery of new drugs and ligands. Essential interactions to induce a desired effect might become available as more generic information is released, and data production will be accelerated as more targets become accessible for study. There are important implications for drug design in the future using predictive methods that are based firmly on direct experimental evidence.

1. Terstappen, G. C. & Reggiani, A. *In silico* research in drug discovery. *Trends Pharmacol. Sci.* **22**, 23–26 (2001).
2. Drews, J. Drug discovery: a historical perspective. *Science* **287**, 1960–1964 (2000).
3. Doyle, D. A. *et al*. The structure of the potassium channel: molecular basis of K+ conduction and selectivity. *Science* **280**, 69–77 (1998).
4. Denker, B. M., Smith, B. L., Kuhajda, F. P. & Agre, P. Identification, purification, and partial characterization of a novel Mr28,000 integral membrane protein from erythrocytes and renal tubes. *J. Biol. Chem.* **263**, 15634–15642 (1988).
5. Tucker, J. & Grisshammer, R. Purification of a rat neurotensin receptor expressed in *Escherichia coli*. *Biochem. J.* **317**, 891–899 (1996).

6. Seifert, R. *et al.* Different effects of Gsa splice variants on β2-adrenoreceptor-mediated signaling. The β2-adrenoreceptor coupled to the long splice variant of Gsa has properties of a constitutively active receptor. *J. Biol. Chem.* **273**, 5109–5116 (1998).
7. Creemers, A. F. L. *et al.* Solid state 15N NMR evidence for a complex Schiff base counterion in the visual G-protein-coupled receptor rhodopsin. *Biochemistry* **38**, 7195–7199 (1999).
8. Vosegaard, T. & Nielsen, N. C. Towards high-resolution solid-state NMR on large uniformly 15N- and [13C, 15N]-labeled membrane proteins in oriented lipid bilayers. *J. Biomol. NMR* **22**, 225–247 (2002).
9. Riek, R., Pervushin, K. & Wüthrich, K. TROSY and CRINEPT: NMR with large molecular and supramolecular structures in solution. *Trends Biochem. Sci.* **25**, 462–468 (2000).

This paper reviews the potential of using new solution-state NMR for the structural determinations of larger (30–100 kDa) purified proteins.
10. Watts, A. *et al.* in *Methods in Molecular Biology: Techniques in Protein NMR* (ed. Downing, K.) (Humana, Totowa, New Jersey, 2004).
11. Roberts, G. C. K. Applications of NMR in drug discovery. *Drug Discov. Today* **5**, 230–240 (2000).
12. Pellecchia, M., Sem, D. S. & Wüthrich, K. NMR in drug discovery. *Nature Rev. Drug. Discov.* **1**, 211–219 (2002). References 11 and 12 are good and comprehensible reviews of solution-state NMR methods to study ligand–target interactions.
13. Shuker, S., Hajduk, P., Meadows, R. & Fesik, S. Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **274**, 1531–1534 (1996).

14. Watts, A. Direct studies of ligand–receptor interactions and ion channel blocking. *Mol. Memb. Biol.* **19**, 267–275 (2002).
15. Watts, A. *et al.* Membrane protein structure determination by solid state NMR. *Nat. Prod. Rep.* **16**, 419–423 (1999).
16. Watts, A. NMR of drugs and ligands bound to membrane receptors. *Curr. Opin. Biotechnol.* **10**, 48–53 (1999).
17. Watts, A. Structural resolution of ligand–receptor interactions in functional, membrane-embedded receptors and proteins using novel, non-perturbing solid state NMR methods. *Pharm. Pharm. Comm.* **5**, 7–13 (1999).
18. Spooner, P. J. R., Rutherford, N., Watts, A. & Henderson, P. J. F. NMR observation of substrate in the binding site of an active sugar H^+ symport protein in native membranes. *Proc. Natl Acad. Sci. USA* **91**, 3877–3881 (1994).
19. Spooner, P. J. R., Veenhoff, L. M., Watts, A. & Poolman, B. Structural information on a membrane transport protein from nuclear magnetic resonance spectroscopy using sequence-selective nitroxide labeling. *Biochemistry* **38**, 9634–9639 (1999)
20. Creuzet, F. Determination of membrane-protein structure by rotational resonance NMR: bacteriorhodopsin. *Science* **251**, 783–786 (1991).
    This was the first example of distance measurements in a membrane receptor using solid-state NMR to determine ligand (retinal) structure.
21. Gröbner, G. *et al.* Observation of light-induced structural changes of retinal within rhodopsin. *Nature* **405**, 810–813 (2000).
22. Ulrich, A. S., Wallat, I., Heyn, M. P. & Watts, A. Re-orientation of retinal in the M-photointermediate of bacteriorhodopsin. *Nature Struct. Biol.* **2**, 190–192 (1995).
23. Carravetta, M. *et al.* Protein-induced bonding perturbation of the rhodopsin chromophore detected by double-quantum solid-state NMR. *J. Am. Chem. Soc.* **126**, 3948–3953 (2004).
24. Blundell, T. L., Jhoti, H. & Abell, C. High-throughput crystallography for lead discovery in drug design. *Nature Rev. Drug Discov.* **1**, 45–54 (2002).
25. Toyoshima, C. & Nomura, H. Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature* **418**, 605–611 (2002).
    This paper presents the first high-resolution structure of a P-type membrane ATPase in which the ligand structure and conformational changes are defined.
26. Watts, J. A., Watts, A. & Middleton, D. A. A model of reversible inhibitors in gastric H^+/K^+-ATPase binding site determined by rotational echo double resonance NMR. *J. Biol. Chem.* **276**, 43197–43204 (2001).
27. Middleton, D. A., Rankin, S., Esmann, M. & Watts, A. Structural insights into the binding of cardiac glycosides to the digitalis receptor revealed by solid-state NMR. *Proc. Natl Acad. Sci. USA* **97**, 13602–13607 (2000).
28. Kim, C. G., Watts, J. A. & Watts, H. Ligand docking in the gastric H+/K+-ATPase — homology modelling of reversible inhibitor sites. *J. Med. Chem.* (in the press).
29. Miyazawa, A., Fujiyoshi, Y., Stowell, M. & Unwin, N. Nicotinic acetylcholine receptor at 4.6Å resolution: transverse tunnels in the channel wall. *J. Mol. Biol.* **288**, 765–786 (1999).
30. Miyazawa, A., Fujiyoshi, Y. & Unwin, N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **424**, 949 (2003).
31. Fraenkel, Y., Gershoni, J. M. & Navon, G. NMR analysis reveals a positively charged hydrophobic domain as a common motif to bound acetylcholine and D-tubocurarine. *Biochemistry* **33**, 644–650 (1994).
32. Williamson, P. T. F., Watts, J. A., Addona, G. H., Miller, K. W. & Watts, A. Dynamics and orientation of N+(CD3)3-bromoacetylcholine bound to its binding site on the nicotinic acetylcholine receptor. *Proc. Natl Acad. Sci. USA* **98**, 2346–2351 (2001).
33. Williamson, P. T. F., Gröbner, G., Spooner, P. J. R., Miller, K. W. & Watts, A. Probing the agonist binding pocket on the nicotinic acetylcholine receptor: a high resolution solid state NMR approach. *Biochemistry* **37**, 10854–10859 (1998).

34. Gallivan, J. P. & Dougherty, D. A. Cation–π interactions in structural biology. *Proc. Natl Acad. Sci. USA* **96**, 9459–9464 (1999).
35. Sabbagh, M., Lukas, R., Sparks, D. & Reid, R. The nicotinic acetylcholine receptor, smoking, and Alzheimer's disease. *J. Alzheimers Dis.* **4**, 317–325 (2002).
36. Shytle, R. *et al.* Cholinergic modulation of microglial activation by α7 nicotinic receptors. *J. Neurochem.* **89**, 337–343 (2004).
37. Terry, A. J. & Buccafusco, J. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. *J. Pharmacol. Exp. Ther.* **306**, 821–827 (2003).
38. Romano, J. A possible explanation for nicotine's neuroprotective effect. *Neurol. Rev.* **12**, 4 (2004).
39. Arehart-Treichel, J. A. Role for nicotine in Alzheimer's, other disorders. *Psych. News* (17 March 2000).
40. Wilens, T. E. *et al.* A pilot controlled clinical trial of ABT-418, a cholinergic agonist, in the treatment of adults with attention deficit hyperactivity disorder. *Am. J. Psych.* **156**, 1931–1937 (1999).
41. Kelly, C. & McCreadie, R. Smoking habits, current symptoms, and premorbid characteristics of schizophrenic patients in Nithsdale, Scotland. *Am. J. Psych.* **156**, 1751–1757 (1999).
42. Pebay-Peyroula, E., Rummel, G., Rosenbusch, J. P. & Landau, E. M. X-ray structure of bacteriorhodopsin at 2.5 angstroms from microcrystals grown in lipidic cubic phases. *Science* **277**, 1676–1681 (1997).
43. Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P. & Lanyi, J. K. Structure of bacteriorhodopsin at 1.55Å resolution. *J. Mol. Biol.* **291**, 899–911 (1999).
44. Gröbner, G. *et al.* Macroscopic orientation of natural and model membranes for structural studies. *Anal. Biochem.* **254**, 132–136 (1997).
45. Kamihira, M. *et al.* Structural and orientational constraints of bacteriorhodopsin in purple membranes determined by oriented-sample solid-state NMR spectroscopy. *J. Struct. Biol.* **149**, 7–16 (2005).
46. Merlitz, H., Burghardt, B. & Wenzel, W. Stochastic tunneling method for high-throughput database screening. *Nanotechnology* **1**, 44–47 (2003).
47. Fraser, D. M., Van Gorkom, L. C. M. & Watts, A. Partitioning behaviour of 1- hexanol into lipid membranes as studied by deuterium NMR spectroscopy. *Biochim. Biophys. Acta* **1069**, 53–60 (1991).
48. Middleton, D. A., Reid, D. G. & Watts, A. A combined quantitative and mechanistic study of drug–membrane interactions using a novel ^2H NMR approach. *J. Pharm. Sci.* **93**, 507–514 (2004).
49. Hu, J. G. *et al.* Early and late M intermediates in the bacteriorhodopsin photocycle: a solid-state NMR study. *Biochemistry* **37**, 8088–8096 (1998).
50. Jaroniec, C. P. *et al.* Measurement of dipolar couplings in a uniformly $^{13}C/^{15}N$ labeled membrane protein: distances between the Schiff Base and aspartic acids in the active site of bacteriorhodopsin. *J. Am. Chem. Soc.* **123**, 12929–12930 (2001).
51. Lemaitre, V. *et al.* New insights onto the bonding arrangements of L- and D-glutamates from solid state 170 NMR. *Chem. Phys. Lett.* **371**, 91–97 (2003).
52. Lansing, J. C., Hu, J. G., Belenky, M., Griffin, R. G. & Herzfeld, J. Solid-state NMR investigation of the buried X-proline peptide bonds of bacteriorhodopsin. *Biochemistry* **42**, 3586–3593 (2003).
53. Jaroniec, C. P. *et al.* Measurement of dipolar couplings in a uniformly ^{13}C-^{15}N-labeled membrane protein: distances between the Schiff Base and aspartic acids in the active site of Bacteriorhodopsin. *J. Am. Chem. Soc.* **123**, 12929–12930 (2001).
54. Castellani, F. *et al.* Structure of a protein determined by solid-state magic angle-spinning NMR spectroscopy. *Nature* **420**, 98–102 (2003).
55. Palczewski, K. *et al.* Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **277**, 687–690 (2000).

56. Opella, S. J. NMR and membrane proteins. *Nature Struct. Biol.* **4** (Suppl.), 845–848 (1997).
    This is a good review of solid-state NMR structure determinations for membrane proteins, which is comprehensively written and well illustrated.
57. Griffin, R. G. Dipolar recoupling in MAS spectra of biological solids. *Nature Struct. Biol.* **5**, 508–512 (1998).
    This paper contains descriptions of dipolar recoupling methods, and is accessible to specialists and nonspecialists alike.
58. Tycko, R. Biomolecular solid state NMR: advances in structural methodology and applications to peptide and protein fibrils. *Annu. Rev. Phys. Chem.* **52**, 575–606 (2001).
59. Thompson, L. K. Solid state NMR studies of the structure and mechanisms of proteins. *Curr. Opin. Struct. Biol.* **12**, 661–669 (2002).
60. Zech, S. G., Olejniczak, E., Hajduk, P., Mack, J. & McDermott, A. E. Characterization of protein–ligand interactions by high-resolution solid-state NMR spectroscopy. *J. Am. Chem. Soc.* **126**, 13948–13953 (2004).
61. Bernstein, J. *Polymorphism in Molecular Crystals* (Oxford Univ. Press, Oxford, 2002).
62. Lemaitre, V., Smith, M. E. & Watts, A. A review of oxygen-17 solid state NMR of organic materials: towards biological applications. *Solid State Nuc. Mag. Res.* **25**, 215–235 (2004).
63. Levitt, M. H., Raleigh, D. P., Creuzet, F. & Griffin, R. G. Theory and simulations of homonuclear spin pair systems in rotating solids. *J. Chem. Phys.* **92**, 6347–6364 (1990).
64. Guillon, T. Introduction to rotational-echo double resonance NMR. *Conc. Mag. Res.* **10**, 277–289 (1998).
65. Caira, M. R. in *Design of Organic Solids* 163–208 (Springer-Verlag, Heidelberg, 1998).
66. Threlfall, T. L. Analysis of organic polymorphs. A review. *Analyst* **120**, 2435– 2460 (1995).
67. Yu, L., Reutzel, S. M. & Stephenson, G. A. Physical characterization of polymorphic drugs: an integrated characterization strategy. *Pharm. Sci. Technol. Today* **1**, 118–127 (1999).
68. Portieri, A., Harris, R. K., Fletton, R. A. & Lancaster, R. W. Effects of polymorphic differences for sulfanilamide, as seen through ^{13}C and ^{15}N solid state NMR, together with shielding calculations. *Magn. Reson. Chem.* **42**, 313–320 (2004).
69. Apperley, D. C. *et al.* Sulfathiazole polymorphism studied by magic-angle spinning NMR. *J. Pharm. Sci.* **88**, 1275–1280 (1999).
70. Midleton, D. A. *et al.* The conformation of an inhibitor bound to gastric proton pump. *FEBS Letts.* **410**, 269–274 (1997).

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