Fatty acid amides are putative endogenous ligands for anaesthetic recognition sites in mammalian CNS

‘To sleep, perchance to dream’ W. Shakespeare

The pioneering work of Meyer and, concurrently but independently, Overton over a century ago, demonstrated that the potency of anaesthetics within a homologous series correlated with their ability to partition out of aqueous solvents into lipids such as octanol. This suggested to several generations of researchers pursuing mechanisms of action, that the molecules were exerting a non-selective physical perturbation of a lipid site in membranes or possibly perturbing the volume or fluidity of the plasma membrane itself. Lipid theories of anaesthetic action have been increasingly marginalized by researchers uncovering substantive evidence that proteins (first the globular luciferases, then, increasingly, native or recombinant ion channels) can bear saturable stereoselective sites for modulation by ‘clinical concentrations’ of a broad spectrum of anaesthetic drugs. Furthermore, these drugs exhibit the same structure–activity profiles in vitro at the channel targets as they do in patients. Perhaps the most attractive model targets for anaesthetic action are the ligand-gated ion channels (LGIC), particularly the nicotinic receptor superfamily. All the members of this family (nicotinic, 5HT3, glycine, and GABA_A receptors) are thought to form fast ion channels by aggregating five subunits around a central hydrophilic pore. This pentameric structure has been confirmed by electron microscopy which suggests that the channels gate (i.e. open when neurotransmitter is bound) via a relatively localized conformational change confined to the core of the protein. Importantly for lipid soluble anaesthetics, during these gating transitions macromolecular subunits themselves do not twist or distort their outer structure at the interface with the membrane bilayer. A plethora of influential papers have recently highlighted the fact that inhibitory ionotropic receptors activated by GABA/glycine have crucial roles in regulating arousal. GABA is used as a transmitter at up to one-third of all CNS synapses. The GABA_A receptor probably represents the closest molecular species to a unifying target for general anaesthetics in the brain. With the cloning of the receptor subunits throughout the 1980s and 1990s we now know that their are many ‘isoforms’ of the GABA_A receptor expressed in the CNS (e.g. see Mckernan and Whiting). Eight GABA subunit classes have been cloned to date, with up to six homologues in each. In the late 1990s it was disclosed that single amino acids (at two discrete intramembranous sites) in ion channel subunits comprising 450 residues conferred sensitivity to volatile anaesthetics, etomidate and ethanol. Importantly, these residues were in transmembrane segments of the pentameric channel complex at sites adjacent to or actually lining the ion channel lumen. The above might be interpreted by some as the definitive unifying solution to the question of cellular actions of anaesthesia but it remains to be seen whether the removal of these sites in vivo will significantly alter the response of whole animals to such depressant drugs. Confirmation that the ‘anaesthetic-sensitive’ β2 subunit has been successfully knocked out in non-lethal mutant mice came in a flurry of abstracts at the European Neuroscience meeting (the largest ever held in the UK) in 2000 in Brighton, England. However, no evidence for or against the relevance of these subunits for anaesthetic action in vivo has been published to date.

In a wider context, anaesthetics are notoriously low-affinity and non-selective drugs that may underpin the agent-dependent spectrum of pharmacological and toxicological effects seen in vivo. Voltage-gated channels and presynaptic processes were historically portrayed as relatively insensitive (being blocked only by ‘heroic’ MAC-multiples). Many of these data were obtained in model (experimentally accessible) preparations such as the squid axon. Thanks to such invertebrate models we know that all voltage-gated pores exist in conformationally distinct modes which underpin action potential shape, unidirectionality and refractoriness: increasingly, it is apparent that the actions/potency of anaesthetics at these pre-synaptic targets are demonstrably state-dependent.

Now it is possible to study cloned voltage-gated Na^+ channels from a variety of species, including humans, under different conditions of neural discharge or in distinctive ionic environments. Mammalian Na^+ channels can be significantly depressed by MAC-equivalent general anaesthetic concentrations if the cells are tonically depolarized or induced to fire rapidly. A variety of voltage-gated calcium channels (VGCCs) are also demonstrably sensitive to block by 1–4 MAC isoflurane. Much of the argument in favour of LGICs as relevant model anaesthetic targets hinges on their relatively high-affinity for anaesthetics (EC50s or IC50s are higher for VGCCs). This correlates better with MAC-equivalent concentrations of anaesthetics in patients at equilibrium. The VGCC experimenters, or independent reviewers, argue that their data are physiologically relevant based on the very steep Ca^{2+} dependence of transmitter-release. The patch clamp technique devised in the early 1980s is applicable even to the smallest mammalian nerve
cell. The pioneering work of Forsythe laid the foundations for impaling both pre-synaptic and post-synaptic cells in the same mammalian CNS synapse. Based on his experiments with metabotropic glutamate receptors, only a tiny proportion of the pre-synaptic Ca\textsuperscript{2+} current needs to be inhibited to impair profoundly the transsynaptic signal.\textsuperscript{14} This reinforces the potential importance of pre-synaptic Ca\textsuperscript{2+}-entry for clinical anaesthetic effect. To summarize, LGICs are only one of a series of anaesthetic-sensitive signalling proteins in the brain. Voltage-gated K\textsuperscript{+} channels, particularly those with ‘twin-pore’ structures\textsuperscript{15} are demonstrably sensitive too. Indeed, elegant mutagenesis experiments have again highlighted important domains for anaesthetic/alcohol recognition on the voltage-gated K\textsuperscript{+} channels.\textsuperscript{16} The NMDA glutamate receptor has long been acknowledged as a high-affinity and stereoselective target for ketamine\textsuperscript{2} and may also be an important target for nitrous oxide\textsuperscript{17} and the experimental anaesthetic, xenon.\textsuperscript{18} What evidence exists for endogenous modulators of arousal which exert their hormonal actions at these anaesthetic drug recognition sites?

For certain structural classes of anaesthetic there is strong, and in some cases, irrefutable evidence for endogenous ligands. A variety of physiological neurosteroids (e.g. see Belelli and colleagues\textsuperscript{19}) have been shown to modulate sites on GABA\textsubscript{A} receptors and these may well influence mood and behaviour as their titres change in menstruation and sexual development. When a veterinary surgeon administers alphaxalone to his moribund charges, is he simply administering a lipid soluble membrane perturbing agent or saturating neural receptors geared to sensing the actions of much lower levels of circulating hormones? Pharmaceutical companies and biophysicists have attempted to restore polar alphaxalone derivatives (with no need for toxic excipients) to medical practice in recent years.\textsuperscript{20} Thanks to the pioneering work of Hughes, Kosterlitz and coworkers, endorphins and enkephalins are now established as endogenous ligands for clinically useful opiate anaesthetics.\textsuperscript{21} Cholesterol has long been known as a depressant, even anaesthetic, molecule in laboratory animals.\textsuperscript{22} Interestingly, much play has been made on the stereoselective actions of anaesthetics at the LGIC targets.\textsuperscript{2} Another membrane lipid component has attracted considerable recent attention as an endogenous depressant. cis-9,10-Octadecenoamide (cOA or ‘oleamide’) was originally isolated from sleep-deprived cats and an enzyme specific for its hydrolysis is present in rat brain.\textsuperscript{25} Synthetic oleamide induced physiological sleep when injected into rats but little is known about its cellular mode of action. High-affinity interactions with recombinant G-protein-linked receptors such as 5HT\textsubscript{2} or 5HT\textsubscript{7} have been reported.\textsuperscript{26} Conflicting evidence suggests that the hypnogenic effects are probably not secondary to activation of G-proteins.\textsuperscript{27} More recent studies demonstrate that oleamide may bind to cannabinoid sites (IC\textsubscript{50} of 10 \mu M) in rodent CNS, that it induces hypolocomotion \textit{in vivo} which is sensitive to the CB1 antagonist SR141716A, but that the brain lipid cannot elicit the same second messenger responses as acknowledged CB1 cannabinoid agonists.\textsuperscript{28} The low-affinity binding and lack of coupling to cAMP both cast doubt on the relevance of a direct interaction with CB1 receptors. To date, none of these putative receptor interactions have been shown to contribute to a net depressant effect for oleamide at a systems level. Experiments in laboratory animals confirm that oleamide induces sleep and biologically active concentrations of oleamide in CSF have been determined.\textsuperscript{29} CB1 antagonists block the oleamide effects on sleep latency in laboratory animals.\textsuperscript{30} The endogenous CB1 ligand anandamide and oleamide are both hydrolysed by the same fatty-acid amidohydrolase enzyme. It has been suggested that the hypnogenic effects of oleamide are not receptor mediated but rather they reflect quenching of this enzyme to increase the titre of anandamide or other endocannabinoids. At the cellular level, oleamide has several interesting parallels with general anaesthetics. Recently, we demonstrated that rat GABA\textsubscript{A} receptors and inhibitory synaptic currents are sensitive to modulation by oleamide (but not equally hydrophobic congeners such as oleic acid or the non-hypnogenic \textit{trans} isomer). Recombinant human GABA\textsubscript{A} receptor isoforms expressed in oocytes were modulated by oleamide and diazepam only if a γ subunit was co-expressed with αβ (but the fatty acid amide response was insensitive to high concentrations of flumazenil, indicating an independent site of action),\textsuperscript{31} see also Yost.\textsuperscript{32} The fact that oleamide and anaesthetics differentially modulate ion channel isoforms in an invariant oocyte membrane strongly suggests that they exert their effects on sterically well-defined sites on the protein, rather than the lipid environment of the cell surface. Oleamide enhances currents through all GABA\textsubscript{A} and glycine subunit isoforms that we have examined to date (with the exception of αβ alone which is not thought to be widely expressed in the brain) but it does not modulate currents through the Rho subunit.\textsuperscript{33} This subunit was originally cloned from retinal tissue but has since been detected at discrete sites in CNS and is not positively modulated (some labs report antagonism) by inhalation anaesthetics.
We have since shown that oleamide concurrently, and with slightly higher affinity, can depress pre-synaptic function, transmitter-release and neuronal burst firing by a state-dependent interaction with the voltage-gated Na⁺-channel (Fig. 1). In voltage-clamped mouse channels, the brain lipid exerts a spectrum of effects closely akin to those exerted by a variety of anaesthetics at clinical concentrations. The depressant molecules exert some degree of tonic block (more pronounced at depolarizing holding potentials), shift inactivation curves strongly in the hyperpolarizing direction and retard recovery from the inactivated channel state. Oleamide, possibly because of its extreme hydrophobicity, does not exhibit frequency-dependent facilitation of block unlike general/local anaesthetics and anticonvulsant drugs. At both the GABA<sub>A</sub> receptor and the Na⁺ channel oleamide is highly stereoselective in its actions. Only the hypnogenic cis form of the molecule exerts a modulatory response (the trans form of the molecule was not effective as a sleep inducer and cannot suppress spontaneous activity in our model cultured neurones). However, the actions of cis oleamide are selective amongst ion channels as it does not block glutamate currents even at high doses and it modulates K⁺ channels only at high concentrations (some were weakly antagonized, other unidentified currents enhanced: to our knowledge, twin-pore channels have yet to be examined but oleamide does not directly alter neuronal or synaptosoneuroosomal resting potential). To summarize the above profile, oleamide exerts stereoselective interactions with target ion channels, which are broadly consistent with its depressant actions and with those of structurally divergent anaesthetics. Like most anaesthetics, it can disrupt post-synaptic function at inhibitory ion channels (but not excitatory channels): pre-synaptic transmitter-release and conduction are reduced by depressing currents through voltage-gated pores. At high concentrations (>20 μM) other groups report that oleamide can deconvolute gap junctions in glia. Gap junctions underpin direct electrical coupling in glial syncitia but are probably more familiar to physiologists or anaesthetists as key components in conducting pathways in the myocardium. Supra-clinical concentrations of anaesthetics can also block gap junctions, for example in fast neural relays underpinning high frequency oscillations in the EEG. If anaesthetics do target similar sites, both the voltage-gated channels and the gap junctions in cardiac tissue could underpin some of the cardiodepressant and arrhythmogenic side-effects seen in patients.

It is already apparent that oleamide itself is not a potent anaesthetic, nor does it strongly synergize anaesthetic drugs in laboratory animals. We know that there is a significant pharmacokinetic and distribution problem when exogenous oleamide is administered to animals. A variety of centres found it difficult to reproduce the original Scripps data on sleep by intraperitoneal administration but recent studies confirm the acute psychomotor depression and hypnogenic effects (e.g. see Cheer and colleagues). An important determinant of activity...

Fig 1 Oleamide is a simple unsaturated fatty acid (C18) amide: the cis form of the molecule (shown above) is an active sleep inducer (cOA) but trans oleamide (tOA) was not hypnogenic in vivo. In common with steroid, barbiturate and volatile anaesthetics, in analogous experiments in our laboratory, the sleep hormone depresses spontaneous activity in cultured neurones and modulates GABA activated chloride currents or prolongs inhibitory synaptic currents. (a) Reversible effects of cOA on responses to 4 s pulses of GABA. Note the large increase in evoked currents is coincident with a drastic reduction in the incidence of synaptic currents (baseline noise). (b) The effects of oleamide such as those of anaesthetics reflect steric conformation: only the cis geometric isomer significantly modulates GABA<sub>A</sub> currents (not shown) or the incidence of synaptic currents in culture as shown in the bar chart (quantified here for inhibitory synaptic currents or IPSCs but excitatory events, EPSCs, were also affected in this way). The inset depicts the effects of 20 μM cOA (*) on averaged synaptic currents compared with pre-treatment and post-washout IPSC profiles. Modified from references. Like anaesthetics, oleamide concurrently targets electrogenesis (pre-synaptic conduction) and depresses transmitter-release from nerve terminals. (c) Bursts of tetrodotoxin-sensitive action potentials are blocked by cOA but not tOA (not shown). The primary spike is not impaired even at high doses. The suppression of these sodium spikes is exerted in the low micromolar range (right). Voltage-clamp experiments confirm that, like a variety of anaesthetics, cOA promotes channel inactivation and retards recovery from the inactivated state. These voltage-clamp data (not shown here) have been published in Anesthesiology.
is the inclusion of carrier lipid in the formulation administered suggesting this may quench degradation of the drug. Under these conditions oleamide can synergize benzodiazepines and barbiturates. Like other amides exposed to serum or CSF amidohydrolases (e.g. local anaesthetics) the molecule will be cleaved readily. Even by direct injection into the ventricular system of the brain, the molecule will not distribute widely or evenly because of its extremely hydrophobic character (it has a calculated log P of >6.5, so should be enriched >1 million fold in the membrane plane). In short, oleamide is made in membranes and degraded there. We can see no reason for it to be secreted from the membrane in large amounts (although we are engaged in experiments to address this issue) and certainly not to equilibrate between bulk CSF and localized sites of action (presumably hypothalamic and brain stem sleep centres). Because of the close analogies with anaesthetics in terms of its in vitro profile at crucial neural ion channels we believe that endogenous lipids of this nature should seriously be considered as endogenous ligands for recognition sites exploited by clinically important depressant drugs.

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