In vitro networks: subcortical mechanisms of anaesthetic action

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Subcortical in vitro studies at the Molecular Mechanisms Conference

There were few presentations at the recent International Conference on Basic and Molecular Mechanisms of Anaesthesia (MAC2001) concerning subcortical anaesthetic actions studied in vitro. In addition to the spinal cord studies from the author’s laboratory presented in detail here and the review of the associated literature, there was one other presentation on the spinal cord slice, which will be covered in the appropriate section below (C. Grasshoff and B. Antkowiak, Effect of volatile anaesthetics on action potential firing of spinal neurones in organotypic slice cultures). In a study of the effects of general anaesthetics on thalamic slices (E. Puil, Thalamic effects of general anaesthetics: similar and dissimilar actions of isoflurane and barbiturates), Puil found that isoflurane in the medial geniculate body shunts tonic firing of both sodium and calcium channel-mediated bursts by increasing leak conductance. Similar actions were observed for high concentrations of pentobarbital, but low concentrations enhanced excitation and bursting. Pentobarbital actions were not blocked by bicuculline. These results are relevant to the role of the thalamus in mediating arousal to auditory stimuli and its blockade by anaesthetics. In a study on cerebellar slices (M. Debus, K. Uerzl and B. Antkowiak, The potency of general anaesthetics to suppress spontaneous neuronal activity in cerebellar acute slices compared to neocortical slice cultures), the authors found that a variety of inhalation agents, ethanol and propofol suppressed mean firing rates more potently in neocortical slices than in cerebellar slices by approximately 5-fold, and the potency ratios among the agents were similar for the two preparations. The results are relevant to the actions of anaesthetics on locomotion and balance.

The role of the spinal cord in anaesthesia

The spinal cord plays a role in two anaesthetic end-points: analgesia and immobility in response to a noxious stimulus. Recent evidence also suggests that ascending traffic from the spinal cord to the brain may also modulate the effects of anaesthetic agents on arousal. Analgesia, meaning the loss of painful sensation before loss of consciousness or of immobility, is clearly a property of some agents but may not be of others. In considering analgesic properties with respect to human pain, it is difficult to separate sedation and anxiolysis from analgesia. Animal models of acute pain, measured as tail-flick latency, suggest that inhalation agents are analgesic at sub-immobilizing concentrations but at very low concentrations may be hyperalgesic. In a model of human experimental pain, isoflurane did not reduce perceived pain intensity. In a tail-pressure test, barbiturates and propofol reduce the threshold for withdrawal at levels below those which prevent movement altogether; however, this may represent hyperreflexia rather than enhanced nociception.

Among all the end-points, the most widely employed for inhalation agents is immobility in response to a noxious stimulus. The development of a standard for anaesthetic potency, defined as the minimum alveolar anaesthetic concentration (MAC) required to prevent movement in 50% of subjects in response to a standardized noxious stimulus, has facilitated the comparison of potency among inhalation agents and served as the gold standard for anaesthetic concentrations relevant to proposed mechanisms of anaesthesia. Because MAC is determined as the loss of motor response to a noxious stimulus, this anaesthetic action has sometimes been equated with antinociception or analgesia. However, antinociception and immobility are probably distinct from each other. Not only does the reflex threshold increase in human experimental pain while sensation remains unchanged, but recent evidence suggests that mouse strains that differ markedly in their responses in pain models display only small differences in MAC.

For many years it was automatically assumed that immobility, like some other anaesthetic end-points, is due
to anaesthetic actions in the brain. However, in the early 1990s seminal studies by Rampil and Antognini and colleagues clearly showed that the spinal cord is the dominant central nervous system locus for determining the MAC for volatile anaesthetic agents. Thus, for the first time anaesthetic mechanisms can be studied at the site known to be relevant to a given end-point and at the relevant concentrations. The concentrations used in experimental studies should match those required to reach the end-point according to the part of the central nervous system studied, otherwise the results are irrelevant. For instance, sites such as the hippocampus, associated with memory, should be studied at the lower anaesthetic concentrations associated with amnesia rather than at MAC levels. For studies in the spinal cord, MAC equivalents are the appropriate concentrations.

**Cellular sites of anaesthetic action within the spinal cord**

The spinal components that may contribute to MAC include the central terminations of sensory primary afferent neurones, interneurones of many types, and the cell bodies and initial axon segments of motor neurones. A simplified schematic diagram of the spinal cord circuitry is shown in Figure 1. The peripheral axons of motor neurones distal to the spinal cord probably do not contribute to immobility, nor do actions at the neuromuscular junction. Conduction in large-diameter myelinated axons, like that in motor neurones, is not sensitive to anaesthetic agents at concentrations relevant to MAC, as many studies have shown. Moreover, conduction in primary sensory nerve axons is relatively unaffected, although impulse generation at peripheral nociceptors may be modified. There are several studies in vivo demonstrating volatile anaesthetic actions on the receptive field and the sensitivities of dorsal horn interneurones, but little has been done in vitro in spinal preparations. Similarly, in vivo studies on the H-reflex document that volatile anaesthetics can depress the simpler monosynaptic circuit from primary afferent nerve terminals to motor neurones. H-reflexes are depressed by volatile agents at subanaesthetic concentrations, but only at high blood concentrations of propofol. Etomidate and ketamine actually increase H-reflex amplitude. The F-wave is an index of motor neurone excitability. The F-wave represents antidromic invasion of central motor neurones that is sufficiently depolarizing to trigger orthodromic impulses after the absolute refractory period has passed. The F-wave may reflect not only intrinsic motor neurone excitability, but also tonic excitatory and inhibitory influences. F-waves are depressed by inhalation agents, including nitrous oxide, at clinically relevant concentrations. Both H- and F-wave depression correlate with loss of movement in response to a noxious stimulus.

**Scope of the review**

This review will focus on anaesthetic actions on motor neurones and on transmitter release from elements presynaptic to them. The hypothesis will be presented that direct actions of anaesthetic agents, in particular volatile agents, on motor neurones contribute importantly to immobility. In this discussion, motor neurones will be treated as a homogeneous population. This is almost certainly an oversimplification as motor neurones vary in size and position according to the muscles they innervate—flexors vs extensors, proximal vs distal, and slow postural vs fast movement muscles. Motor units differ in properties such as speed of adaptation to a sustained stimulus. In mammals there is also a separate population of small motor neurones that innervate muscle spindles. The extent to which these populations of motor neurones differ in response to anaesthetic agents is uncertain.

**Early studies in the spinal cord**

Most early basic neurophysiological studies in vertebrates were performed in spinal cord motor neurones, which became the cell type used most widely for understanding vertebrate neurophysiology. Interpretation of experimental results was facilitated by several advantages of the vertebrate spinal cord. The separation of afferent input from motor output in dorsal and ventral roots permitted a defined input by stimulating a dorsal root; extracellular recordings could be made from ventral roots in the knowledge that this reflected the responses of motor neurones. After the introduction of microelectrode intracellular recording, the large size of motor neurones made them nearly the only vertebrate neurone that offered advantages comparable to those of the large axons and cell bodies of crustaceans and molluscs. Functionally, a cell impaled by blind probing could be unambiguously identified as a motor neurone by activating it antidromically via the ventral root.

Much of the initial knowledge of anaesthetic actions was developed by gruelling in vivo studies requiring hours of surgical preparation and painstaking maintenance of the experimental animal. In a landmark series of such studies, Somjen demonstrated that ether and thiopental depressed the monosynaptic excitatory postsynaptic potential in all motor neurones, elevated the threshold for impulse initiation in approximately half, and had no consistent effect on resting membrane potential. There were no discernible effects on conduction in the primary afferent nerve terminals. The results of his studies led to the hypothesis that these two anaesthetics decrease reflex responses predominantly by depressing excitatory synaptic transmission to motor neurones, with an uncertain and variable contribution from an increase in threshold; depression of excitatory synaptic transmission was postulated to be due to postsynaptic changes, although a decrease in excitatory transmitter release could not be excluded. With only minor
modifications as a result of the proliferation of the receptors and ion channels that are now known to influence neurones, hypotheses such as these still form the nucleus of the debate concerning anaesthetic actions in the spinal cord.

The techniques used in these difficult in vivo studies were superseded in the early 1970s by the in vitro hippocampal slice preparation. The hippocampal slice became the dominant neuronal preparation just in time to participate in and benefit from the development of new knowledge regarding the multiplicity of neurotransmitters and the complexity of determinants of excitability. However, almost simultaneously with the growth of hippocampal slice studies, Otsuka and his colleagues developed an in vitro preparation of the neonatal rat spinal cord, which we and others have used extensively in studies of anaesthetic and analgesic actions. Later, Takahashi and Konnerth showed it...
was possible to study visually identified motor neurones in spinal cord slices, initially by sharp electrode recording and eventually with whole-cell patch clamp. This preparation is now in widespread use.

Factors governing motor neurone excitability

This section outlines receptors and ion channels that determine motor neurone excitability and the anaesthetic actions on them where these are known.

Intrinsic properties

All neurones maintain a resting potential difference between the inside and the outside of the cell membrane. The ultimate cause of this is active ion transport, predominantly of sodium ions from the inside to the outside of the cell, and the passive redistribution of potassium ions to the cell interior. However, the more immediate contributor is the selective potassium permeability of voltage-independent (non-gated) ion channels. Increased activity of these channels, by increasing the conductance to potassium, will move the resting potential towards the potassium equilibrium potential and away from the threshold of impulse initiation. In addition, by lowering membrane resistance these channels will short-circuit excitatory synaptic input and render it less effective. An anaesthetic agent that increased the passive flow of current through such channels would thus reduce excitability. Channels of particular current interest are tandem pore potassium channels. At least some members of this class of channels have a response to volatile anaesthetic agents that would move excitability in this direction.

Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel. Some types of sodium channels are sensitive to volatile anaesthetic agents at reasonable concentrations. A persistent inward sodium current in hypoglossal motor neurones that has a pH sensitivity similar to that of the TASK-1 tandem pore potassium channel.

Motor neurones respond to persistent depolarization above threshold by generating a train of action potentials. The frequency and number of impulses in the train are limited by calcium-dependent potassium channels; alcohols, ketamine and a barbiturate reduce current through channels of this type but volatile anaesthetics exert little effect at anaesthetically relevant concentrations.

Synaptic inputs: excitatory

Most of the fast ligand-gated excitatory synaptic input to motor neurones is mediated by receptors of the α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) subtype (GluR1–4); however, at least in the very young rat, spinal cord N-methyl-d-aspartate (NMDA) receptors provide a greater contribution to the depolarization than is observed in hippocampal cells. Although some kainate receptors, particularly GluR5 and KA1, are present on motor neurones, kainate receptors provide a vanishingly small contribution to excitatory input (J. Knape and J. J. Kendig, unpublished data). Motor neurones are themselves cholinergic, but there appears to be little contribution from nicotinic receptors to excitatory input; the response to the application of nicotinic agonists is very small (J. Knape and J. J. Kendig, unpublished data). It is not certain whether 5-HT3 receptors are present on motor neurones.

In addition to fast excitatory transmission, there are slower forms of excitatory transmission mediated by metabotropic (G-protein-coupled) receptors of various types. These include serotonin, neurokinin (NK) 1-3, α1- and α2-adrenergic, dopamine, adenosine, cholinergic muscarinic and metabotropic glutamate receptors. The functions of these receptors on motor neurones are not certain, and some may be inhibitory rather than excitatory.

Synaptic inputs: inhibitory

Unlike other parts of the central nervous system, the major ionotropic inhibitory transmitter in the spinal cord is glycine, although GABA also contributes; there is evidence that the two ligands of inhibitory chloride channels are coreleased at the same synaptic terminals. Motor neurones are subject to tonic inhibitory input, as evidenced by spontaneous miniature synaptic currents, and also to both feed-forward and feedback inhibition. Feed-forward inhibition permits coordinated movement by enforcing alternate relaxation of flexor and extensor muscles, and prevents the exaggerated reflexes characteristic of some CNS disorders. Feedback inhibition is represented by the classic Renshaw cell, innervated by a collateral branch from the motor neurone axon and in turn forming inhibitory synaptic connections with the motor neurone. The early component of this inhibition is due to glycinerergic input and the later component to GABA. In addition to the fast ligand-gated chloride channels, there are other inhibitory inputs to motor neurones mediated by second messenger-linked receptors, including those for GABA<sub>B</sub> receptors. There is a vast literature on anaesthetic actions on GABA<sub>A</sub> and glycine receptors, but little on slower modulatory inhibitory channels.
Methods for studying spinal actions of anaesthetics in vitro: extracellular

The sum of the responses of a population of motor neurones can be recorded from an electrode placed over a ventral root in close proximity to the ventral horn, with stimuli applied to a dorsal root. This can be done in vitro with intact spinal cords taken from very young rats. Many of the early studies on anaesthetic actions cited above employed this method in vivo. In addition to the usual advantages of controlled conditions for in vitro recording, the method has the advantages of employing a purely sensory orthodromic volley and the capability of measuring subthreshold motor neurone responses. However, the neuronal circuits still essentially constitute a black box, giving the method the advantage of revealing everything the entire spinal cord can do but the disadvantage, in mechanistic terms, that changes induced by anaesthetics cannot be attributed to one element or another. Extracellular recording, by its nature, does not produce meaningful absolute values for the amplitudes of the responses recorded, so changes must be expressed as percentages of the control amplitude for each experiment. Furthermore, under most experimental conditions only relatively fast and large transients can be observed, again giving limited insight into underlying changes in resting potential or membrane resistance in individual motor neurones. One type of extracellular recording, in which potentials in a ventral root are measured across a sucrose gap of very high resistance, partially resolves these limitations, although again only average changes can be measured, not absolute values of potentials in individual motor neurones. The method thus obscures variability in the population of motor neurones and is influenced predominantly by the largest motor neurones in the pool. However, this method was used in a very influential paper that demonstrated hyperpolarization in motor neurones of the spinal cord of the frog, measured as the algebraic sum of the responses of the total population of motor neurones to a broad range of anaesthetics, including barbiturates, ether and α-chloralose. The hyperpolarization was not blocked by GABA_A antagonists (glycine antagonists were not used) and was hypothesized to be the result of an increase in potassium conductance, because parallel experiments on hippocampal neurones showed hyperpolarization accompanied by a conductance increase.

The monosynaptic reflex and its underlying excitatory postsynaptic potential

Stimulating a lumbar dorsal root and recording from the corresponding ipsilateral ventral root elicits the classic monosynaptic reflex (Fig. 1A), observed as a compound action potential in the ventral nerve root. Thresholds for eliciting this response are so low that little of the underlying excitatory postsynaptic potential (EPSP) can be seen before it is obliterated by the action potential. Offsetting the stimulating and recording electrodes by one or two segments permits a large subthreshold EPSP to be recorded without contamination by the compound action potential. The fast population EPSP in motor neurones is mediated by both AMPA and NMDA receptors; a combination of AMPA-selective and NMDA-selective antagonists almost completely abolishes it. Motor neurones are subject to tonic inhibition via both GABA_A and glycine receptors; application of the GABA_A antagonist bicuculline or the glycine antagonist strychnine elevates the response.

Anesthetic actions on extracellularly recorded responses are summarized in Table 1. The population EPSP and the monosynaptic reflex are sensitive to volatile anaesthetic agents, including halothane, isoflurane, enflurane and ethanol, at concentrations equivalent to MAC or lower. Concentrations that inhibit the monosynaptic reflex correlate well with MAC. An experimental anaesthetic, cyclobutane, depresses the monosynaptic reflex, whereas a related non-immobilizing compound does not.

### Table 1 Summary of anaesthetic actions in the spinal cord. Where a class of agent is not mentioned, the relevant experiments have not been done

<table>
<thead>
<tr>
<th>Property</th>
<th>Anaesthetic action</th>
</tr>
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<tbody>
<tr>
<td>Motor neurone</td>
<td></td>
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<tr>
<td>Resting potential</td>
<td>Variable increase (volatiles, barbiturates) or no change (ethanol)</td>
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<tr>
<td>Conductance</td>
<td>Variable increase (volatiles) or no change (ethanol)</td>
</tr>
<tr>
<td>Threshold</td>
<td>Increase (volatiles, ethanol, barbiturates)</td>
</tr>
<tr>
<td>Glutamate currents</td>
<td>Decrease (volatiles)</td>
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<tr>
<td>Glycine currents</td>
<td>Prolongation, no amplitude change (volatiles)</td>
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<tr>
<td>Terminals presynaptic to motor neurones</td>
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<tr>
<td>Glycinergic</td>
<td>Increase in spontaneous transmitter release but only when sodium channels blocked, otherwise no change or decrease (volatiles)</td>
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<tr>
<td>Glutamatergic</td>
<td>Decrease in spontaneous transmitter release under both conditions (volatiles and ethanol)</td>
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<tr>
<td>Total inhibitory charge transfer</td>
<td>Increase only when sodium channels blocked, otherwise no change or decrease (volatiles)</td>
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<tr>
<td>Synaptic transmission to motor neurones</td>
<td></td>
</tr>
<tr>
<td>Monosynaptic reflex</td>
<td>Decrease (volatiles and ethanol, not barbiturates or ketamine)</td>
</tr>
<tr>
<td>Slow ventral root potential</td>
<td>Decrease (all agents), more sensitive than monosynaptic reflex</td>
</tr>
<tr>
<td>Roles of inhibitory neurotransmitters (GABA, glycine)</td>
<td>Contribution to anaesthetic depression of synaptic transmission, but not to depression of direct glutamate-evoked currents in motor neurone (volatiles and ethanol)</td>
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depress both the AMPA and NMDA receptor-mediated components of the EPSP, but with some apparent selectivity for the NMDA component. The monosynaptic reflex is not diminished by barbiturates, propofol, α2-adrenergic agonists, opiates, ketamine, and barbiturate, depressed monosynaptic reflexes. However, the propofol results were obtained on a background of barbiturate anaesthesia, which might be expected to exert additive effects with propofol.

The slow ventral root potential

The slow ventral root potential is a complex polysynaptic response that requires the activation of small-diameter sensory nerves for its full expression (Fig. 1A). Because of this property, its evocation by true noxious stimuli and its sensitivity to analgesic agents, it is believed to be related to nociception. The slow ventral root potential has an early component sensitive to NMDA receptor antagonists and a late component that appears to be mediated by a variety of metabotropic receptors.

The slow ventral root potential is more sensitive to anaesthetic agents than the fast EPSP. The late metabotropic receptor-mediated component is selectively sensitive to α2-adrenoceptor agonists, whereas volatile anaesthetic agents, ketamine, midazolam, and alcohols show some selectivity for the early component. It is not certain where the receptors that mediate these anaesthetic actions are located. Certainly there are NMDA receptors on motor neurones, and possibly also metabotropic receptors of various types. However, it is also probable that at least some of the anaesthetic depression of the slow ventral root potential results from anaesthetic actions on interneurones presynaptic to the motor neurones (Fig. 1A). This question cannot be resolved by studies on the intact cord.

Information from genetically engineered mice

Studies on population-evoked ventral root responses in the intact cord are limited in the extent to which mechanistic explanations can be derived. However, some progress can be made. We have investigated responses to enflurane in spinal cords from mice that lack the β3 subunit of the GABA receptor, which behavioural studies have shown to have an increased enflurane requirement to prevent movement in response to a noxious stimulus. However, ventral root responses in spinal cords from the null mutants did not differ from those from cords from wild-type animals in their sensitivity to enflurane. The contributions of GABA receptors to enflurane actions did change, however; bicuculline significantly attenuated enflurane’s depressant actions in cords from wild-type mice but not in mutant cords. The results suggest that EPSP depression results from anaesthetic actions on multiple targets in the spinal cord, and that the mutation caused an unpredicted shift in the proportions of targets in the mix. GABA receptors became less important, but in order to maintain the same sensitivity other targets must have played a greater role in the mutants than in the wild-type animals. In the same studies, glycine receptors appeared to be up-regulated in the mutants. These receptors also contributed slightly to anaesthetic actions on the EPSP, but to the same extent in wild-type and mutant animals. Thus, some other, unidentified receptors took on a greater role in anaesthetic depression in the mutant animals. The results are theoretically highly interesting, but illustrate the riskiness of assuming that global knockout mutations will provide simple solutions to the puzzle of anaesthetic mechanisms.

Recording from single motor neurones in the spinal cord slice

The disadvantages of extracellular recording are resolved by studies of single motor neurones, most commonly in recent times using patch-clamp techniques to record from cell bodies. The method of preparing slices from the spinal cord, combined with visualization of cells in the cord via infrared illumination on closed circuit TV and fluorescent labelling, allows the identification of large cells in the ventral horn as motor neurones. With a different technique, using antidromic activation via a ventral root, motor neurones can be identified functionally in the intact or semi-intact cord.

When motor neurones are stimulated via a dorsal root (or in slices via an electrode placed in the dorsal root entry zone) (Fig. 1B and C), the changes induced by anaesthetics can be directly related to studies of population-evoked responses in the intact cord, but with greater insight into their mechanistic basis. Furthermore, by evoking responses in motor neurones directly with glutamate, meanwhile blocking presynaptic impulse activity, it is possible to bypass presynaptic elements and unambiguously identify anaesthetic actions on motor neurones themselves (Fig. 1D). As well as advantages, there are some disadvantages to single-cell recording. In the intact cord, finding and identifying motor neurones by blind probing is cumbersome and the yield is low. In slices, visual control makes establishing a recording easy, but with much of the connectivity stripped away important elements may be lost.

Dorsal root-evoked responses are the single-neurone counterpart of the population ventral root responses evoked by dorsal root stimulation in the intact cord. Although it is possible in the intact cord to examine the slow depolariza-
Presynaptic impulse activity and bicuculline to block GABA<sub>A</sub> receptors in voltage-clamp conditions in the presence of tetrodotoxin to block spinal cord neurones. (A) The excitatory postsynaptic potential evoked by electrical stimulation in the dorsal root entry zone is depressed by enflurane at an anaesthetic concentration. (B) Enflurane 1 MAC also depresses the inward current evoked by glutamate puffs (arrows) under voltage-clamp conditions in the presence of tetrodotoxin. The same is true when glycine receptors are also blocked. Thus, some of the depressant actions of enflurane result from direct actions on glutamate currents in motor neurones themselves, independent of the presynaptic circuitry and actions on inhibitory receptors.

Actions that are the counterparts of population slow ventral root potentials, in spinal cord slices any such responses are small because of the diminished input. Most studies have therefore examined relatively fast events, which correspond to the short-latency population EPSP and early polysynaptic responses. In addition to examining excitatory synaptic transmission, single-cell studies using whole-cell patch techniques in current-clamp mode or intracellular sharp electrodes can look at changes in intrinsic excitability by examining resting membrane potential, conductance, threshold and the number of impulses evoked by depolarization to a given level. Studies in voltage-clamp mode can exclude changes in response resulting from resting membrane potential changes, at least in the cell body and proximal dendrites; clamp control in distal dendrites is problematic.

**Anaesthetic actions on intrinsic motor neurone excitability**

Anaesthetic actions on individual motor neurones are summarized in Table 1. Using intracellular electrodes, Takenoshita and Takahashi<sup>67</sup> found that halothane hyperpolarized motor neurone cell membranes in rats by several millivolts while increasing input conductance. The threshold for spike initiation did not change. The authors concluded that the larger current required for impulse initiation was due to the hyperpolarization and the increase in conductance, which they attributed to an increase in potassium permeability. In studies with whole-cell patch electrodes, our studies also showed that halothane consistently hyperpolarized motor neurones, with a reduction in the number of impulses evoked by depolarizing current injections, which could be attributed to the hyperpolarization. However, there was no significant change in input resistance (G. Cheng and J. J. Kendig, unpublished data). Ethanol also decreased the number of impulses evoked by a given level of depolarizing input current, but without changes in resting potential, whereas input resistance increased, as did threshold.<sup>73</sup> Enflurane also hyperpolarized mouse spinal cord neurones, but again with no significant change in input resistance.<sup>13</sup> In a paper presented at the MAC2001 conference (C. Grasshoff and B. Antkowiak, Effect of volatile anaesthetics on action potential firing of spinal neurones in organotypic slice cultures), the spontaneous activity of unidentified neurones induced by low magnesium was also reduced by enflurane, halothane and sevoflurane. Because the studies were extracellular, there is no information on resting potential changes or conductance. The early studies by Somjen cited above suggested that, for ether and thiopental, hyperpolarization was inconsistent. Our studies show that hyperpolarization varies among agents but there is no consistent change in membrane conductance. Thus, reduction in the number of impulses in a train and increase in threshold appear to have different causes for different agents, and our laboratory differs from others in finding no decrease in membrane resistance.

**Anaesthetic actions on glutamatergic excitation**

As in the intact cord, single-cell studies show that volatile anaesthetic agents and ethanol depress the fast EPSP and the corresponding excitatory postsynaptic current (Fig. 2A).<sup>13</sup> <sup>67</sup> <sup>72</sup> <sup>73</sup> Enflurane and ethanol depress both AMPA and NMDA receptor-mediated components of EPSPs and currents.<sup>13</sup> <sup>72</sup>

When presynaptic impulses are blocked by tetrodotoxin to cut out presynaptic circuitry and excitatory potentials and currents are evoked by brief pulses of glutamate, then ethanol and enflurane depress glutamate-evoked potentials and currents.<sup>13</sup> <sup>72</sup> This result shows that at least some of the actions of these agents can be attributed to direct actions on the motor neurones themselves, in addition to any effects mediated via presynaptic elements.

It has been a dominant theory that enhancement of activity at GABA<sub>A</sub> receptors is an important component, perhaps the only component, of the mechanism of action of a wide variety of anaesthetic agents.<sup>24</sup> <sup>25</sup> <sup>69</sup> Although there has been a recent retreat from the universality of this view,<sup>15</sup> it remains pervasive in discussions of molecular mechanisms of anaesthesia. For the spinal cord, similar or greater importance is attached to the enhancement of glycine receptor-mediated inhibition. In order to test whether postsynaptic actions on either or both of these receptors
account for anaesthetic depression of glutamate-evoked responses in motor neurones, both receptors were blocked by their respective antagonists. In the presence of bicuculline, strychnine or both, ethanol and enfurane still depressed both AMPA and NMDA types of glutamate-evoked currents (Fig. 1b). Application of any of the antagonists, alone or in combination, did not change the sensitivity of glutamate-evoked currents to the anaesthetic agents. This result shows that volatile anaesthetics can exert depressant actions on spinal motor neurones independent of actions on either of the inhibitory chloride channels, and equally on currents mediated by both major subtypes of glutamate receptors. The result does not, however, demonstrate that these anaesthetics act directly on the glutamate receptors, as the depression of currents may be mediated indirectly via an action elsewhere.

### Actions on glycinergic inhibitory transmission

To define pre- and postsynaptic anaesthetic actions at glycinergic synapses, we investigated the effects of volatile anaesthetic agents on spontaneous and evoked glycinergic currents in spinal cord motor neurones. The volatile anaesthetic agents enfurane, isoflurane and halothane significantly increased the frequency of glycinergic miniature inhibitory postsynaptic currents (mIPSCs). However, without tetrodotoxin, isoflurane and halothane had no effect and enfurane decreased spontaneous IPSC frequency. All the anaesthetics prolonged the decay time constant (τ) of both spontaneous and glycine-evoked currents without increasing the amplitude. With tetrodotoxin the total charge transfer was increased, but without tetrodotoxin the charge transfer was unchanged (isoflurane and halothane) or decreased (enfurane). Enfurane-induced increases in mIPSC frequency were not significantly affected by Cd²⁺ (50 μM), thapsigargin (1–5 μM), or KB-R7943 (5 μM). KB-R7943 and thapsigargin together abolished the enfurane-induced increase in mIPSC frequency. Thus, there are opposing facilitatory and inhibitory actions of volatile anaesthetics on glycine release that depend on calcium homeostatic mechanisms and sodium channels respectively. Under normal conditions (no tetrodotoxin), the absolute amount of glycinergic inhibition does not increase. The contribution of tonic glycinergic inhibition to anaesthesia may depend on its duration rather than its absolute magnitude.

Comparative studies on glutamate miniature excitatory currents mediated by AMPA receptors show only a decrease in frequency with enfurane whether or not sodium channels are blocked (G. Cheng and J. J. Kendig, unpublished data). Thus, anaesthetic actions on spontaneous transmitter release are transmitter-specific, a finding which implies differences in the presynaptic terminals or in the release machinery for glutamate versus glycine, or in both.

### Relative roles of anaesthetic actions on motor neurones and presynaptic elements

Although the studies outlined in the preceding section show clearly that anaesthetic agents can act directly on motor neurones to depress responses to transmitter and to reduce the probability of impulse generation, the methods of isolating postsynaptic responses preclude the assignment of relative sensitivity. Direct application of glutamate does not mimic glutamatergic synaptic transmission precisely. The concentration of glutamate applied does approximate the millimolar concentrations released into the synaptic cleft, but exogenous glutamate also activates extrasynaptic receptors and the time course of action is different. In spite of these limitations, the results do show that motor neurone responses to glutamate are depressed at the same concentrations of anaesthetic that depress circuit-mediated synaptic transmission in the intact cord. Thus, the question of relative importance is like the chicken and the egg. If the motor neurone cannot respond, in a sense it does not matter whether or not presynaptic input is also reduced, and vice versa.

There is a difference between synaptically evoked responses and glutamate-evoked currents with respect to the contributions of GABAₐ and glycine receptors to anaesthetic actions. Blockade of these inhibitory chloride channels does not alter the anaesthetic sensitivity of motor neurones when the probe is exogenously applied glutamate, but does attenuate anaesthetic actions measured as depression of the monosynaptic EPSP. The attenuation is not large, particularly in the case of glycinergic receptors, and clearly the response is still depressed in the presence of antagonists to these receptors. It might be argued that the ability of actions on inhibitory channels to modify glutamate-evoked responses is limited when spontaneous activity is blocked. However, there is considerable tonic release, particularly of glycine, even under these conditions, and both bicuculline and strychnine increase the amplitude of glutamate-evoked currents.

### Relationship between studies on motor neurones and movement in vitro

In view of the role of motor neurones as the final common path that integrates all upstream input into a move–no move decision, anaesthetic actions that limit the ability of motor neurones to generate a train of impulses in response to an excitatory transmitter are directly relevant to the anaesthetic end-point of immobility in response to a noxious stimulus. Results in vivo are in agreement with a limited role for inhibitory chloride channels in blocking movement. Block of these channels by intrathecal application of bicuculline and strychnine increases anaesthetic requirement by a maximum of approximately 40%. As the reports of the studies cited above make clear, there are multiple targets for anaesthetic actions, both on motor neurones and presynaptic elements.
neurones themselves and on elements presynaptic to them. Anaesthetics depress the ability of motor neurones to generate action potentials, by acting on a number of different ion channels that may vary from agent to agent. Some of these may generate hyperpolarization, but this is not universal and is not a prerequisite for reducing impulse generation for at least some agents. The preceding section suggests that a number of voltage-gated and voltage-independent channels regulate excitability. It is probable that not all the targets that modulate intrinsic excitability have been identified.

It is clear that actions of anaesthetic agents on GABA<sub>A</sub> and glycine receptors are important. However, in particular for volatile agents, they are not the sole or even the major contributors to anaesthetic depression of motor neurone excitability. Actions on these receptors may be more important for some i.v. agents, including propofol and barbiturates, and there is evidence that enhancement of GABA-mediated responses in the spinal cord is more prominent for these agents than for ethanol and isoflurane.14 31 55 74

Actions on the glutamate receptors found on motor neurones are probably important. For ethanol and enflurane there is no apparent selective sensitivity for NMDA vs AMPA currents. Selective actions on NMDA vs AMPA currents have been reported for xenon but not isoflurane in another preparation.15 However, there is still the caution that there is as yet no identified site for volatile agents on the glutamate receptor itself, and anaesthetic actions on receptor currents may be indirect. Ketamine, on the other hand, almost certainly exerts its actions, on motor neurones as elsewhere, predominantly as a non-competitive antagonist at NMDA receptors.

There are large areas to which little attention has been paid but will probably be important in the future. In particular, anaesthetic actions on the intracellular metabolic pathways that lead to changes in receptor phosphorylation constitute an area of research that has hardly been touched. In addition, anaesthetic actions on receptors coupled to G-proteins have not been investigated extensively.

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