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Are mesopontine cholinergic neurons either necessary or sufficient components of the ascending reticular activating system?

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The reticular activating system is thought to be composed of one or more thalamo-cortical afferents whose activation results in desynchronization of the electroencephalogram. In recent years, a strong body of correlative evidence has accumulated suggesting that mesopontine cholinergic neurons are a key component of the reticular activating system. However, despite intense study, several critical predictions of the hypothesis remain unfulfilled, and it is still not possible to conclude that mesopontine cholinergic neuronal activity is either necessary or sufficient for generation of desynchrony. Specific criteria required to satisfy this hypothesis are put forth, and potential experimental approaches required are outlined. Such rigorous treatment of this issue will assist in maintaining the rapid pace of advance in this field.

Key words: cholinergic neurons / reticular activating system / electroencephalogram

EVER SINCE THE pioneering studies of Moruzzi and Magoun,[1] it has been hypothesized that within the brain there exists one or more neuronal systems whose function is to modulate global brain activity. A major advance came when Shute and Lewis applied the acetylcholinesterase histochemical technique to brain tissue and revealed the presence of a putative cholinergic component of the ascending reticular activating system.[2] Subsequent studies using choline acetyltransferase immunohistochemistry confirmed that neurons in the laterodorsal and pedunculopontine tegmental nuclei were indeed cholinergic[3-7], and firmly cemented the concept that mesopontine cholinergic neurons might indeed contribute to the reticular activating system.

In the intervening years, much effort has been devoted to study of the biology of these neurons, with particular emphasis upon their role as mediator of the reticular activating system. In the paragraphs below we review the salient evidence relevant to this issue and find that while supportive data exists, it is as of yet impossible to conclude that transmitter release from the axon terminals of mesopontine cholinergic neurons is either sufficient or necessary for the function of the reticular activating system.

At the outset, it is important to define what is meant by the term 'reticular activating system'. Over the years, the reticular activating system has variously been described as both conceptual heuristic and a *bona fide* anatomical entity. Moreover, a number of functions have fallen under the umbrella of its moniker, either implicitly or explicitly, adding further confusion to the issue. For the purposes of this review, we define the reticular activating system as the neuronal system(s) whose function is to modify thalamo-cortical function such that EEG desynchrony ensues.

The precise neuronal elements involved in generating the EEG are not known with certainty. However, over the past decade a strong body of evidence has accumulated indicating that thalamic activity is fundamental to the process. The prevailing view is that thalamic relay neurons exist in two states, a relatively depolarized state in which tonic firing can be evoked, and a relatively hyperpolarized state in which burst firing predominates.[8,9] Generally speaking, when thalamic relay neurons are in burst mode the EEG is synchronized and when they are in tonic mode it is desynchronized. Thus, the first criteria to be fulfilled by neurons contributing to the ascending reticular activating system is that they project to and depolarize thalamic relay neurons sufficiently to bring them into the tonic mode of activity. Mesopontine cholinergic neurons fully satisfy this criteria, as they provide a massive innervation of the thalamus[3-7] and synapse[10] upon relay neurons. Not only is the anatomical connectivity of these neurons appropriate, but physiological studies also support the hypothesis: exogenous

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acetylcholine depolarizes thalamic relay neurons in brain slices[11] and similar effects are seen *in vivo* following stimulation of the region of the mesopontine tegmentum in which cholinergic neurons are localized.[12-14]

It should be recognized that mesopontine cholinergic neurons are not exclusive in this regard: noradrenergic, serotonergic, histaminergic, and in some species basal forebrain cholinergic neurons all project to the thalamus,[15] and can excite thalamic relay neurons either directly or indirectly.[16] Similar effects can even be seen by stimulation of a metabotropic glutamatergic cortico-thalamic pathway,[17] and there may be other afferent systems not yet described which play similar roles. What appears to be unique about the mesopontine cholinergic neurons is that the thalamus represents their major target, whereas it only represents one of many for the other systems listed. Nonetheless, at the present time it must be considered that numerous neuronal systems may contribute to the reticular activating system. One important goal for the future of the field is to determine which systems do indeed constitute the reticular activating system, and what their precise contributions are.

A second requirement of any set of neurons contributing to the reticular activating system is that they should exhibit relatively high levels of activity when the EEG is desynchronized, namely during waking and REM sleep, and conversely should be relatively inactive during slow wave sleep when EEG synchrony is seen. This requirement would seem to eliminate consideration of noradrenergic, serotonergic and histaminergic neurons, at least as mediators of EEG desynchrony during REM sleep; all three sets of neurons exhibit the classic behavioral neurophysiological profile of firing regularly during waking and falling silent during REM sleep.[18,19] On the other hand, it is impossible to rule out a role for these neurons during waking, and indeed it seems likely that they do contribute to the process of EEG desynchrony during the waking state.

Electrophysiological recordings obtained from the mesopontine tegmentum have indicated that the majority of neurons in the region show increased activity whenever the EEG is desynchronized.[20-22] Unfortunately, these data are not definitive as the region is both electrophysiologically and neurochemically heterogeneous and the exceedingly difficult task of determining the transmitter status of such neurons *in vivo* has not been achieved. Moreover, a subset of neurons in this region appear to fire selectively during REM sleep.[20-22] Such data appeared to be consistent with experiments demonstrating powerful inhibitory effects of both serotonin[23,24] and noradrenaline[25] upon identified mesopontine cholinergic neurons in brain slices. Given that noradrenergic and serotonergic neurons are at their most active during waking when the EEG is desynchronized,[18] it was hypothesized[26] that mesopontine cholinergic neurons *in vivo* might be inhibited during waking and active only during REM sleep. In this scenario, EEG desynchrony would arise by different mechanisms in different states, by the action of non-cholinergic thalamic afferents during waking, and by the action of mesopontine cholinergic afferents during REM sleep.

In order to shed light upon this question, we turned to *in-vivo* microdialysis to measure release of acetylcholine in the rat thalamus across behavioral states, and found acetylcholine release to be high during both waking and REM sleep, and significantly lower during slow wave sleep.[27] Although such studies are not commonly carried out in the rat brain, in this case it provided information difficult to obtain in other species where in addition to the prominent innervation of the thalamus from the mesopontine cholinergic cell mass, there also exists a modest but not insignificant innervation of the thalamus originating from the basal forebrain.[28,29] This is not an issue in the rat brain, where only the thalamic reticular nucleus receives an innervation from the basal forebrain,[30] with the remaining cholinergic innervation deriving from the brainstem. We further confirmed that the acetylcholine release which was measured indeed derived from the brainstem afferents by infusing a retrograde tracer through the microdialysis probe, thereby determining the innervation of the region of the thalamus sampled by the microdialysis probe. Following immunohistochemical processing, it was observed that 95% of the cholinergic neurons labelled in the brain were found in the mesopontine tegmentum, indicating that the acetylcholine release measured in the thalamus derived in large part from the terminals of mesopontine cholinergic neurons. Thus, it seems clear that the activity of mesopontine cholinergic neurons co-varies with EEG desynchrony, and that cholinergic neurons are active both during waking and REM sleep. In addition to providing insight about the behavioral neurophysiology of mesopontine cholinergic neurons, these data would suggest that any inhibitory effects of serotonin and noradrenaline during the waking state are largely overcome by excitatory inputs, such as those mediated by NMDA receptors.[31]

Taken together, these observations provide correlative evidence in support of the hypothesis that mesopontine cholinergic neurons contribute to the reticular activating system. However, the fact that other cell groups can subserve similar functions under at least some conditions[32] and the overall increasing sophistication of the field now requires us to ask the more probing question of whether or not activity in mesopontine cholinergic neurons is necessary and/or sufficient for generating desynchrony of the EEG.

First, we shall consider the issue of necessity. If mesopontine cholinergic neuronal activity is necessary for EEG desynchrony (assuming the rest of the neuronal elements required are intact and functional), then blockade of the action of acetylcholine in the thalamus should block EEG desynchrony. In fact, it has long been known that systemic administration of the antimuscarinic compound atropine blocks EEG desynchrony during most waking behaviors,[33] but the locus of the effect has never been determined. Although technically challenging, in theory one could apply an antimuscarinic compound locally within the thalamus and then document any resultant changes in the EEG. Such an experiment has yet to be performed.

One confound associated with such an experiment is the implicit assumption that it is acetylcholine release which is critical. In fact, although frequently described as cholinergic neurons, these cells co-express a variety of neuroactive agents including substance P, corticotropin releasing factor, gastrin-releasing peptide, natriuretic peptides, and nitric oxide synthase.[34-37] Thus, in order to thoroughly investigate this issue, one may have to block not only cholinergic receptors in the thalamus, but perhaps receptors for any other neuroactive agents which may be released from the axon terminals of these neurons as well.

A second aspect of the necessity theory predicts that elimination of the parental cell bodies in the mesopontine tegmentum should eliminate EEG desynchrony. It is true that transection of the neuraxis at the level of the mesopontine tegmentum abolishes EEG desynchrony during waking,[38] but such treatments are clearly not selective. More intriguing have been the results of studies which have attempted to specifically lesion the regions of the brainstem core where cholinergic perikarya are located, as this manipulation has surprisingly little effect upon EEG desynchrony.[39] These experiments are still not definitive, as only 60–70% loss of mesopontine cholinergic neurons was achieved, but they do cast some doubt upon the hypothesis that cholinergic neuronal activity is *necessary* for EEG desynchrony. In fact, the most parsimonious interpretation is that either (1) activation of mesopontine cholinergic neurons is not functionally related to EEG desynchrony; or (2) in the absence of cholinergic neuronal activity, EEG desynchrony may occur via other means. Resolution of this question still awaits the development of a means of selectively lesioning mesopontine cholinergic neurons. Such an experiment would go a long way towards clarifying the requirement of these neurons for EEG desynchrony.

The second question of importance is sufficiency. The most direct test of this hypothesis would be to stimulate the mesopontine cholinergic neurons and induce EEG desynchrony. This experiment was first performed nearly 50 years ago by Moruzzi and Magoun in their original pioneering studies, and has been repeated many times since. However, the specificity of this manipulation remains a critical issue, for several reasons: (1) the region is neurochemically heterogeneous; (2) the brainstem contains myriad fiber tracts and the extent to which the stimulation spreads is unknown; and (3) similar effects can be obtained when stimulating a variety of regions of brainstem reticular core. Once again, selective stimulation of mesopontine cholinergic neurons is required. Moreover, even though blockade of muscarinic receptors can block EEG desynchrony evoked by electrical stimulation of the brainstem, the locus of this effect is difficult to determine, and may even be intracortical.[40,41] A second prediction of the sufficiency hypothesis is that exogenous application of acetylcholine to the thalamus will induce EEG desynchrony. Again, this experiment has never been carried out, but sensitive use of *in-vivo* microdialysis as a means of local drug application may go some way in addressing this issue. In addition, one must remain aware of the question of co-localized neurotransmitters (see previously) as a possible confound in interpreting the results of any such experiments.

What is the significance of determining whether or not mesopontine cholinergic neurons are necessary and/or sufficient for generating desynchrony of the EEG? Such experiments will tell us a great deal about the organization of the reticular activating system(s), and both the necessary and sufficient arms of the hypothesis have different stores to tell. It seems likely (although not proven) that mesopontine cholinergic neurons will not be necessary for EEG desynchronization, but rather that activation of more than one thalamo-cortical afferent system can result in EEG

desynchrony. If this is indeed the case, one can then begin to ask more subtle questions about the functional roles of each different thalamo-cortical afferent system. For example, the present discussion has used the relatively digital (and somewhat simplistic) issue or whether the EEG is synchronized or desynchronized. If multiple systems can evoke this 'switch', one can then move to the more complex question of behavioral arousal, and ask whether activation of these different neurotransmitter systems has differing behavioral sequelae. The sufficiency issue is really much more critical in determining whether or not mesopontine cholinergic neurons even contribute to the reticular activating system. This is a particularly surprising conclusion, considering the intense scrutiny this system has received in recent years. Given both the ubiquity of the use of the EEG as a diagnostic tool in clinical neurology and the increasing maturity of the field, rigorously addressing this issue is now a necessity.

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