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AROUSAL FROM SLICES TO HUMANS:

Translational studies on sleep-wake control

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Abstract

Most psychiatric and neurological disorders exhibit sleep disorders, and in some cases presage the disease. Study of the control of sleep and waking has the potential for making a major impact on a number of disorders, making translational neuroscience research on this area critical. One element of the reticular activating system (RAS) is the pedunculopontine nucleus (PPN), which is the cholinergic arm of the RAS, and projects to the thalamus to trigger thalamocortical rhythms and to the brainstem to modulate muscle tone and locomotion. We developed a research program using brainstem slices containing the PPN to tell us about the cellular and molecular organization of this region. In addition, we developed the P13 midlatency auditory evoked potential, which is generated by PPN outputs, preparation in freely moving rats. This allows the study of PPN cellular and molecular mechanisms at the level of the whole animal. We also study the P50 midlatency auditory evoked potential, which is the human equivalent of the rodent P13 potential, allowing us to study processes detected in vitro, confirmed in the whole animal, and tested in humans. This translational research program led to the discovery of a novel mechanism of sleep-wake control, pointing the way to a number of new clinical applications in the development of novel stimulants and anesthetics.

Keywords

Brainstem slices; Electrical coupling; Gap junctions; Modafinil; P13 potential; P50 potential

1. Introduction

The P50 potential is a midlatency auditory, click stimulus-evoked, response recorded from the vertex that occurs at a latency of 40–70 msec in the human. The P50 potential has three main characteristics, a) it is present during waking and rapid eye movement (REM) sleep, but not during deep slow-wave sleep [1] (i.e. it is sleep state-dependent, occurring during cortical electroencephalographic (EEG) synchronization of fast oscillations, mainly in the low gamma band range (20–40 Hz); but not during cortical synchronization of slow oscillations, <10 Hz), b) is blocked by the cholinergic antagonist scopolamine (i.e. may be mediated, at least in part, by cholinergic neurons) [2], and c) undergoes rapid habituation at stimulation rates greater than 2 Hz (i.e. is not manifested by a primary afferent pathway, but perhaps by multi-synaptic, low security synaptic elements of the reticular activating system (RAS)) [3]. The P50 potential, but none of the earlier latency primary auditory evoked

potentials, thus diminishes and disappears with progressively deep stages of sleep and then reappears during REM sleep [4]. This suggests that at least one generator of the P50 potential is functionally related to states of arousal, prompting the idea that the potential is generated, at least in part, by cholinergic mesopontine cells, especially in the pedunculopontine nucleus (PPN), which are known to be preferentially active during waking and REM sleep, but inactive during slow-wave sleep [5].

The P50 potential has been shown to exhibit characteristic abnormalities, especially in habituation, in various psychiatric and neurological disorders, all of which are marked, and even presaged, by sleep disorders. Using a paired stimulus paradigm, schizophrenic patients did not inhibit the response to the second click stimulus under conditions in which normal subjects do show such reduced responsiveness, i.e. there was a decrease in sensory gating [6]. Schizophrenic patients show such sleep-related symptoms as reduced slow wave sleep, reduced REM sleep latency, exaggerated startle response and hallucinations (reviewed in 7). We reported the presence of decreased habituation, or a sensory gating deficit, of the P50 potential in another disorder marked by abnormalities of arousal and excitability, posttraumatic stress disorder (PTSD), in both male combat veterans and female rape victims [8]. PTSD is also marked by such sleep-wake state-related abnormalities as increased REM sleep drive, hyperarousal, hallucinations and exaggerated startle response (reviewed in 7). A similar decrease in sensory gating of the P50 potential was observed in patients with depression [9], and in the late stages of Parkinson's disease (PD), suggesting that a sensory gating deficit can be present in these subjects, who also suffer from sleep dysregulation [10]. Studies in another neurodegenerative disorder suggest that sensory gating of this potential is also decreased in Huntington's Disease (HD), which is marked by sleep dysregulation and exaggerated startle response [11]. The P50 potential also appears to undergo changes in *amplitude* as a result of other pathological states, being reduced in amplitude in patients with Alzheimer's disease (AD) [12], autism [13] and narcolepsy [14], which is characterized by daytime somnolence, cataplexy, sleep paralysis and hypnagogic hallucinations. In very general terms, then, the P50 potential is upregulated (increased amplitude and/or decreased sensory gating) in disorders that are marked by upregulation of RAS output, and downregulated in disorders marked by decreased RAS output.

In the rat, the waveforms following an auditory stimulus are the brainstem auditory evoked response (BAER), the P7 potential at 7 msec latency, the P13 potential at 13 msec latency, and the P25 potential at 25 msec latency. The midlatency auditory evoked P13 potential appears to be the rodent equivalent of the human P50 potential and the feline "wave A". It is the only midlatency auditory evoked response that has the same three characteristics as the human P50 potential and feline "wave A" as described above [15]. Since the P13 potential can be elicited after decerebration, or after ablation of the primary auditory cortex bilaterally, it appears to have a subcortical origin [7]. Convincing evidence for the subcortical origin of the vertex-recorded P13 potential is that injections of various neuroactive agents known to inhibit PPN neurons, when injected into the posterior midbrain, will reduce or block the P13 potential, while not affecting the primary auditory P7 potential. Noradrenergic, gabaergic, serotonergic and cholinergic agents are all known to inhibit PPN neurons [7], and injection of each of these agents in the region of the PPN reduced or blocked the P13 potential in a dose-dependent manner [16]. Moreover, interventions which modulate arousal such as various anesthetics, head injury and ethanol, all selectively reduce or block the P13 potential in a dose-dependent manner [17].

PPN neurons, many of which are cholinergic [18], increase firing during waking ("Wake-on") and REM sleep ("REM-on"), or both ("Wake/REM-on"), but decrease firing significantly during slow-wave sleep (SWS) [19–21], suggestive of increased excitation during activated states, exactly like the P50 potential in humans and the P13 potential in

rodents. The following describes how findings in single cells in the PPN in vitro led to the testing of a novel stimulant on the P13 potential in whole animals, with parallel effects on the P50 potential in humans. These studies allow the design of studies transitioning easily between the bench, the bedside, and back to the bench. Novel mechanisms for the control of sleep and waking promise to lead to the development of new stimulants and anesthetics.

2. Experimental Procedures

The Animal Electrophysiology Core Facility of the Center for Translational Neuroscience (CTN) allows us to perform patch clamp recordings (using a Nikon FN-1 microscope with water immersion lenses, fluorescence, and differential contrast optics, paired Sutter microdrives, and Axon Instruments dual 700B amplifiers) [22], interface chamber recordings (using a Nikon FN-1 microscope with non-water immersion lenses, BSC slice chamber, and Grass P511 amplifiers), and P13 potential recordings (using LabView software, Grass P511 amplifiers, and a recording chamber) [15–17], as previously described. The Histology Core Facility of the CTN allows immunocytochemical labeling of neurobiotin and cholinergic cell markers [22], while the Imaging Core Facility of the CTN allows us to generate high-resolution images in order to perform quantitative morphometry using a Nikon C-1 confocal microscope with NIS Elements software (Fig. 1) [22]. The Molecular Core Facility of the CTN allows us to analyze connexin 36 (Cx 36) protein levels and expression (Light Cycler RT-PCR, Luminex assays), as previously described [22]. The Human Electrophysiology Core Facility of the CTN allows us to record the human P50 midlatency auditory evoked potential and other measures, as previously described [8–11, 14].

3. Results

We recently discovered the presence of electrical coupling in the PPN [22]. We can detect dye coupling between cells or inject both cells in order to visualize points of contact (Fig. 1A). These two cells were each patch clamped and neurobiotin in the pipette allowed to enter the cells. These two PPN cells were determined to be electrically coupled (current steps applied to one cell were evident in the other cell in the presence of TTX, an effect blocked by gap junction blockers). The slice was processed for immunocytochemical labeling of neurobiotin. In addition, we study output cells of PPN by injecting retrobeads (green dots) into a target of the PPN, such as the intralaminar thalamus (Fig. 1B). Two days later, we cut brainstem slices and record in the region projecting to the injection site. Under fluorescence microscopy, we can detect retrobeads (green dots) and record from known output cells. After recording, we processed for neurobiotin in the recorded cell (violet), and also for immunolabeling to detect cholinergic PPN neurons (red). Note that these were 400 μ m slices processed without further cutting. This increases background labeling but allows us to reconstruct most of the injected neuron in relation to cholinergic and retrogradely labeled cells, i.e. in output neurons. We also detected protein levels and mRNA of the neuronal gap junction protein connexin 36 (Cx 36) (not shown). We punched (1 mm diameter punches) the nucleus in question and analyzed the tissue for Cx 36 protein. Our study showed that Cx 36 was present at high levels early in development (10 days of age) and decreased dramatically during the developmental decrease in REM sleep (30 days). The decrease was evident in all of the RAS nuclei analyzed (PPN, subcoeruleus-SubC, and intralaminar thalamus/parafascicular nucleus-Pf).

Modafinil is approved for use in treating excessive sleepiness in narcolepsy, daytime sleepiness due to obstructive sleep apnea, and to shift work sleep disorder, and is also prescribed in a number of neuropsychiatric conditions. Many publications on this agent acknowledge that the mechanism of action of modafinil is unknown, although there is

general agreement that it increases glutamatergic, adrenergic and histaminergic transmission and decreases GABAergic, transmission [23]. Modafinil was recently found to increase electrical coupling between cortical interneurons, thalamic reticular neurons, and inferior olive neurons [24]. Following pharmacologic blockade of connexin permeability, modafinil restored electrotonic coupling. The effects of modafinil were counteracted by the gap junction blocker mefloquine. These authors proposed that modafinil may be acting in a wide variety of cerebral areas by increasing electrotonic coupling in such a way that the high input resistance typical of GABAergic neurons was reduced. Basically, electrical coupling may contribute to action potential synchronization and network oscillations, to coordination and reinforcement of IPSPs, and to coincidence detection in inhibitory networks [25]. We confirmed the fact that modafinil appears to increase electrical coupling in the RAS by showing that, in the absence of action potentials or fast synaptic transmission, modafinil decreased the input resistance of electrically coupled RAS cells, an effect blocked by the gap junction antagonists carbenoxolone or mefloquine [22]. These effects were evident in the absence of changes in resting membrane potential or of changes in the amplitude of induced EPSCs. We hypothesized that increased electrical coupling of GABAergic RAS neurons by modafinil may decrease their input resistance and, consequently, GABA release, thus disinhibiting output cells, perhaps accounting for its stimulant properties. These findings in general suggest that increasing electrical coupling may promote states of synchronization of sleep-wake rhythms, thus controlling changes in state through increased coherence, especially at gamma band frequency. The presence of electrical coupling in the RAS, which may act with known transmitter interactions to generate ensemble activity, provides new and exciting directions for the field of sleep-wake control.

We then determined if injections of modafinil into the PPN would affect the amplitude of the P13 potential in the awake, freely moving rat, and if any induced stimulant effect could be blocked by pretreatment, locally at the level of the PPN, with gap junction antagonists [26]. We found that modafinil, when injected into the PPN, increased arousal levels in a dose-dependent manner as determined by the amplitude of the P13 potential in the rat, an effect blocked by the gap junction antagonist mefloquine, suggesting that one mechanism by which modafinil increased arousal may have been by increasing electrical coupling (Fig. 2 left). Additional studies showed that carbenoxolone, another gap junction blocker, also negated the stimulant action of modafinil. This further confirmed that a novel mechanism of sleep-wake control includes gap junctions [22]. The fact that anesthetics like halothane and propofol block gap junctions and also induce anesthesia, make this finding relevant for the field of anesthesia.

Our studies on the human P50 potential showed that oral modafinil increased the amplitude of the P50 potential, suggesting that the amplitude of this waveform can be used as a measure of arousal level in the human (Fig. 2 right). The effect was present 1 hr after the drug and peaked 2 hr after administration (Fig. 2 right A). The same effect, i.e. increased amplitude, but peaking 1 hr after administration, was observed in the vertex-recorded auditory midlatency P13 potential after oral administration of 3 mg/kg modafinil in intact rats (Fig. 2 right B). A similar increase in amplitude of the P13 potential was observed to peak 35 min after intracranial administration of modafinil into the region of the PPN (Fig. 2 right C). This study indicated that modafinil could increase P13 potential amplitude by acting locally within the RAS. The use of parallel preparations, the human P50 potential and the rodent P13 potential, allow investigation of arousal systems in humans as well as invasive/interventional studies in animals on the same neurological substrate [27]. Animal models of a number of disorders are being used in the CTN to study changes in P13 potential characteristics that parallel those of the human P50 potential in the same disorder.

The study of the same system from slices to whole animals to humans represents a consistent bench-to-bedside approach. However, we are going back to the bench to further study the effects of modafinil on single cells and population responses in slices. During activated states (waking and paradoxical sleep), EEG responses are characterized by low amplitude, high frequency oscillatory activity in the gamma band range (~20–100 Hz). Gamma frequency oscillations have been proposed to participate in sensory perception [28, 29], and it has been suggested that such coherent events occur at cortical [30] or thalamocortical [31] levels. Similar oscillations are present in the hippocampus [32], and cerebellum [33]. We carried out a study to determine if PPN neurons also exhibit gamma band activity in terms of action potential frequency or subthreshold oscillations in single cells, and if the population as a whole shows gamma band activity when pharmacologically activated. Fig. 3A shows whole-cell patch clamp recordings in PPN neurons held at –60 mV in current clamp, while depolarizing current steps were used to induce firing of action potentials. We used 500 msec duration current steps with 1 sec between steps. Firing frequency was determined by measuring the interspike interval between the first two, middle two and last two action potentials in the current step. We found that the average maximal firing frequency in PPN neurons was 50 $\pm$ 16 Hz. That is, PPN neurons, regardless of depolarizing amplitude, fired at gamma band frequency, and exhibited subthreshold oscillations at the same frequencies (not shown). PPN neurons showed this tendency regardless of cell type or response to cholinergic input. We then used an interface chamber to record population responses in the PPN after application of transmitters known to modulate the PPN. Cholinergic input into the PPN was tested using different concentrations of cholinergic agonist carbachol (CAR). We applied different concentrations of CAR to the PPN and discovered that rhythmic oscillations could be induced by CAR in a dose-dependent manner (not shown). CAR induced peaks of activity at theta and gamma band (Fig. 4 A, B). Significantly, when we applied modafinil, the agent by itself did not produce a specific response, but when CAR was reapplied after 20 min of exposure to modafinil, the response to CAR was amplified (Fig. 4 B, C, D). The increased coherence enabled by modafinil is evident in the event related spectral perturbations (ERSPs) generated from the population responses (Fig. 4 C vs D). This suggests that modafinil-induced increases in electrical coupling may amplify the responses of PPN neurons to transmitter inputs. In general, these results suggest that a) gamma band activity appears to be part of the intrinsic membrane properties of PPN neurons, b) the population as a whole generates gamma band activity under the influence of specific transmitters, and c) modafinil appears to amplify the response to some transmitters. These studies as a whole allow extrapolation of results from slices to predict responses in vivo in whole animals and humans, a powerful set of translational research tools.

4. Discussion

The studies described represent a coordinated program of research spanning cellular electrophysiology, systems neurobiology, and human neurophysiology, all aimed at investigating mechanisms of sleep-wake control that have a number of clinical applications. A major target of this effort is the new stimulant modafinil, which is being used for the treatment of a number of disorders, despite the fact that its mechanism of action was unknown until recently. Our work confirmed previous studies in other brain regions [24], that its mechanism of action is to increase electrical coupling in RAS centers [22]. Our findings in slices showed that the effects of modafinil could be blocked by two different gap junction blockers, each of which has side effects but only one effect in common. This allowed us to test the effects of modafinil in the whole animal, and specifically on a process subserved by RAS centers, the sleep state-dependent P13 potential. Its effects were similarly blocked by the same two gap junction blockers, confirming that the arousing effects of this agent could be due to increased electrical coupling. Further studies on the sleep state-

dependent P50 potential in humans showed that this agent induces similar increases in arousal. These preliminary studies now establish this method for a number of studies on clinical populations. For example, we found that a sub-population of ex-preterm adolescents exhibit decreased arousal levels compared to gender- and age-matched ex-term adolescents [34]. This work, aimed at alleviating the long-term effects of low birth weight, identified three subpopulations, one with profound hypoarousal, that could respond to treatment with this stimulant. A number of additional studies are ongoing in which we use the P50 potential to tell us about any dysregulation in level of arousal and/or sensory gating deficit, and determine if modafinil or another agent may have a salutary effect. Of course, having access to the rodent P13 potential allows us to test a number of agents on animals. We can also investigate cellular mechanisms, and even molecular expression of gap junction proteins in whole animals or slices containing the PPN.

Recent studies showed that single cell and population activity in the PPN shows maximal activation at gamma band range (responses plateau at this level and do not keep increasing with increasing depolarization or stimulation). Moreover, when these cells are depolarized, the responses persist at gamma band frequency, suggesting that the system is geared to fire maximally when activated by arousing stimuli, at gamma band frequencies. This observation suggests that a similar mechanism to that in the cortex for achieving temporal coherence is present in the PPN and perhaps its subcortical targets such as the intralaminar/parafascicular and subcoeruleus nuclei. We suggest that, rather than participating in the temporal binding of sensory events, gamma band activity generated in the PPN may help stabilize coherence related to arousal, providing a stable activation state during waking and paradoxical sleep. Much work is needed to support this speculation, but the intriguing findings described here certainly provide a starting point for such investigations. The translational research potential of this observation is considerable, allowing us to study basic arousal mechanisms and how they impact higher-level cognitive function related to sensory perception and binding. It is evident that modafinil potentiates coherence, opening the possibilities for therapeutic intervention on a number of additional disorders.

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Figure 1.

Identification of PPN neurons. A. Two patch clamped PPN cells found to be electrically coupled using electrophysiological criteria. Reconstruction using a confocal microscope revealed several potential points of contact. Calibration bar 40 μm . B. Patch clamped PPN cell (magenta) immunocytochemically labeled for neurobiotin from the recording pipette that was found to have retrobeads (greenish white beads) previously injected into the intralaminar thalamus, indicating that the recorded neuron was a thalamic projection PPN neuron. The same cell was also immunocytochemically labeled as cholinergic (red). Calibration bar 50 μm .

Figure 2.

Effects of modafinil on rodent P13 potential and human P50 potential. Left. Percent change in average P13 potential amplitude after saline (X), and after modafinil (MOD) at 300 μ M (filled circles), as well as after mefloquine (MEF) alone at 25 μ M (filled squares) and MEF at 25 μ M followed 20 min later by MOD at 300 μ M (open squares) compared to pre-injection control for each agent. One-way ANOVA comparison between MEF alone and saline induced significant decreases at 5, 10, 15, 25, 35 ($p < 0.01$), and 45 min ($p < 0.05$) (asterisks not shown for clarity), but pretreatment with MEF blocked the effects of MOD at all time points (open squares) and was not different from saline (X). When a three factor, two way analysis (pre- and post injection values at all time points after MOD vs MEF vs MEF+MOD between MOD at 300 μ M (filled circles) and MEF+MOD (open squares)) was done, significant increases were present comparing MOD vs MEF+MOD at 10 and 15 min ($*p < 0.05$) and 25–55 min ($**p < 0.01$) (upper asterisks), suggesting the presence of MEF suppressed the increases seen with MOD alone. When MEF vs MEF+MOD were compared, significant decreases were present only at 10 and 35 min ($**p < 0.01$) (lower asterisks). Significant differences between MOD alone and MEF alone were present at 10–55 min ($p < 0.01$, asterisks not shown for clarity). Right. A. Human P50 potential before (left panel), 1 hr after modafinil (middle panel), and 2 hr after modafinil (right panel). Note the persisting increase in amplitude after oral modafinil (100 mg) in humans. B. Rodent P13 potential before (left panel), 30 min after modafinil (middle panel), and 1 hr after modafinil. Note the

persisting increase in amplitude after oral modafinil (4 mg/kg) in rats. C. Rodent P13 potential before (left panel), 10 min after modafinil (middle panel), and 35 min after modafinil (right panel). Note the persisting increase in amplitude after intracranial (PPN injection 300 μ M) modafinil.

Figure 3.

Gamma band activity in whole-cell recorded PPN cells. A. Increasing steps of current (increase of 30 pA per step, each step was 500 ms in duration, 1 sec between steps, records truncated) caused cells to fire action potentials at higher frequencies. This cell fired maximally at 54 Hz, which is within the gamma frequency range. B. Graph showing the firing frequency of ten cells at each current step (small bullets) and of the average of the 50 recorded cells (large red bullets, $R^2 = 0.994$). The average maximal firing frequency was at the 180 pA current step, where cells fired at the average rate of 50 ± 16 Hz for all cells. C. Graph showing the firing frequency of each cell at the 180 pA current step, divided according to cell type (black dots). The average firing frequency of each cell type is also shown (red bullets). Type I neurons ($n=17$) fired significantly faster than type II ($n=16$) or III ($n=16$) cells [$*p < 0.05$], but type II and III neurons did not fire action potentials significantly faster than one another [ns, $p > 0.05$]. The average ($\pm$ SD) maximal firing frequency for type I, II, and III neurons were: 58 ± 15 Hz, 45 ± 15 Hz, and 46 ± 16 Hz, respectively.

Figure 4.

Population responses in the PPN. A. Representative population recordings during control (black), after carbachol (CAR, 50 μ M, red), after MOD (300 μ M, green), and after additional CAR (blue). B. Sample power spectra during control (minute 2, black), during CAR (red, minute 9), after MOD (green, minute 14), and after additional CAR (blue, minute 9). Note the activation of activity at specific frequencies including theta and gamma by CAR, and their increase after pretreatment with MOD and subsequent application of CAR. C. MatLab generated event related spectral perturbations (ERSPs) during initial CAR application. Note density of activation in the theta and gamma ranges beginning 3 min after the beginning of CAR application. D. MatLab generated ERSPs after MOD pretreatment and during subsequent CAR application. Note the increase in density at specific peaks of activity in the theta and gamma band range. Increased density suggests increased coherence at the same frequencies.