Brief Communication

N-Type Calcium Channel α_{1B} Subunit ($Ca_V2.2$) Knock-Out Mice Display Hyperactivity and Vigilance State Differences

Carsten T. Beuckmann,[1,2,4] **Christopher M. Sinton,**[3] **Norimasa Miyamoto,**[1,4] **Mitsuhiro Ino,**[1] **and Masashi Yanagisawa**[2,4]
[1]Eisai Company Ltd., Tsukuba, 300-2635 Ibaraki, Japan, and [2]Howard Hughes Medical Institute and Departments of [3]Internal Medicine and [4]Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Differential properties of voltage-dependent Ca^{2+} channels have been primarily ascribed to the α_1 subunit, of which 10 different subtypes are currently known. For example, channels that conduct the N-type Ca^{2+} current possess the α_{1B} subunit ($Ca_v2.2$), which has been localized, *inter alia*, to the piriform cortex, hippocampus, hypothalamus, locus coeruleus, dorsal raphe, thalamic nuclei, and granular layer of the cortex. Some of these regions have been previously implicated in metabolic and vigilance state control, and selective block of the N-type Ca^{2+} channel causes circadian rhythm disruption. In this study of $Ca_v2.2^{-/-}$ knock-out mice, we examined potential differences in feeding behavior, spontaneous locomotion, and the sleep–wake cycle. $Ca_v2.2^{-/-}$ mice did not display an overt metabolic phenotype but were hyperactive, demonstrating a 20% increase in activity under novel conditions and a 95% increase in activity under habituated conditions during the dark phase, compared with wild-type littermates. $Ca_v2.2^{-/-}$ mice also displayed vigilance state differences during the light phase, including increased consolidation of rapid-eye movement (REM) sleep and increased intervals between non-REM (NREM) and wakefulness episodes. EEG spectral power was increased during wakefulness and REM sleep and was decreased during NREM sleep in $Ca_v2.2^{-/-}$ mice. These results indicate a role of the N-type Ca^{2+} channel in activity and vigilance state control, which we interpret in terms of effects on neurotransmitter release.

Key words: **mouse; calcium; locomotion; REM sleep; vigilance state; electroencephalogram; EEG**

Introduction

Sleep is an active process that reflects changes in specific ascending neurotransmitter systems from the pons–midbrain via the thalamus to the cortex. Thalamic relay neurons project widely to the cortex to regulate activity and synchronization of cortical neurons. Changes in vigilance state, between rapid-eye movement (REM) sleep, non-REM (NREM) sleep, and wakefulness, thus reflect thalamic propagation to the cortex of differential activity in pontine and hypothalamic nuclei (Steriade et al., 1993).

Activating inputs to the thalamus include monoaminergic brainstem nuclei, cholinergic pontine and basal forebrain nuclei, and histaminergic midbrain nuclei. The pontine cholinergic neurons reside in the pedunculopontine tegmental and laterodorsal tegmental nuclei and can be divided into two major groups: wake–REM-active and REM-active neurons. REM-active neurons are inhibited by noradrenaline from the locus coeruleus (LC) and serotonin from the dorsal raphe nucleus (DR). The monoaminergic neurons of LC and DR therefore discharge most actively during wakefulness, slow during NREM sleep, and become quiescent during REM sleep (McGinty and Harper, 1976; Aston-Jones and Bloom, 1981).

Voltage-dependent Ca^{2+} channels (VDCCs) regulate calcium influx into cells, a process that mediates many neuronal changes, including neurotransmitter release (Dunlap et al., 1995). Several types of neuronal VDCCs have been differentiated on the basis of voltage and blockade by specific agents (Randall and Tsien, 1995), but three types, P/Q-type, N-type, and R-type, are predominantly expressed in neurons (Olivera et al., 1985; Ertel et al., 2000). N-type Ca^{2+} channels are critically involved in neurotransmitter release from central neurons, including glutamate (Luebke et al., 1993), γ-aminobutyric acid (Horne and Kemp, 1991), acetylcholine (Wessler et al., 1990), dopamine (Woodward et al., 1988; Herdon and Nahorski, 1989; Turner et al., 1993), and noradrenaline (Dooley et al., 1988). Molecular studies have revealed that the α_{1B} ($Ca_V2.2$) gene encodes the subunit specific for the N-type Ca^{2+} channel, and mice lacking the α_{1B} subunit could therefore be a useful tool for studying neurotransmitter mechanisms. Therefore, they have been adopted in modeling disorders attributable to sympathetic nerve dysfunction (Ino et al., 2001) and for studying pain-related disorders (Hatakeyama et al., 2001; Kim et al., 2001; Saegusa et al., 2001).

The hypothalamic, thalamic, and mesencephalic expression pattern of the α_{1B} subunit (Tanaka et al., 1995) includes nuclei known to be involved in sleep–wake regulation. Furthermore, the N-type Ca^{2+} channel contributes to both excitatory and inhibitory synaptic transmission in rat hypothalamic neurons (Zeilhofer et al., 1996), suggesting that this channel might be related to

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Correspondence should be addressed to Dr. Carsten T. Beuckmann, Discovery Research Laboratories I, Eisai Company Ltd., Tokodai 5-1-3, Tsukuba, 300-2635 Ibaraki, Japan. E-mail: c-beuckmann@hhc.eisai.co.jp.

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Table 1. Food and water consumption of $Ca_v2.2^{-/-}$ mice and $Ca_v2.2^{+/+}$ littermates

	+/+	−/−	+/+	−/−
Food intake (gm/12 hr)	0.77 ± 0.02	0.96 ± 0.11	3.6 ± 0.22	3.28 ± 0.1
Food access (counts/12 hr)	597 ± 197	654 ± 303	1833 ± 406	1665 ± 341
Feeding duration (sec/12 hr)	784 ± 189	1102 ± 517	2753 ± 643	2737 ± 290
Water intake (ml/12 hr)	1.2 ± 0.1	1.5 ± 0.2	5 ± 0.4	5 ± 0.1
Water access (licks/12 hr)	436 ± 64	563 ± 168	3663 ± 311	3530 ± 217

Data (mean ± SEM) are itemized separately for light phase (white) and dark phase (gray). $n = 4$ for each genotype. Food was standard 6% fat rodent chow.

homeostatic, motivational, and rhythmical behavior patterns. In fact, ω-conotoxin GVIA, a specific N-type calcium channel blocker, disrupts the circadian rhythm in rats (Nakagawa et al., 1979; Masutani et al., 1995). Here we characterized the α_{1B} subunit gene null mice using measures of spontaneous locomotor activity and electronencephalographic (EEG)–electromyographic (EMG) characterization of vigilance states. We show that the absence of the N-type Ca^{2+} channel results in a hyperactive phenotype and changes in vigilance state transitions. To our knowledge, this is the first report concerning a relationship between this Ca^{2+} channel and sleep–wake behavior.

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas Southwestern Medical Center at Dallas and were strictly in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice with a nonfunctional α_{1B} subunit of VDCCs ($Ca_v2.2^{-/-}$ knock-out mice) had been established previously by disrupting the coding region of the α_{1B} gene via insertion of a selection marker (PGKneoA+) (Ino et al., 2001). For the present experiments, male $Ca_v2.2^{-/-}$ knock-out mice (backcross generation, $n = 12$ into C57BL/6NCrj) and their male $Ca_v2.2^{+/+}$ wild-type littermates were housed under constant conditions with a 12 hr light/dark cycle at 24 ± 1°C and had free access to food and water.

Food and water consumption

Four $Ca_v2.2^{-/-}$ and four weight-matched wild-type littermates at 20 weeks of age were habituated to a cage containing a running wheel (Vital View; Mini-Mitter, Bend, OR) for 7 d before initiating measurement of food and water consumption that continued for 7 d. Amounts of food and water consumed were determined every 12 hr at the photo-period (i.e., 12 hr light/dark cycle) boundary. Access frequency to food and water, as well as duration of bouts of feeding, were automatically recorded on-line to a computer.

Spontaneous locomotor activity

Novel conditions. Eight $Ca_v2.2^{-/-}$ and eight weight-matched wild-type 29-week-old littermates were individually introduced into an open-field arena (ENV-510; 27 × 27cm; Med Associates, St. Albans, VT) for 12 min, during which time locomotor activity was recorded.

Habituated conditions. Six $Ca_v2.2^{-/-}$ and six weight-matched wild-type 22-week-old littermates were habituated to an open-field arena (Opto-Varimex; 42.5 × 42.5cm; Columbus Instruments, Columbus, OH) for 12 hr during the dark period. Locomotor activity was then recorded for three consecutive days on a 12 hr light/dark cycle, during which time mice could feed and drink *ad libitum*. Data for each mouse were averaged over 3 d before being grouped by genotype.

Vigilance state determination

At 22 weeks, six $Ca_v2.2^{-/-}$ and six weight-matched wild-type littermates were deeply anesthetized (80 mg/kg of ketamine, 8 mg/kg of xylazine, i.p.) and surgically implanted with recording electrodes under sterile conditions, as described previously (Chemelli et al., 1999). EEG signals were recorded unilaterally from fronto-occipital electrode pairs, positioned 1.1 mm rostral and 1.45 mm lateral from bregma, and 3.5 mm caudal and 1.45 mm lateral from bregma. EMG signals were recorded from the nuchal musculature. Mice were allowed to recover from surgery and were habituated to recording conditions for 2 weeks before EEG–EMG signals were archived as 20 sec epochs for three consecutive 24 hr periods, as described previously (Chemelli et al., 1999). For vigilance state analysis, EEG–EMG data were visually analyzed by two independent observers who were blinded to genotype, using standard criteria for rodent vigilance state classification (Radulovacki et al., 1984). Data for each mouse were averaged over 3 d before being grouped according to genotype.

EEG power spectral analysis

The EEG frequency distribution was analyzed by power spectral analysis [i.e., fast Fourier transform (FFT)] into 1 Hz bins from 1 to 32 Hz. For derivation of power spectra for each vigilance state, FFT data for 100 representative artifact-free epochs from 12 hr recordings for each photo period (50 for REM sleep) were averaged for each animal, normalized to a spectral density function by dividing each bin by the total average power for that mouse over the respective photo period, and then averaged across three recording periods. Finally, means of these spectral density functions were derived over all animals for each genotype. Statistical analysis was by Student's *t* test, and the null hypothesis was rejected at $p < 0.05$.

Results

Food and water consumption

Mice were more active and consumed more food and water during the dark phase than in the light phase. No significant differences between $Ca_v2.2^{-/-}$ mice and their $Ca_v2.2^{+/+}$ littermates were noted for any feeding parameter (Table 1) or body weight measure. Weights at the end of the habituation period were 27.5 ± 1 and 26.9 ± 1 gm ($Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$ mice, respectively) and 27.9 ± 1 and 27.1 ± 0.8 gm after 1 week of the feeding study. $Ca_v2.2^{-/-}$ mice therefore showed no overt metabolic phenotype.

Spontaneous locomotor activity

Spontaneous locomotor activity was monitored under novel conditions during 12 min of the light phase and under habituated conditions during three consecutive 24 hr periods. As expected, both genotypes under habituated conditions showed a higher total activity count (ambulation, repetitive behavior, and rearing combined) during the dark phase than in the light phase (Fig. 1). However, under novel conditions, as well as under habituated conditions in the dark phase, $Ca_v2.2^{-/-}$ mice showed significantly more activity (20 and 95%, respectively) than their wild-type littermates (Fig. 1). The genotypes showed no difference in weights before or after these activity studies.

Vigilance state determination

Table 2 displays vigilance state parameters for $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$ mice classified separately for light and dark phases. No significant differences in vigilance state parameters between $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$ littermates were observed during the dark phase, although a trend toward increased wakefulness at the expense of both sleep states was noted. However, during the light phase, $Ca_v2.2^{-/-}$ mice displayed several differences. Although

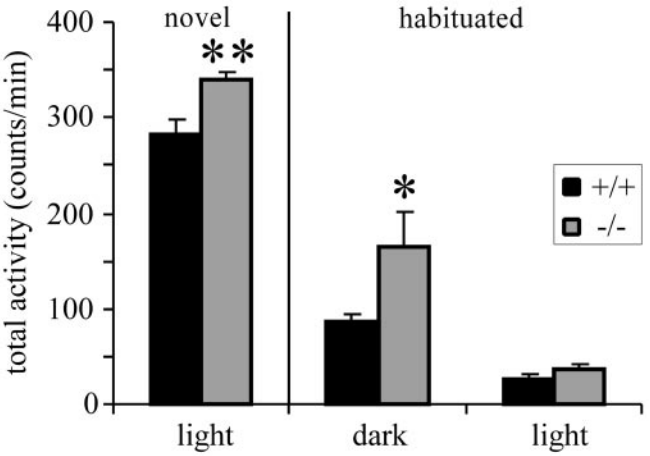

Figure 1. Spontaneous locomotor activity in $Ca_v2.2^{-/-}$ mice and $Ca_v2.2^{+/+}$ wild-type littermates. Activity counts (mean ± SEM) were determined under novel conditions for 12 min during the light phase ($n = 8$ per genotype) and under habituated conditions for 3 consecutive days ($n = 6$ per genotype). Significant differences are indicated by one asterisk ($p < 0.05$) or two asterisks ($p < 0.01$).

total REM sleep time was not changed, mean REM sleep episode duration was increased with a concomitant decrease in the number of REM sleep episodes. REM sleep latency was also increased, as well as the mean inter-REM sleep interval. In association with these differences in REM sleep, we also noted an increase in the average intervals of occurrence of NREM sleep and wakefulness episodes in $Ca_v2.2^{-/-}$ mice, compared with $Ca_v2.2^{+/+}$ controls. Together, these changes in vigilance state parameters indicate decreased vigilance state fragmentation in the $Ca_v2.2^{-/-}$ mice during the light phase.

EEG power spectral analysis

Both genotypes demonstrated an increase in EEG spectral power during wakefulness in the light phase compared with the dark phase (Fig. 2*A*). In contrast, during NREM sleep, higher power was observed during the dark phase within each genotype (Fig. 2*B*), but during REM sleep, no differences in regard to the photo period were observed (Fig. 2*C*). However, a comparison between genotypes revealed important differences in the EEG power spectra of $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$ mice. These differences were independent of the photo period, and spectra for light and dark phases were therefore pooled for this comparison. $Ca_v2.2^{-/-}$ mice had greater spectral power during wakefulness (mean average power per 1 Hz bin, 0.79 ± 0.04 vs 0.59 ± 0.03 for $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$, respectively; $p = 0.004$) (Fig. 2*A*) and during REM sleep (mean average power per 1 Hz bin, 0.73 ± 0.05 vs 0.6 ± 0.03 for $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$, respectively; $p = 0.026$) (Fig. 2*C*). During NREM sleep, this effect was reversed, and $Ca_v2.2^{-/-}$ mice showed less EEG spectral power than controls (mean average power per 1 Hz bin, 1.27 ± 0.05 vs 1.59 ± 0.06, for $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$, respectively; $p = 0.002$) (Fig. 2*B*). Statistical comparisons between EEG power of both genotypes in each 1 Hz bin showed that regions 4–9 and 13–20 Hz (wakefulness), 2 Hz bin and regions 4–32 Hz (NREM sleep), and regions 2–8 and 14–16 Hz (REM sleep) were significantly different ($p < 0.05$). Furthermore, during REM sleep, the power spectrum maximum shifted ~1 Hz lower in $Ca_v2.2^{-/-}$ mice (i.e., peak frequency bin was between 7 and 8 Hz in $Ca_v2.2^{-/-}$ mice and between 8 and 9 Hz in $Ca_v2.2^{+/+}$ mice) (Fig. 2*C*). A shift in the power spectral maximum was also noted in NREM sleep (Fig. 2*B*), but this could reflect the change in overall power in this vigilance state.

These state-dependent differences in EEG power did not result from an overall difference in electrode positioning or recorded signal between genotypes, because total EEG spectral power averaged over the complete 24 hr recording period was identical in both genotypes (572 ± 63 μV^2 for $Ca_v2.2^{-/-}$ and 569 ± 40 μV^2 for $Ca_v2.2^{+/+}$; $p = 0.97$). Thus, the noted differences between $Ca_v2.2^{-/-}$ and $Ca_v2.2^{+/+}$ mice were specific for the respective vigilance states.

Discussion

Results from this study demonstrate that $Ca_v2.2^{-/-}$ mice are more spontaneously active during the dark phase and also respond to a novel environment with more activity. From the vigilance state measures, we also noted a significant consolidation of REM sleep events during the light phase, and this was associated with increased intervals between successive episodes of NREM sleep and wakefulness. Thus, when compared with $Ca_v2.2^{+/+}$ mice, sleep is more consolidated in knock-out mice during the normal sleep phase, although the total time spent in NREM and REM sleep is essentially identical between both genotypes. In contrast, during the normally active dark phase, we noted a nonsignificant tendency toward more time spent in wakefulness in the $Ca_v2.2^{-/-}$ mice, with a corresponding decrease in total NREM and REM sleep times. The latter result, in conjunction with the observed hyperactivity, increased EEG spectral power during wakefulness, and decreased EEG spectral power and frequency during NREM sleep in the $Ca_v2.2^{-/-}$ mice, is an indication of a mouse that is tonically more alert and vigilant during the dark phase.

Several VDCCs, of which the P/Q-type, N-type, and R-type are predominantly expressed, regulate neuronal processes that depend on calcium influx. The N-type calcium channel has been shown to play a particularly significant role in neurotransmitter release (Dunlap et al., 1995; Catterall, 1998; Mochida et al., 1998), and its absence from $Ca_v2.2^{-/-}$ mice (Ino et al., 2001) is therefore most likely to affect this aspect of neuronal function. However, the widespread distribution of the N-type calcium channel throughout the neuroaxis indicates that more studies are now required to elucidate the exact mechanism by which $Ca_v2.2^{-/-}$ mice maintain increased vigilance. Thus, only speculative suggestions are currently possible. However, the N-type channel is densely expressed in the LC and DR regions (Tanaka et al., 1995), and both are associated with ascending monoaminergic activating systems. The absence of the N-type channel in these regions in $Ca_v2.2^{-/-}$ mice could therefore increase the baseline discharge rate of monoaminergic neurons. Indeed, the N-type channel has been implicated in local feedback inhibition of raphe neurons by serotonin (Bayliss et al., 1997), and absence of the channel may therefore be sufficient to reduce the normal inhibitory effect of axon collaterals. The same mechanism could be invoked in the LC (Singewald and Philippu, 1998). Alternatively, the action of glutamatergic excitatory afferents to this region (Kawahara et al., 1999; Szabo and Blier, 2001) may be potentiated in $Ca_v2.2^{-/-}$ mice. In support of this hypothesis, increased baseline activity in the ascending monoaminergic systems would elevate spontaneous motor activity, both during normal wakefulness and under novel conditions, as we observed in $Ca_v2.2^{-/-}$ mice.

However, the increase in EEG spectral power during REM sleep recorded in $Ca_v2.2^{-/-}$ mice, primarily in the θ frequency range (centered at 8–9 Hz), cannot be attributable to a direct effect of the ascending monoaminergic systems, because these

Table 2. Vigilance state parameters recorded from $Ca_v2.2^{-/-}$ mice and $Ca_v2.2^{+/+}$ wild-type littermates

	REM sleep		NREM sleep		Awake	
	+/+	−/−	+/+	−/−	+/+	−/−
Total time (minutes)	47 ± 3.5	51.3 ± 2	440 ± 9	441 ± 10	232 ± 13	226 ± 11
Episode duration (seconds)	69.3 ± 4	113 ± 4**	289 ± 14	333 ± 24	168 ± 13	177 ± 19
Number of episodes	41.5 ± 4.1	27.3 ± 0.7**	92.3 ± 4.4	82.2 ± 7.9	84.3 ± 4.6	80.4 ± 7.7
Average occurrence (minutes)	26.1 ± 2.2	29.6 ± 1.1	12.6 ± 0.7	16.6 ± 0.6**	22.2 ± 1.3	26.9 ± 1.3*
REM latency (minutes)	9 ± 0.1	11.3 ± 0.8*				
Inter-REM interval (minutes)	16.4 ± 1.8	24 ± 0.7**				
Total time (minutes)	14.9 ± 2	11.6 ± 2.2	253 ± 31	218 ± 35	451 ± 32	489 ± 37
Episode duration (seconds)	56.7 ± 5.9	54 ± 5.6	279 ± 17	298 ± 29	574 ± 114	839 ± 249
Number of episodes	15.9 ± 1.9	14.1 ± 3.3	53.9 ± 5.6	44.7 ± 7.2	52.3 ± 5.6	44.7 ± 7.2
Average occurrence (minutes)	83.6 ± 13.2	109.6 ± 30	23.4 ± 3.1	31.6 ± 9.9	30.7 ± 6.2	39.1 ± 13.1
REM latency (minutes)	9.4 ± 0.4	10.4 ± 0.8				
Inter-REM interval (minutes)	45.8 ± 4.3	63.7 ± 15.3				

Data (mean ± SEM) are itemized separately for light phase (white) and dark phase (gray). Asterisks indicate differences between (+/+) ($n = 8$) and (−/−) ($n = 8$) mice (*$p < 0.05$; **$p < 0.01$).

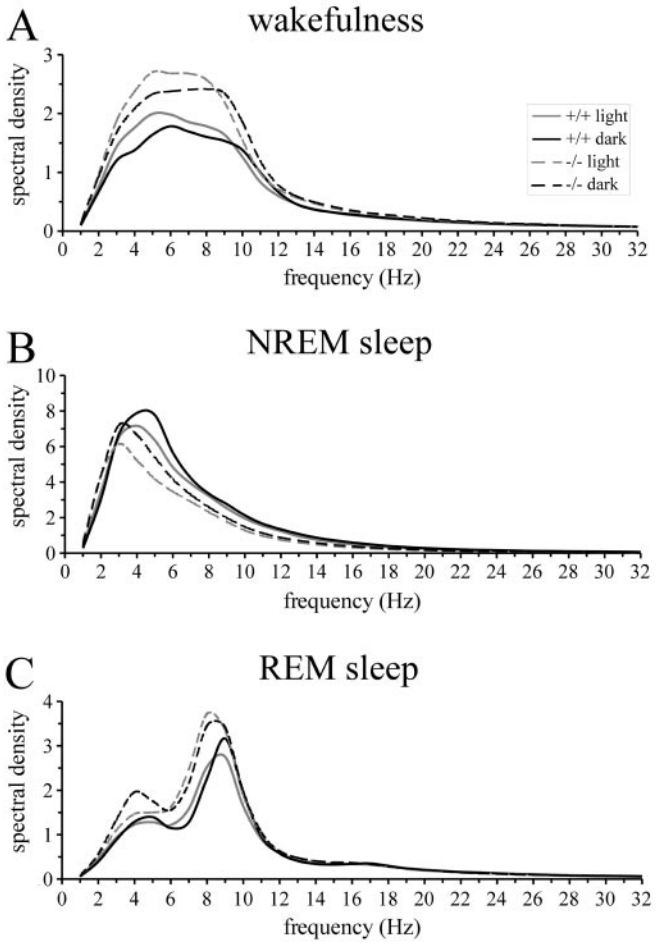

Figure 2. EEG power spectra for $Ca_v2.2^{-/-}$ mice ($n = 6$; dashed lines) and $Ca_v2.2^{+/+}$ littermates ($n = 6$; solid lines). Spectra were extracted from selected epochs and normalized to the average signal strength of each individual animal before being averaged over all animals of the respective genotype. *A–C*, Spectra itemized separately for light (gray) and dark (black) phases. *A*, Wakefulness. *B*, NREM sleep. *C*, REM sleep. Note that spectral power for wakefulness and REM sleep is increased in $Ca_v2.2^{-/-}$ mice, but it is decreased during NREM sleep in this genotype. REM sleep and NREM sleep spectra of the $Ca_v2.2^{-/-}$ mice also show a shift in the peak frequency of approximately −1 Hz.

cells are essentially quiescent during this state (McGinty and Harper, 1976; Aston-Jones and Bloom, 1981). The principal frequency component in the θ range during REM sleep is driven through the septum and recorded from the hippocampus (Vinogradova, 1995; Vertes and Kocsis, 1997). The interburst interval of septal pacemaker cells sets the frequency of the θ rhythm (Brazhnik and Vinogradova, 1986), and this interval is determined primarily by ascending cholinergic brainstem activating systems (Vertes, 1981). Hence, the reduction in θ frequency noted in $Ca_v2.2^{-/-}$ mice, and thus an increase in the interburst interval of septal pacemaker cells, might be related to reduced brainstem activation during REM sleep. However, the N-type calcium channel is also localized to all hippocampal fields as well as the medial and lateral septum (Tanaka et al., 1995), and it is likely that the absence of the channel in these areas would modulate both the power and frequency of hippocampal θ. Future studies are required to address these possibilities.

The consolidation of vigilance states, specifically during the light phase in $Ca_v2.2^{-/-}$ mice with a primary effect on REM sleep, is a potentially critical result of this study. Although the changes in cellular discharge patterns at the thalamic and cortical levels that are associated with each vigilance state have been established (Steriade et al., 1993), little is known about the mechanisms by which the switch from one vigilance state to another occurs. One possibility is that the presence of the N-type channel in thalamic relay nuclei (Chung et al., 2000) could affect these mechanisms. Recently, however, the orexin neuropeptides have been implicated in the transition between vigilance states, and particularly in the boundary conditions for the occurrence of REM sleep (Saper et al., 2001). Therefore, it is an intriguing possibility that the absence of the N-type calcium channel may affect either the presynaptic release of orexin onto neurons that control the sleep–wake cycle or the postsynaptic effects of orexin on these neurons. Involvement of the N-type channel has in fact been demonstrated previously for the postsynaptic effect of orexin on ventral tegmental dopamine cells (Uramura et al., 2001). Future experiments will therefore address the possibility that extracellular orexin levels are affected in $Ca_v2.2^{-/-}$ mice or that orexin production is different in these mice. Understanding the mechanisms by which orexin changes the discharge rate of postsynaptic neurons that modulate the sleep–wake cycle could advance our understanding of vigilance state transitions. $Ca_v2.2^{-/-}$ mice may therefore play an important role in these future studies.

Of interest in the $Ca_v2.2^{-/-}$ mouse, and indeed generally in any knock-out model, is the question of possible developmental compensatory effects, particularly on other VDCCs. However, previous *in vitro* electrophysiological studies in $Ca_v2.2^{-/-}$ mice have shown that, although N-type currents are abolished in superior cervical ganglion and dorsal root ganglion neurons, other Ca^{2+} currents, including the L-type, P/Q-type, and R-type, are

not different from wild-type controls in these cells (Hatakeyama et al., 2001; Ino et al., 2001). This work, which will now be repeated with central neurons, suggests that any developmental compensatory effects may not implicate other VDCCs. Additionally, we will examine the effect of introcerebroventricular administration of the specific N-type channel blocker, ω-conotoxin GVIA, in $Ca_v2.2^{+/+}$ mice. This should produce a phenotype similar to that of $Ca_v2.2^{-/-}$ mice. In contrast, a similar experiment with $Ca_v2.2^{-/-}$ mice should not change the phenotype. These studies, in combination with specific experiments to examine the potential upregulation of mRNA of other Ca^{2+} channel α_1 subunits, will provide additional information on compensatory effects in this knock-out model.

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