BASIC SCIENCE

Sleep Homeostatic and Waking Behavioral Phenotypes in *Egr3*-Deficient Mice Associated with Serotonin Receptor 5-HT$_2$ Deficits

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Study Objective: The expression of the immediate early gene early growth response 3 (*Egr3*) is a functional marker of brain activity including responses to novelty, sustained wakefulness, and sleep. We examined the role of this gene in regulating wakefulness and sleep.

Methods: Electroencephalogram/electromyogram (EEG/EMG) were recorded in *Egr3-/-* and wild-type (WT) mice during 24 h baseline, 6 h sleep disruption and 6 h recovery. Serotonergic signaling was assessed with 6 h EEG/EMG recordings after injections of nonselective 5-HT$_2$ antagonist (clozapine), selective 5-HT$_2$ antagonists (5-HT$_{2A}$; MDL100907 and 5-HT$_{2BC}$; SB206553) and a cocktail of both selective antagonists, administered in a randomized order to each animal.

Results: *Egr3-/-* mice did not exhibit abnormalities in the timing of wakefulness and slow wave sleep (SWS); however, EEG dynamics in SWS (suppressed 1–3 Hz power) and in quiet wakefulness (elevated 3–8 Hz and 15–35 Hz power) differed in comparison to WT-mice. *Egr3-/-* mice showed an exaggerated response to sleep disruption as measured by active wakefulness, but with a blunted increase in homeostatic sleep drive (elevated 1–4 Hz power) relative to WT-mice. *Egr3-/-*mice exhibit greatly reduced sedative effects of clozapine at the electroencephalographic level. In addition, clozapine induced a previously undescribed dissociated state (low amplitude, low frequency EEG and a stable, low muscle tone) lasting up to 2 h in WT-mice. *Egr3-/-* mice did not exhibit this phenomenon. Selective 5-HT$_{2A}$ antagonist, alone or in combination with selective 5-HT$_{2BC}$ antagonist, caused EEG slowing coincident with behavioral quiescence in WT-mice but not in *Egr3-/-* mice.

Conclusion: *Egr3* has an essential role in regulating cortical arousal, wakefulness, and sleep, presumably by its regulation of 5-HT$_2$ receptors.

Keywords: cortical arousal, early growth response, sedation, serotonin

Citation: Grønli J, Clegern WC, Schmidt MA, Nemri RS, Rempe MJ, Gallitano AL, Wisor JP. Sleep homeostatic and waking behavioral phenotypes in *Egr3*-deficient mice associated with serotonin receptor 5-HT$_2$ deficits. *SLEEP* 2016;39(12):2189–2199.

Significance

Sleep facilitates the maintenance of plastic processes established during wakefulness. Wakefulness or sleep can modify expression of transcriptional regulatory genes. Unlike other plasticity-related immediate early genes, expression of early growth response (Egr) 3 is elevated also during recovery sleep after enforced wakefulness. Here, we found that *Egr3*-deficient mice exhibit normal timing of spontaneous wakefulness and sleep; however, electroencephalography indicated suppression of homeostatic sleep drive. *Egr3*-deficiency resulted in more active wake during experimental sleep disruption. Pharmacological probing indicated that 5-HT$_{2A}$ receptor signaling promotes cortical arousal, and that *Egr3*-deficient mice are deficient in 5-HT$_{2A}$ receptor-mediated signaling. These observations indicate a role for *Egr3* and its transcriptional target, the 5-HT$_{2A}$ receptor, in cortical arousal and the response to clozapine.

INTRODUCTION

One function ascribed to sleep is to facilitate the long-term maintenance of plastic processes initiated during wakefulness, e.g., Aton et al.[1] The mechanisms whereby it does so remain to be ascertained. Expression of immediate early genes (IEGs) changes significantly between sleep and wakefulness, encoding long-term synaptic changes across the sleep/wake cycle.[2,3] The early growth response (Egr) genes *Egr1* and *Egr3* are upregulated by synaptic activity[4,5] and regulate target genes such as the plasticity-associated *Arc* gene important in protein synthesis-dependent synaptic plasticity during sleep.[6] *Egr3* expression is upregulated in the cerebral cortex in response to prolonged wakefulness.[2,7,8] However, unlike *Egr1* and other transcriptional regulatory IEGs, *Egr3* messenger RNA (mRNA) is reported to be elevated also during recovery sleep.[7]

Egr3-deficient mice exhibit hyperarousal to novelty and impaired learning and memory.[9,10] There are no gross abnormalities in brain development, basal synaptic transmission, or density of hippocampal neurons.[9] However, *Egr3*-deficiency results in a 70% reduction in cortical serotonin 5-HT$_{2A}$ receptor binding compared to wild-type (WT) mice[11] and abolishes sleep deprivation-induced upregulation of 5-HT$_{2A}$ receptor expression.[8] Of the many serotonin receptor subtypes, the 5-HT$_2$ receptors play an important role in promoting arousal and wakefulness.[12–14] Mice lacking 5-HT$_{2A}$ receptors exhibit potentiated behavioral reactivity in situations involving novelty,[15] express more wakefulness and less slow wave sleep (SWS), and exhibit attenuated responses to sleep disruption indicating a sleep homeostatic phenotype.[14] *Egr3*-deficient mice exhibit a profound resistance to the sedating effects of clozapine and other second-generation antipsychotic medications at the behavioral level, but not to the locomotor suppressive effects of first-generation antipsychotic agents.[11,16] This effect is shown to be mediated, at least in part, by 5-HT$_{2A}$ receptors,[11] and 5-HT$_{2A}$ receptor-deficient mice display the same phenotype.[17]

If *Egr3* modulates the expression of plasticity-associated genes and serotonergic signaling in a physiologically relevant manner, it may be essential for waking behavior, sleep timing, and the electroencephalographic manifestations of sleep need. It may additionally influence the electroencephalographic response to sedating agents known to act on 5-HT$_2$ receptors, such as clozapine. We performed studies on sleep in baseline

conditions and assessed the response to pharmacological manipulations of serotonergic signaling in *Egr3*-deficient mice (*Egr3-/-*) mice. We report that despite normal timing of spontaneous wakefulness and sleep in *Egr3-/-* mice, the electroencephalogram exhibits abnormalities in frequency ranges associated with homeostatic sleep drive. Additionally, their behavioral and electroencephalographic responses to both sleep deprivation and pharmacological probing of serotonergic function are distinct from those of WT mice. *Egr3* has an essential role in regulating behavior and cortical arousal, at least in part by regulation of 5-HT_2 receptors.

METHODS

Ethical Approval

This study was approved by the institutional animal care and use committee of Washington State University (Protocol Number: 3932) and conducted in accordance with National Research Council guidelines and regulations controlling experiments in live animals (Institute of Laboratory Animal Resources, 1996).

Animals

Egr3-/- mice were originally reported by Tourtellotte and Milbrandt.[18] In the current study, WT and *Egr3-/-* mice were generated from breeding of *Egr3+/-* pairs transferred from the colony of Gallitano-Mendel et al.[16] where the *Egr3* null allele had been backcrossed to C57BL/6 mice more than 20 generations. *Egr3-/-* mice were identified by polymerase chain reaction genotyping. Mice of both sexes (sleep and clozapine experiment: 16 WT, 7 male and 15 *Egr3-/-*, 6 male; 5-HT_2 antagonist experiment: 8 WT, 2 male and 7 *Egr3-/-*, 3 male) were used. All animals were housed in a 12 h light/12 h dark cycle with food and water available ad libitum.

Surgery Procedures

Animals were anesthetized with isoflurane (5% induction; 1% to 3% to maintain 0.5 to 1 Hz respiration rate) for implantation of bilateral frontal stainless steel electroencephalography (EEG) electrodes with bregma coordinates AP = +1.0 mm, ML = ± 1.5 mm and AP = −3.0 mm, ML = +1.5 mm for reference electrode. Electromyography (EMG) electrodes were implanted bilaterally in the nuchal muscle. Analgesic (buprenorphine; 0.1 mg/kg, subcutaneously) and anti-inflammatory (flunixin meglumine; 0.1 mg/kg, subcutaneously) agents were given 2 days after surgery. At least 2 weeks were allowed following surgery for recovery.

Experimental Sleep Protocol

Mice were acclimatized to the cylindrical recording cage (diameter 25 cm × height 20 cm) for 1 night before 24-h baseline EEG/EMG recordings starting at lights-on (Zeitgeber, ZT0). Immediately after baseline recording, 6 h sleep disruption was enforced by a rotating bar disturbing the animals every 5 to 6 sec in the base of the cage[19] and undisturbed sleep recorded over the next 6 h. Animals were then returned to colony housing for a period of 72 h or more prior to initiation of pharmacological experiments.

Experimental Pharmacological Protocol

The order in which treatments were administered was randomized individually for each subject in both pharmacological experiments. EEG/EMG were recorded for 6 h after injections administered at the start of the active phase (dark period). A washout period of 24 h or more was allowed between two consecutive treatments. The washout periods were more than 10 times the half-lives of clozapine (110 min[20]) and the 5-HT_{2A} antagonist MDL100907 (71 min[21]) and exceeded the duration of previously reported EEG effects (5-HT_{2BC};SB206553: 3 h[14]) by eightfold. The animals were treated in matched pairs of the same sex and body weight and opposite genotype.

The clozapine experiment included three injections (0, 3.5 and 7 mg/kg clozapine, intraperitoneally) to each animal. Clozapine (Sigma-Aldrich, C6305, St. Louis, MO) was dissolved in 1.2 N HCl and diluted to 2 mg/mL in sterile water before being stored in aliquots at −20°C. Aliquots were thawed at 37°C more than 1 h before use and diluted to their final concentration in a saline/disodium phosphate solution with a final pH of 6.5–7. Vehicle was identically prepared without the addition of clozapine.

The 5-HT_2 experiment included injections of vehicle, 5-HT_{2A} antagonist (MDL100907; 5 mg/kg), 5-HT_{2BC} antagonist (SB206553; 5 mg/kg) and a cocktail of both antagonists (5 mg/kg each). Agents, doses, and vehicle were based on prior work examining their effects in 5-HT_{2A}–deficient mice.[14] MDL100907 [R-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol] (Tocris, 4173, Bristol, UK) and SB206553 [N-3-pyridinyl-3,5-dihydro-5-methyl-benzo(1,2-b:4,5-b′)dipyrrole-1(2 H)carboxamide hydrochloride] (Tocris, 661, Bristol, UK) were dissolved in 5.5% glucose solution containing 0.005% acetic acid (v/v).

Classification of Sleep and Wakefulness States

Acquired EEG/EMG signals were amplified (Pinnacle Technology part #8201), digitalized, and collected via a printed-circuit-board (PCB)-based preamplifier (Pinnacle Technology part #8200-K1-SL), at a sampling frequency of 400 Hz. Sleep stages were classified by visual inspection and power spectral analysis was performed offline with Neuroscore (version 2.0.1, Data Sciences Inc., St.Paul, US). Wake and sleep were classified in 10-sec epochs by an experienced scorer blind to the experimental conditions. The EEG signal was filtered with high-pass: 0.5 Hz, low pass: 30 Hz, and EMG signal high-pass filtered at 10Hz.[22] The following stages were scored: wake with high frequency, low voltage activity in the EEG, and high/moderate activity in EMG; SWS with 11–16 Hz spindles and slow wave activity (SWA; 1–4 Hz) and rapid eye movement (REM) sleep with theta (5–8 Hz) activity and low neck muscle EMG. Wakefulness was subdivided into quiet wakefulness (QW) or active wakefulness (AW) using EMG peak-to-peak amplitude of all wake epochs across the 12-h recording. QW was defined as 33rd percentile or less and AW 66th percentile or higher of all wake EMG peak-to-peak amplitude values. The criteria used are shown to be sufficient to exclude epochs with high locomotor activity from QW, and to demonstrate unique electrophysiological changes between QW and AW, across frequency bands and cortices.[23]

Table 1—Effect of genotype on baseline sleep timing (%).

State	Phase	*Egr3+/+*	*Egr3-/-*	P value
Wake	Dark	61.2 ± 11.6	60.5 ± 12.1	NS
	Light	38.2 ± 7.5*	42.1 ± 7.8*	NS
SWS	Dark	34.3 ± 10.2	34.5 ± 10.7	NS
	Light	50.4 ± 7.5*	49.4 ± 7.9*	NS
REMS	Dark	4.2 ± 2.2	4.9 ± 2.3	NS
	Light	11.1 ± 3.0*	8.2 ± 3.2*	0.008

Time spent in wakefulness and sleep across the dark/light cycle in wild type mice (n = 16) and *Egr3-/-* mice (n = 15). P value indicates significant differences between the genotypes. Asterisks indicate significant differences between light and dark phase: * < 0.05. Sex differences were not detected. NS, non-significance.

Table 2—Effect of sleep disturbance on sleep timing (minutes/6 hours).

State	Day	*Egr3+/+*	*Egr3-/-*	P
Wake	Baseline	143 ± 42	157 ± 43	NS
	SD	263 ± 46*	329 ± 48*	< 0.001
SWS	Baseline	181 ± 36	175 ± 37	NS
	SD	94 ± 44*	31 ± 45*	< 0.001
REMS	Baseline	35 ± 13	26 ± 14	NS
	SD	3 ± 7*	0 ± 7*	NS

Effect of 6 hours sleep disturbance on wakefulness and sleep in *Egr3-/-* mice (n = 15) and wild type mice (n = 16). P value indicates significant differences between the genotypes. Asterisks indicate significant differences compared to baseline: * < 0.05. Sex differences were not detected. NS, non-significance.

Quantitative EEG

Power spectral analysis was performed offline by fast Fourier transform (FFT) analysis on unfiltered EEG signals on each 10-sec epoch segregated into 2-sec intervals with a Hamming window and 50% overlap, yielding an average power value for the 10-sec epoch. Artifacts were removed in two steps: (1) visual inspection of EEG signals where all epochs containing electrical artifacts were excluded; and (2) an automated algorithm detecting epochs with values exceeding the mean value by at least 8 standard deviations. In all files, fewer than 5% of epochs were eliminated from analysis. To account for individual differences while measuring sleep homeostatic changes in response to sleep loss, EEG spectral power tracked across QW episodes during sleep disruption was normalized to the average values within each animal's 24 h baseline recording.

Statistics

The software package MATLAB (The MathWorks Inc., Natick, USA) was used for analysis and modelling. Statistica (version12, StatSoft Inc) was used for statistical analyses. Markers of homeostatic sleep drive during 6 h of sleep disruption were normalized to 24 h baseline condition. Latencies to AW and QW were tested with *t*-tests for independent measures. Genotype specificity of the dissociated state was tested with Pearson chi-square test. All other parameters were analysed with repeated-measures analysis of variance with the factors 'genotype' and 'sex' as between subjects factors and the time interval analyzed and pharmacological agent as within subject factors. All significant main and interaction effects were followed up with Fisher least significant difference *post hoc* test. Significance level was $\alpha \leq 0.05$. Sex did not show a statistical main effect or interaction with genotype, unless otherwise stated.

RESULTS

Egr3-/- Mice Exhibit Different EEG Dynamics in Waking and Sleep States

Spontaneous sleep exhibited the typical diurnal rhythm of sleep and wakefulness in both genotypes. The percent of time spent in wakefulness and SWS was similar between the two genotypes. *Egr3-/-* mice spent less time in REM sleep than WT mice during the inactive (light) phase (genotype × dark/light phase: $F_{1,27} = 6.73$; $P = 0.015$, see Table 1). However, across 24 h, the percent time spent in REM sleep did not differ between *Egr3-/-* mice (6.6 ± 0.6%) and WT mice (7.6 ± 0.6%). The duration of the REM sleep episodes was longer in *Egr3-/-* (1.4 ± 0.1 min) than in WT mice (1.1 ± 0.1 min; genotype; $F_{1,27} = 7.49$; $P = 0.011$). Other state consolidation measures (duration of wake and SWS episodes, number of brief awakenings lasting less than 20 sec during sleep) did not significantly vary as a function of *Egr3*-deficiency.

Profiles of the EEG power in the 1- to 20-Hz range from the different wake and sleep states yielded a significant difference between genotypes for QW, SWS, and REM sleep (genotype × 1 Hz bin: $F_{19, 513} > 2.49$, $P < 0.001$, all). Figure 1 illustrates that *Egr3-/-* mice exhibited a suppressed EEG power in the 1- to 3-Hz range during SWS and an increased EEG power in the 3- to 8-Hz range in QW and REM sleep, compared to WT. Additionally, *Egr3-/-* mice demonstrated elevated beta (15–35 Hz) activity in all states, except from AW, relative to WT mice (Figure 1, insets).

Heightened Arousal during Sleep Disruption in Egr3-/- Mice

Six hours of sleep disruption, beginning at ZT0, resulted in significant sleep loss in both genotypes compared to time matched baseline recordings (Table 2). Notably, the *Egr3-/-* mice spent more time awake (329 ± 48 min) than WT mice (263 ± 46 min; genotype: $F_{1,29} = 11.4$; $P = 0.002$). This effect may reflect a heightened reactivity to the novelty of a rotating bar in the cage to enforce wakefulness, as the sleep disruption also resulted in different waking behavior during SD (genotype × time; AW: $F_{11,319} = 1.89$, $P = 0.039$ and QW: $F_{11,319} = 4.60$, $P < 0.001$). As illustrated in Figure 2A and 2B, *Egr3-/-* mice exhibited a shorter latency to AW and spent more time in AW during the first 3.5 h of sleep disruption compared to WT mice. Moreover, latency to QW was greater in *Egr3-/-* mice and *Egr3-/-* mice spent less time in QW than WT during the first 0.5–1.5 h of sleep disruption. Also, in line with existing behavioral data of hyperarousal when exposed to novelty,[10] the AW behavior in *Egr3-/-* mice dissipated over time, demonstrating habituation.

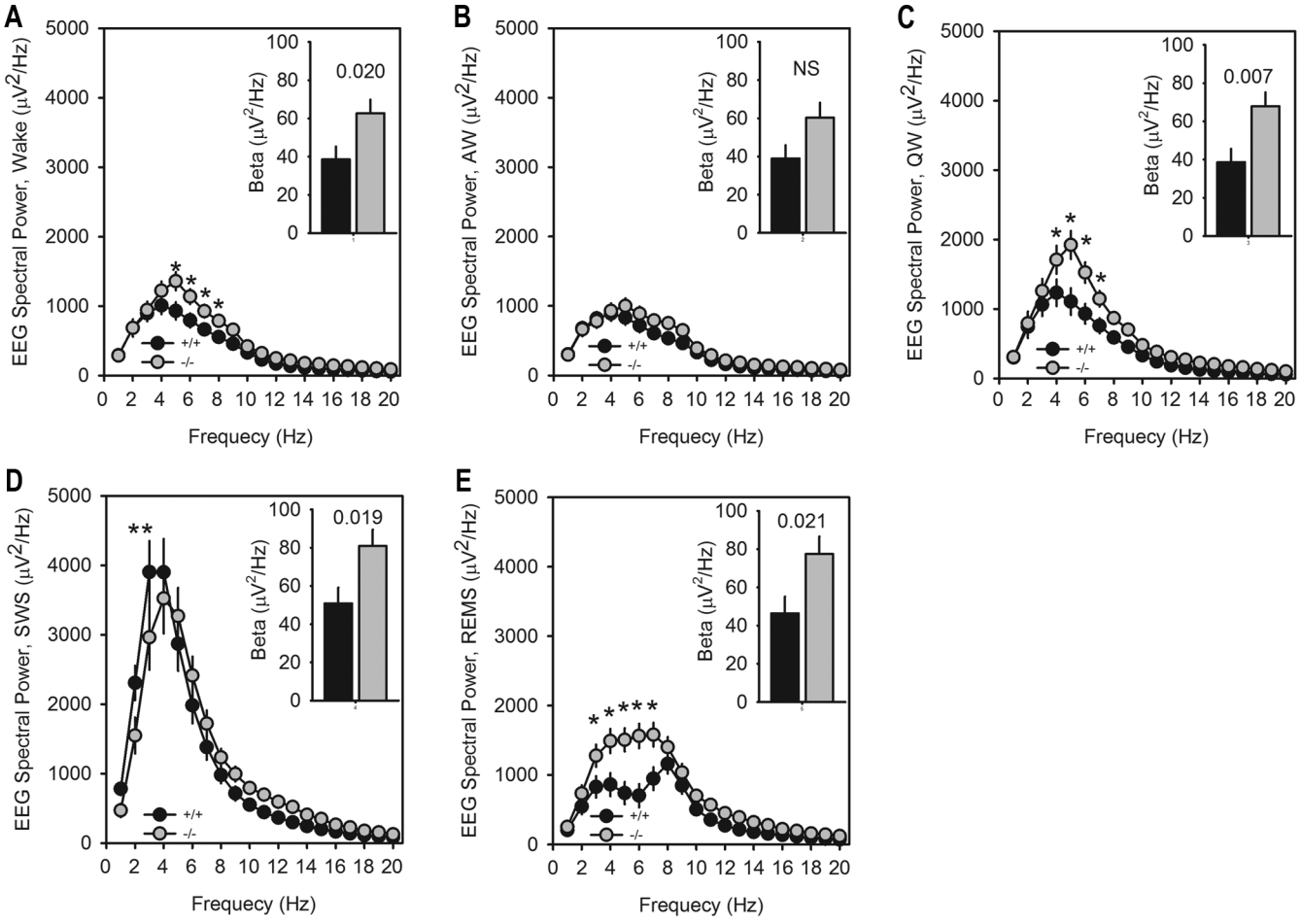

Figure 1—Effect of *Egr3* disruption on the electroencephalographic power profiles of the different wake and sleep states. Mean values ± standard error of the mean in the 1 to 20 Hz and beta (15–35 Hz) range across the 24 h baseline recording are shown. Asterisks indicate significant differences between *Egr3*-deficient mice (n = 15) and wild type mice (n = 16). Sex differences were not detected.

Attenuated Homeostatic Sleep Drive in SWS of Egr3-/- Mice after Sleep Disruption

Despite the more robust sleep loss and more AW behavior during sleep disruption in *Egr3-/-* mice than WT mice, there was no genotype effect in EEG hallmarks of accumulated sleep drive measured in QW epochs during the sleep disruption. The elevation of SWA, theta, and beta power during QW exhibited a progressive, time-dependent increase in both genotypes across sleep disruption (time: $F_{13,312} > 2.10$, $P < 0.014$, genotype × time: $P > 0.24$, all bands. Figure 2C illustrates the dynamics of the EEG changes during the sleep disruption when normalized to 24 h baseline condition. Also, the insets in Figure 2 show that absolute EEG power in the theta and beta bands in QW epochs during sleep disruption was elevated in *Egr3-/-* mice relative to WT mice.

The recovery sleep of *Egr3-/-* mice demonstrated a more blunted response to sleep loss than WT mice (genotype × baseline / SD × 0.5 h: $F_{11,209} = 2.22$; $P = 0.015$). Figure 2D illustrates that sleep disruption in WT mice induced elevated SWA for 3 h, before returning to time matched baseline level. *Egr3-/-* mice demonstrated elevated SWA for 2 h. SWA across the 6 h of recovery sleep was 32% lower in the *Egr3-/-* mice relative to the WT mice, but not significantly reduced ($P = 0.075$).

Egr3-/- Mice are Relatively Resistant to Clozapine-Induced Suppression of AW and Sedation

The sedating effect of clozapine, measured both behaviorally and in the EEG, was significantly attenuated in the *Egr3-/-* mice compared to WT mice. Clozapine suppressed AW in the WT mice, an effect significantly attenuated in the *Egr3-/-* mice (genotype: $F_{1,69} = 9.8$, $P = 0.003$). Figure 3A illustrates that compared to vehicle treatment, WT mice increased their latency to 2 min of accumulated AW sevenfold when receiving low-dose and 13-fold at high-dose clozapine. In contrast, *Egr3-/-* mice increased latency to AW only after high-dose clozapine compared to vehicle treatment.

Figure 3B illustrates that the effects of clozapine on time spent in wake and sleep also differentiated *Egr3-/-* and WT mice (genotype × time: $F_{5,115} > 2.99$, $P < 0.014$, both AW and QW). High-dose clozapine reduced time in AW throughout the first 5 h after administration in WT mice, an effect that lasted for only 2 h in *Egr3-/-* mice, compared to vehicle. Low-dose clozapine suppressed time in AW for 2 h and 1 h, in WT and

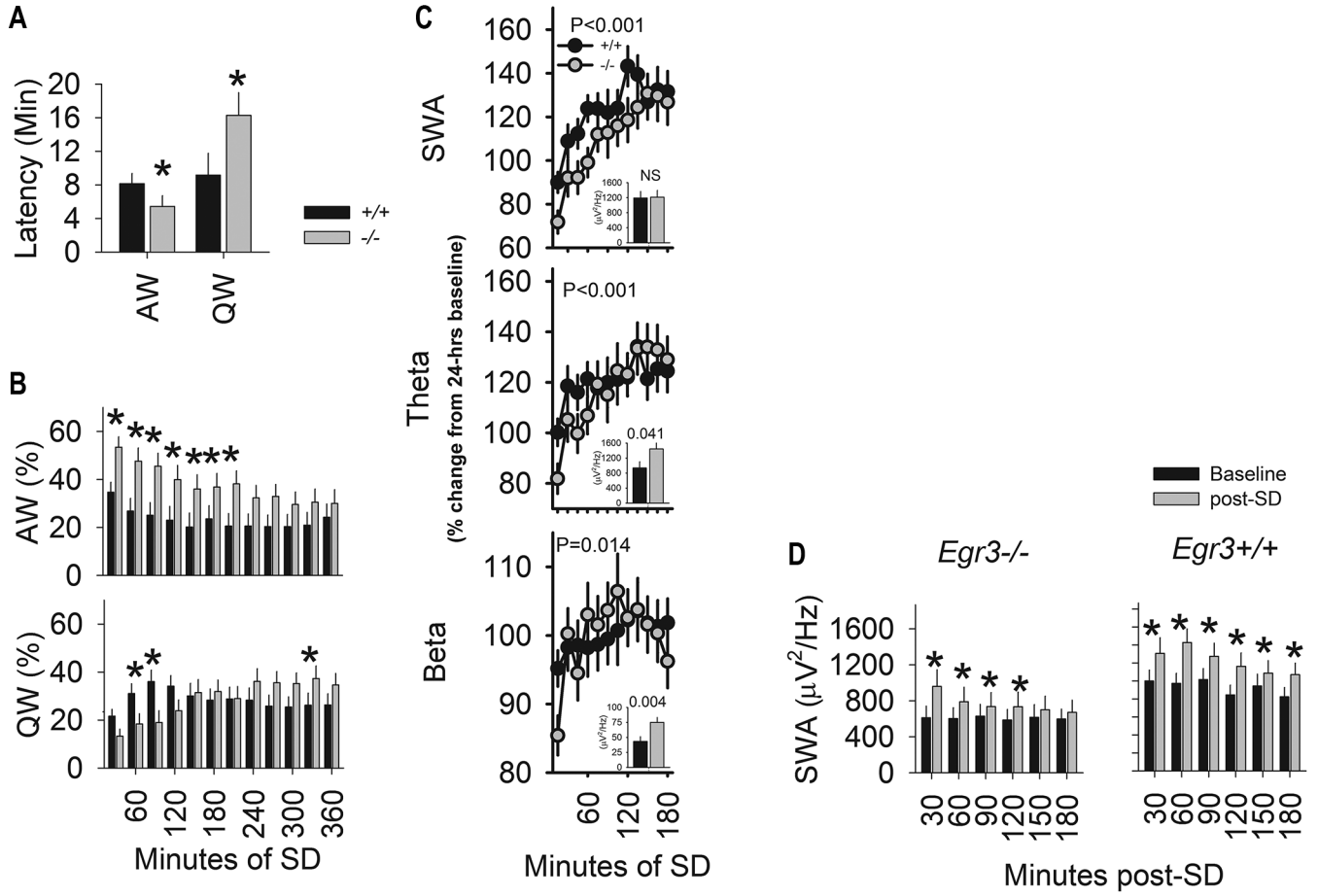

Figure 2—Response to 6 h of sleep disruption. Waking behavior. **(A)** Latency to 2 min accumulated active wakefulness (AW) and quiet wakefulness (QW) and **(B)** time spent in AW and QW during the session of sleep disruption (SD). Markers of homeostatic sleep drive. **(C)** Progressive increase in slow wave activity (SWA; 1–4 Hz), theta (5–8 Hz), and beta (15–35 Hz) activity during quiet wakefulness over the time course of SD. The insets show the absolute electroencephalogram power. Recovery sleep. **(D)** Sleep intensity (SWA) during recovery sleep and time matched baseline condition. Asterisks indicate significant differences between *Egr3*-deficient mice (n = 15) and wild-type mice (n = 16). Data are shown as mean values ± standard error of the mean. Squares indicate significant differences compared to baseline level. P values indicate main effect of time (repeated-measures analysis of variance). Sex differences were not detected.

*Egr3-/-*mice, respectively. Concurrently, time in QW increased the first 3 h after receiving high-dose clozapine in WT mice and the first 2 h in *Egr3-/-* mice. Low-dose clozapine increased time in QW for 2 h and 1 h in WT mice and *Egr3-/-* mice, respectively. Clozapine induced slowing of the waking EEG, an effect modulated by genotype in a dose-dependent fashion (genotype × dose × EEG frequency: $F_{38,1121} = 2.08$, $P < 0.001$; Figure 3C). In WT mice, SWA (1 to 4 Hz) was elevated 80% after receiving high dose clozapine compared to vehicle. *Egr3-/-* mice exhibited a nonsignificant elevation of 20% compared to vehicle. Low-dose clozapine did not significantly alter SWA in either genotype compared to vehicle.

Only WT mice showed typical[24] clozapine-induced changes in time spent in SWS (genotype × time: $F_{5,315} > 2.6$, $P < 0.027$), see Figure 3B. WT mice spent more time in SWS when receiving high-dose compared to vehicle treatment. The low-dose treatment showed only an increase in SWS during the hour immediately after treatment. Notably, *Egr3-/-* mice did not exhibit significant alterations in sleep timing after receiving clozapine compared to vehicle treatment. REM sleep timing was unaffected by treatment in both genotypes.

EEG slowing by clozapine was specific to wakefulness as SWA in SWS and REM sleep was not modulated by genotype, dose, or their interaction. Thus, *Egr3-/-* mice are relatively resistant to the blockade of arousal-promoting mechanisms by clozapine, whether the effect is measured behaviorally,[16] by the timing of wakefulness and sleep, or by electroencephalographic changes during wake.

Clozapine Induces a Dissociated State in WT Mice But Not in *Egr3-/-* Mice

In addition to changes in conventionally defined wakefulness and sleep, clozapine induced marked alterations in the waking EEG/EMG in a subset of WT mice (high dose: 8 of 12 and low dose: 2 of 12), see Figure 4. We defined this state as dissociated state (DS) as it was characterized by low amplitude EEG (typically associated with wake), predominantly with low frequency activity (less than 5 Hz, typically associated with SWS), and low muscle tone (typically associated with sleep). Moreover, DS

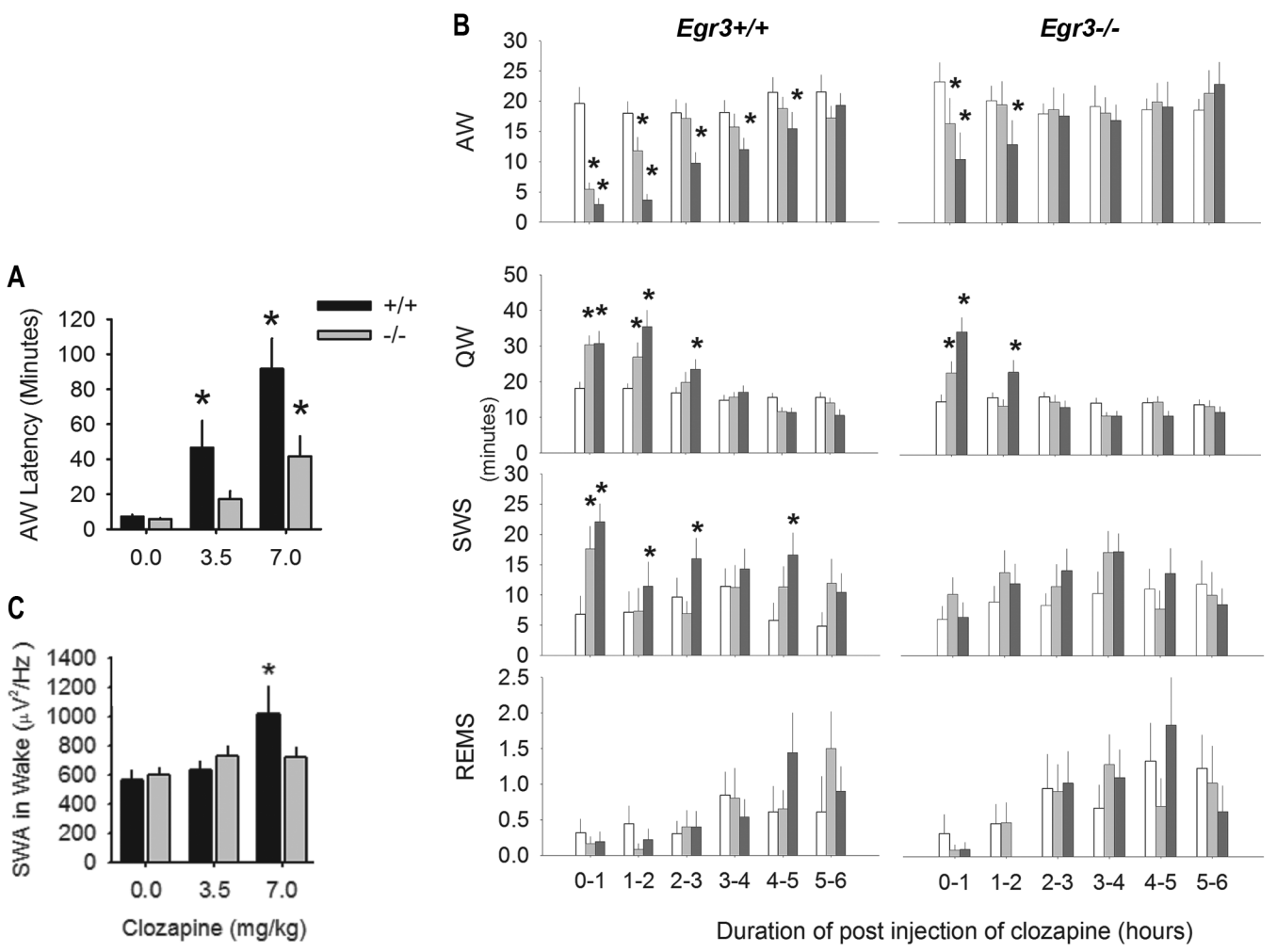

Figure 3—Clozapine-induced changes in waking and sleep in *Egr3*-deficient mice (n = 12) and wild-type mice (n = 13). **(A)** Latency to 2 min accumulated active wakefulness (AW) after vehicle or clozapine administration. **(B)** Time spent in wakefulness and sleep and **(C)** slowing of the waking electroencephalogram (SWA; 1–4 Hz) during the 2-h interval after clozapine administration. In panel B, white bars = vehicle, light gray bars = 3.5 mg/kg clozapine, and dark gray bars = 7.0 mg/kg clozapine. Data are shown as mean values ± SEM. Asterisks indicate significant differences compared to vehicle treatment. Sex differences were not detected. QW, quiet wakefulness; REMS, rapid eye movement sleep; SWA, slow wave activity; SWS, slow wave sleep.

occurred in uninterrupted intervals with longer duration (from 29 to 128 min) than SWS episodes in WT mice (typically less than 2 min). The duration of DS was paradoxically less after receiving the higher dose than the lower dose. This phenomenon was not observed in any of the *Egr3-/-* mice at either dose of clozapine (genotype effect, $\chi_1^2 = 12.75$, $P < 0.001$, Figure 4A).

The waking EEG response to clozapine in WT mice was distinct from both QW and AW states, by virtue of the simultaneous elevation of SWA and suppression of higher frequencies (more than 6 Hz; see Figure 4B). These polysomnographic data indicate that suppression of AW and the sedating effect of clozapine is a mixed and a remarkable stable state distinct from wakefulness (characterized by low amplitude EEG and high amplitude EMG) and sleep (characterized by transient episodes of high amplitude SWA and low muscle tone). *Egr3*-deficient mice did not exhibit this phenomenon: EEG power was elevated relative to WT mice at frequencies > 4 Hz after both 3.5 and 7 mg/kg clozapine.

Egr3-/- Mice Exhibit Deficits in Response to Serotonergic Agents Blocking Behavioral and Cortical Arousal

5-HT_2 antagonists were administered immediately prior to dark onset to measure the contribution of 5-HT_{2A} and 5-$HT_{2B/C}$ receptors to arousal. Blocking 5-HT_2 receptors induced a strong behavioral sedation manifested by suppression of AW in WT mice. Figure 5A illustrates that compared to vehicle, WT mice increased their latency to 2 min of accumulated AW fivefold after combined treatment of both 5-HT_{2A} and 5-HT_{2BC} antagonists (treatment × genotype: $F_{3,33} = 3.6$, $P = 0.024$). This effect of combined treatment was abolished in *Egr3-/-* mice. Treatment with 5-HT_{2A} antagonist increased the latency to AW, significantly in *Egr3*-deficient mice.

Changes in time spent in AW after treatments with 5-HT_2 antagonists were restricted to WT mice. WT mice exhibited a reduction in time spent in AW the first hour after all three 5-HT_2 antagonist treatments; treatment with 5-HT_{2BC} antagonist induced a prolonged effect by suppressing time in AW

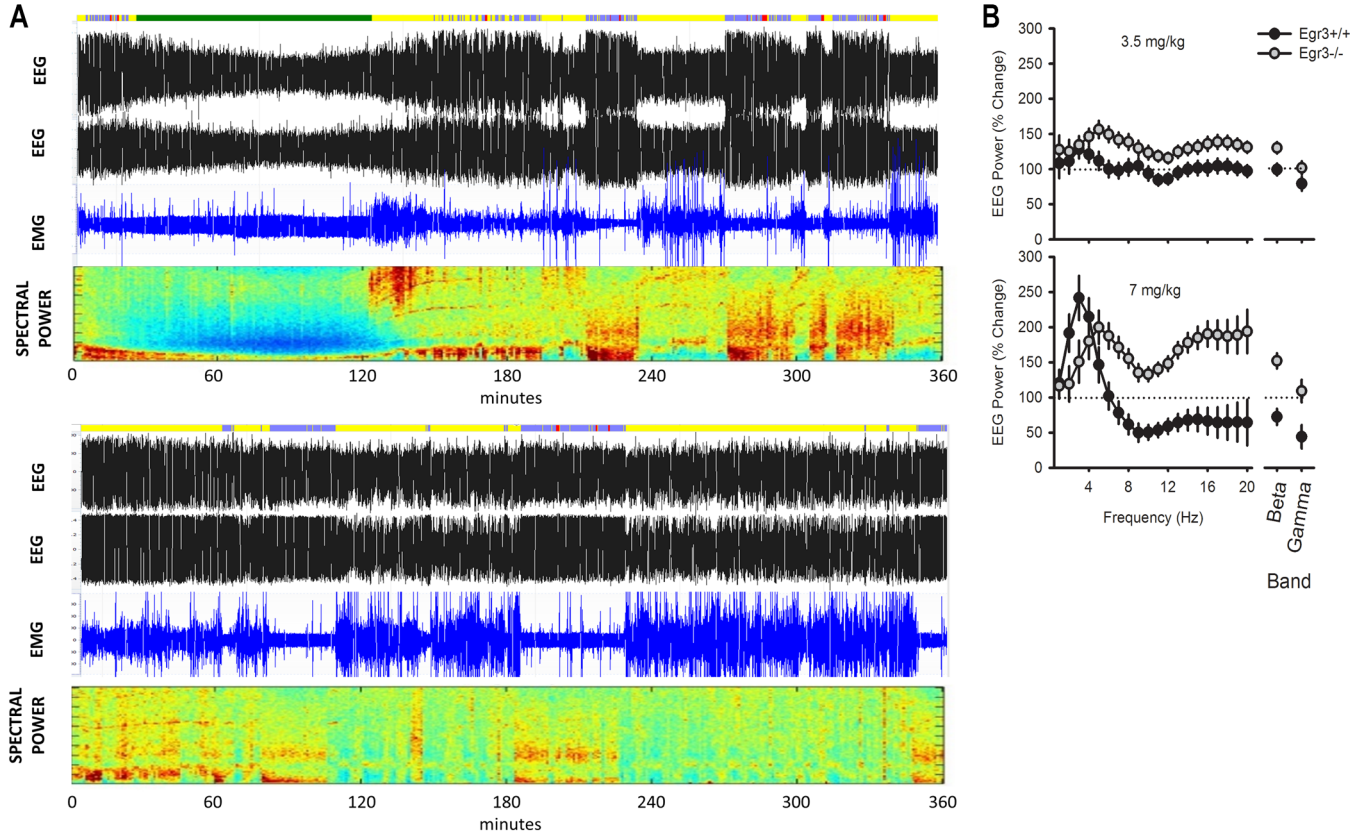

Figure 4—*Egr3*-deficient mice show no clozapine-induced dissociated state (DS). **(A)** 6 h polysomnographic traces after administration of 7mg/kg clozapine upper; wild-type mice exhibiting the DS and lower; *Egr3-/-* mice not exhibiting DS. The multicolored band at the top of each record indicates state; yellow = wake; purple = SWS; red = REMS; green = dissociated. Bilateral frontal electroencephalogram (EEG) are shown in black, neck electromyography in blue, and the lower color maps show spectral power in the 0–40 Hz range independently of sleep state. **(B)** EEG power profiles in the 1 to 20 Hz, beta (15–35 Hz) and gamma (80–90 Hz) range across 2 h recording after administration of clozapine in *Egr3*-deficient mice (n = 15) and wild-type mice (n = 16). Data are shown as mean values ± standard error of the mean Sex differences were not detected.

during the whole 6 h recording period, compared to vehicle treatment. *Egr3-/-* mice did not exhibit significant alterations in time spent in AW after receiving any of the 5-HT_2 antagonists. See Figure 5B.

WT mice increased time spent in QW the first hour after all the treatments compared to vehicle. The increase in QW after treatment with 5-HT_{2A} antagonist lasted for 2 h, 5-HT_{2BC} antagonist also increased time in QW in the periods 2 to 3 h and 4 to 6 h after treatment. The combined treatment exhibited a biphasic response of time spent in QW; first an acute increase then a decrease 4 to 6 h after the injection. *Egr3-/-* mice exhibited only modest alterations for time spent in QW. See Figure 5C. Time in SWS and REM sleep was not affected in any genotype by 5-HT_2 antagonist treatments (time × genotype: $F_{5,195} < 0.45$, $P > 0.81$ and treatment × time × genotype: $F_{15,195} < 1.08$, $P > 0.38$).

Notably, changes in EEG power induced by 5-HT_2 antagonist treatment were robust in WT mice and nearly absent in *Egr3-/-* mice. The effect in WT mice was restricted to QW (treatment × EEG frequency × genotype: $F_{57,741} = 2.66$, $P < 0.00$) and not apparent in AW ($P = 0.166$, Figure 5D). In WT mice, 5-HT_2 antagonists elevated the EEG power in the lower frequencies during QW (4 to 6 Hz for 5-HT_{2A} antagonist alone and 2 to 5 Hz for co-administration with 5-HT_{2BC} antagonist), compared to vehicle. 5-HT_{2BC} antagonist alone suppressed EEG power in the 3- to 5-Hz range. *Egr3-/-* mice did not exhibit significant elevation of EEG power in the same frequency range with either treatment. Mice of both genotypes exhibited suppression of power in the 9- to 10-Hz range after co-administration of the 5-HT_{2A} and 5-HT_{2BC} antagonists. In sum, *Egr3*-deficient mice were resistant to 5-HT_2 modulation of wakefulness, both at the behavioral and the electroencephalographic levels.

DISCUSSION

This article investigated the functional consequences of genetic disruption of *Egr3* and serotonergic signaling in wakefulness and sleep. Despite the fact that *Egr3*-deficient mice appear to have normal timing of spontaneous wakefulness and SWS, the EEG exhibits abnormalities in frequency ranges associated with homeostatic sleep drive. Additionally, their behavioral and EEG responses to both sleep deprivation and pharmacological probing of serotonergic function are distinct from those of WT mice.

The high intensity SWA and beta activity in *Egr3-/-* mice in the baseline condition was similar to that of WT mice in a

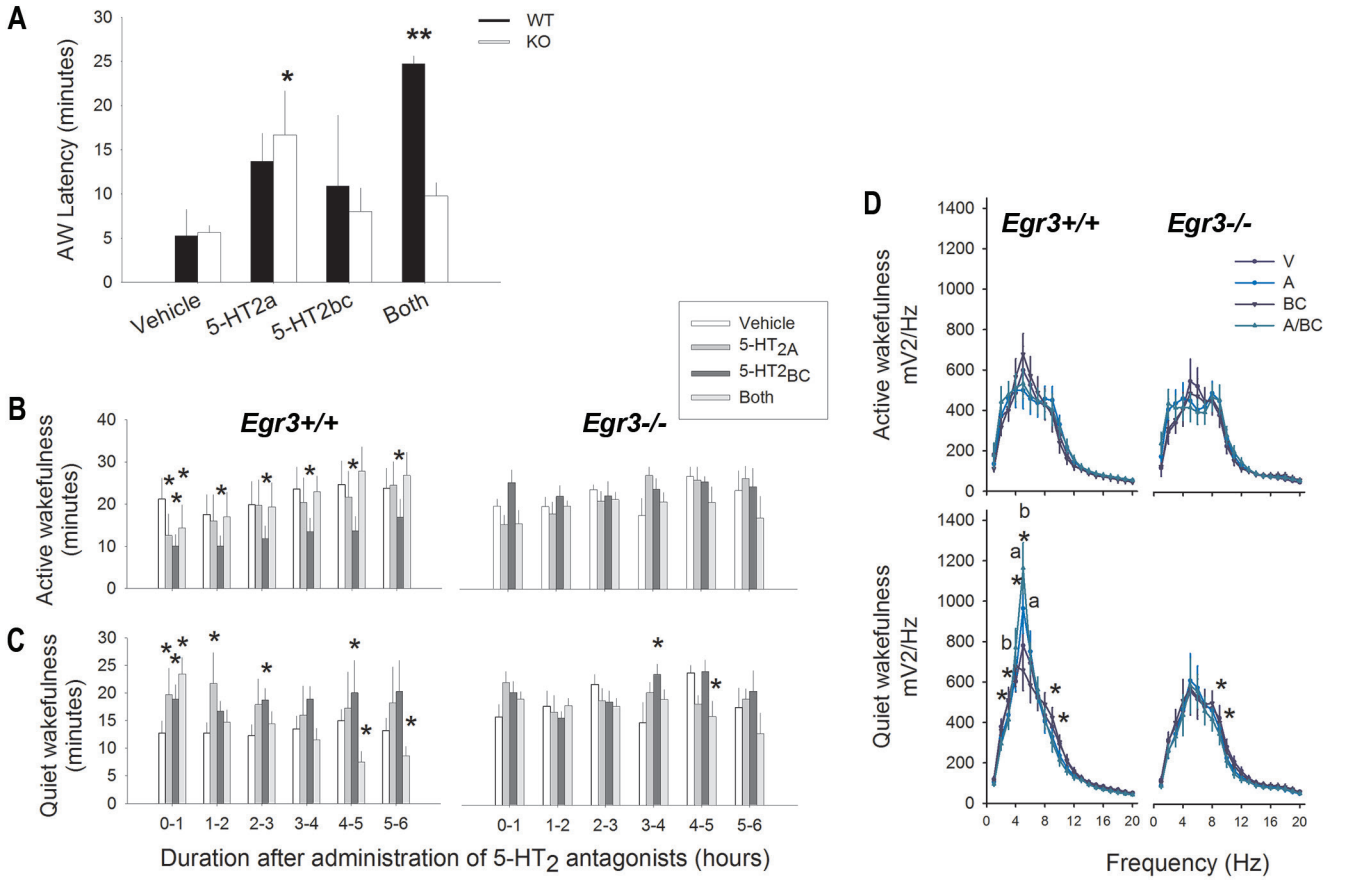

Figure 5—Changes in waking after 5-HT$_2$ antagonists treatment (5 mg/kg) in *Egr3*-deficient mice (n = 7) and wild-type mice (n = 8). **(A)** Latency to 2 min accumulated active wakefulness (AW). **(B,C)** Time spent in active wakefulness and quiet wakefulness after vehicle. Asterisks indicate significant differences compared to vehicle treatment. **(D)** Waking EEG spectral power. a, b, and asterisks indicates significant differences compared to vehicle treatment, 5-HT$_{2A}$, 5-HT$_{2BC}$ or both antagonist, respectively. Sex differences were not detected.

condition of high sleep drive,[23] suggesting a reduced level of arousal during QW in *Egr3*-deficient mice relative to WT mice. During sleep disruption,

Egr3-/- mice exhibited a rise in homeostatic sleep drive equal to WT mice. Yet, the *Egr3-/-* mice showed a sleep phenotype with a suppressed SWA intensity in SWS, both during baseline condition and during recovery sleep from sleep disruption. Also, the sleep intensity during recovery sleep was blunted. This difference contrasts with the acute behavioral response of *Egr3-/-* mice, which exhibited more AW than WT mice at the outset of sleep disruption. Active waking behavior normally potentiates an increase in the homeostatic sleep drive.[25] Cortical *Egr3* and other IEG mRNAs (like *Egr1*) are consistently found to be upregulated during prolonged wakefulness.[2,7,8] However, unlike *Egr1* and other IEGs, *Egr3* mRNA is reported to be elevated also during recovery sleep.[7] Although *Egr3* is reported to be a late response gene,[5] the increased IEG mRNA expression during 4 h of recovery sleep is rare and may underlie the abnormalities found in the response of *Egr3-/-* mice to sleep disruption.

Neuronal firing patterns, neuromodulators, and cerebral metabolism change across states of arousal and sleep. 5-HT$_2$ signaling plays an important role in the regulation of arousal and timing of sleep.[13] Here, we show that *Egr3-/-* mice exhibit a striking resistance to a suppression of EEG power by a pharmacological blockade of the arousal-promoting circuits in the central nervous system, both by clozapine (a leading atypical antipsychotic and a potent 5-HT$_2$ receptor antagonist), and by a selective 5-HT$_{2A}$ antagonist. These findings are in line with prior studies in *Egr3*-deficient mice showing resistance to locomotor suppression to similar pharmacological treatment.[11,16] In WT mice, clozapine caused sedation as reported in rats[24] and blockade of the 5-HT$_2$ receptors produced a prolonged inhibition of AW and low-frequency activity. These effects were not detected in *Egr3-/-* mice.

If the EEG abnormalities documented in *Egr3-/-* mice are indeed consequent to 5-HT$_2$ signaling deficits, then similar deficits would be expected in 5-HT$_2$ receptor-deficient mice. 5-HT$_{2A}$-/- mice have been subjected to sleep[14] and behavioral[15] studies, and do exhibit phenotypes similar to *Egr3-/-* mice. *Egr3-/-* mice[10] and 5-HT$_{2A}$-/- mice[15] exhibit heightened reactivity in situations involving novelty. Both strains show attenuated enhancement of SWA in recovery sleep after sleep disruption.[14] Pharmacologically, both *Egr3-/-* and 5-HT$_{2A}$-/- mice fail to respond to the 5-HT$_{2A}$ antagonist MDL100907 across sleep and wakefulness. These parallels indicate that 5-HT$_{2A}$ deficits

may play an important role in the behavioral[11,16] and sleep phenotypes demonstrated in *Egr3*-deficient mice. Yet, there is one key difference between *Egr3*-/- and 5-HT_{2A}-/- mice in sleep regulation. Whereas 5-HT_{2A}-/- mice exhibit reduced time spent in SWS and elevated time awake in baseline conditions,[14] we did not find such abnormalities in SWS and wake timing in *Egr3*-/- mice. This difference may reflect the localization and/or extent of 5-HT_{2A} deficits, given the importance of subcortical serotonergic signaling in regulating sleep timing. The 5-HT_{2A} signaling in *Egr3*-/- mice is reduced in frontal cortex[11], but this may differ across brain regions. 5-HT_{2A} signaling is absent in all tissues in 5-HT_{2A}-/-mice. In addition, our data are a strong indicator that functions and/or expression of 5-HT_{2B} and/or 5-HT_{2C} receptors is perturbed in *Egr3*-deficient mice.

The importance of 5-HT_2 signaling in maintaining cortical arousal is illustrated in the WT mice by reduced time spent in AW and EEG slowing in QW after 5-HT_2 blockade. Additionally, resemblance of the EEG spectral profile of *Egr3*-deficient mice in QW at baseline to that of WT mice after sleep disruption, and the failure of 5-HT_{2A} blockade to potentiate this phenotype are indicative of a crucial function for (tonic) 5-HT_{2A} signaling in maintaining cortical arousal. The 5-HT_{2A} receptor is enriched in pyramidal cells of the cerebral cortex, relative to the other G protein-coupled receptors that are activated by serotonin. G protein activation by 5-HT_{2A} facilitates excitation of neurons. Cell type-specific manipulations of 5-HT_{2A} receptors will be necessary to determine whether direct effects on pyramidal cell excitability underlie the effects of 5-HT_{2A} receptors on arousal. The function of this receptor in maintenance of arousal may be particularly important during protracted wake, because expression of 5-HT_{2A} receptors increases during enforced wake, at the mRNA level in rodents[8] and at the level of receptor availability in human subjects.[26] If so, antagonism of the receptor would have increasingly robust EEG and behavioral effects in parallel with increasing sleep need during sleep disruption. This possibility was not addressed in the current study, as MDL100907 was administered immediately after the light phase of the LD cycle, a time when sleep need is at its nadir in mice.

Sedation is a common side effect of antipsychotic agents.[27] Our data demonstrate that clozapine not only suppresses AW and slows the waking EEG, as reported in humans[28] and rodents,[24] but additionally induces marked alterations in their waking EEG/EMG lasting up to 2 h following treatment. We defined this state as dissociated state (DS) because it was a mixture of extremely low amplitude EEG (typically associated with wake) and low muscle tone (typically associated with sleep). The EEG during DS was paradoxically composed primarily of low-frequency activity (less than 5 Hz), albeit at an amplitude far lower than normally occurred during SWS, and with significant suppression of higher (5 to 20 Hz) oscillations that predominate in wake. All of these EEG/EMG effects of clozapine were significantly attenuated in mice deficient in *Egr3*. The clozapine-induced DS was a remarkably stable state and is likely a direct consequence of continuous suprathreshold receptor occupancy by clozapine. The reason that some and not all WT mice exhibited DS is unclear. The effect was not explained by sex, previous exposure to clozapine or distinct litters. Inconsistent EEG responses to clozapine treatment have also been reported in rats,[24] and systematic differences between responding and non-responding rats were not described.

The neurobiological mechanisms responsible for the sedative effect of clozapine had historically been attributed to binding of the drug to histamine H1 receptors; however, prior studies demonstrating that *Egr3*-/- mice and 5-HT_{2A}-/- mice[17] display resistance to this sedation elucidated a key role for the 5-HT_{2A} receptor in this prominent side effect of clozapine and other second-generation antipsychotic agents.[11,16] The suppression of AW in WT mice could be explained by blockade of 5-HT_2-mediated arousal promoting neurotransmission,[27] but selective blockade of 5-$HT2_{2A}$ signaling or simultaneous blockade of 5-$HT2_{2A}$/5-$HT2_{2BC}$ signaling did not mimic the DS observed after clozapine. Clozapine is pharmacologically complex. Effects on sleep regulatory mechanisms including gamma-aminobutyric acid (GABA)ergic, adenosinergic, and glutamatergic signaling may also be involved in the response to clozapine.[11,17] *Egr3* regulates $GABA_A$ receptor subunit expression[29,30] in addition to 5-HT_{2A} receptor expression and function.[8,11] Clozapine antagonizes $GABA_A$ binding and produces a functional antagonism of $GABA_A$ receptors at synapses.[31] Currently most treatments of insomnia potentiate GABAergic transmission.[32] Adenosine, acting via A1 receptors, is a key factor in the homeostatic control of sleep.[33,34] The role of *Egr3* in regulating A1 receptors is still not known, but warrants further investigations. Studies further investigating 5-HT_2, $GABA_A$, glutamatergic, and adenosinergic signaling in *Egr3*-deficient mice may provide valuable information about regulation of cortical arousal in the face of accumulating sleep need.

Egr3-/- mice display hyperarousal to novel and stressful stimuli and to handling. Compared to WT mice, *Egr3*-/- mice respond to a novel environment (open field) with a behavioral hyperarousal that attenuates within 50 min,[10] and to gentle handling for 90 sec with increased plasma corticosterone.[10] Therefore, in an effort to minimize stress during the enforced waking and to exclude locomotion and hyperarousal to novelty as a confounding variable in sleep timing and EEG power spectra, the current experiment applied a rotating bar in the home cage instead of gentle handling for the 6 h sleep disruption. Our comparison of active and quiet wake states confirms that hyperarousal occurs in the novel situation posed by the rotating bar in the home cage environment, but also the attenuation of this hyperarousal over time. To our knowledge, there is no study comparing stress responses (corticosterone, adrenocorticotropic hormone, other hormones or physiological outcome) in the rotating bar protocol against other sleep disruption protocols such as gentle handling. Induction of sleep disruption by the rotating bar was successful, as sleep was reduced by 91% (albeit with transient intervals of sleep), and the rising of sleep pressure was evident from increase in SWA and theta activity in the 6 h of sleep disruption protocol. Nonetheless, whether any hormonal responses to novelty outlast behavioral responses in the rotating bar environment and whether they contribute to differences in the sleep phenotype is not certain.

One measure of significance from the current results that remains to be evaluated in more detail is the effect of *Egr3*-deficiency on REM sleep timing (reduced amount in the inactive phase) and EEG spectra (higher intensity of SWA and theta activity). Impaired REM sleep may explain the profound impairments in cued-associative learning/memory in *Egr3-/-* mice.[9,10] This memory task is REM sleep-dependent and depends upon the hippocampus,[35] where *Egr3* is highly expressed and is critical for synaptic plasticity.[9,10] The fact that *Egr3* directly regulates at least one critical gene regulating learning and memory, *Arc*, suggests a potential mechanism by which *Egr3* may influence REM sleep-dependent learning and memory consolidation.

In conclusion, the current results identify *Egr3* as a wake-sleep related transcriptional regulator of considerable functional significance. Deficits in 5-HT_2 receptor-mediated signaling are likely to mediate, at least in part, this phenotype.

REFERENCES

1. Aton SJ, Suresh A, Broussard C, Frank MG. Sleep promotes cortical response potentiation following visual experience. Sleep 2014;37:1163–70.
2. Thompson CL, Wisor JP, Lee C, et al. Molecular and anatomical signatures of sleep deprivation in the mouse brain. Front Neurosci 2010;4:165.
3. Cirelli C, Tononi G. Differential expression of plasticity-related genes in waking and sleep and their regulation by the noradrenergic system. J Neurosci 2000;20:9187–94.
4. Li L, Carter J, Gao X, Whitehead J, Tourtellotte WG. The neuroplasticity-associated arc gene is a direct transcriptional target of early growth response (Egr) transcription factors. Mol Cell Biol 2005;25:10286–300.
5. O'Donovan KJ, Baraban JM. Major Egr3 isoforms are generated via alternate translation start sites and differ in their abilities to activate transcription. Mol Cell Biol 1999;19:4711–8.
6. Lyford GL, Yamagata K, Kaufmann WE, et al. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 1995;14:433–45.
7. Terao A, Greco MA, Davis RW, Heller HC, Kilduff TS. Region-specific changes in immediate early gene expression in response to sleep deprivation and recovery sleep in the mouse brain. Neuroscience 2003;20:1115–24.
8. Maple AM, Zhao X, Elizalde DI, McBride AK, Gallitano AL. Htr2a Expression responds rapidly to environmental stimuli in an Egr3-dependent manner. ACS Chem Neurosci 2015;6:1137–42.
9. Li L, Yun SH, Keblesh J, et al. Egr3, a synaptic activity regulated transcription factor that is essential for learning and memory. Mol Cell Neurosci 2007;35:76–88.
10. Gallitano-Mendel A, Izumi Y, Tokuda K, et al. The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. Neuroscience 2007;148:633–43.
11. Williams AA, Ingram WM, Levine S, et al. Reduced levels of serotonin 2A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. Neuropsychopharmacology 2012;37:2285–98.
12. Dugovic C. Functional activity of 5-HT2 receptors in the modulation of the sleep/wakefulness states. J Sleep Res 1992;1:163–8.
13. Portas CM, Bjorvatn B, Ursin R. Serotonin and the sleep/wake cycle: special emphasis on microdialysis studies. Prog Neurobiol 2000;60:13–35.
14. Popa D, Lena C, Fabre V, et al. Contribution of 5-HT2 receptor subtypes to sleep-wakefulness and respiratory control, and functional adaptations in knock-out mice lacking 5-HT2A receptors. J Neurosci 2005;25:11231–8.
15. Weisstaub NV, Zhou M, Lira A, et al. Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science 2006;313:536–40.
16. Gallitano-Mendel A, Wozniak DF, Pehek EA, Milbrandt J. Mice lacking the immediate early gene Egr3 respond to the anti-aggressive effects of clozapine yet are relatively resistant to its sedating effects. Neuropsychopharmacology 2008;33:1266–75.
17. McOmish CE, Lira A, Hanks JB, Gingrich JA. Clozapine-induced locomotor suppression is mediated by 5-HT2A receptors in the forebrain. Neuropsychopharmacology 2012;37:2747–55.
18. Tourtellotte WG, Milbrandt J. Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. Nat Genet 1998;20:87–91.
19. Wisor JP, Clegern WC, Schmidt MA. Toll-like receptor 4 is a regulator of monocyte and electroencephalographic responses to sleep loss. Sleep 2011;34:1335–45.
20. Aitchison KJ, Jann MW, Zhao JH, et al. Clozapine pharmacokinetics and pharmacodynamics studied with Cyp1A2-null mice. J Psychopharmacol 2000;14:353–9.
21. Scott DO, Heath TG. Investigation of the CNS penetration of a potent 5-HT2a receptor antagonist (MDL 100,907) and an active metabolite (MDL 105,725) using in vivo microdialysis sampling in the rat. J Pharm Biomed Anal 1998;17:17–25.
22. Wisor JP, Rempe MJ, Schmidt MA, Moore ME, Clegern WC. Sleep slow-wave activity regulates cerebral glycolytic metabolism. Cereb Cortex 2012;23:1978–87.
23. Gronli J, Rempe MJ, Clegern WC, Schmidt M, Wisor JP. Beta EEG reflects sensory processing in active wakefulness and homeostatic sleep drive in quiet wakefulness. J Sleep Res 2016;25:257–68.
24. Sorge S, Pollmacher T, Lancel M. Clozapine alters sleep-wake behavior in rats. Neuropsychopharmacology 2004;29:1462–9.
25. Vyazovskiy VV, Tobler I. The temporal structure of behaviour and sleep homeostasis. Plos One 2012;7:e50677.
26. Elmenhorst D, Kroll T, Matusch A, Bauer A. Sleep deprivation increases cerebral serotonin 2A receptor binding in humans. Sleep 2012;35:1615–23.
27. Miller AL. Combination treatments for schizophrenia. CNS Spectr 2004;9(9 Suppl 9):19–23.
28. Gunther W, Baghai T, Naber D, Spatz R, Hippius H. EEG alterations and seizures during treatment with clozapine. A retrospective study of 283 patients. Pharmacopsychiatry 1993;26:69–74.
29. Raible DJ, Frey LC, Cruz Del Angel Y, Russek SJ, Brooks-Kayal AR. GABA(A) receptor regulation after experimental traumatic brain injury. J Neurotrauma 2012;29:2548–54.
30. Roberts DS, Raol YH, Bandyopadhyay S, et al. Egr3 stimulation of GABRA4 promoter activity as a mechanism for seizure-induced up-regulation of GABA(A) receptor alpha4 subunit expression. Proc Natl Acad Sci U S A 2005;102:11894–9.
31. Michel FJ, Trudeau LE. Clozapine inhibits synaptic transmission at GABAergic synapses established by ventral tegmental area neurones in culture. Neuropharmacology 2000;39:1536–43.
32. Luppi PH, Peyron C, Fort P. Not a single but multiple populations of GABAergic neurons control sleep. Sleep Med Rev. 2016 Mar 12. [Epub ahead of print].
33. Alanko LO, Laitinen JT, Stenberg D, Porkka-Heiskanen T. Adenosine A1 receptor-dependent G-protein activity in the rat brain during prolonged wakefulness. Neuroreport 2004;15:2133–7.
34. Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW. Control and function of the homeostatic sleep response by adenosine A1 receptors. J Neurosci 2009;29:1267–76.
35. Silvestri AJ. REM sleep deprivation affects extinction of cued but not contextual fear conditioning. Physiol Behav 2005;84:343–9.

SUBMISSION & CORRESPONDENCE INFORMATION

Submitted for publication March, 2016
Submitted in final revised form June, 2016
Accepted for publication August, 2016
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DISCLOSURE STATEMENT

This was not an industry supported study. This research was supported by National Institutes of Health grants RO3NS082973 and RO1NS078498, and National Institute of Mental Health Award (MH097803). There was no off-label or investigational use. Dr. Gallitano has financial interest in Proteon Therapeutics Inc. The other authors have indicated no financial conflicts of interest.