A balanced clock: network plasticity in the central mammalian clock

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AGING AFFECTS GABAERGIC FUNCTION AND CALCIUM LEVELS IN MAMMALIAN CENTRAL CLOCK

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ABSTRACT

Aging impairs the function of the central circadian clock in mammals, the suprachiasmatic nucleus (SCN), leading to a reduction in the output signal. The weaker timing signal from the SCN results in a decline in rhythm strength in many physiological functions, including sleep-wake patterns. Accumulating evidence suggests that the reduced amplitude of the SCN signal is caused by a decreased synchrony among the SCN neurons. The present study was aimed to investigate the hypothesis that the excitation/inhibition (E/I) balance plays a role in synchronization within the network. Using calcium (Ca^{2+}) imaging, the polarity of Ca^{2+} transients in response to GABA stimulation in SCN slices of old mice (20-24 months) and young controls were studied. We found that the amount of GABAergic excitation was increased, and that concordantly the E/I balance was higher in SCN slices of old mice when compared to young controls. Moreover, we showed an effect of aging on the baseline intracellular Ca^{2+} concentration, with higher Ca^{2+} levels in SCN neurons of old mice, indicating an alteration in Ca^{2+} homeostasis in the aged SCN. We conclude that the change in GABAergic function, and possibly the Ca^{2+} homeostasis, in SCN neurons may contribute to the altered synchrony within the aged SCN network.

1. INTRODUCTION

In mammals, the suprachiasmatic nucleus (SCN) functions as a master circadian clock that drives 24 h rhythms in both physiology and behavior. Based on molecular feedback loops, individual SCN neurons generate ~24 h rhythms in gene expression and cellular processes that in turn regulate electrical activity rhythms (Buhr & Takahashi, 2013; Hastings *et al.*, 2018). This circadian rhythmicity is maintained when the SCN neurons are isolated, demonstrating that single cells function as cell autonomous oscillators (Welsh *et al.*, 1995; Reppert & Weaver, 2002; Welsh *et al.*, 2010). Through synchronization and coupling, these individual SCN neurons produce a coherent output signal in ensemble electrical activity with a peak in the subjective day and a trough in the subjective night, which is conveyed to other brain areas and the periphery (Ramkisoensing & Meijer, 2015). Misalignment of single cell oscillators leads to a disruption or loss of SCN rhythm at the tissue level and consequently to malfunction of peripheral clocks. This can have detrimental effects on human health and is associated with, for instance, cancer, cardiovascular, metabolic, and immune disorders (Roenneberg & Merrow, 2016; Patke *et al.*, 2019). Aging promotes such circadian dysfunction by impacting the clock machinery on different levels (Buijink & Michel, 2020). Vice versa, the dysfunctional circadian clock has detrimental effects on the course of aging and is a risk factor for age-related diseases (Kondratova & Kondratov, 2012; Fonseca Costa & Ripperger, 2015). Understanding age-related mechanisms of clock dysfunction can therefore help identifying targets to intervene in this vicious cycle.

In both human and animal models, age-related changes often lead to a reduction in behavioral activity levels and fragmented sleep-wake rhythms (Dijk & Duffy, 1999; Hofman & Swaab, 2006; Farajnia *et al.*, 2012), a longer latency to re-entrain to shifted light-dark schedules (Biello, 2009; Froy, 2011), and the inability to adapt to a different photoperiod (Buijink *et al.*, 2020). These behavioral deficits are likely the effect of age-related attenuation of the timing signal generated by the SCN network (Oster *et al.*, 2003; Farajnia *et al.*, 2014a).

Both in vivo and ex vivo studies showed a significant reduction in the amplitude of the ensemble electrical activity rhythm of the aged SCN (Satinoff *et al.*, 1993; Watanabe *et al.*, 1995; Nakamura *et al.*, 2011; Farajnia *et al.*, 2012), which can partly be explained by decreased synchronization within the SCN network. Ex vivo electrophysiological recordings of subpopulations in SCN slices of aged mice showed redistribution of phases with a second cluster in the middle of the night. In contrast, peaks in SCN electrical activity in young control slices only clustered around the middle of the day (Farajnia *et al.*, 2012).

An important neurotransmitter that plays a role in synchronization within the SCN network is γ-aminobutyric acid (GABA), which is expressed in almost all SCN neurons (Moore & Speh, 1993; Abrahamson & Moore, 2001). Although the precise role for GABA in the process of synchronization is still under debate (Ono *et al.*, 2020), the number of GABAergic synaptic terminals in the SCN are diminished by 26% due to aging (Palomba *et al.*, 2008) and GABAergic postsynaptic currents are reduced in frequency and amplitude (Nygard *et al.*, 2005; Farajnia *et al.*, 2012). Interestingly, GABA has the ability to act both as an inhibitory and excitatory neurotransmitter in SCN neurons and therefore contributes to plasticity in the excitatory/inhibitory (E/I) balance within the SCN

(Albus *et al.*, 2005; Choi *et al.*, 2008). A narrow control over the E/I balance in neuronal networks is known to be critical for proper brain function and E/I imbalance – often caused by a reduction in GABAergic activity – has been correlated with aging-related deficits and the pathogenesis of several neurodegenerative diseases (Rissman & Mobley, 2011; Legon *et al.*, 2016; Tran *et al.*, 2019; Bruining *et al.*, 2020). The possible effects of aging on the polarity of GABAergic signaling and the corresponding E/I balance in the SCN have not yet been studied.

Here, we investigated the aging effect on the GABAergic E/I balance by measuring intracellular calcium levels. Specifically, we determined the polarity of Ca^{2+} transients in response to GABA stimulation in SCN slices of old mice (20-24 months) and young controls during the day, and tested our hypothesis that aging leads to an increment in excitatory responses to GABA. We confirmed that the E/I balance shifted towards more excitation in SCN slices of old mice compared to young controls. Furthermore, we found an effect of aging on the baseline intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The cells measured from the SCN slices of old mice showed a higher $[Ca^{2+}]_i$ during the day compared to the cells from the young controls.

2. MATERIALS AND METHODS

2.1. Animals and housing

Young (2 – 4 months, N = 11) and old (20 – 24 months, N = 9) male C57BL/6 mice (Janvier Labs, Saint-Berthevin, France) were housed in a climate controlled environment (21 °C, 40-50% humidity) with full-spectrum diffused lighting with an intensity between 50 and 100 lux (Osram truelight TL) and ad libitum access to food and water throughout the experiment. The mice were kept in groups of 2 to 4 mice on an equinoctial photoperiod of 12:12 h light-dark (LD 12:12) cycle. Mice older than 20 months received, in addition to the regular food, hydration and nutritional gels as supportive care. The animals were kept under these conditions for at least 4 weeks prior to the ex vivo experiments. Ex vivo experiments were performed within a 4 h interval centered around the middle of the day. All animal experiments were performed in accordance with the regulations of the Dutch law on animal welfare, and the institutional ethics committee for animal procedures of the Leiden University Medical Center (Leiden, The Netherlands) approved the protocol (AVD 1160020185524; PE. 18.113.07).

2.2. Slice preparation

After decapitation, brains were quickly removed and placed into modified ice-cold artificial cerebrospinal fluid (ACSF), containing (in mM): NaCl 116.4, KCl 5.4, NaH_2PO_4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1, $MgCl_2$ 4, $NaHCO_3$ 23.8, glucose 15.1, and 5 mg/L gentamycine (Sigma Aldrich, Munich, Germany) and saturated with 95% O_2 – 5% CO_2. Coronal hypothalamic slices containing the SCN (250 µm) were cut using a vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany) and sequentially maintained in a holding chamber containing regular, oxygenated ACSF ($CaCl_2$ increased to 2 mM and without $MgCl_2$). The slices were incubated in a water bath (37 °C) for 30 minutes and were then maintained at room temperature until the start of the recordings.

2.3. Ca^{2+} imaging

Neurons in brain slices were bulk-loaded with the ratiometric, membrane permeable Ca^{2+} indicator dye fura-2-acetoxymethyl ester (Fura-2-AM). First, the slices were transferred from the holding chamber to a 35 mm petri dish and reviewed under the microscope. The side of the slide that contained (the larger part of) the SCN was placed up and the ACSF was removed from the petri dish. One drop of highly concentrated Fura-2-AM (998 µM) was placed on the SCN of each slice for 1 minute after which 10 mL of a mix of ACSF containing 7 µM Fura-2-AM was added to the slices. The slices stayed in this mixture for 1 hour on room temperature while maintained saturated with 95% O_2 – 5% CO_2. The slices were then rinsed four times with fresh ACSF before being transferred back into the holding chamber where they stayed another hour on room temperature. After this loading protocol, the slices were moved (one by one) to a recording chamber (RC-26G, Warner Instruments, Hamden, CT, USA) mounted on the fixed stage of an upright fluorescence microscope (Axioskop 2-FS Plus, Carl Zeiss Microimaging, Oberkochen, Germany) and constantly perfused with oxygenated ACSF (2.5 mL/min) at room temperature. The indicator dye was excited alternatively at wavelengths of 340 and 380 nm by means of a monochromator (Polychrome V, TILL Photonics; now FEI Munich GmbH, Munich, Germany). Emitted light (505 nm) was detected by a cooled CCD camera (Sensicam, TILL Photonics; now FEI Munich GmbH, Munich, Germany), and images were acquired at 2 second intervals. The slices settled in the recording chamber for at least 5 minutes before the start of the recordings. After one minute of baseline recording, GABA (200 µM, 15 s) was applied locally using an eight-channel pressurized focal application system (ALA-VM8, ALA scientific instruments, NY, USA), and Ca^{2+} transients were recorded. After two GABA pulses, 1 minute apart, and another minute of recording in which the Ca^{2+} transients returned to baseline, ACSF containing elevated levels of K^+ (20 mM, 15 s) was applied to identify healthy, responding neurons. Cells with at least 10% increase in $[Ca^{2+}]_i$ in response to K^+ were considered to be healthy. The experiments as well as the analysis were accomplished using imaging software (TILLvision, TILL Photonics; now FEI Munich GmbH, Munich, Germany).

2.4. Data analysis and statistics

Single-wavelength images were background subtracted and ratio images (340/380) were generated. Region of interest-defined cells and mean ratio values were determined, from which the intracellular Ca^{2+} concentration was calculated. Neuronal Ca^{2+} responses were further analyzed using IGOR Pro (WaveMetrics, Portland, OR, USA). Cells with an amplitude less than 10% of baseline values in response to elevated levels of K^+, or cells with instable (rising or falling) baselines or baselines > 600 nM were excluded from analyses. The transient responses in Ca^{2+} concentration within the first seconds after the stimulation were evaluated, with responses smaller than ± 10% of baseline values defined as non-responding cells. GABA-evoked responses showing Ca^{2+} transients with a decrease in amplitude of more than 10% from baseline were considered inhibitory and responses with an increase of more than 10% from baseline were defined as excitatory. Cells that showed both excitatory and inhibitory responses after a single GABA stimulation were defined as biphasic. Per animal, one to three SCN slices were analyzed and the Ca^{2+} responses to GABA application were

measured in typically 60 – 160 cells. For each animal, the distribution of the different types of responses and the E/I ratio were determined. To calculate the E/I ratio, the number of cells that responded excitatory was divided by the number of cells that responded inhibitory for per animal (i.e. slices from 1 animal were pooled) and averaged per group. In total we measured 924 cells in 27 slices from 9 old mice and 1204 cells in 26 slices from 11 young mice.

Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). The effect of age on the GABAergic response types was tested per category using the Mann-Whitney U test. The effect on the E/I ratios was tested with two-sided, unpaired t-tests. Lastly, the effect of age on baseline $[Ca^{2+}]_i$ and amplitudes of the GABAergic responses were tested with two-sided, unpaired t-tests with Welch's correction. Differences with $P \leq 0.05$ were considered significant.

3. RESULTS

3.1. GABAergic excitation increased in old SCN

We investigated the effect of aging on the GABAergic activity in SCN neurons by recording GABA-induced single cell Ca^{2+} transients in SCN slices from old (20-24 months) and young (2-4 months) mice (Figure 1A). In each SCN slice, we recorded a combination of transient increases, decreases, or no changes in $[Ca^{2+}]_i$ in response to GABA application. In a another subset of SCN neurons we recorded a biphasic response to GABA. The amplitude of the GABAergic responses did not differ between the old and the young SCN neurons (Figure S1, inhibition; old: -66.53 ± 5.39, n = 433, young: -59.87 ± 1.82, n = 691, *P = 0.242*, excitation; old: 58.90 ± 4.32, n = 313, young: 62.73 ± 5.03, n = 322, *P = 0.869*). The percentages of the different GABAergic response types differed between old and young animals. Old SCN slices exhibited significantly more excitatory responses to GABA as compared to SCN slices of young controls (Figure 1B, old: 32.99 ± 3.17 %, n = 9, young: 23.41 ± 2.29 %, n = 11, *P = 0.038*). This increase in GABAergic excitation leads to an increase in the E/I balance in old SCN slices, as compared to young SCN slices (Figure 1C, old: 0.80 ± 0.12, n = 9, young: 0.48 ± 0.07, n = 11, *P = 0.034*). These results show that aging affects the polarity of responses to GABA.

3.2. Age-related increase in GABAergic excitation mainly in posterior part of the SCN

Several studies have shown that the amount of GABAergic excitation varies between the different SCN areas in rats (Albus *et al.*, 2005; Choi *et al.*, 2008; Irwin & Allen, 2009). Therefore, we tested whether there were regional differences in the distribution of the GABAergic response types along the anteroposterior and the dorsoventral axis. There were no significant differences in GABAergic responses between the dorsal and ventral SCN, both for the old and young mice (Figure S2). GABAergic response type only showed significant spatial differences in old SCN, but not young, along the anteroposterior axes with more excitatory responses in the posterior slices, as compared to the anterior slices (Figure 2A, 2C and 2D, old; excitation; anterior: 19.66 ± 1.83 %, n = 8, posterior: 45.05 ± 6.24 %, n = 9, *P = 0.003*, young; excitation; anterior: 16.37 ± 2.33 %, n = 11, posterior: 19.98 ± 4.14 %, n = 5, *P = 0.473*).

When comparing young and old, the posterior part of the SCN was the only region showing significant differences with more GABAergic excitation and less inhibition (Figure 2C and 2D,

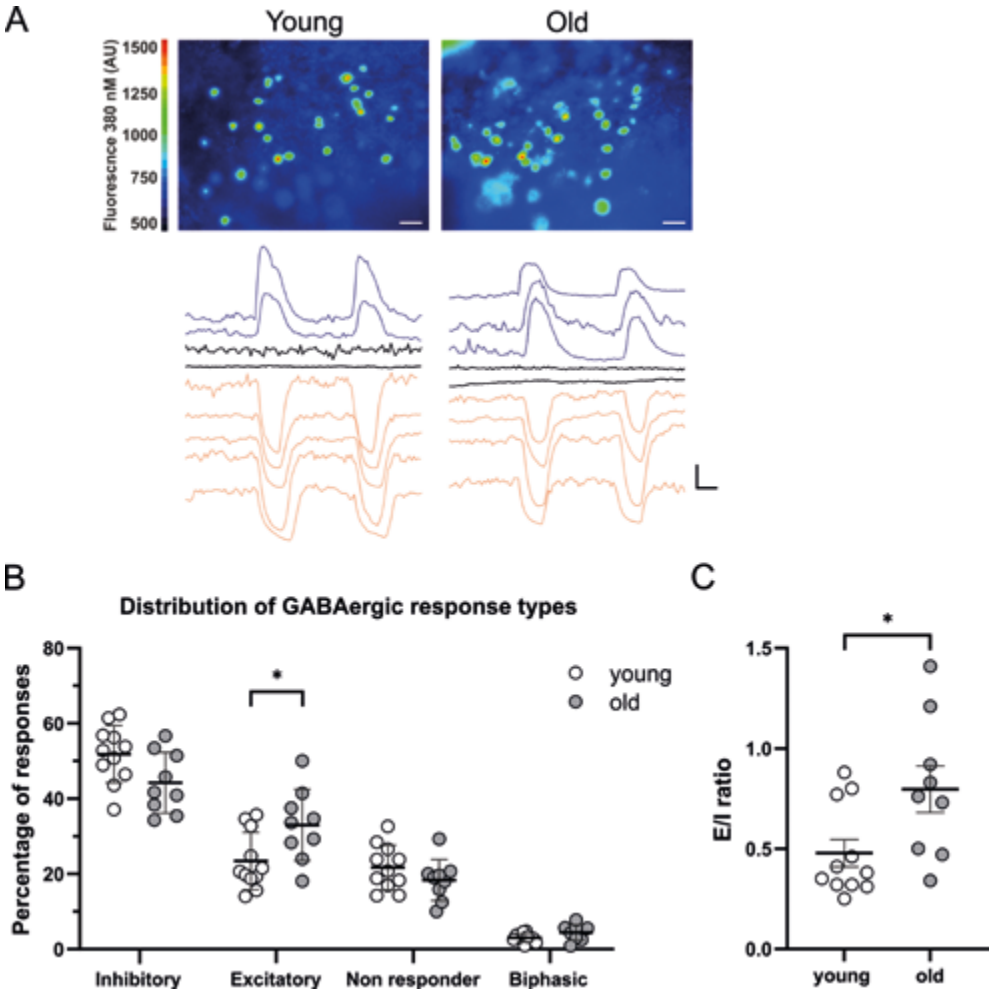

Figure 1. More GABAergic excitation in SCN slices from old mice. A. Upper panels: examples of fura-2-AM loaded SCN neurons in slices from young (left) and old (right) mice. Color scale indicates fluorescence at 380 nm excitation in arbitrary units (Scale bar, 20 µm). Lower panels: example traces of Ca^{2+} transients in response to two GABA administrations recorded from one SCN slice from a young (left) and old (right) mouse. Excitatory responses are shown in blue, inhibitory responses in orange and non-responding cells in black (Scale bars, 50 nM, 20 s). B. The percentages of inhibitory, excitatory, non-responding, and biphasic cells. Each dot represents the mean percentage of responses per response type per SCN. Every single dot in one response type category adds up to 100% together with the corresponding dots in the other categories. C. E/I ratio's in young and old mice, determined by dividing the number of excitatory responses by the number of inhibitory responses measured from each SCN. Filled dots represent values from young mice (n = 11) and open dots represent values from old (n = 9) mice. Bars indicate mean ± SEM. * *P < 0.05*, distribution of GABAergic response types: Mann-Whitney U test for each category, E/I ratio: unpaired t-test with Welch's correction.

excitation; old: 45.05 ± 6.24 %, n = 9, young: 19.98 ± 4.14 %, n = 5, *P = 0.007*, inhibition; old: 36.81 ± 5.53 %, n = 9, young: 60.62 ± 4.89 %, n = 5, *P = 0.007*). In the anterior slices the differences between old and young were smaller, but the calculated E/I balance was significantly different between young

and old in both posterior as well as anterior SCN slices (Figure 2E, anterior; old: 0.52 ± 0.09, n = 8, young: 0.29 ± 0.04, n = 11, *P = 0.041*, posterior; old: 1.08 ± 0.20, n = 8, young: 0.36 ± 0.10, n = 5, *P = 0.010*). No differences in GABAergic responses or E/I balance were observed in the central part of the SCN slices of young and old mice (Figure 2B and 2E, E/I ratio; old: 0.79 ± 0.19, n = 10, young: 0.73 ± 0.12, n = 10, *P = 0.767*).

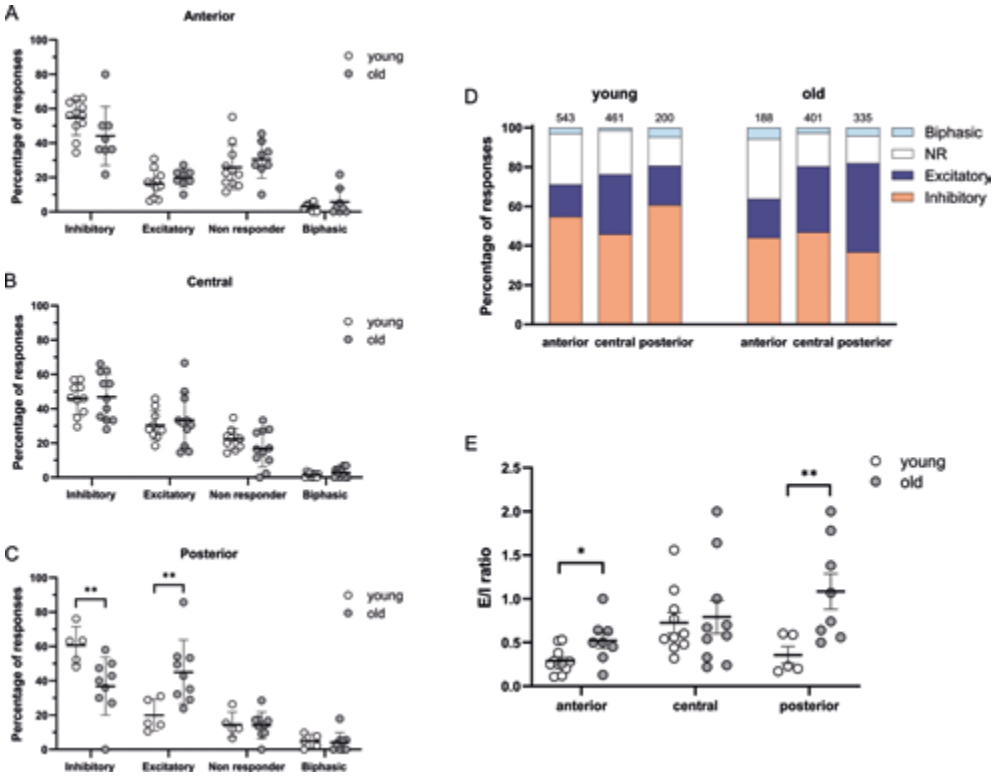

Figure 2. Spatial differences in GABAergic responses along the anteroposterior axis. A, B + C. The percentages of inhibitory, excitatory, non-responding, and biphasic cells in the anterior (A), central (B), and posterior (C) part of the young and old SCN. Each dot represents the mean percentage of responses per response type per SCN. Every single dot in one response type category adds up to 100% together with the corresponding dots in the other categories. D. Distribution of GABAergic response types for the anterior, central, and posterior part of the young (left) and the old (right) SCN. Orange represents the percentage of inhibitory responses, dark blue represents excitatory responses, white represents non-responding cells, and light blue represents biphasic responses. The value on top of the bar shows the total number of cells measured. E. E/I ratios per SCN region in young and old mice, determined by dividing the number of excitatory responses by the number of inhibitory responses measured from different parts of the SCN along the anteroposterior axis. Filled dots represent values from young mice and open dots represent values from old mice. Bars indicate mean ± SEM. * *P < 0.05*, ** *P < 0.01*. distribution of GABAergic response types: Mann-Whitney U test for each category, E/I ratio: unpaired t-test with Welch's correction.

3.3. Intracellular Ca^{2+} concentration is higher in old SCN

To examine whether the baseline $[Ca^{2+}]_i$ changes with aging, we compared $[Ca^{2+}]_i$ (determined by the average $[Ca^{2+}]_i$ in a 20 s interval before GABA application) in SCN neurons from old and young mice during the day. We found higher baseline $[Ca^{2+}]_i$ levels in cells of the old SCN, when compared to the young SCN neurons (Figure 3A, old: 150.17 ± 3.78 nM, n = 924, young: 132.09 ± 2.28 nM, n = 1204, *P < 0.0001*). We also wondered whether this increase was specific for cells with a certain GABAergic response. In comparison with young SCN neurons, baseline $[Ca^{2+}]_i$ was higher in old SCN cells that exhibited GABAergic inhibitory and excitatory responses, but not in the non-responding or the biphasic cells. Baseline $[Ca^{2+}]_i$ was also higher in both old and young SCN neurons that exhibited GABAergic inhibitory responses, when compared to excitatory responses (Figure 3B, inhibition; old: 160.80 ± 6.80 nM, n = 419, young: 134.10 ± 2.78 nM, n = 632, *P = 0.0003*, excitation; old: 135.10 ± 4.75 nM, n = 296, young; 116.10 ± 3.88 nM, n = 270, *P = 0.002*, inh vs exc old: *P = 0.002*, young: *P = 0.0002*). These results suggest that Ca^{2+} homeostasis in the SCN neurons is affected by age and the GABAergic response type is correlated to the baseline $[Ca^{2+}]_i$ in young and old SCN.

4. DISCUSSION

In this study we examined the effect of aging on the GABAergic E/I balance and on intracellular Ca^{2+} levels in the mammalian pacemaker. Our results demonstrate that aging affects the response polarity, and thus the function, of GABA, which is the most abundant neurotransmitter in the SCN.

We measured significantly more GABAergic excitatory responses in SCN slices from old mice when compared to slices from young controls. Particularly, in the posterior part of the old SCN we found significantly more excitation and significantly less inhibition compared to the young SCN. Accordingly, we recorded increased E/I ratios in SCN slices from old mice. We also demonstrate that

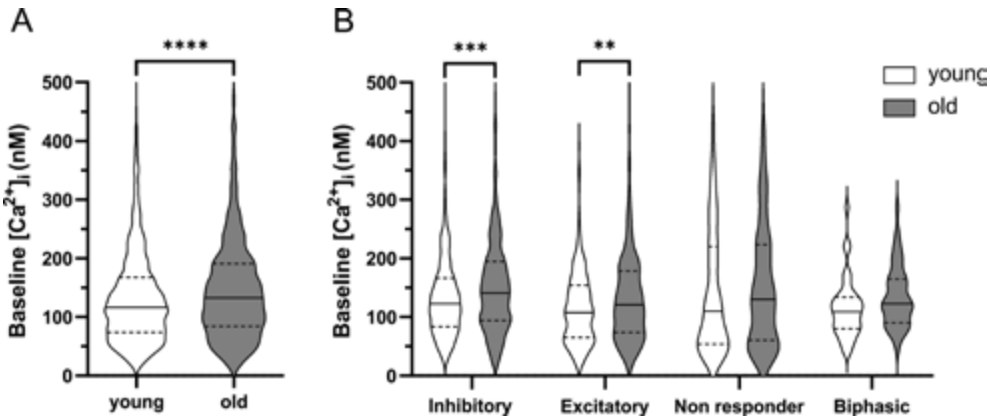

Figure 3. Baseline $[Ca^{2+}]_i$ is higher in old SCN neurons. A. Violin plots show baseline $[Ca^{2+}]_i$ levels (nM) from all SCN neurons measured in slices from young (n = 1204) and old (n = 924) mice. B. Violin plots show baseline $[Ca^{2+}]_i$ levels (nM) from all SCN neurons measured categorized per GABAergic response type. White violins represent data from young mice and grey violins represent data from old mice. Violin plots show median and quartiles, ** *P < 0.01*, *** *P < 0.001*, **** *P < 0.0001*, unpaired t-test with Welch's correction.

the baseline $[Ca^{2+}]_i$ is higher in SCN cells from old mice, compared to young mice. This is interesting considering that Ca^{2+} is an important intracellular signaling molecule and critical for molecular rhythm generation (Lundkvist *et al.*, 2005).

GABA is known to elicit both inhibitory and excitatory responses in the central clock (Choi *et al.*, 2008; Irwin & Allen, 2009; Farajnia *et al.*, 2014b) and the polarity of GABAergic activity can switch depending on the subregion of the SCN (Albus *et al.*, 2005; Choi *et al.*, 2008; Irwin & Allen, 2009; DeWoskin *et al.*, 2015; Myung *et al.*, 2015). We found spatial differences in the distribution of the different GABAergic response types, with a significant increase in excitatory responses along the anteroposterior axis in the old, but not the young SCN (Figure 2A-D). In slices from both young and old mice, we did not find a difference in the polarity of the GABAergic responses between the ventral and dorsal SCN (Figure S2). The lack of regional differences in GABAergic responses between anterior, central, and posterior SCN slices of young controls, or between dorsal and ventral SCN slices is comparable to a previous study examining the effect of photoperiod on the GABAergic responses in the mouse SCN (Farajnia *et al.*, 2014b). The spatial organization that we observed in C57BL/6 mice differed from the spatial pattern previously observed in rat, in which there are regional differences found along the dorsoventral axis (Albus *et al.*, 2005; Choi *et al.*, 2008; Irwin & Allen, 2009). We have no interpretation for this difference, but it adheres to functional and anatomical differences that are also observed between mice and rats (Morin *et al.*, 2006).

The reported increase in GABAergic excitation in the old SCN suggests an increase in $[Cl^-]_i$, since the polarity of the GABAergic signal depends in part on the $[Cl^-]_i$ and the relationship between the chloride equilibrium potential (E_{Cl^-}) and the membrane potential (V_m) (Kaila, 1994; Ben-Ari, 2002). Protein expression of the cation-chloride co-transporter responsible for the influx of chloride, the NKCC1, displays circadian rhythmicity in hamsters under constant conditions and is regulated by environmental lighting conditions, as NKCC1 protein levels in the SCN of hamsters housed in constant light are higher than of hamsters entrained to 14:10 LD cycles or under constant darkness (McNeill *et al.*, 2020). There is no clear consensus on the distribution of co-transporter expression in the SCN. NKCC1 protein expression is shown to be higher in the dorsal part of the rat SCN at night, with no differences in subregion during the day (Choi *et al.*, 2008), however, in hamsters exposed to constant darkness or 14:10 LD cycles, NKCC1 expression is higher in the ventral SCN compared to the dorsal part (McNeill *et al.*, 2020). Another study shows higher $[Cl^-]_i$ during the day than during the night in both the ventral and dorsal mouse SCN neurons. Additionally, the KCCs – the extruders of chloride – plays a major role in $[Cl^-]_i$ regulation, while NKCC1 has a relatively minor role (Klett & Allen, 2017). Our results suggest that the expression of NKCC1 and/or KCC2 is also affected by aging, since we demonstrated an alteration in the polarity of the GABAergic response in the old SCN.

4.1. E/I balance and synchronization within the SCN network

The increase in excitatory responses in the old SCN may be part of mechanisms that contribute to the degree of synchronization within the SCN network. Both in vivo and ex vivo studies showed a significant reduction in the amplitude of the ensemble electrical activity rhythms of the aged SCN that is likely the consequence of decreased synchronization within the SCN network (Satinoff *et al.*,

1993; Watanabe *et al.*, 1995; Nakamura *et al.*, 2011; Farajnia *et al.*, 2012). Ex vivo electrophysiological recordings showed changes in neuronal phase distribution in SCN slices of aged mice (Farajnia *et al.*, 2012) and these alterations at the network level underlie the diminished SCN output signal. Although the mechanisms that regulate neuronal phase distribution in the SCN are still unknown, there are studies suggesting that an increase in E/I balance might be responsible for modulating the phase distribution (Farajnia *et al.*, 2014b; Rohr *et al.*, 2019). GABA is expressed in almost all SCN neurons and is, because of its dual action as inhibitor and activator, an important contributor to the E/I balance in the SCN (Albus *et al.*, 2005; Choi *et al.*, 2008). Even though evidence suggest that GABA is involved in phase adjustment and synchronization of the SCN network, no consensus exists on the precise role of GABA in network synchronization (Ono *et al.*, 2020). Our results contribute to this debate by showing increased levels of GABAergic excitation in an SCN network that is in a more desynchronized state (Farajnia *et al.*, 2012), suggesting GABA regulation is involved in phase distribution. Other studies also suggest that GABA does not promote synchrony, or works as a destabilizer or phase desynchronizer within the SCN, but these studies do not distinguish between GABAergic inhibition or excitation (Aton *et al.*, 2006; Freeman *et al.*, 2013). Whether there is an actual causal link between either the E/I balance and synchronization, or whether this reflects aging-related compensatory mechanisms for the alterations at the network level, still needs further investigation.

Alterations in the SCN network are involved in aging, as well as in the adaptation to photoperiods. As with aging, exposure to a long day photoperiod causes more phase dispersal in the SCN (VanderLeest *et al.*, 2007; Brown & Piggins, 2009; Buijink *et al.*, 2016) and a switch in the polarity of GABAergic activity from inhibition to excitation in many SCN neurons (Farajnia *et al.*, 2014b). Farajnia et al. proposes that the relation between GABAergic inhibition and excitation may contribute to the photoperiod-induced phase adjustments within the network. Our results show an increase in the number of excitatory GABAergic responses and an increase in the E/I balance in the old SCN (Figure 1), similar to the changes in the SCN of mice entrained to a long photoperiod, and thus our data support this hypothesis.

4.2. Plasticity in E/I balance in the SCN

The polarity of the GABAergic response in the SCN, and thus the E/I balance, can vary depending on time of day or the photoperiod to which the animals are exposed (Albus *et al.*, 2005; Choi *et al.*, 2008; Farajnia *et al.*, 2014b). In addition, a recent study showed that lighting conditions affect the circadian regulation and levels of NKCC1 protein expression, and thus the action of GABA (McNeill *et al.*, 2020). The plasticity in GABAergic function is thought to support adaptation to environmental conditions. It is not clear whether the excitatory action of GABA in aging is also functional by contributing to a compensatory mechanism to reorganize the neuronal network of the SCN, or else, is a consequence of a loss of function in the aging SCN. Moreover, it remains to be investigated whether there is still plasticity in the E/I balance in the SCN of old mice, or if the increase that we show here is static and irreversibly changed in the SCN network. In an recent study we were able to show that the aging SCN is still able to adapt its molecular clock to different

photoperiods as well as the young, suggesting that the SCN network is still flexible in aging (Buijink *et al.*, 2020).

4.3. Aging and intracellular Ca^{2+} levels in the SCN

One important intracellular component involved in phase adjustment is Ca^{2+}, which we determined in our baseline measurements before GABA application. Therefore, we were able to compare baseline $[Ca^{2+}]_i$ of SCN neurons from slices of old and young mice. Our results show that the baseline $[Ca^{2+}]_i$ is higher in SCN cells from old mice, compared to young controls (Figure 3) which is in accordance with previous studies in other brain areas (Galla *et al.*, 2020; Uryash *et al.*, 2020; Mozolewski *et al.*, 2021). In both young and old SCN slices, the neurons that showed GABAergic inhibition exhibited the highest baseline calcium levels. GABA induced Ca^{2+} transients can depend on baseline $[Ca^{2+}]_i$ (Irwin & Allen, 2009). A possible cause for the relationship between baseline $[Ca^{2+}]_i$ and GABAergic response type could be the different levels of electrical activity of the neuron. At a higher firing rate, the $[Ca^{2+}]_i$ baseline would be increased due to influx of Ca^{2+} through voltage-activated Ca^{2+} channels and an inhibitory input would have a larger effect compared to a silent neuron. Also, it is plausible that a neuron with a low or high $[Ca^{2+}]_i$ may not be able to further lower or raise $[Ca^{2+}]_i$ after a GABAergic stimulus, respectively. Our evidence implies that both baseline calcium levels and calcium homeostasis are altered in the old SCN which could further impair cellular phase adjustments. Even though Ca^{2+} is one of the most essential and well-studied signaling molecules, surprisingly little is known about the influence of aging on the Ca^{2+} signaling or Ca^{2+} homeostasis in the SCN. Studies in other brain areas have focused on the contribution of plasma membrane Ca^{2+} pumps, intracellular stores like the endoplasmic reticulum, and the mitochondria in aged neurons or neurodegenerative disorders (Supnet & Bezprozvanny, 2010; Zaidi *et al.*, 2018; Calvo-Rodriguez *et al.*, 2020; Trombetta-Lima *et al.*, 2021). It should be noted though, that the SCN neurons can already tolerate higher levels of $[Ca^{2+}]_i$ when compared to other brain areas (Diekman *et al.*, 2013). Given the essential role of calcium in both intracellular signaling pathways and rhythm generation and its association with multiple neurodegenerative disorders (Foster, 2007; Berridge, 2013), restoring calcium signaling in old SCN neurons could be an interesting target for therapy.

Additionally, maintenance of an adequate balance of excitation and inhibition could benefit healthy aging. Several studies have shown a shift in E/I balance, with heightened neuronal activity in the hippocampus or prefrontal cortex due to decreased inhibitory networks (Legon *et al.*, 2016; Tran *et al.*, 2019). This loss of inhibition, and thus an increased E/I ratio, was correlated to aging and the pathogenesis of neurodegenerative disorders (Rissman & Mobley, 2011; Bruining *et al.*, 2020). Moreover, the E/I ratio increased in aged rats with impaired memory function, however aged rats with unimpaired memory function had similar hippocampal E/I ratios as young controls, showing that a proper balance between inhibition and excitation is crucial for maintaining memory performance during aging (Tran *et al.*, 2019) and stresses the importance of an adequate balance in the aged brain. Our study demonstrates that the E/I balance of the neuronal network of the central clock is also challenged by aging with potential consequences for clock function. The stabilization of SCN E/I ratio to a healthy range in aging will not only benefit SCN network properties, but may

also counteract the detrimental effects of the clock on neurodegenerative diseases (Leng *et al.*, 2019; Fifel & De Boer, 2021).

REFERENCES

1. Abrahamson, E.E. & Moore, R.Y. (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Res.*, 916, 172-191.
2. Albus, H., Vansteensel, M.J., Michel, S., Block, G.D. & Meijer, J.H. (2005) A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr. Biol.*, 15, 886-893.
3. Aton, S.J., Huettner, J.E., Straume, M. & Herzog, E.D. (2006) GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons. *Proc. Natl. Acad. Sci. U. S. A.*, 103, 19188-19193.
4. Ben-Ari, Y. (2002) Excitatory actions of gaba during development: the nature of the nurture. *Nature reviews. Neuroscience*, 3, 728-739.
5. Berridge, M.J. (2013) Dysregulation of neural calcium signaling in Alzheimer disease, bipolar disorder and schizophrenia. *Prion*, 7, 2-13.
6. Biello, S.M. (2009) Circadian clock resetting in the mouse changes with age. *Age (Dordrecht, Netherlands)*, 31, 293-303.
7. Brown, T.M. & Piggins, H.D. (2009) Spatiotemporal heterogeneity in the electrical activity of suprachiasmatic nuclei neurons and their response to photoperiod. *J. Biol. Rhythms*, 24, 44-54.
8. Bruining, H., Hardstone, R., Juarez-Martinez, E.L., Sprengers, J., Avramiea, A.E., Simpraga, S., Houtman, S.J., Poil, S.S., Dallares, E., Palva, S., Oranje, B., Matias Palva, J., Mansvelder, H.D. & Linkenkaer-Hansen, K. (2020) Measurement of excitation-inhibition ratio in autism spectrum disorder using critical brain dynamics. *Sci. Rep.*, 10, 9195.
9. Buhr, E.D. & Takahashi, J.S. (2013) Molecular Components of the Mammalian Circadian Clock. In Kramer, A., Merrow, M. (eds) *Circadian Clocks*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 3-27.
10. Buijink, M.R., Almog, A., Wit, C.B., Roethler, O., Olde Engberink, A.H., Meijer, J.H., Garlaschelli, D., Rohling, J.H. & Michel, S. (2016) Evidence for Weakened Intercellular Coupling in the Mammalian Circadian Clock under Long Photoperiod. *PLoS One*, 11, e0168954.
11. Buijink, M.R. & Michel, S. (2020) A multi-level assessment of the bidirectional relationship between aging and the circadian clock. *J. Neurochem.*
12. Buijink, M.R., Olde Engberink, A.H.O., Wit, C.B., Almog, A., Meijer, J.H., Rohling, J.H.T. & Michel, S. (2020) Aging Affects the Capacity of Photoperiodic Adaptation Downstream from the Central Molecular Clock. *J. Biol. Rhythms*, 35, 167-179.
13. Calvo-Rodriguez, M., Hernando-Pérez, E., López-Vázquez, S., Núñez, J., Villalobos, C. & Núñez, L. (2020) Remodeling of Intracellular Ca(2+) Homeostasis in Rat Hippocampal Neurons Aged In Vitro. *Int. J. Mol. Sci.*, 21.
14. Choi, H.J., Lee, C.J., Schroeder, A., Kim, Y.S., Jung, S.H., Kim, J.S., Kim, D.Y., Son, E.J., Han, H.C., Hong, S.K., Colwell, C.S. & Kim, Y.I. (2008) Excitatory actions of GABA in the suprachiasmatic nucleus. *J. Neurosci.*, 28, 5450-5459.
15. DeWoskin, D., Myung, J., Belle, M.D., Piggins, H.D., Takumi, T. & Forger, D.B. (2015) Distinct roles for GABA across multiple timescales in mammalian circadian timekeeping. *Proc. Natl. Acad. Sci. USA*, 112, E3911-3919.
16. Diekman, C.O., Belle, M.D., Irwin, R.P., Allen, C.N., Piggins, H.D. & Forger, D.B. (2013) Causes and consequences of hyperexcitation in central clock neurons. *PLoS Comput. Biol.*, 9, e1003196.
17. Dijk, D.J. & Duffy, J.F. (1999) Circadian regulation of human sleep and age-related changes in its timing, consolidation and EEG characteristics. *Ann. Med.*, 31, 130-140.
18. Farajnia, S., Deboer, T., Rohling, J.H., Meijer, J.H. & Michel, S. (2014a) Aging of the suprachiasmatic clock. *Neuroscientist*, 20, 44-55.
19. Farajnia, S., Michel, S., Deboer, T., vanderLeest, H.T., Houben, T., Rohling, J.H., Ramkisoensing, A., Yasenkov, R. & Meijer, J.H. (2012) Evidence for neuronal desynchrony in the aged suprachiasmatic nucleus clock. *J. Neurosci.*, 32, 5891-5899.
20. Farajnia, S., van Westering, T.L.E., Meijer, J.H. & Michel, S. (2014b) Seasonal induction of GABAergic excitation in the central mammalian clock. *Proc. Natl. Acad. Sci. USA*, 111, 9627-9632.
21. Fifel, K. & De Boer, T. (2021) The circadian system in Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy. *Handb. Clin. Neurol.*, 179, 301-313.

22. Fonseca Costa, S.S. & Ripperger, J.A. (2015) Impact of the circadian clock on the aging process. *Front. Neurol.*, 6, 43.
23. Foster, T.C. (2007) Calcium homeostasis and modulation of synaptic plasticity in the aged brain. *Aging cell*, 6, 319-325.
24. Freeman, G.M., Krock, R.M., Aton, S.J., Thaben, P. & Herzog, E.D. (2013) GABA networks destabilize genetic oscillations in the circadian pacemaker. *Neuron*, 78, 799-806.
25. Froy, O. (2011) Circadian rhythms, aging, and life span in mammals. *Physiology (Bethesda)*, 26, 225-235.
26. Galla, L., Redolfi, N., Pozzan, T., Pizzo, P. & Greotti, E. (2020) Intracellular Calcium Dysregulation by the Alzheimer's Disease-Linked Protein Presenilin 2. *Int. J. Mol. Sci.*, 21.
27. Hastings, M.H., Maywood, E.S. & Brancaccio, M. (2018) Generation of circadian rhythms in the suprachiasmatic nucleus. *Nat. Rev. Neurosci.*, 19, 453-469.
28. Hofman, M.A. & Swaab, D.F. (2006) Living by the clock: The circadian pacemaker in older people. *Ageing Research Reviews*, 5, 33-51.
29. Irwin, R.P. & Allen, C.N. (2009) GABAergic signaling induces divergent neuronal Ca2+ responses in the suprachiasmatic nucleus network. *Eur. J. Neurosci.*, 30, 1462-1475.
30. Kaila, K. (1994) Ionic basis of GABAA receptor channel function in the nervous system. *Prog. Neurobiol.*, 42, 489-537.
31. Klett, N.J. & Allen, C.N. (2017) Intracellular Chloride Regulation in AVP+ and VIP+ Neurons of the Suprachiasmatic Nucleus. *Sci. Rep.*, 7, 10226.
32. Kondratova, A.A. & Kondratov, R.V. (2012) The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.*, 13, 325-335.
33. Legon, W., Punzell, S., Dowlati, E., Adams, S.E., Stiles, A.B. & Moran, R.J. (2016) Altered Prefrontal Excitation/Inhibition Balance and Prefrontal Output: Markers of Aging in Human Memory Networks. *Cereb. Cortex*, 26, 4315-4326.
34. Leng, Y., Musiek, E.S., Hu, K., Cappuccio, F.P. & Yaffe, K. (2019) Association between circadian rhythms and neurodegenerative diseases. *Lancet Neurol.*, 18, 307-318.
35. Lundkvist, G.B., Kwak, Y., Davis, E.K., Tei, H. & Block, G.D. (2005) A calcium flux is required for circadian rhythm generation in mammalian pacemaker neurons. *J. Neurosci.*, 25, 7682-7686.
36. McNeill, J.K., Walton, J.C., Ryu, V. & Albers, H.E. (2020) The Excitatory Effects of GABA within the Suprachiasmatic Nucleus: Regulation of Na-K-2Cl Cotransporters (NKCCs) by Environmental Lighting Conditions. *J. Biol. Rhythms*, 35, 275-286.
37. Moore, R.Y. & Speh, J.C. (1993) GABA is the principal neurotransmitter of the circadian system. *Neurosci. Lett.*, 150, 112-116.
38. Morin, L.P., Shivers, K.Y., Blanchard, J.H. & Muscat, L. (2006) Complex organization of mouse and rat suprachiasmatic nucleus. *Neuroscience*, 137, 1285-1297.
39. Mozolewski, P., Jeziorek, M., Schuster, C.M., Bading, H., Frost, B. & Dobrowolski, R. (2021) The role of nuclear Ca2+ in maintaining neuronal homeostasis and brain health. *J. Cell Sci.*, 134.
40. Myung, J., Hong, S., DeWoskin, D., De Schutter, E., Forger, D.B. & Takumi, T. (2015) GABA-mediated repulsive coupling between circadian clock neurons in the SCN encodes seasonal time. *Proc. Natl. Acad. Sci. USA*, 112, E3920-3929.
41. Nakamura, T.J., Nakamura, W., Yamazaki, S., Kudo, T., Cutler, T., Colwell, C.S. & Block, G.D. (2011) Age-related decline in circadian output. *J. Neurosci.*, 31, 10201-10205.
42. Nygard, M., Hill, R.H., Wikstrom, M.A. & Kristensson, K. (2005) Age-related changes in electrophysiological properties of the mouse suprachiasmatic nucleus in vitro. *Brain Res. Bull.*, 65, 149-154.
43. Ono, D., Honma, K.I. & Honma, S. (2020) GABAergic mechanisms in the suprachiasmatic nucleus that influence circadian rhythm. *J. Neurochem.*
44. Oster, H., Baeriswyl, S., Van Der Horst, G.T. & Albrecht, U. (2003) Loss of circadian rhythmicity in aging mPer1-/-mCry2-/- mutant mice. *Genes Dev.*, 17, 1366-1379.
45. Palomba, M., Nygård, M., Florenzano, F., Bertini, G., Kristensson, K. & Bentivoglio, M. (2008) Decline of the Presynaptic Network, Including GABAergic Terminals, in the Aging Suprachiasmatic Nucleus of the Mouse. *J. Biol. Rhythms*, 23, 220-231.
46. Patke, A., Young, M.W. & Axelrod, S. (2019) Molecular mechanisms and physiological importance of circadian rhythms. *Nature Reviews Molecular Cell Biology*.

47. Ramkisoensing, A. & Meijer, J.H. (2015) Synchronization of Biological Clock Neurons by Light and Peripheral Feedback Systems Promotes Circadian Rhythms and Health. *Front. Neurol.*, 6, 128.
48. Reppert, S.M. & Weaver, D.R. (2002) Coordination of circadian timing in mammals. *Nature*, 418, 935-941.
49. Rissman, R.A. & Mobley, W.C. (2011) Implications for treatment: GABAA receptors in aging, Down syndrome and Alzheimer's disease. *J. Neurochem.*, 117, 613-622.
50. Roenneberg, T. & Merrow, M. (2016) The Circadian Clock and Human Health. *Curr. Biol.*, 26, R432-443.
51. Rohr, K.E., Pancholi, H., Haider, S., Karow, C., Modert, D., Raddatz, N.J. & Evans, J. (2019) Seasonal plasticity in GABAA signaling is necessary for restoring phase synchrony in the master circadian clock network. *eLife*, 8.
52. Satinoff, E., Li, H., Tcheng, T.K., Liu, C., McArthur, A.J., Medanic, M. & Gillette, M.U. (1993) Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? *Am. J. Physiol.*, 265, R1216-1222.
53. Supnet, C. & Bezprozvanny, I. (2010) Neuronal calcium signaling, mitochondrial dysfunction, and Alzheimer's disease. *J. Alzheimers Dis.*, 20 Suppl 2, S487-498.
54. Tran, T., Bridi, M., Koh, M.T., Gallagher, M. & Kirkwood, A. (2019) Reduced cognitive performance in aged rats correlates with increased excitation/inhibition ratio in the dentate gyrus in response to lateral entorhinal input. *Neurobiol. Aging*, 82, 120-127.
55. Trombetta-Lima, M., Sabogal-Guáqueta, A.M. & Dolga, A.M. (2021) Mitochondrial dysfunction in neurodegenerative diseases: A focus on iPSC-derived neuronal models. *Cell Calcium*, 94, 102362.
56. Uryash, A., Flores, V., Adams, J.A., Allen, P.D. & Lopez, J.R. (2020) Memory and Learning Deficits Are Associated With Ca(2+) Dyshomeostasis in Normal Aging. *Front. Aging Neurosci.*, 12, 224.
57. VanderLeest, H.T., Houben, T., Michel, S., Deboer, T., Albus, H., Vansteensel, M.J., Block, G.D. & Meijer, J.H. (2007) Seasonal encoding by the circadian pacemaker of the SCN. *Curr. Biol.*, 17, 468-473.
58. Watanabe, A., Shibata, S. & Watanabe, S. (1995) Circadian rhythm of spontaneous neuronal activity in the suprachiasmatic nucleus of old hamster in vitro. *Brain Res.*, 695, 237-239.
59. Welsh, D.K., Logothetis, D.E., Meister, M. & Reppert, S.M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron*, 14, 697-706.
60. Welsh, D.K., Takahashi, J.S. & Kay, S.A. (2010) Suprachiasmatic nucleus: cell autonomy and network properties. *Annu. Rev. Physiol.*, 72, 551-577.
61. Zaidi, A., Adewale, M., McLean, L. & Ramlow, P. (2018) The plasma membrane calcium pumps-The old and the new. *Neurosci. Lett.*, 663, 12-17.

SUPPLEMENTARY MATERIALS

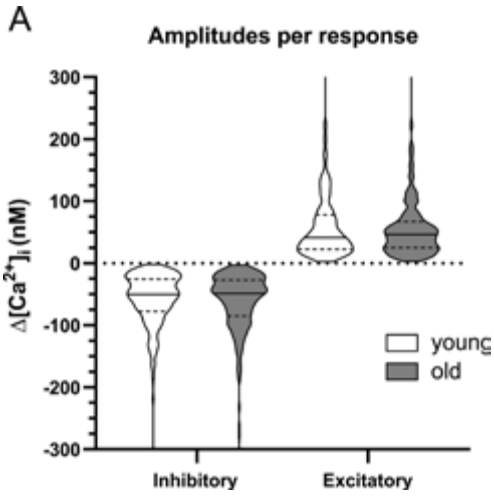

Figure S1. Amplitudes of GABAergic responses. A. Violin plots show amplitudes (delta $[Ca^{2+}]_i$) of all SCN neurons that responded in an inhibitory and excitatory manner. White violins represent data from SCN slices from young mice and grey violins represent data from SCN slices from old mice. Unpaired t-test with Welch's correction, n.s.

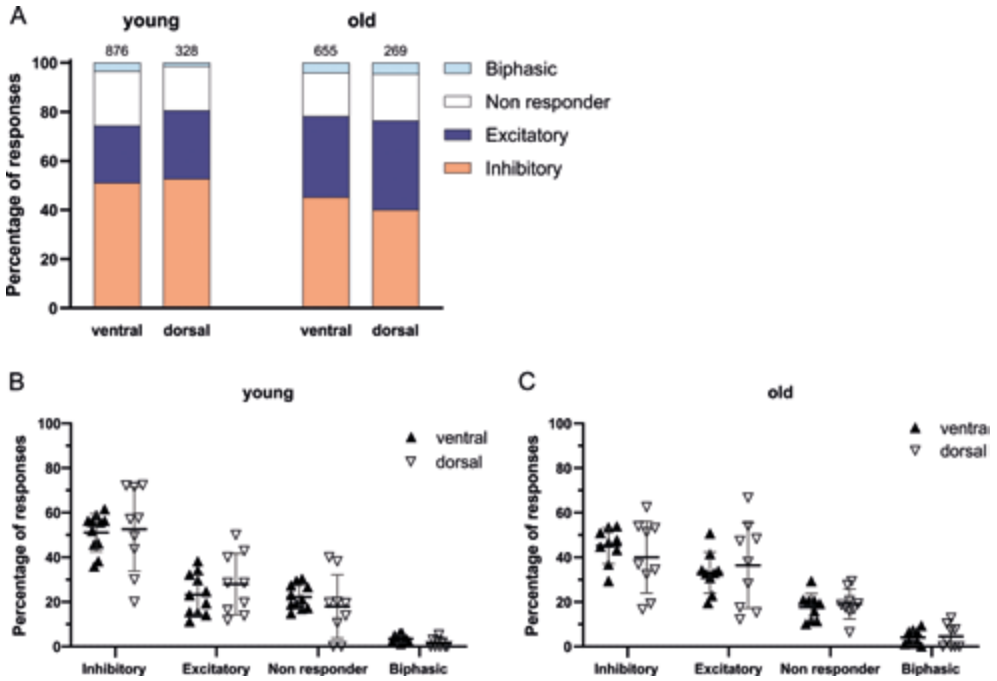

Figure S2. GABAergic responses along the dorsoventral axis. A. Distribution of GABAergic response types for the ventral and dorsal part of the young (left) and the old (right) SCN. Orange represents the percentage of inhibitory responses, dark blue represents excitatory responses, white represents non-responding cells, and light blue represents biphasic responses. The value on top of the bar represents the total number of cells measured. B + C. The percentages of inhibitory, excitatory, non-responding, and biphasic cells in the dorsal and ventral part of the young (B) and old (C) SCN. Each dot represents the mean percentage of responses per response type per SCN position. Every single dot in one response type category adds up to 100% together with the corresponding dots in the other categories. Filled triangles represent values from the ventral part and open triangles represent values from the dorsal part of the SCN. Bars indicate mean ± SEM. Mann-Whitney U test, n.s.