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1 **Bi-directional communication between pontine nucleus incertus and the medial septum is carried**
 2 **out by electrophysiologically distinct neuronal populations**

3 Abbreviated title:

4 **Electrophysiological heterogeneity of NI neurons**

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26

27 **Abstract**

28 Theta oscillations are one of the brain rhythms involved in memory formation, sensorimotor
29 integration and control of locomotion and behavioural states. Generation and spatiotemporal
30 synchronisation of theta oscillations rely on the interactions between nuclei forming a large neural
31 network, part of which is pontine nucleus incertus (NI).

32 Here we identified distinct populations of NI neurons, based on the relationship of their firing
33 to the hippocampal waves, with the special focus on theta oscillations, and the direction and type of
34 interaction with the medial septum (MS) in male, urethane anaesthetised rats. By recording NI
35 neuronal firing and hippocampal LFP we described NI neurons that fire action potentials in a theta
36 phase-independent or theta phase-locked and delta wave-independent or delta wave-locked
37 manner. Among hippocampal activity independent NI neurons, irregular, slow-firing and regular, fast-
38 firing cells were observed, while hippocampal oscillations/waves locked NI neurons could be of
39 bursting or non-bursting type. By projection specific opto-tagging, we revealed that only fast-firing
40 theta phase-independent NI neurons innervate the MS, rarely receiving feedback information. In
41 contrast, majority of theta bursting NI neurons were inhibited by MS stimulation and this effect was
42 mediated by direct GABAergic input.

43 Described NI neuronal populations differ in reciprocal connections with the sept
44 hippocampal system, plausibly forming separate neuronal loops. Our results suggest that theta
45 phase-independent NI neurons participate in theta rhythm generation through direct innervation of
46 the MS, while theta-bursting NI neurons transmit further the rhythmic signal received from the MS to
47 stabilise and/or strengthen rhythmic activity in other structures.

48

49 **Significance Statement**

50 The generation and spatiotemporal synchronisation of theta oscillations rely on the
51 interactions between nuclei forming a large neural network, part of which is the pontine nucleus
52 incertus (NI). Here we describe that within NI there are populations of neurons that can be
53 distinguished based on the relationship of their firing to the hippocampal theta oscillations and delta
54 waves. We show that these neuronal populations largely do not have reciprocal connections with the
55 septohippocampal system, but form separate neuronal loops. Our results suggest that MS projecting,
56 fast-firing theta phase-independent NI neurons may participate in theta rhythm generation through
57 direct innervation of the MS, while theta-bursting NI neurons may transmit further the rhythmic
58 signal received from the MS to other structures.

59

60 **1. Introduction**

61 Theta rhythm is one of the most abundant brain oscillatory phenomena, characterised by
 62 frequency in the 3-12Hz band. This rhythm can be observed at the level of the electrical activity of
 63 neuronal populations (e.g. local field potential; Bland, 1986), in the rhythmic pattern of action
 64 potential firing of multiple or single neurons (e.g. rhythmic theta bursts; Bland et al., 2002; Di Prisco
 65 et al., 2002), and in the oscillations of neuronal membrane potential (Bland et al., 2002; Nunez et al.,
 66 1987; Strata, 1998). Theta rhythms present at various levels of the brain's organisation may interfere
 67 with each other and synchronise themselves in a large number of interconnected (directly and/or
 68 indirectly) structures (Buzsaki and Moser, 2013). Theta oscillations, spatio-temporally synchronised in
 69 nuclei forming functional networks, are a source of timing information used for memory coding and
 70 retrieval, sensorimotor integration, and processing of spatial information (Bland, 1986; Buzsaki,
 71 1989; Buzsaki, 2002; Huerta and Lisman, 1993; Oddie and Bland, 1998; O'Keefe and Recce, 1993;
 72 Pavlides, et al. 1988; Vertes, 2005).

73 Several lines of evidence have shown that frequency and amplitude of hippocampal theta
 74 oscillations depend on the synergistic action of the bursting and tonic firing of theta-ON neurons
 75 located in the nuclei of the ascending brainstem hippocampal synchronising pathway. Tonically firing
 76 theta-ON neurons predominate at the brainstem levels of this system, while bursting theta-ON
 77 neurons are more likely to be found at the higher brain levels (Vertes et al., 2004). However, it has
 78 been shown that already at the level of brainstem and midbrain nuclei, bursting theta-ON neurons
 79 can be found, plausibly participating in the synchronisation and functional coupling of widely
 80 distributed structures responsible for different mnemonic functions (Di Prisco et al., 2002; Kocsis et
 81 al., 2001; Ma et al., 2013).

82 An important element of the ascending brainstem hippocampal synchronising pathway is
 83 nucleus incertus (NI; Brown and McKenna, 2015; Korotkova et al., 2018; Ma et al., 2018; Ryan et al.,
 84 2011). NI is a group of large, mostly GABAergic neurons located below the IV ventricle, expressing
 85 several markers, including relaxin-3 (RLN3), neuromedin B (NMB) and type 1 corticotropin-releasing

factor receptor (Banerjee et al., 2010; Lu et al., 2020; Ma et al., 2013). NI neurons strongly innervate elements of the septohippocampal system of the mammalian brain (Goto et al., 2001; Olucha Bordonau et al., 2003; Teruel-Marti et al., 2008). In the rat medial septum, afferents from NI form dense plexus of symmetric (putatively inhibitory) terminals that synapse on the hippocampus-projecting neurons (Olucha Bordonau et al., 2012). Activation or inactivation of NI has been shown to induce or attenuate hippocampal theta rhythm, respectively (Nunez et al., 2006). Strong coherence between the NI local field potential and hippocampal theta has been described, as well as theta phase-locked firing of some NI neurons (Cervera Ferri et al., 2011; Ma et al., 2013). Interestingly, Martinez-Bellver et al. (2017) showed that there is a causal interaction between firing of NI neurons and the septohippocampal system. Recent studies have shown that there is great heterogeneity within the NI, both at the functional, neurochemical and connectivity level. It was shown that the optogenetic activation of a subpopulation of NMB expressing NI neurons increases, whereas optogenetic inhibition of these cells decreases the power of hippocampal theta rhythm (Lu et al., 2020). On the other hand – as shown by another group – optogenetic activation of the GABAergic NI neurons, which also includes NMB-expressing cells decreased hippocampal theta power (Nasirova et al., 2020; Szonyi et al., 2019). These data show that the manipulation of different populations of NI neurons has different effects, both at the hippocampal theta and the behavioural levels. However, the electrophysiological identity of the NI neuronal subpopulations, their connections with the septohippocampal, and their relationship with hippocampal theta rhythm have not been well defined to date.

Therefore, the aim of our study was to identify NI neuronal populations that innervate and/or receive innervation from the medial septum and to determine the temporal relationship of their firing with the hippocampal theta oscillations and delta waves, which will better define neuronal circuits involved in hippocampal synchronisation.

111 **2. Materials and methods**

112 *2.1. Experimental Design*

113 **Animals and ethical approval.** The experiments were performed on male Sprague-Dawley rats
 114 at the age of 8 – 12 weeks and weight of 300 – 400 g. The animals were bred at the Institute
 115 of Zoology and Biomedical Research Animal Facility at the Jagiellonian University in Krakow under
 116 standard conditions (12:12 light/dark cycle, light on at 08:00 h; temp. $20 \pm 2^{\circ}\text{C}$; humidity $50 \pm 10\%$)
 117 with food and water *ad libitum*. The experiments were approved by the 2nd Local Institutional Animal
 118 Care and Use Committee (Krakow, Poland) and carried out in accordance with the EU Directive
 119 2010/63/EU on the protection of animals used for scientific purposes. All efforts were made to
 120 minimise pain and discomfort, as well as the number of animals used in the procedures.

121 **Viral vectors injections.** The anaesthesia was induced by intraperitoneal (i.p.) injection of ketamine
 122 (100 mg/kg; Ketamina; Biowet, Poland) and xylazine (10 mg/kg; Sedazin; Biowet, Poland) mixture.
 123 To maintain anaesthesia, an additional 30% of the original dose of ketamine was injected during
 124 the procedure if needed. An anti-inflammatory drug was injected subcutaneously at the beginning
 125 of the surgery (0.4% in saline; 0.1 ml/100 g animal body weight; Tolfedine; Vétoquinol; Biowet,
 126 Poland). After the disappearance of the corneal reflex and withdrawal response to paw pinch,
 127 the animal was mounted on the stereotaxic frame (SF-1450AP; ASI Instruments Inc., MI, USA)
 128 on the standard ear and incisor bars. During the whole procedure, the animal's body temperature
 129 was maintained at $37 \pm 0.5^{\circ}\text{C}$ by an automatic heating pad (TCP-02; WMT, Poland). The eyes were
 130 protected from drying by the application of eye drops (Starazolin HydroBalance; Polpharma, Poland).
 131 After cutting the skin and exposing the bones of the skull vault, a trepanation hole was drilled to
 132 allow stereotactic access to the medial septum. A glass microcapillary tube (VITREX, Denmark) was
 133 processed on a vertical puller (PE-21; Narishige Scientific Instrument Lab., Japan) to obtain an ultra-
 134 thin tip that was then broken to the final diameter of 40 – 50 μm . The injection needle prepared in
 135 this way was tightly connected to a Hamilton's syringe (1 μl ; Hamilton; Bonaduz, Switzerland) and the
 136 whole system was filled with paraffin oil (Sigma-Aldrich, Poland). The tip of the microinjection

137 capillary was backfilled with the viral vector just before it was lowered into the target structure. The
 138 medial septum of seventeen rats (8 for *in vivo* and 9 for *ex vivo* recordings; see below) was injected
 139 with an adeno-associated viral vector carrying genes for channelrhodopsin-2 (ChR2) and enhanced
 140 yellow fluorescent protein (EYFP), both expressed under the control of human synapsin (hSyn)
 141 promoter (AAV2-hSyn-hChR2(H134R)-EYFP; donated by Karl Deisseroth; UNC Vector Core, NC, USA;
 142 Zhang et al., 2006). The medial septum of fifteen rats was injected with a retrograde adeno-
 143 associated viral vector carrying genes for Chronos and green fluorescent protein (GFP), both
 144 expressed under the control of synapsin promoter (pAAV-Syn-Chronos-GFP; donated by Edward
 145 Boyden; Addgene viral prep #59170-AAVrg; Addgene, MA, USA; Klapoetke et al., 2014). The following
 146 coordinates were used for medial septum injections: AP: +0.6 mm, ML: 0 mm, DV: -7.2 mm (1st
 147 injection) and -6.6 mm (2nd injection) ventral from bregma (300 nl were injected at each ventral
 148 point). Finally, the scalp was sutured and the wound was covered with spray dressing (Nanosilver;
 149 Bioton, Poland) to promote healing. After surgery, the animals were allowed to recover for two
 150 weeks; their condition was monitored daily until the final experiment.

151 ***In vivo* preparation.** All *in vivo* electrophysiological experiments were performed on urethane
 152 anaesthetised rat preparation (urethane i.p. injection; 1.4 g/kg diluted in saline; Sigma-Aldrich,
 153 Poland). The anaesthetised animal was mounted in the stereotaxic frame (SF-1450AP, ASI
 154 Instruments Inc.; Warren, MI, USA) on the standard ear and incisor bars. The tracheotomy was
 155 performed to reduce the mechanical instability of the brainstem resulting from the respiratory
 156 movements of the animal. During the whole experiment, the electrocardiogram (ECG) was
 157 monitored, and the body temperature was held at $37 \pm 0.5^\circ\text{C}$ using an automatic heating pad (TCP-
 158 02; WMT, Poland). After cutting the skin and exposing the bones of the skull vault, the bregma point
 159 was set below the level of the lambda point (usually 2.3 mm) to obtain a rostral inclination of the
 160 skull of 15° , which allowed access to the nucleus incertus bypassing the confluence of superior
 161 sagittal sinus and transverse sinuses. Craniotomies were made above the areas of insertion of the
 162 electrodes and optical fibres, and after cleaning, the exposed brain surface was protected with a

163 drop of paraffin oil (Sigma-Aldrich, Poland). The stereotactic coordinates of the examined brain
 164 structures were taken from the rat brain atlas (Paxinos and Watson, 2007), and then recalculated
 165 taking into account the rostral inclination of the skull.

166 **Recording of the hippocampal local field potential.** Local field potentials (LFP) were recorded with
 167 a blunt cut, Teflon coated stainless-steel wire (o.d.: 200 μ m; Science Products, Germany) from
 168 stratum lacunosum moleculare of the hippocampal CA1 field (rostral angle 15°, AP: -4.6 mm, ML: 3
 169 mm, DV: -1.9 mm from the bregma). To verify the placement of the electrode, an electrolytic lesion
 170 (current of 0.6 mA applied for 1 s) was made at the end of the recording. In the case of experiments
 171 with optogenetics, the LFP recording electrode was attached to the skull with dental cement
 172 (Duracryl Plus; SpofaDental, Czech Republic).

173 **Multichannel *in vivo* recordings from the NI.** The 32-channel multielectrode array (MEA; 4 shanks,
 174 8 electrodes per shank; model A4x8-10mm-50-200-177; NeuroNexus, USA) was positioned
 175 in the nucleus incertus using the following stereotaxic coordinates: AP: -9.2 – -9.7 mm, ML: 0.1 –
 176 0.4 mm, DV: -7.5 – -7.2 mm from bregma (Paxinos and Watson, 2007). The spontaneous firing
 177 activity of NI neurons was recorded in one position in each animal. The extracellular signal picked up
 178 by MEA was digitised (40 kHz per channel), wide-band filtered (0.77 – 7500 Hz) and stored on a hard
 179 drive using OmniPlex D Neural Data Acquisition System (Plexon Inc., USA). To reconstruct the
 180 position of each MEA, its shanks were covered with a fluorescent dye (DiI; Thermo Fisher Scientific,
 181 USA) before brain implantation.

182 **Optogenetic stimulation of the MS during the multichannel recording of NI electrical activity *in***
 183 ***vivo*.** Three weeks after the viral vector injection into the medial septum (AAV2-hSyn-hChR2(H134R)-
 184 EYFP; as described above), the NI neurons' responses to medial septum optogenetic stimulation were
 185 observed. The LFP signal was recorded from the stratum lacunosum moleculare of the hippocampal
 186 CA1 field and electrical activity of NI neurons was extracellularly recorded with the multielectrode
 187 array, as described above. For the optogenetic stimulation, the optical fibre (core $\varnothing$ = 100 μ m,
 188 NA = 0.22; Thorlabs, NJ, USA) was implanted so that its tip was aimed at the MS

(coordinates of the tip: right angle 10°, AP: 0.7 mm, ML: 0.7 mm, DV: -5.6 mm from the bregma; Paxinos and Watson, 2007). To verify the placement of the MEA and optical fibre, both were covered with a fluorescent dye (Dil; Thermo Fisher Scientific, USA) before brain implantation. The optical fibre was connected to a blue laser light source (wavelength: 473 nm; model: MBL-III-473 with PSU-III-LED controller; CNI Optoelectronics Technology Co., Ltd., China). The light power never exceeded 10 mW, as measured with the optical power meter (model: PM-100D equipped with S121C photodiode; ThorLabs, NJ, USA) at the tip of the optical fibre. Temporal parameters of the light pulses were digitally controlled by a micro1401 mk II laboratory interface and Spike2 software running a custom written script (Cambridge Electronic Design, UK). Protocols of optogenetic stimulation of MS included three frequencies from the theta band: 4 Hz (4 x 100 ms light pulses), 8 Hz (8 x 40 ms light pulses), 12 Hz (12 x 20 ms light pulses), one high-frequency pattern: 20 Hz (40 x 10 ms light pulse) and one single pulse (50ms) pattern. The given frequencies are based on intervals between light pulses' onsets. Stimulation at each frequency/pattern was applied at least 50 times at 10 s intervals.

Ex vivo recording: tissue preparation, data acquisition and optogenetic stimulation of medial septum originating terminals. Whole-cell patch clamp electrophysiological recordings were performed as previously described (Kania et al., 2020). Male Sprague-Dawley rats were deeply anesthetised with isoflurane (AErrane; Baxter, Poland) and decapitated between 02:00-03:00 ZT. The brains were collected in ice-cold, low-sodium, high-magnesium ACSF, containing (in mM): 185 sucrose, 25 NaHCO₃, 3 KCl, 1.2 NaH₂PO₄, 2 CaCl₂, 10 MgSO₄ and 10 glucose (pH = 7.4; osmolality = 290 – 300 mOsmol/kg) and cut into 250 µm thick coronal sections on a vibrating microtome (VT 1000S; Leica Instruments, Germany). Sections containing the NI were transferred to an incubation chamber containing carbogenated, warm (32 °C) ACSF, containing (in mM): 118 NaCl, 25 NaHCO₃, 3 KCl, 1.2 NaH₂PO₄, 2 CaCl₂, 1.3 MgSO₄ and 10 glucose (pH = 7.4; osmolality 290 – 300 mOsmol/kg). After a recovery period (90 – 120 min), the slices were placed in a recording chamber, where the tissue was perfused (2 ml/min) with carbogenated, warm (32 °C) ACSF of the same composition as in the incubation chamber. Medial septum was also cut into 250 µm thick coronal section on a vibrating

215 microtome and viral vectors injection site was verified using Axio Imager M2 fluorescent microscope
 216 with an A-Plan 10×/0.25 objective. Recording micropipettes (7-9 MΩ; measured in the recording
 217 chamber filled with ACSF) were fabricated from borosilicate glass capillaries (BF100-78-10; Sutter
 218 Instruments, CA, USA) using a horizontal puller (P-97; Sutter Instruments, CA, USA) and filled with
 219 intrapipette solution containing (in mM): 145 potassium gluconate, 2 MgCl₂, 4 Na₂ATP, 0.4 Na₃GTP, 5
 220 EGTA, 10 HEPES (pH = 7.3; osmolality 290 – 300 mOsmol/kg) and biocytin (0.05%, for subsequent
 221 immunofluorescent identification of recorded neurons). The calculated liquid junction potential was
 222 +15 mV, and this value was subtracted from the recorded signal prior to the analysis. NI neurons
 223 were approached with a tip of the recording micropipette under visual control using an upright
 224 microscope (Examiner D1; Zeiss, Germany) equipped with video-enhanced infrared differential
 225 interference contrast. Cell-attached and subsequent whole-cell configurations were obtained using a
 226 negative pressure delivered by mouth suction. Voltage clamp recording and data acquisition were
 227 performed using an SEC 05LX amplifier (NPI, Germany) and Micro 1401 mk II (Cambridge Electronic
 228 Design, UK) converter operated by Signal and Spike2 software (Cambridge Electronic Design, UK) .
 229 Analogue signal filtered (low-pass filter: 3 kHz) and digitised (20 kHz). All drugs used in the *ex vivo*
 230 experiments were applied via perfusion system. Voltage-clamp recordings (command potential –50
 231 mV) were performed in standard ACSF or ACSF containing gabazine (5 μM; cat.no. 1262; Tocris,
 232 Poland) – a selective GABA_A receptor antagonist. As the calculated reversal potential for Cl⁻ currents
 233 for the solutions used in this study was -90mV, outward currents recorded at –50 mV holding
 234 potential were considered inhibitory (iPSCs, inhibitory postsynaptic currents), while inward currents
 235 were considered excitatory (ePSCs, excitatory postsynaptic currents). The tip of the optical fibre (core
 236 ∅ = 200 μm, NA = 0.5; Thorlabs, NJ, USA) was positioned approximately 100 μm above the surface of
 237 the brain slice, aiming at the tip of the recording micropipette positioned within the NI. The optical
 238 fibre was connected to a blue light emitting LED (wavelength: 465 nm; PlexBright LED Module;
 239 Plexon Inc., USA). The power of the LED output was controlled by the current source (PlexBright LED
 240 Driver LD-1; Plexon Inc., TX, USA) and never exceeded 10 mW, as measured with the optical power

meter (PM-100D equipped with S121C photodiode; ThorLabs, NJ, USA) at the tip of the optical fibre. The temporal parameters of the light pulses were digitally controlled by a micro1401 mk II laboratory interface and Spike2 software running a custom written script. Optogenetic stimulation of MS originating terminals within the NI consisted of at least 20 x 5 ms light pulses applied at 10 s interval. After the experiment brain slices were fixed and processed for histological examination as described below.

Optogenetic identification of NI neurons innervating the MS. Three weeks after the retrograde viral vector injection into the medial septum (pAAV-hSYN-Chronos-GFP; as described above), the NI neurons innervating the MS were optogenetically identified. The LFP signal was recorded from hippocampal CA1 field and electrical activity of NI neurons was extracellularly recorded with the multielectrode array, as described above. The optical fibre (core $\varnothing$ = 200 μ m, NA = 0.5; Thorlabs, NJ, USA) was implanted above the NI (coordinates of the tip: caudal angle 30°, AP: -9.6, ML: 0 mm, DV: -7.2 from bregma; Paxinos and Watson, 2007). The MEA and the optical fibre, before implantation, were covered with a fluorescent dye (DiI and DiD respectively; Thermo Fisher Scientific, USA) to allow subsequent verification of their location in the tissue. The optical fibre was connected to a blue light emitting LED (wavelength: 465 nm; model: PlexBright LED Module; Plexon Inc., USA). The power of the LED output was controlled by the precise current source (model: PlexBright LED Driver LD-1; Plexon Inc., USA) and never exceeded 12 mW, as measured with the optical power meter (model: PM-100D equipped with S121C photodiode; ThorLabs, NJ, USA) at the tip of the optical fibre. Protocols of optogenetic stimulation of NI included eight patterns, each consisting of 50 light pulses: 5 ms pulses applied at frequencies: 10, 30 and 50 Hz and 50 ms pulses applied at 1 Hz. A neuron was considered to be directly driven by optogenetic stimulation (i.e., optogenetically tagged) if it responded with 100% fidelity to 10 and 30 Hz pulses, at least 90% fidelity to 50 Hz pulses, and responded with high-frequency discharges (> 50Hz) to laser light pulses of 50 ms duration.

Histological verification, immunohistochemical staining and image acquisition. At the end of each *in vivo* experiment, the animals were transcardially perfused with PBS followed by 4% formaldehyde

267 solution. The extracted brains (*in vivo* experiments) or brain slices (*ex vivo* experiments) were kept
 268 overnight in 4% formaldehyde solution at 4 °C. The brains were cut into 50 µm coronal slices
 269 on the vibrating microtome (model: VT1000S; Leica, Germany) and the brain slices from the *ex vivo*
 270 experiments were processed histologically without further sectioning. To visualise biocytin filled
 271 neurons recorded *ex vivo*, fixed free-floating sections were blocked and permeabilised with 10%
 272 normal donkey serum (NDS) and 0.6% Triton X-100 in PBS, respectively, at 4 °C overnight or for 3 h
 273 at room temperature. Subsequently, after washing in PBS, the sections were incubated
 274 with ExtrAvidin®-Cy3™ (1:200), 2% NDS and 0.3% Triton X-100 in PBS for 48 up to 72 h at 4 °C and,
 275 after several washing steps (in PBS), the slices were mounted on to glass slides and coverslipped with
 276 Fluoroshield with DAPI (Sigma-Aldrich, Poland). The brain sections from the *in vivo* experiments
 277 involving pAAV-Syn-Chronos-GFP injection to the MS (see above) were immunostained against GFP
 278 to improve the visibility of cells transfected with the viral vector. Sections were placed in 10%
 279 normalised donkey serum (NDS; Abcam, UK) and 0.6% TritonX-100 (Sigma-Aldrich, Poland) in PBS
 280 for 1 h at room temperature to block the non-specific binding sites and permeabilisation,
 281 respectively. After washing in PBS, the slices were incubated for 24 h with rabbit anti-GFP antibodies
 282 (1:1000; Abcam, UK). Then, after another washing step, the sections were incubated overnight
 283 in the secondary donkey anti-rabbit AlexaFluor 488 antibody solution (1:400; Jackson
 284 ImmunoResearch, UK). After final rinsing, the sections were placed on slides and closed using
 285 Fluoroshield with DAPI (Sigma-Aldrich, Poland). The sections were imaged with a fluorescent
 286 microscope (Axio Imager.M2, camera: AxioCam MRm; objectives: A-Plan 10×/0.25
 287 and EC-Plan-Neofluar 20×/0.25 Zeiss, Germany).

288 2.2. Data processing and analysis

289 All data were processed and analysed offline, except for the initial filtration of recorded
 290 signals as described above. The extracellular signal from the MEA recording spots located within the
 291 NI (as demonstrated by histological verification) was digitally filtered (high-pass filter: 300 Hz)
 292 and subjected to spike detection in the Kilosort2 software (Pachitariu et al., 2016). The filtered signal

293 and spike times of the sorted units were converted to the SON file format using a custom written
 294 MATLAB script (MathWorks, MA, USA), and then the quality of spike sorting was inspected and
 295 verified manually in Spike2 software (Cambridge Electronic Design, UK). The units that had
 296 percentage of refractory period violations ($[(\text{inter-spike intervals} < 2 \text{ ms} / \text{total number of intervals}]$
 297 $\times 100)$ higher than 0.1%, were rejected from further analysis. The final analysis of electrophysiological
 298 signal was carried out in NeuroExplorer and MATLAB software (NexTechnologies, CO, USA). Theta
 299 and delta epochs were identified based on percentage of spectral power exceeding 50% in theta (3 –
 300 6 Hz) and delta band (0.1 – 3 Hz), respectively, calculated for consecutive 4 s windows of the LFP
 301 signal recorded from the CA1 field (as described above). The full band (0-500Hz) power spectral
 302 density was calculated with Fast Fourier Transform applied to the LFP signal with Welch's method (4
 303 s windows). Zero phases (i.e., peak negativity) of theta and delta waves were detected with
 304 NeuroExplorer software algorithm (NexTechnologies, CO, USA) based on the method described
 305 elsewhere (Klausberger et al., 2003). Using the custom MATLAB script, each spike generated by the
 306 NI neuron was assigned a theta oscillation phase determined using the Hilbert transform. Based on
 307 the obtained values, the activity of each neuron was subjected to circular statistics using the MATAB
 308 CircStat toolbox (Berens, 2009) to determine whether, and if so, at which phase of hippocampal
 309 theta oscillation the given neuron preferentially generates action potentials. It was assumed that the
 310 firing of a neuron is theta phase-independent if the probability of generating action potential was
 311 uniformly distributed around the hippocampal theta cycle ($p_R > 0.05$, Rayleigh's test for uniformity)
 312 or direction vector length $r < 0.2$ (at $p_R \leq 0.05$). If the probability was non-uniformly distributed
 313 around the hippocampal theta cycle ($p_R \leq 0.05$) and the length of the direction vector was ≥ 0.2 , the
 314 neuron was identified as theta phase-locked (Lasztóczy et al., 2011). Theta phase preference of
 315 specific NI neuron populations was determined and compared using the Watson-Williams test
 316 (Watson and Williams, 1956). To determine whether the neuronal activity was locked with delta
 317 waves the peri-delta wave zero phase Z-score histograms (10ms bins) were generated using
 318 NeuroExplorer. The obtained histograms were analysed using custom-made MATLAB script. To each

319 histogram a 5th degree polynomial was fitted and the times and amplitudes of minimum and
 320 maximum closest to zero-phase were extracted. When at least one peak exceeded z-score value of
 321 2.575 (99% confidence level) the neuronal activity was considered correlated with the delta wave.

322 For each NI neuron, the median firing rate in theta and delta epochs was determined. The
 323 firing pattern of the recorded neuron (regular, irregular, bursting and non-bursting) was determined
 324 based on interspike interval and autocorrelation histograms generated for theta epochs. Bursts of
 325 action potentials and the parameters describing them were detected using a custom MATLAB script
 326 based on MLIB toolbox (Stüttgen, 2020). For each neuron, in each subsequent theta epoch, the spike
 327 density function (SDF; gaussian kernel, width = 25 ms) and its mean value were calculated. The
 328 beginning of the burst was set on the first spike after the SDF exceeded the mean value, and the end
 329 of the burst was set on the last spike before the SDF returned below the mean value. The following
 330 parameters describing theta bursts and intensity of bursting were determined for each NI bursting
 331 neuron: length of the burst, number of spikes in the burst, intraburst frequency, bursting rate (i.e.,
 332 bursts fired in time unit), % of spikes generated in the bursts (i.e., [no of spikes in bursts/total
 333 number of spikes] * 100%), interburst interval and coefficient of variation of the interburst intervals.

334 To determine if neuron firing was correlated with each other, cross-correlograms (10ms bin,
 335 ± 0.25 s delay) of activity in pairs of simultaneously recorded neurons using the NeuroExplorer were
 336 generated. The obtained cross-correlograms were analysed using custom-made MATLAB script. A
 337 seventh-degree polynomial was fitted to each cross-correlogram and the time and amplitude of the
 338 maximum closest to the zero lag were extracted, as well as the minimum preceding it. When at least
 339 one peak exceeded z-score value of 2.575 (99% confidence level) the neuronal activity of the pair was
 340 considered correlated with one another.

341 To check whether the optogenetic stimulation of the MS elicited the reactions of NI neurons
 342 in vivo, the peristimulus spike density function (Gaussian kernel, width = 50 ms) histograms were
 343 prepared using custom- Spike2 software (Cambridge Electronic Design, UK). Then, using custom-
 344 made MATLAB scripts, the signal was divided into episodes of activity either above or below mean

345 firing rate in the baseline, and next, their lengths and mean firing rates were extracted. Following, a
346 multivariate Gaussian distribution was fitted to both parameters of baseline episodes and anomaly
347 detection for the post-baseline episodes was performed. As a criterium for the detection the
348 Mahalanobis distance was used; its value was set to 5.9915 which is equivalent to the Chi-square
349 distribution-derived probability of 0.05 for two variables. The episodes lying outside of the
350 distribution which latencies were shorter than 50 ms from the stimulation onset were classified as
351 effects; the adjacent significant episodes were merged.

352 The amplitude of the light-evoked post synaptic currents recorded during the *ex vivo*
353 experiments was determined based on the average response to 20 consecutive pulses (Spike2
354 software, Cambridge Electronic Design, UK). The distribution of the obtained parameters was tested
355 for normality using the Shapiro-Wilk test. All values, because of their non-normal distribution, were
356 presented as medians and ranged between the 1st and 3rd quartiles. Statistical evaluation of the
357 differences between the medians was made using the Wilcoxon test (for paired data) or the Mann-
358 Whitney test (for unpaired data) implemented in GraphPad Prism software (version 6; GraphPad
359 Software, Inc., CA, USA). Differences with $p < 0.05$ were considered statistically significant.

360

361 3. Results

362 3.1. Electrophysiological characterisation of NI neurons in vivo

363 3.1.1. Firing rate of the NI neurons during cortical activation and SWA

364 The position of the microelectrode array in the nucleus incertus (NI) has been confirmed
 365 in 16 animals (Fig. 1A). The activity of 197 NI neurons (no. of neurons/animal: average = 12,
 366 range = <5, 28>) was recorded over at least one full, spontaneous cycle of brain state alternation,
 367 covering the phase of cortical activation and slow-wave activity (SWA). The firing rate of the majority
 368 of NI neurons (87%, 171/197) was significantly higher during spontaneous cortical activation than
 369 during SWA state (theta-ON neurons; activation: median = 19.6 Hz, 1st & 3rd quartile: 8.7 & 38.7 Hz;
 370 SWA: median = 9.8 Hz, 1st & 3rd quartile: 4.0 & 21.2 Hz; n = 171, Z = 11.34, p < 0.0001, Wilcoxon
 371 matched pairs test). The firing rate of the minority of NI neurons (3%, 6/197) did not change across
 372 the brain states (theta unrelated; activation: median = 5.4 Hz, 1st & 3rd quartile: 3.9 & 26.9 Hz;
 373 SWA: median = 3.9 Hz, 1st & 3rd quartile: 2.4 & 37.3 Hz; n = 6, Z = 0.10, p = 0.92, Wilcoxon matched
 374 pairs test) or was significantly lower during the activation comparing to the SWA state (theta-OFF
 375 neurons; 10%, 20/197; activation: median = 6.5 Hz, 1st & 3rd quartile: 3.5 & 16.6 Hz; SWA: median =
 376 14.9 Hz, 1st & 3rd quartile: 6.3 & 22.2 Hz; n = 20, Z = 2.80, p < 0.01, Wilcoxon matched pairs test).

377 3.1.2. Firing of NI neurons in relation to the phase of hippocampal theta oscillation

378 Theta oscillations in the local field potential in the stratum lacunosum moleculare of the CA1
 379 region of the hippocampus, observed during brain activation, were the basis for determining
 380 whether NI neurons generate action potentials in a theta phase-locked manner. 59% of recorded NI
 381 neurons (117/197) fired action potentials in theta phase-independent manner (Fig. 2A and 2C;
 382 theta phase-independent neurons; $p_R > 0.05$, Rayleigh's test for uniformity or $p_R \leq 0.05$ and direction
 383 vector length $r < 0.2$), whereas firing of 41% of recorded NI neurons (80/197) was significantly locked
 384 with the phase of the hippocampal theta oscillations (Fig. 2B and 2D; theta phase-locked neurons;
 385 $p_R < 0.05$, Rayleigh's test for uniformity and direction vector length $r \geq 0.2$). Population of theta
 386 phase-locked NI neurons, observed in our study, had a significant preference to fire action potentials

at the rising phase of the hippocampal theta oscillation recorded from the hippocampal CA1 field (mean direction = 134.0 ± 7 deg., direction vector length = 0.55, $p_R \ll 0.001$, $n = 80$). The phase preference of theta phase-locked NI neurons was not correlated either with their anteroposterior, lateromedial or dorsoventral position in the nucleus incertus ($r = 0.09$, $p = 0.35$, $r = 0.33$, $p = 0.07$ and $r = -0.09$, $p = 0.44$ respectively; Zar, 1999).

3.1.3. Firing patterns of the NI neurons during hippocampal theta oscillation

In each group of NI neurons, both theta phase-independent and theta phase-locked, two additional subpopulations could be clearly differentiated, based on their pattern of electrical activity.

Theta phase-independent NI neurons

In the population of theta phase-independent NI neurons ($n = 117$), the majority (61%, 71/117) were characterised by irregular, slow-firing of action potentials (Fig. 3A), whereas a minority (39%, 46/117) were regular, fast-firing neurons (Fig. 3B). Regular, fast-firing NI neurons fired action potentials significantly faster and more regularly compared to the irregular, slow-firing neurons (Tab.1).

Theta phase-locked NI neurons

In the theta phase-locked group of NI neurons ($n = 80$), the majority (69%, 55/80) generated bursts of action potentials, as indicated by a bimodal distribution of interspike intervals and autocorrelation function plots with first and second order peaks (Fig. 3C). A smaller proportion of theta phase-locked NI neurons (31%, 25/80) generated a non-bursting pattern of electrical activity (Fig. 3D). Bursting, theta phase-locked NI neurons fired action potentials significantly faster compared to the non-bursting, theta phase-locked neurons (Tab.1). The vast majority of theta phase-locked neurons was located in the nucleus incertus *pars dissipata* (Fig. 1C and 1D; laterality $> 200 \mu\text{m}$), i.e., all bursting neurons (100%, 55/55) and all but two non-bursting neurons (92%, 23/25). Population of theta bursting NI neurons had a strong preference to fire action potentials at the rising phase of the hippocampal theta oscillation (Fig. 4A and 4B; mean direction = 126.0 ± 6.8 deg.,

direction vector length = 0.70, $pR < 0.001$, $n = 55$). On the other hand, non-bursting, theta phase-locked NI neurons, had uniformly distributed phase preferences and thus, this population of NI neurons shown no significant preference to fire action potentials at a particular phase of the hippocampal theta oscillation (Fig. 4A and 4C; mean direction = 169.8 ± 16.8 deg., direction vector length = 0.34, $pR = 0.051$, $n = 25$). The analysis of the correlation of parameters describing the strength of generated bursts and intensity of bursting with firing of NI theta bursting neurons was performed. It revealed that intraburst frequency and the number of spikes generated per burst were positively, linearly correlated with the firing rate of a neuron ($R^2 = 0.96$ and $R^2 = 0.94$, respectively; Fig. 5A and 5B), while the burst length did not show significant correlation (linear, $R^2 = 0.01$ or exponential, $R^2 = 0.52$; Fig. 5C). At the same time, bursting rate, interburst interval and CV of interburst interval were exponentially correlated with the firing rate (positive correlation, $R^2 = 0.82$ and negative correlations $R^2 = 0.94$ and $R^2 = 0.75$, respectively; Fig. 5D, 5E and 5F), while the percent of spikes generated in bursts did not show significant correlation (neither linear, $R^2 = 0.01$ nor exponential, $R^2 = 0.29$; Fig. 5G).

3.1.4. Cross-correlation of NI neuronal firing during hippocampal theta oscillation

A cross-correlation analysis of activity in pairs of simultaneously recorded neurons (during cortical activation), belonging to the same or different populations of NI neurons (as described above), was performed (Fig. 6). It revealed that firing of all theta bursting NI neurons is highly synchronised (100%, 128/128 pairs of neurons, peak z-score > 2.575 ; median peak z-score = 39.8, 1st & 3rd quartile: 24.5 & 56.6, median peak time = 0.02s, 1st & 3rd quartile: -0.01 & 0.04; Fig. 6A). Similarly, majority of non-bursting, theta phase-locked neurons fired in synchrony with theta bursting ones (95%, 98/103 pairs of neurons, peak z-score > 2.575 ; median peak z-score = 11.7, 1st & 3rd quartile: 7.5 & 20.8, median peak time = 0.04s, 1st & 3rd quartile: 0.01 & 0.06; Fig. 7B). Only less than half of analysed pairs of non-bursting, theta phase-locked neurons was synchronised (47%, 14/30 pairs of neurons, peak z-score > 2.575) what resulted in insignificant synchrony within this population of NI neurons (median peak z-score = 1.9, 1st & 3rd quartile: 0.4 & 3.3, median peak time = -0.02s,

439 1st & 3rd quartile: -0.05 & 0.02; Fig. 6C). At the same time, a vast majority of analysed pairs of theta
 440 phase-independent NI neurons displayed asynchronous firing (cross-correlation peak z-score <
 441 2.575): 93% (127/136) of paired irregular firing neurons (Fig. 6D), 95% (225/237) of paired regular &
 442 irregular firing neurons (Fig. 6E) and 99% (91/92) of paired regular firing neurons (Fig. 6F).

443 3.1.5. Firing of NI neurons in relation to the delta waves during SWA

444 Slow-wave activity observed in the local field potential in the CA1 region of the hippocampus
 445 was the basis for determining whether NI neurons generate action potentials in a delta wave-
 446 dependent manner. Majority of recorded NI neurons (73%; 144/197) fired action potentials
 447 independently of delta waves (Fig. 7A and 7C; delta wave-independent neurons; peak z-score of delta
 448 wave zero-phase triggered PSTH < 1.96), whereas firing of 27% of recorded NI neurons (53/197) was
 449 significantly locked with the hippocampal delta waves (Fig. 7B and 7D; delta wave-locked neurons;
 450 peak z-score of delta wave zero-phase triggered PSTH peak ≥ 1.96). The latter population of neurons
 451 showed the highest probability of generating action potentials on the ascending phase of the delta
 452 wave, i.e., after its negative peak (median lag = 0.15 s; 1st & 3rd quartile: 0.09 & 0.21 s). Of the 144
 453 delta wave-independent NI neurons, the majority (69%; 100/144) were neurons that, during cortical
 454 activation, were also theta phase-independent (85% of the theta phase-independent population;
 455 100/117). The remaining 44 (31%) delta wave-independent NI neurons, during cortical activation
 456 were theta phase-locked (55% of the theta phase-locked population; 44/80). Of the 53 delta wave-
 457 locked NI neurons, the majority (68%; 36/53) were neurons that, during cortical activation, were
 458 theta phase-locked (45% of the theta phase-locked population; 36/80). The remaining 17 (32%) delta
 459 wave-locked NI neurons, during cortical activation were theta phase-independent (15% of the theta
 460 phase-independent population; 17/117).

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466 **3.1.6. Firing patterns of the NI neurons during hippocampal SWA**

467 In each group of NI neurons, both delta wave-independent and delta wave-locked, two
 468 additional subpopulations could be clearly differentiated, based on their pattern of electrical activity.

469 **Delta wave-independent NI neurons**

470 In the population of delta wave-independent NI neurons ($n = 144$), the vast majority (82%,
 471 118/144) were characterised by irregular, slow-firing of action potentials (Fig. 8A), whereas a
 472 minority (18%, 26/118) were regular, fast-firing neurons (Fig. 8B). Regular, fast-firing NI neurons fired
 473 action potentials significantly faster and more regularly compared to the irregular, slow-firing
 474 neurons (Tab.2).

475 **Delta wave-locked NI neurons**

476 In the delta wave-locked group of NI neurons ($n = 53$), the majority (62%, 33/53) generated a
 477 non-bursting pattern of electrical activity (Fig. 8C). A smaller proportion of delta wave-locked NI
 478 neurons (38%, 20/53) generated bursts of action potentials, as indicated by a distribution of
 479 interspike intervals (dominant peak at short intervals and substantially smaller and slowly decaying
 480 count of longer intervals) and autocorrelation plot with first order peak sharply centered around zero
 481 (Fig. 8D). Bursting, delta wave-locked NI neurons fired action potentials significantly faster compared
 482 to the non-bursting, delta wave-locked neurons (Tab. 2).

483 **3.1.7. Cross-correlation of NI neuronal firing during SWA**

484 A cross-correlation analysis of activity in pairs of simultaneously recorded neurons (during
 485 cortical SWA), belonging to the same or different populations of NI neurons (as described above),
 486 was performed (Fig. 9). It revealed that firing of all delta bursting NI neurons is highly synchronised
 487 (100%, 19/19 pairs of neurons, peak z-score > 2.575 ; median peak z-score = 13.3, 1st & 3rd quartile:
 488 7.4 & 17.0, median peak time = 0.01s, 1st & 3rd quartile: -0.01 & 0.02; Fig. 9A). Less than half of
 489 non-bursting, delta wave-locked neurons fired in synchrony with delta bursting ones (44%, 25/57

490 pairs of neurons, peak z-score > 2.575) what resulted in insignificant synchrony between these two
 491 populations of NI neurons (median peak z-score = 2.2, 1st & 3rd quartile: 1.0 & 3.8, median peak
 492 time = -0.03s, 1st & 3rd quartile: -0.09 & 0.01; Fig. 9B). Similarly, only 39% (16/41) of analysed pairs
 493 of non-bursting, delta wave-locked neurons were synchronised (peak z-score > 2.575) what resulted
 494 in insignificant synchrony within this population of NI neurons (median peak z-score = -0.03, 1st &
 495 3rd quartile: -0.09 & 0.01, median peak time = 0.01s, 1st & 3rd quartile: -0.04 & 0.05; Fig. 9C). At the
 496 same time, a vast majority of analysed pairs of delta wave-independent NI neurons displayed
 497 asynchronous firing (cross-correlation peak z-score < 2.575): 78% (459/588) of paired irregular firing
 498 neurons (Fig. 9D), 99% (265/268) of paired regular & irregular firing neurons (Fig. 9E) and all (29/29)
 499 of paired regular firing neurons (Fig. 9F).

500 3.2. Nucleus incertus neurons' responses to input from the medial septum

501 3.2.1. Responses of the NI neurons to optogenetic stimulation of the medial septum *in vivo*

502 The effective transfection of the medial septum (AAV2-hSyn-hChR2(H134R)-EYFP) as well
 503 as the position of the multielectrode array within the NI and the optical fibre above the MS was
 504 confirmed in four rats (Fig. 10A and 10B). The spontaneous activity of 63 nucleus incertus neurons
 505 (no. of neurons/animal: average = 16, range = <7, 28>) and their responses to the optogenetic
 506 stimulation of the medial septum were recorded. Responses of 37 neurons were recorded during
 507 both, cortical activation and SWA, and the remaining neurons in only one of the cortical states.
 508 During cortical activation, majority (86%, 6/7) of bursting theta phase-locked neurons were inhibited
 509 by the MS stimulation (Fig. 10C and 10E; Tab. 3) and cessation of each light pulse evoked inhibition
 510 was followed by rebound burst of action potentials. Theta phase-locked non-bursting neurons
 511 responded to MS activation with excitation and inhibition in equal proportions (40%, 2/5; Fig. 10C
 512 and 10E; Tab. 3). It is noteworthy that only a minority of theta bursting and non-bursting neurons
 513 (14%, 1/7 and 20%, 1/5, respectively) did not respond to MS stimulation, and at the level of
 514 population, theta phase-locked NI neurons showed inhibitory response followed by rebound
 515 excitation (Fig. 10E). Interestingly, MS stimulation with a series of pulses at frequencies in the theta

band (4, 8, 12Hz) resulted in the synchronization of the theta bursting NI neurons to stimulation frequency (Fig. 11A). Conversely, high-frequency stimulation of the MS disrupted theta bursting of the NI neurons (Fig. 11B and 11C). On the other hand, 56% (9/16) and 44% (7/16) of theta phase independent, regular fast-firing neurons and irregular slow-firing neurons, respectively, did not responded to the MS optogenetic stimulation (Fig. 10C; Tab. 3). At the same time, excitatory and inhibitory responses were observed in a similar proportion in both theta-independent neuron populations (Fig. 10C; Tab. 3), resulting in no response at the population level (Fig. 10E). During cortical SWA, stimulation of MS inhibited majority of delta-bursting NI neurons, while among non-bursting delta wave-locked cells only excitatory responses were observed (Fig. 10D; Tab. 3). However, due to the small number of recorded neurons, it was not possible to conclusively determine whether, if at all, delta wave-locked neurons show a dominant response at the population level (Fig. 10F). In contrast, delta wave-independent neurons, although more frequently responded to MS stimulation with inhibition than excitation (25% vs. 15% and 44% vs. 11% respectively for irregular and regular firing neurons; Fig. 10D; Tab. 3), did not show a dominant response at the population level (Fig. 10F). Among NI neurons, whose responses to MS stimulation were observed in both cortical states ($n = 37$), 19% (7/32) were excited, 24% (9/37) were inhibited and 30% (11/37) did not respond in both, cortical activation and SWA (Fig. 10G). Relatively small fraction of NI neurons responded to MS stimulation in only one cortical state, and none changed the direction of the response between cortical states (Fig. 10G).

3.2.2. Responses of the NI neurons to optogenetic stimulation of the medial septum originating terminals – *ex vivo* recordings

To investigate the functional properties of the MS innervation of the NI, whole-cell voltage clamp recordings were performed using brain slices from animals that were intra-MS injected with AAV2-hSyn-hChr2(H134R)-EYFP (Fig. 12A). In 15 out of 55 recorded NI neurons, optogenetic stimulation of MS originating fibres evoked inhibitory postsynaptic currents (IPSCs; Fig. 12B and 12C) and only one neuron responded with excitatory postsynaptic currents (EPSCs). IPSCs evoked by MS

542 fibre stimulation had short latency (2.5 ± 0.3 ms, $n = 15$), they disappeared at chloride reversal
 543 potential (-90 mV; 9/9 neurons; Fig. 12C), and were blocked by the selective GABA_A receptor
 544 antagonist gabazine ($5 \mu\text{M}$; 7/7 neurons; Fig. 12D and 12E). No differences were detected in the
 545 proportions of inhibition/excitation and IPSCs/EPSCs evoked by MS axonal endings stimulation
 546 observed in *in vivo* and *ex vivo* recordings, respectively ($p < 0.05$; two-tailed, Fisher's exact test).

547 3.3. Optogenetic identification of the NI neurons that innervate the medial septum

548 In animals whose medial septum was transfected with the retrograde viral vector
 549 (pAAV-hSYN-Chronos-GFP; Fig. 13A), the direct responses of 22 NI neurons to optogenetic
 550 stimulation were observed (Fig. 13B; 10 animals; opto-tagged NI neurons/animal: average = 2.2,
 551 range = $<1, 5>$). All NI neurons identified as innervating medial septum belonged to the regular, fast-
 552 firing theta phase-independent type of cells (100%, 22/22; Fig. 13D) that, during cortical SWA fired in
 553 delta wave-independent manner (59%, 13/22 were irregular, slow firing; 21%, 9/22 were regular
 554 slow firing).

555 4. Discussion

556 In the current study we identified two distinct NI neuronal populations, based on the
 557 relationship of their firing to the phase of the hippocampal theta rhythm and the direction and
 558 interaction with the medial septum: theta phase-independent and theta phase-locked neurons.
 559 Theta phase independent NI neurons included irregular, slow-firing and regular, fast firing cells.
 560 Theta phase locked neurons had a strong preference to fire action potentials at the rising phase of
 561 the hippocampal theta oscillation recorded from the stratum lacunosum moleculare of the
 562 hippocampal CA1 field (SLM). Interestingly, theta phase-locked NI neurons could be of bursting or
 563 non-bursting type. Theta-bursting NI neurons may have been omitted in previous studies due to the
 564 localisation of these cells mainly in the lateral parts of the NI (Ma et al., 2013). During the cortical
 565 SWA, based on the relation of action potential firing to hippocampal delta waves, we were able to
 566 distinguish two groups of NI neurons: delta wave-independent and delta wave-locked. Delta wave-

567 independent NI neurons could be of irregular, slow-firing or regular, fast firing type and delta wave-
 568 locked neurons could be of bursting and non-bursting type. Delta wave-locked NI neurons fired
 569 action potentials preferentially on the ascending phase of the delta waves. In line with previous
 570 studies, for most of the NI neurons we noted an increased firing during cortical activation and a
 571 decreased discharge rate in the presence of slow-wave activity (Nunez et al., 2006; Martinez-Bellver
 572 et al., 2015). By projection specific opto-tagging, we determined that only fast firing theta phase-
 573 independent NI neurons innervate the medial septum (MS). On the other hand, among neurons of
 574 this type, majority was unresponsive to optogenetic stimulation of the MS. In contrast, majority of
 575 theta bursting NI neurons were inhibited by the MS stimulation, and this effect was mediated by
 576 direct GABAergic input.

577 The knowledge gathered to date about nucleus incertus implies that it is a strong modulator
 578 of theta oscillations, however the involvement of NI in theta rhythm generation seems to be
 579 complex. Fibres originating from the NI heavily innervate the MS, and a recent study showed that
 580 GABAergic, including parvalbumin expressing, and glutamatergic MS neurons express mRNA for
 581 cognate relaxin-3 receptors, which makes these neurons a possible target of NI innervation (Albert-
 582 Gasco et al., 2018; Haidar et al., 2019; Olucha-Bordonau et al., 2012). Importantly, MS parvalbumin
 583 GABAergic neurons were shown to rhythmically hyperpolarise hippocampal interneurons and
 584 promote theta oscillations (Hangya et al., 2009). The latest studies revealed that NMB expressing NI
 585 neurons project to the MS and are involved in the control of locomotion, arousal, and hippocampal
 586 theta oscillations (Lu et al., 2020; Nasirova et al., 2020). Given that half of the NMB neurons co-
 587 express RLN3, it is not surprising that the relaxinergic input from the NI to the MS was also shown to
 588 be involved in theta rhythm modulation (Ma et al., 2009; Olucha-Bordonau et al., 2012; Ma et al.,
 589 2013; Haidar et al., 2017; Albert-Gasco et al., 2018; Szonyi et al., 2019). However, until now, the
 590 electrophysiological features of the NI neurons innervating the MS were unknown. Our results show
 591 that only regular, fast-firing theta phase-independent NI neurons project to the medial septum. In
 592 the light of the research by Szonyi et al. (2019), who showed that the NI neurons innervating MS give

593 collaterals to the oriens-lacunosum-moleculare (OLM) neurons of the hippocampus, it can be
 594 assumed that the regular, fast-firing NI neurons identified by us can also innervate this population of
 595 hippocampal interneurons (Fig. 14).

596 Our data show that majority of the recorded regular, fast-firing theta phase-independent
 597 neurons innervating the MS were insensitive to the MS stimulation, and only the minor fractions
 598 responded with relatively weak excitation or inhibition. This indicates that the NI neurons innervating
 599 the MS do not receive strong MS feedback. At the same time, majority of theta-bursting neurons
 600 responded to the optogenetic stimulation of the MS with initial inhibition followed by a burst of
 601 action potentials. This rebound excitation was evident during rhythmic stimulation of the MS with
 602 frequencies across the theta band. Additionally, high-frequency stimulation eliminated spontaneous
 603 theta bursts, but still synchronised the NI neurons to the frequency of stimulation, which indicates
 604 strong control of theta-bursting NI neurons by the input from the MS. Based on these results, it is
 605 likely that the NI bursting neurons are innervated by one of the two identified GABAergic medial
 606 septal bursting neuron populations, one of which is coupled to the peak and the other to the trough
 607 of hippocampal theta-wave (observed in the SLM; Borhegyi et al., 2004; Fig. 14).

608 Theta bursting MS neurons, by direct innervation of the hippocampal interneurons, basket
 609 and OLM cells (Fig. 14), cause their rhythmic hyperpolarisations followed by rebound bursts. A similar
 610 mechanism may underlie the rhythmic activity of the NI bursting neurons that receive MS
 611 innervation, as described in our study. This is further supported by our observation that higher
 612 strength of bursts and intensity of bursting occurred in NI neurons having higher firing rates, while
 613 the proportion of action potentials generated in bursts and length of bursts were not correlated with
 614 the firing rate. Moreover, the temporal convergence of the bursts of the NI neurons and
 615 hippocampal basket cells allows to hypothesise that both these populations may be innervated by
 616 the same population of rhythmically bursting GABAergic MS neurons (Fig. 14). In this way, theta-
 617 bursting NI neurons can use the pace received from the MS to efficiently participate in the
 618 synchronisation of oscillatory activity in the brain areas involved in locomotion or memory formation

619 (Goto et al., 2001; Olucha-Bordonau et al., 2003). Directional information flow between the NI and
 620 the hippocampus and coherence between the field activity observed in both structures during theta
 621 oscillations suggest that the hippocampus is one of the target structures of NI theta-bursting cells
 622 (Cervera-Ferri et al., 2011; Martinez-Bellver et al., 2017).

623 Additionally, our in vivo recordings showed that a fraction of the non-bursting theta phase-
 624 locked and irregular, slow-firing theta phase-independent NI neurons responded to the MS
 625 stimulation, and their responses were heterogeneous (inhibition or excitation). At the same time, our
 626 ex vivo results revealed that MS fibres optostimulation evoked inhibitory, GABA_A-dependent
 627 postsynaptic currents in a vast majority (all but one) of the recorded NI neurons. This suggests that
 628 the direct input from MS into NI is largely inhibitory. Concurrently, a recent study in mice has shown
 629 that stimulation of MS terminals within the NI elicited both GABA and glutamate dependent
 630 responses (Lu et al., 2020). These results indicate possible species differences in MS-NI innervation,
 631 as previously shown for some of the features of the NI originating RLN/RXFP3 system in mice and rats
 632 (Ma et al., 2017a).

633 Several studies have reported that NI activation or inhibition affects theta oscillations (Nunez
 634 et al., 2006; Martinez-Bellver et al., 2017; Ma et al., 2017b; Szonyi et al., 2019; Lu et al., 2020), but
 635 the results remain inconclusive. Some of the discrepancies may result from methodological
 636 differences, such as the used species (mouse/rat), state of the animal (anaesthetised/freely moving),
 637 stimulation method (electrical/optogenetic) or the group of activated cells (GABAergic/RLN3-
 638 positive/NMB-positive). In one of the recent studies, all GABAergic NI neurons were optogenetically
 639 activated with light pulses applied at 25 Hz (Szonyi et al., 2019), which resulted in a decrease in the
 640 power of hippocampal theta oscillations. Such stimulation protocol – while inducing activity of
 641 regular, fast-firing theta phase-independent NI neurons that is close to their physiological rate and
 642 pattern of firing – at the same time can desynchronise rhythmic firing of the theta-bursting NI
 643 neurons. If, as we postulate from our observations, the function of theta bursting NI neurons is to
 644 enhance the synchronisation in theta frequencies, the tonic activity of these neurons, forced by

645 optogenetic stimulation should weaken the theta oscillation strength. This explanation is further
646 supported by the results reported by Lu et al. (2020), who increased the power of hippocampal theta
647 oscillations by selectively stimulating the NI neuronal population innervating the MS (and therefore
648 not theta-bursting NI neurons, as we know from our observations), with light pulses at a frequency
649 and pattern similar to their physiological firing (our study; Lu et al., 2020). The current paper and
650 previous data (Lu et al., 2020; Nunez et al., 2006) indicate that the innervation of the MS by regularly
651 (tonically) firing NI neurons has a facilitating and/or gating function in the brainstem originating
652 mechanism of theta rhythm induction. In line with these results, Ma et al. (2016), using activation of
653 the NI via hM3Dq-DREADD, showed that NI neurons depolarisation – plausibly maintaining their
654 firing pattern – caused an increase in theta power. In the case of the theta-bursting population of
655 neurons, this probably led to the generation of more action potentials in bursts and possible
656 transition of irregularly bursting neurons into the rhythmic theta-bursting firing mode.

657 Hippocampal theta oscillations result from rhythmic changes in polarisation of the principal
658 cells, induced by the activity of hippocampal interneurons, which in turn are influenced by signals
659 from the medial septum. In our proposed model, two distinct neuronal populations, identified by us
660 in the NI, may be involved in hippocampal theta rhythm generation in different ways: regular, fast-
661 firing theta phase-independent NI neurons innervating the MS may have permissive function, while
662 theta-bursting NI neurons are likely involved in synchronising theta rhythm of other brain structures
663 (Fig. 14). In summary, the results described in this paper shed new light on the interpretation of
664 previous observations and provide the basis for a more precise incorporation of the nucleus incertus
665 in the mechanism of generation and/or modulation of septohippocampal theta oscillations.

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 789

Figures captions

Fig. 1. Histological verification of the positioning of the recording microelectrode array (MEA) in the nucleus incertus (NI) and reconstruction of the location of different types of neurons recorded within the NI. **A, left**, an example of the Dil (red) filled traces of the four shanks of the MEA (indicated by the white arrows) terminating on the ventral border of the NI (the brain section is coronal and the MEA penetration was done at a caudal angle of 15°; see Materials and methods section). **Right**, reconstruction of the location of all recorded NI neurons ($n = 197$). **B**, Reconstruction of the location of theta phase-independent neurons (**top**; $n = 117$) divided into irregular, slow-firing (**middle**; $n = 71$) and regular, fast-firing (**bottom**; $n = 46$) neurons. **C**, location of theta phase-locked (**top**; $n = 80$) neurons in the NI divided into bursting (**middle**; $n = 55$) and non-bursting (**bottom**; $n = 25$) neurons. The size of the circles indicates the number of neurons recorded at a given location (○, ○, ○, ○ – 1, 2, 3, 4 neurons respectively). The dotted line indicates the midline. The location of the NI neurons is presented on one side, but the recordings were made from both sides of the brain. **D**, histogram showing the distribution of different types of NI neurons along anterior-posterior (AP), medial-lateral (ML) and dorsal-ventral (DV) axes (bin = 100 μm). Scale bars = 200 μm . Nlc – nucleus incertus *pars compacta*, Nld – nucleus incertus *pars dissipata*, 4V – 4th ventricle, Cb – cerebellum, DTg – dorsal tegmental nucleus, LC – locus coeruleus, mlf – medial longitudinal fasciculus, RtTg – reticulotegmental nucleus of the pons.

Fig. 2. Two types of nucleus incertus neurons that can be distinguished on the basis of their preference to fire action potentials in the hippocampal theta phase-independent or theta phase-locked manner. **A**, an exemplary NI neuron that fires action potentials in the theta phase-independent manner ($p_R > 0.05$ or $p_R \leq 0.05$; $r < 0.2$). **B**, an exemplary neuron with strong preference ($p_R < 0.05$; $r \geq 0.2$) to fire at the specific phase of the ongoing hippocampal theta oscillation. **Top**, scatter plots showing the position of individual action potentials (dots) around two cycles of the

816 hippocampal theta oscillation. **Middle**, circular plots showing the histogram of the angular
 817 distribution of action potentials around the cycle of the hippocampal theta oscillation. Red solid lines
 818 show direction vectors. The red, dotted line circles indicate the length of the direction vector equal
 819 to 0.2. **Bottom**, histograms showing the distribution of action potential firing probability around two
 820 cycles of the hippocampal theta oscillation. The red, dashed line depicts idealised hippocampal theta
 821 oscillation. The trough of the hippocampal theta rhythm was defined as the zero phase (see
 822 Materials and methods section). Bins (all histograms) = 10° . In the case of both neurons, analysis of
 823 200s of firing recorded during cortical activation is shown. r – direction vector length; p_R – confidence
 824 level of Rayleigh’s test for uniformity. **C** and **D**, the preference to theta phase of all recorded theta
 825 phase-independent NI neurons and theta phase-locked NI neurons, respectively, during two theta
 826 cycles. **Top**, heat maps of the probability to fire action potentials at a specific hippocampal theta
 827 phase for theta phase-independent (**C**; $n = 117$) and theta phase-locked (**D**; $n = 80$) neurons.
 828 The probability of action potential generation is colour coded. The neurons are sorted according to
 829 the phase of the probability peak. **Bottom**, the median (blue line) phase preference of the two
 830 groups of NI neurons shown above. Light blue area indicates interquartile range; the red, dashed line
 831 illustrates idealised hippocampal theta oscillation.

832
 833 **Fig. 3.** Electrophysiological types of recorded NI neurons ($n = 197$). **Centre**, pie chart showing
 834 the proportion of the different types in the total NI neuron population. **Left**, theta phase-
 835 independent neurons consist of two subgroups: irregular, slow-firing neurons (**A**) and regular, fast-
 836 firing neurons (**B**). **Right**, theta phase-locked neurons consist of two subgroups: bursting neurons (**C**)
 837 and non-bursting neurons (**D**). In each panel, histograms of interspike intervals (left) and
 838 autocorrelation function plots (right) during cortical activation are shown for a representative neuron
 839 of each type. The theta wave and action potentials generated by an idealised neuron of a given type
 840 are schematically presented below the histograms. Bins (all histograms) = 10 ms.

841

842

843 **Fig. 4.** The preference of non-bursting and bursting, theta phase-locked NI neurons to fire action
 844 potentials at a specific phase of the hippocampal theta. **A**, circular plot showing the phase preference
 845 of individual bursting (blue; $n = 55$) and non-bursting (green; $n = 25$) neurons. Solid lines show the
 846 mean direction vector of a specific neuronal population (coded by colour). Whiskers represent
 847 circular standard errors of the mean directions. The red, dotted line circle indicates the length of the
 848 direction vector equal to 0.2. Red asterisk denotes a significant phase preference of population of
 849 theta bursting NI neurons, r – direction vector length, p_R – confidence level of Rayleigh's test for
 850 uniformity. Heat maps of the probability to fire action potentials at a specific hippocampal theta
 851 phase for theta phase-locked bursting (**B**) and theta phase-locked non-bursting (**C**) neurons. The
 852 neurons are sorted according to the phase of the probability peak. **Bottom**, the median (blue and
 853 green lines) phase preference of the two groups of NI neurons shown above. Light coloured area
 854 indicates interquartile range; the red, dashed line illustrates idealised hippocampal theta oscillation.
 855 The trough of the hippocampal theta rhythm was defined as the zero phase.

856

857 **Fig. 5.** The correlation of the parameters describing the strength of the generated bursts (green
 858 circles): **A**, intraburst frequency; **B**, no. of spikes per burst; **C**, length of burst, and the intensity of
 859 bursting (blue circles): **D**, bursting rate; **E**, interburst interval (IBI); **F**, CV of interburst interval (CV_{IBI});
 860 **G**, percent of spikes fired in bursts (%SFB), with the median firing rate of NI theta bursting neurons (n
 861 $= 55$). Asterisks and fitted curves, drawn with a solid line, indicate significant correlation ($R^2 \geq 0.7$),
 862 whereas fitted curves, drawn with dotted line, indicate insignificant correlation ($R^2 < 0.7$). Individual
 863 R^2 values are given on each plot. Type of fit used is colour coded: linear – violet, exponential – red.
 864 Note that in case of insignificant correlations, linear and exponential fits were performed. The blue
 865 circles with the whiskers on the side of each graph shows the median, 1st & 3rd quartiles (Q1 & Q3)
 866 of the analysed parameters.

867

Fig. 6. Cross-correlation analysis of spontaneous activity, observed during cortical activation, of theta phase-locked NI neurons: **A**, theta bursting vs. theta bursting (T-bst. vs. T-bst.); **B**, non-bursting vs. theta bursting (non-bst. vs. T-bst.); **C**, non-bursting vs. non-bursting (non-bst. vs. non-bst.) and theta phase-independent NI neurons: **D**, irregular firing vs. irregular firing (irreg. vs. irreg.); **E**, regular firing vs. irregular firing (reg. vs. irreg.); **F**, regular firing vs. regular firing (reg. vs. reg.). **Top**, heatmaps showing cross-correlations of activity of NI theta bursting (T-bst.) neuron pairs. Cross-correlations are sorted by the time of the peak closest to zero-lag. Cross-correlation with significant peak values (z-score >2.575 ; above 99% confidence level) are shown first (from top). The bar on the right indicates the proportion of pairs of neurons whose activity was correlated (light red) and uncorrelated (grey). Note, that all theta bursting neuronal pairs were significantly cross-correlated. **Middle**, times and square roots of z-score values of the cross-correlograms' positive peaks (closest to zero-lag; red dots) and preceding it negative peaks (blue dots). Light grey area indicates range of insignificant z-score values (range ± 2.575 ; equivalent to $\pm 99\%$ confidence limits). **Bottom**, the median (blue line) of all cross-correlograms shown above. Light blue area indicates interquartile range.

Fig. 7. Two types of nucleus incertus neurons that can be distinguished on the basis of their preference to fire action potentials in the hippocampal delta wave-independent or delta wave-locked manner. **A**, an exemplary NI neuron that fires action potentials in the delta wave-independent manner (peak z-score of delta wave zero-phase triggered PSTH < 1.96). **B**, an exemplary neuron with strong preference to fire in a hippocampal delta wave-wave locked manner (peak z-score of delta wave zero-phase triggered PSTH peak ≥ 1.96). **Top**, scatter plots showing the position of individual action potentials (dots) around delta wave negative peak. **Bottom**, histograms showing the distribution of action potential firing around delta wave negative peak. The red, dashed line depicts idealised hippocampal delta wave. Light grey area indicates range of insignificant z-score values (range ± 1.96 ; equivalent to $\pm 95\%$ confidence limits). Bins = 10ms. **C** and **D**, The relation between firing of all recorded delta wave-independent (**C**) and delta wave-locked (**D**) NI neurons and negative

894 peak of hippocampal CA1 delta waves. **Top**, heat maps showing z-score values of delta wave zero-
 895 phase triggered PSTH (10ms bins) for delta wave-independent (**C**; $n = 144$) and delta wave-locked (**D**;
 896 $n = 53$) neurons. The z-score values are colour coded. The neurons are sorted according to the time
 897 of the z-value peak. **Bottom**, the median (blue line) delta wave zero-phase triggered PSTH of the two
 898 groups of NI neurons shown above. Light blue area indicates interquartile range; the red, dashed line
 899 illustrates idealised hippocampal delta wave.

900
 901 **Fig. 8.** Electrophysiological types of recorded NI neurons ($n = 197$). **Centre**, pie chart showing
 902 the proportion of the different types in the total NI neuron population. **Left**, delta wave-independent
 903 neurons consist of two subgroups: irregular, slow-firing neurons and regular, fast-firing neurons.
 904 **Right**, delta wave-locked neurons consist of two subgroups: non-bursting neurons and bursting
 905 neurons. In each panel, histograms of interspike intervals (left) and autocorrelation function plots
 906 (right) during hippocampal SWA are shown for a representative neuron of each type. Fragment of
 907 hippocampal LFP is shown at the bottom of each panel. Vertical, lines indicate action potentials
 908 generated by simultaneously recorded different types of NI neurons. Bins (all histograms) = 10 ms.

909
 910 **Fig. 9.** Cross-correlation analysis of spontaneous activity, observed during cortical SWA, of delta
 911 wave-locked NI neurons: **A**, delta bursting vs. delta bursting (D-bst. vs. D-bst.); **B**, non-bursting vs.
 912 delta bursting (non-bst. vs. D-bst.); **C**, non-bursting vs. non-bursting (non-bst. vs. non-bst.) and delta
 913 wave-independent NI neurons: **D**, irregular firing vs. irregular firing (irreg. vs. irreg.); **E**, regular firing
 914 vs. irregular firing (reg. vs. irreg.); **F**, regular firing vs. regular firing (reg. vs. reg.). **Top**, heatmaps
 915 showing cross-correlations of activity of NI theta bursting (T-bst.) neuron pairs. Cross-correlations are
 916 sorted by the time of the peak closest to zero-lag. Cross-correlation with significant peak values (z-
 917 score > 2.575 ; above 99% confidence level) are shown first (from top). The bar on the right indicates
 918 the proportion of pairs of neurons whose activity was correlated (light red) and uncorrelated (grey).
 919 Note, that all theta bursting neuronal pairs were significantly cross-correlated. **Middle**, times and

920 square roots of z-score values of the cross-correlograms' positive peaks (closest to zero-lag; red dots)
 921 and preceding it negative peaks (blue dots). Light grey area indicates range of insignificant z-score
 922 values (range ± 2.575 ; equivalent to $\pm 99\%$ confidence limits). **Bottom**, the median (blue line) of all
 923 cross-correlograms shown above. Light blue area indicates interquartile range.

924
 925 **Fig. 10.** Responses of different types of NI neurons to the optogenetic stimulation of the MS during
 926 hippocampal theta and SWA. **A**, Schemes illustrating the injection of viral vector into the MS and the
 927 experimental procedure conducted 3 weeks after the injection. **B**, Histological verification of the
 928 positioning of the optical fibre above the medial septum and the recording microelectrode array
 929 (MEA) in the nucleus incertus. **Top**, an example of the Dil (red) filled trace of the optical fibre
 930 terminating above the MS transfected with the viral vector (green). aca – anterior commissure,
 931 anterior part, cc – corpus callosum, Ctx – cerebral cortex, CPu – caudate putamen, LV – lateral
 932 ventricle, MS – medial septal nucleus. **Bottom**, an example of the Dil (red) filled traces of the four
 933 shanks of the MEA. Light grey spots represent electrodes positioned within the NI, whereas dark grey
 934 spots are localised outside the nucleus incertus. Nlc – nucleus incertus pars compacta, Nld – nucleus
 935 incertus *pars dissipata*, 4V – 4th ventricle, Cb – cerebellum, DTg – dorsal tegmental nucleus, mlf –
 936 medial longitudinal fasciculus. **C** and **D**, heatmaps showing the responses of different types of NI
 937 neurons to MS stimulation during cortical activation and SWA, respectively. The values of PSTHs
 938 (10ms bins; normalized to baseline) are colour coded. Vertical dotted lines indicate MS stimulation
 939 onset and offset (laser light: 473nm, <10mW; 50ms). Light blue, bright red, and gray bars to the right
 940 of the heatmaps indicate the proportion of inhibited, excited and non-responding neurons,
 941 respectively. **E** and **F**, response of selected populations of NI neurons to MS stimulation during
 942 cortical activation and SWA, respectively. Responses of theta-locked, theta-independent, theta
 943 bursting, delta-wave-locked and delta wave-independent populations of NI neurons are shown. Blue
 944 line – median PSTH value; light blue area indicates interquartile range; cyan bar indicates the
 945 stimulation time. **G**, Pie chart showing the proportions of response types of NI neurons whose

946 responses to MS stimulation were observed in both cortical states (blue: inhibition, red: excitation,
947 gray: no response).

948

949

950 **Fig. 11.** **A**, scatter plots (**top**) and histograms (**bottom**) showing the exemplary response of theta-
951 bursting NI neuron to an optogenetic stimulation at three different theta frequencies (4, 8 and 12
952 Hz). Note that in all cases the neuron reacted with inhibition followed by a rebound excitation. **B**,
953 responses of the same NI theta-bursting neuron to high-frequency stimulation of the MS (20 Hz).
954 Representative fragments of the raw signal and separated spikes (red) of the recorded NI neuron. **C**,
955 histograms of interspike intervals (**top**) and autocorrelation function plots (**bottom**) of exemplary
956 theta-bursting NI neuron (shown on **A** and **B**) before (**left**), during (**middle**; marked with light blue
957 colour) and after (**right**) the high frequency, optogenetic stimulation of the MS. Cyan coloured bars
958 indicate the laser light (473nm; <10 mW) pulses. Bins (all histograms) = 10 ms.

959

960 **Fig. 12.** *Ex vivo* responses of the NI neurons to optogenetic stimulation of the medial septum (MS)
961 originating axonal endings. **A**, schematic representation of the experimental design showing viral
962 vectors injection procedure and subsequent MS axonal endings optogenetic stimulation and whole-
963 cell voltage clamp recording of the NI neurons. **B**, Reconstruction of the location of biocytin filled NI
964 neurons in which iPSCs (blue circles), ePSCs (red circle) or no change (grey circles) in response to
965 optogenetic stimulation of the MS originating axonal endings were recorded. **C**, Average ($\pm$ SD) iPSC
966 (20 stimulations) recorded from the NI neuron following optogenetic stimulation of MS axonal
967 endings at command potential of -50 mV and its disappearance at -90 mV. **D**, Average ($\pm$ SD) iPSC
968 (20 stimulations) recorded from the NI neuron following optogenetic stimulation of MS axonal
969 endings under control conditions (sACSF), its complete disappearance in the presence of GABA_A
970 receptor antagonist - gabazine (5 μ M) and partial return after drug washout. **E**, Distribution of iPSCs'

971 amplitudes in the control conditions (sACSF), during gabazine application and washout (n = 7). Cyan
 972 coloured bars indicate the laser light pulses (465 nm; 5 ms, 10 s interval; <10 mW).

973

974 **Fig. 13.** Identification of nucleus incertus neurons innervating the medial septum. **A**, schemes
 975 illustrating the injection of retrograde viral vector into the MS and the experimental procedure
 976 conducted 3 weeks after the injection. **B**, fragments of raw signal showing responses to different
 977 optogenetic stimulation protocols: single pulse lasting 50 ms (**top**) and short, 5ms pulses at
 978 increasing frequencies (10, 30 and 50 Hz). **C**, overlaid averaged spike waveforms from the baseline
 979 (black) and from the periods of light stimulation (cyan). **D**, histogram of interspike intervals (**left**) and
 980 autocorrelation function plot (**right**) of exemplary theta phase-independent, regular, fast firing NI
 981 neuron that was optogenetically identified as MS projecting (shown on **B** and **C**). Bins = 10ms. Cyan
 982 coloured bars indicate the laser light pulses (473 nm; <10 mW).

983

984 **Fig. 14.** The postulated nucleus incertus contribution to hippocampal theta rhythm generation. **A**, a
 985 simplified diagram showing the nuclei of the septohippocampal system, the nucleus incertus and
 986 their interconnections. The abbreviations inside the ovals indicate the cell type: ACh – cholinergic,
 987 GABA – GABAergic, Glu – glutamatergic within medial septum/vertical limb of the diagonal band
 988 nucleus (MS/DBv), PC – pyramidal cell, BASK – basket cell, OLM – oriens-lacunosum-moleculare cell
 989 within the hippocampus and Fast Reg – regular, fast-firing theta phase-independent, Slow Irreg –
 990 irregular, slow-firing theta phase-independent, BST – bursting, theta phase-locked, non-BST – non-
 991 bursting, theta phase-locked within the nucleus incertus. The arrows endings indicate excitatory and
 992 the perpendicular endings indicate inhibitory connections. **B**, Idealised hippocampal theta oscillation
 993 observed in the stratum lacunosum moleculare of the hippocampal CA1 region and the schematic
 994 temporal distribution of firing of theta-bursting neurons in: medial septum (blue and green bars),
 995 nucleus incertus (purple bars), hippocampal OLM cells (orange bars) and hippocampal basket cell
 996 (cyan bars).

Tab. 1. Firing of different types of NI neurons during state of cortical activation.

		median FR [Hz]	1 st ; 3rd quartile [Hz]	Mann-Whitney test	CV	1 st ; 3rd quartile	Mann-Whitney test
cortical activation	Tph-indep.	irregular, slow-firing (n = 71)	7.7	5.2; 17.6	p < 0.0001* U = 543.0	0.68	0.51; 0.9
		regular, fast-firing (n = 46)	26.5	16.0; 37.4		0.19	0.17; 0.21
	Tph-locked	bursting (n = 55)	42.6	27.5; 59.7	p < 0.0001* U = 39.0	1.1	0.9; 1.34
		non-bursting (n = 25)	5.5	4.5; 11.6		0.8	0.7; 1.0

Tab. 2. Firing of different types of NI neurons during state of cortical SWA.

		median FR [Hz]	1 st ; 3rd quartile [Hz]	Mann-Whitney test	CV	1 st ; 3rd quartile	Mann-Whitney test
cortical SWA	Dw.-indep.	irregular, slow-firing (n = 118)	6.5	3.0; 12.6	p < 0.0001* U = 317.0	1.0	0.84; 1.4
		regular, fast-firing (n = 26)	29.4	24.1; 35.2		0.17	0.15; 0.19
	Dw.-locked	bursting (n = 20)	20.8	17.4; 33.1	p < 0.0001* U = 106.0	1.1	1.0; 1.2
		non-bursting (n = 33)	9.0	3.8; 15.5		0.9	0.76; 1.1

Tab. 3. Parameters of NI neurons' responses to the medial septum optogenetic stimulation.

		response type	fraction (count)	FR change [%]	latency to max [ms]	duration [ms]
cortical activation	Tph-locked	bursting (n = 7)	inhib	86% (6)	-45 (-53; -41)	42.5 (35; 85.3)
			excit	0%	-	-
			no resp.	14% (1)	-	-
		non-bursting (n = 5)	inhib	40% (2)	-65	57
			excit	40% (2)	76	46
			no resp.	20% (1)	-	-
	Tph-indep.	irregular, (n = 16)	inhib	31% (5)	-58 (-71; -25)	68 (34.5; 91)
			excit	25% (4)	63 (43; 99)	51.5 (47.8; 102.5)
			no resp.	44% (7)	-	-
		regular, (n = 16)	inhib	25% (4)	-12 (-32; -5)	38.5 (25.3; 51.8)
			excit	19% (3)	25 (11; 33)	29 (23; 46)
			no resp.	56% (9)	-	-
cortical SWA	Dw.-locked	bursting (n = 4)	inhib	75% (3)	-74 (-74; -54)	92 (48; 109)
			excit	25% (1)	74	63
			no resp.	0%	-	-
		non-bursting (n = 4)	inhib	0%	-	-
			excit	50% (2)	77	40.5
			no resp.	50% (2)	-	-
	Dw.-indep.	irregular, (n = 20)	inhib	25% (5)	-63 (-75; -33)	46 (38; 56)
			excit	15% (3)	104 (102; 106)	54 (36; 123)
			no resp.	60% (12)	-	-
		regular, (n = 9)	inhib	44% (4)	-17 (-20; -7)	48 (30.8; 63)
			excit	11% (1)	14	30
			no resp.	45% (4)	-	-

A - theta phase-independent NI neuron

B - theta phase-locked NI neuron

C - theta phase-independent NI neurons

D - theta phase-locked NI neurons

theta phase-independent NI neurons: 59% (n=117)

theta phase-locked NI neurons: 41% (n=80)

Theta phase-locked NI neurons

Theta phase-independent NI neurons

A - delta wave-independent NI neuron

B - delta wave-locked NI neuron

C - delta wave-independent NI neurons

D - delta wave-locked NI neurons

delta wave-independent NI neurons: 73% (n=144)

delta wave-locked NI neurons: 27% (n=53)

Delta wave-locked NI neurons

Delta wave-independent NI neurons