Coordinated electrical activity in the olfactory bulb gates the

oscillatory entrainment of entorhinal networks in neonatal mice

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ABSTRACT

While the developmental principles of sensory and cognitive processing have been

extensively investigated, their synergy has been largely neglected. During early life, most

sensory systems are still largely immature. As a notable exception, the olfactory system

reaches full maturity during intrauterine life, controlling mother-offspring interactions and

neonatal survival. Here, we elucidate the structural and functional principles underlying the

communication between olfactory bulb (OB) and lateral entorhinal cortex (LEC) – the

gatekeeper of limbic circuitry – during neonatal mouse development. Combining

optogenetics, pharmacology, and electrophysiology *in vivo* with axonal tracing, we show that

mitral cell-dependent discontinuous theta bursts in OB drive network oscillations and time the

firing in LEC via axonal projections confined to upper cortical layers. Pharmacological

silencing of OB activity diminishes entorhinal oscillations. Moreover, odor exposure boosts

OB-entorhinal coupling at fast frequencies. Thus, early OB activity shapes the maturation of

entorhinal circuits.

<u>**Running title:**</u>

Olfactory control of entorhinal development

<u>**Keywords:**</u>

Development, olfactory, entorhinal, oscillations, optogenetics, mitral cells, connectivity

INTRODUCTION

Coordinated patterns of electrical activity periodically entrain developing neuronal networks

in rhythms with a broad frequency spectrum. These patterns have been proposed to critically

shape brain maturation (1-3). Experimental evidence supporting this hypothesis has been

mainly provided for sensory systems. For example, in the visual and auditory systems,

spontaneous activity from sensory periphery (i.e., retina or cochlea) controls the formation of

cortical representations underlying stimulus perception (4, 5). Theta band spindle bursts and

gamma oscillations in the developing somatosensory system promote thalamo-cortical

connectivity and maturation of coupling with the motor system (6, 7). Overall, the

discontinuous oscillatory activity in sensory cortices during development has multifold origin,

including stimulus-independent activation in the periphery and entrainment of local cortical

circuits via chemical and electrical synapses (1, 8).

While less investigated, limbic circuits show similar patterns of coordinated activity

during early development, with discontinuous theta bursts (4-12 Hz) and superimposed fast

episodes (20-40 Hz) in beta-gamma frequency (9-13). Theta bursts facilitate unidirectional

communication from the CA1 area of intermediate/ventral hippocampus (HP) to the prelimbic

subdivision of the prefrontal cortex (PFC) via glutamatergic projections (14). As a

consequence of hippocampal theta drive, pyramidal neurons in local prelimbic circuits

generate beta-low gamma oscillations (15). Theta coupling between neonatal PFC and HP is

controlled by the lateral entorhinal cortex (LEC) that densely projects to both areas (11). The

complex organization of limbic circuits at early age raises the question, which mechanisms

control the gatekeeper function of LEC during early development. Similar to sensory

systems, the neonatal LEC could be driven by spontaneous activity from the sensory

periphery. Indeed, the adult LEC receives direct input from the olfactory bulb (OB) that, in

contrast to other sensory systems, bypasses the thalamus (16, 17). Mitral and tufted cells

(MTCs) represent the sole OB output neurons. Rather than simply relaying information, these

neurons are embedded in a complex network that controls odor information coding (18, 19).

The axons of MTCs terminate in entorhinal layer I on apical dendrites of layer II/III pyramidal

and stellate cells (20), which in turn form the perforant path projection to the hippocampal

formation (21, 22). Layer II/III neurons in LEC project back to OB (23), yet distinct entorhinal

populations are differently engaged in feedforward and feedback signaling during odor

processing (24). Thereby, odor-evoked activity in the adult controls the gateway function of

LEC interfacing HP and neocortical regions (25, 26).

While the sense of smell serves fundamental functions in newborn animals (27), the

role of olfactory inputs and OB activity for limbic circuit maturation remains unknown. Since

other sensory systems are still immature during early life – and thus their impact on limbic

circuits is negligible – this knowledge gap appears even more striking. Rodent pups are

blind, deaf and have limited sensorimotor abilities until the end of the second postnatal week

(28, 29). In contrast, the olfactory system maturates early and is considered to be fully

functional at birth, providing the major sensory stimulus in neonatal rodents. We hypothesize

that both odor-dependent and -independent coordinated activity in OB control the

entrainment of entorhinal networks during neonatal development. Here, we combine

optogenetics, electrophysiology, and pharmacology *in vivo* with anatomical tracing in

neonatal mice (postnatal day (P) 8-10) to elucidate the olfactory control of the functional

maturation of entorhinal circuits.

RESULTS

OB and LEC are reciprocally connected in neonatal mice

In mice, MTCs mature during intrauterine life and their axons reach cortical targets during the first two postnatal weeks (30). This time window coincides with the period of strong gating of prefrontal-hippocampal networks by theta activity in LEC. To detail on the spatial patterns of connectivity between OB and LEC in P8-10 mice, we performed an in-depth investigation of axonal projections from MTCs to LEC and, *vice versa*, of entorhinal projections to OB. First, we used Tbet-cre;R26-tdTomato mice (n=4) for intact-brain imaging of long-range projections by electrophoretic tissue clearing and confocal fluorescence microscopy (Fig 1A, B). In these mice, MTCs are genetically tagged (Fig 1C) (31). Already at P8, the lateral olfactory tract (LOT) comprising MTC axons appeared fully developed and reached the posterior part of the cerebrum, including piriform cortex (PIR) and LEC (Fig 1A, D). As previously shown in adult rats (32), MTC axons were mainly confined to layer I of neonatal LEC (Fig 1D). Retrograde tracing with Fluorogold (FG) injected into the LEC of P3-4 mice confirmed the direct connectivity (Fig 1E). No differences between dorsal and ventral OB were detected with respect to the density of MTC projections to LEC.

Second, we assessed the spatial organization of feedback projections from LEC to OB. Unilateral injection of FG confined to OB of P3-4 mice (n=12) led to bright fluorescent back-labeling of parental cell bodies in ipsilateral LEC that project to OB of P8-10 mice (Fig 1F). Their density was lower when compared to the cells detected in ipsilateral PIR. Most labeled neurons were located in layer II and III (88.40%, 259/293, 3 pups, 11 sections). To examine the neurochemical identity of entorhinal neurons projecting to OB, we counter-stained the FG-labeled neurons for GABA and CamKII. While most OB-projecting neurons (99.66%, 292/293) were negative for GABA, hence glutamatergic, a small fraction 0.34%, 1/293) was GABA-positive. Similarly, CamKII staining revealed that the large majority, but not all FG-labeled cells, were glutamatergic (data not shown). These data indicate that top-down projections from LEC to OB can be either excitatory or inhibitory, as recently described for adult mice (33).

Taken together, the results of morphological investigation show that afferent and

efferent projections couple neonatal LEC and OB. While glutamatergic MTCs axons target

entorhinal layer I, glutamatergic and few GABAergic neurons in superficial layers of LEC

innervate the OB.

Continuous respiration-related activity and discontinuous theta bursts entrain the

neonatal OB

Despite abundant data on morphological development, the functional maturation of OB is still

largely unknown. In contrast to the retina and cochlea, which lack stimulus sensitivity at early

stages of postnatal development and only generate spontaneous activity, the OB processes

olfactory input already at birth (34). To elucidate the patterns of activity in the neonatal OB,

we performed multi-site extracellular recordings of local field potential (LFP) and multiple unit

activity (MUA) from the mitral cell layer in the dorsal and ventral OB of P8-10 mice *in vivo*

(n=49). Signal reversal between the internal plexiform layer (IPL) and external plexiform layer

(EPL), as well as the large MTC spikes served as physiological markers for confirming the

position of recording electrode set according to stereotaxic coordinates. In addition, the

location of DiI-labeled electrodes was confirmed after histological investigation *post mortem*

(Fig 2A, S1 Fig A, B).

Two patterns of coordinated activity were detected in OB (Fig 2B, C). First, we

recorded continuous low amplitude oscillations with slow frequency peaking at 2-4 Hz. Given

their temporal correlation and frequency overlap with respiration (median frequency: 2.37 Hz,

iqr: 2.12-2.70 of chest movements) (Fig 1C, D), we defined this activity as respiration-related

rhythm (RR). The RR reversed at the level of MTC layer and had larger amplitudes in EPL

and glomerular layer when compared to the activity in MTC layer (data not shown). Its

temporal relationship to the phase of the respiratory cycle differed between layers; the peak

of RR cycle in the granule cell layer (GCL) and its trough in EPL and glomerular layer

correlated with exhalation. Second, we recorded discontinuous high amplitude oscillatory

events with spindle shape in the neonatal OB (Fig 2B, C). These events had faster

frequencies when compared to RR with a peak within theta frequency band (4-12 Hz) (Fig

2D). Given their resemblance in shape and frequency dynamics to previously characterized

oscillatory events in neonatal cortical areas (9-11, 35), these events were classified as theta

bursts.

As reported for adult OB, prominent spiking characterized neonatal MTCs. Analysis of

single unit activity (SUA) after principal component analysis (PCA)-based sorting of units

revealed that the majority (80%) of spikes occurred during theta bursts. The firing rate during

bursts (median: 1.36 Hz, iqr: 0.25-3.23 Hz) was significantly (p=3.65x10^{-7}, Wilcoxon signed-

rank test, n=34 cells from 14 animals) augmented when compared to non-bursting periods

(median: 0.44 Hz, iqr: 0.09-1.48 Hz) (Fig 2E). To assess the temporal relationship between

oscillatory OB rhythms and MTC firing, we estimated the coupling strength between SUA and

RR as well as between SUA and theta bursts by calculating the pairwise phase consistency

(PPC), a bias-free measure of rhythmic neuronal synchronization (36). Both rhythms similarly

timed MTC firing (RR: median PPC: 0.21, iqr: 0.20-0.22 vs. theta burst: median PPC: 0.21,

iqr: 0.20-0.21., p=0.1664, Wilcoxon signed-rank test, 2 outliers removed, n=32 cells, Fig 2F).

In adults, dorsal and ventral OB subdivisions have distinct physiology and function.

MTC axons that originate in the dorsal OB are known to strongly project to amygdala and

mediate innate odor responses, whereas ventral OB accounts for processing of learned

odorants (37, 38). To assess whether distinct activity patterns entrain the dorsal vs. ventral

OB at neonatal age, we compared RR and theta bursts from both subdivisions (S1 Fig). The

power of RR was similar in both sub-divisions (dorsal: median 233.12 µV², iqr: 153.42-

418.09, n=7; ventral: median 335.75 µV², iqr: 195.91-452.52, n=10; p=0.54, Wilcoxon rank-

sum test). Similarly, theta burst occurrence (dorsal: median 4.65 bursts/min, iqr: 3.87-5.55;

ventral: median 5.09 bursts/min, iqr: 4.27-5.30, p=0.74, Wilcoxon rank-sum test), duration

(dorsal: median 6.76 s, iqr: 4.44-8.86 s; ventral: median 3.54 s, iqr: 1.65-5.01 s; p=0.09,

Wilcoxon rank-sum test), amplitude (dorsal: median 73.80 µV, iqr: 59.56-75.37; ventral:

median 66.59 µV, iqr: 59.10-72.37; p=0.67, Wilcoxon rank-sum test) and relative power

(dorsal: median 493.98 Hz, iqr: 430.71-763.00; ventral: median 452.63 Hz, iqr: 395.3-1071.6,

p=0.96, Wilcoxon rank-sum test) were comparable across OB subdivisions. These data

indicate that the dorsal and ventral OB show similar activity at early postnatal age. Except

otherwise indicated, further investigation focused on the ventral OB subdivision, taking into

account its role for learning processes in relation with the limbic system (38).

Coordinated patterns in the sensory periphery have been reported to critically depend

on the brain state, diminishing or even disappearing in the presence of anesthetics (39, 40).

In contrast, early oscillations in the developing brain have often been investigated in the

presence of urethane anesthesia (9, 35, 41, 42). Rodent pups spend most of the time

sleeping. The sleep-mimicking action of urethane might explain the similar patterns of

neuronal activity previously observed in anesthetized and sleeping rodent pups (14, 43). To

assess the urethane influence on RR and theta bursts, we recorded from both ventral (n=12)

and dorsal OB (n=6) of neonatal mice before and after urethane injection. Anesthesia did not

change the overall structure of OB activity, with continuous RR and discontinuous theta

bursts persisting (S2 Fig A, S1 Table). Both the power of RR and the occurrence of theta

bursts remained unchanged (S2B Fig). However, urethane anesthesia profoundly reduced

theta burst duration (S2B Fig), augmenting those time windows lacking theta band activity

and therefore, the fragmented appearance of neonatal activity in OB (S2B Fig).

These data indicate that, independent of OB subdivision and brain state, the neonatal

OB shows two main patterns of early oscillatory activity, continuous RR activity and

discontinuous theta bursts.

Mechanisms underlying the generation of continuous and discontinuous oscillatory

activity in the neonatal OB

To elucidate the mechanisms contributing to the generation of continuous RR and

discontinuous theta bursts in the OB of neonatal mice, we used two experimental

approaches. First, the temporal coupling between respiration and continuous 2-4 Hz

oscillations in OB suggests that nasal air flow contributes to RR generation. To test this

hypothesis, we reduced the nasal air flow by unilateral naris occlusion in P8-10 pups (n=12)

using a previously developed protocol (44, 45). MUA and oscillatory activity of OB were

recorded before and after naris occlusion with silicon adhesive (data not shown). While

unilateral deprivation did not change the overall structure of OB activity patterns, it reduced

the RR power from 396.05 µV² to 293.30 µV² (baseline: iqr 232.58-570.88 µV²; occlusion: iqr

136.10-410.14 µV², p=0.0009, Wilcoxon signed-rank test). By contrast, the theta bursts in OB

were not affected by naris occlusion (baseline: median: 643.45 Hz, iqr: 342.6-1009.7;

occlusion: median 700.35 Hz, iqr 284.5-1240.8; p=0.91, Wilcoxon signed-rank test).

Correspondingly, the firing rate during RR (baseline: median 1.18 Hz, iqr 0.26-2.45) as well

as coupling strength (i.e. PPC) between units and RR (baseline: median 8.50×10^{-4}, iqr 0-

0.0084, one outlier removed) decreased after naris occlusion (firing rate: occlusion: median

0.75 Hz, iqr 0.25-1.91, p=0.021, Wilcoxon signed-rank test; coupling strength: occlusion:

median -3.09×10^{-5}, iqr $-2.76^{\times 104}-1.56 \times 10^{-4}$; p=0.049, Wilcoxon signed-rank test, one outlier

removed). The temporal structure (coupling strength for baseline: median 2.09×10^{-4}, iqr: -

0.0001-0.0015; occlusion: median -1.19×10^{-4}, iqr $-3.54 \times 10^{-4}-1.59 \times 10^{-4}$; p=0.19, Wilcoxon

signed-rank test, one outlier removed) of OB firing in relationship with theta bursts remained

unchanged after naris occlusion. Thus, RR activity, but not theta bursts critically depends on

nasal air flow.

The second experimental approach aimed at assessing the role of MTCs, the OB

projection neurons, to the generation of coordinated patterns of oscillatory activity. For this,

we selectively manipulated MTC firing by light in P8-10 pups bred from crossing hemizygous

Tbet-cre mice with R26-homozygous R26-ArchT-EGFP mice. By these means, MTCs of cre-

positive mice selectively expressed the proton pump ArchT fused with EGFP. Already at P8,

the fusion protein expression was robust both in MTC somata (S3A Fig) and axonal

projections targeting LEC, PIR and posterior cortical amygdala (Fig 3A). Cre-negative mice

were used as controls.

In a first experiment, we tested the efficiency of light-dependent MTC silencing in

neonatal OB by performing whole-cell patch-clamp recordings from biocytin-filled EGFP-

positive neurons (n=7 cells) in coronal slices containing the OB of P8-10 R26-heterozygous

Tbet-cre;R26-ArchT-EGFP mice (n=5) (S3A Fig). Yellow light pulses (595 nm, 5 s, 0.2-0.6

mW) triggered MTC hyperpolarization from -49.96 mV to -58.39 mV (baseline: iqr -57.28-

45.61 mV; light administration: iqr -63.10-48.89 mV, Wilcoxon signed-rank test, p=0.0078)

and, consequently, inhibition of firing (S3B Fig). Since MTCs are strongly interconnected

within local circuits, we tested whether light pulses caused MTC silencing also in the

presence of synaptic inputs. To mimic such inputs, we paired the light stimulation with

depolarizing current pulses of different intensities. Upon injections ≤ 60 pA, light stimulation

still efficiently blocked action potential discharge in ArchT-EGFP-expressing MTCs (S3C

Fig).

Next, we assessed the contribution of MTC firing to the patterns of oscillatory activity

in OB by performing extracellular recordings of LFP and MUA in OB of P8-10 R26-

heterozygous cre-positive (n=12) and cre-negative (n=11) Tbet-cre;ArchT-EGFP mice. Upon

in vivo light stimulation (Fig 3A), the majority (64.58%, 31/48) of MTCs responded with a

pronounced firing rate decrease from a median of 1.2 Hz (iqr 0.66-2.26) before to 0.45 Hz

(iqr 0.13-0.99) during light exposure. None of the units augmented the firing during

illumination and only few units (4.14%, 2/48) showed a post-stimulus firing increase (Fig 3D).

Some units (31.25%, 15/48), most likely non-MTCs located close to the mitral cell layer, did

not respond to light stimulation. Local silencing of MTCs modified the coordinated activity of

OB. The properties of RR and theta bursts (theta burst power: p=0.23, Wilcoxon rank-sum

test) were largely similar in cre-negative and cre-positive mice under control conditions (i.e.

no light stimulation). Only the power of RR activity was slightly different (RR power: p=0.03,

Wilcoxon rank-sum test). Upon light stimulation the RR power in cre-positive pups did not

change (pre stimulus: median 92.27 μV^2, iqr 80.12-122.36; during stimulus: median 86.99

μV^2, iqr 71.96-100.06, p=0.2324, Wilcoxon signed-rank test, one outlier removed). In

contrast, theta power in cre-positive pups significantly decreased during light stimulation (pre

stimulus: median 89.73 μV^2, iqr 57.28-100.73; during stimulus: median 70.58 μV^2, iqr 45.70-

87.91, p=0.0049, Wilcoxon signed-rank test, one outlier removed). The theta responses to

light differed between cre-positive (median 0.84 μV^2, iqr 0.81-0.89) and cre-negative pups

(median 0.99 μV^2, iqr 0.93-1.20, p=0.0024, Wilcoxon rank-sum test, 3 outliers from

expression group removed), whereas for RR during light stimulus was similar in the two

groups (cre-positive, median 0.91, iqr 0.82-1.0; cre-negative pups median 0.96, iqr 0.93-1.05,

p= 0.3447, Wilcoxon rank-sum test, 1 outlier from control group, 2 outliers from expression

group removed) (Fig 3E, F).

These data show that RR and theta bursts in the neonatal OB have different origin.

While RR critically depends on nasal air flow, MTC activity is necessary for the entrainment

of OB in theta bursts.

Theta bursts in OB drive discontinuous oscillations and time the firing in the neonatal

LEC

The presence of both direct axonal MTC-to-LEC projections and early patterns of oscillatory

activity in OB led to the question of their relevance for the emergence of functional

assemblies in the neonatal LEC. In contrast to the documented relevance of entorhinal

output for developing limbic circuits (11), the role of sensory inputs for the functional

maturation of LEC is still unknown.

Multi-site extracellular recordings of LFP and MUA from the layer II/III of LEC from

P8-10 mice *in vivo* (n=11) (Fig 4A) confirmed the previously reported presence of

discontinuous theta bursts with large amplitude (median 154.14 µV, iqr 101.10-191.65) and a

duration of 5.15 s (iqr 4.13-8.48) (Fig 4B-D). They appear superimposed on a slow rhythm

(2-4 Hz) that continuously entrains the neonatal LEC and has been overlooked in previous

investigations. This slow pattern of activity that was present both during theta bursts (median

area power 526.25 μV^2, iqr 307.68-1171.85) and "silent" periods (median area power 86.57

μV^2, iqr 52.55-344.43), temporally correlated with the simultaneously recorded respiration

and was therefore, classified as entorhinal RR. These results demonstrate that the

respiration-entrained brain rhythms, a powerful mechanism of long-range coupling (46),

emerge early during development. Beside oscillatory patterns, neonatal LEC generates

prominent firing concentrated during theta bursts (median 0.42 Hz, iqr 0.22-0.86 vs. non-

bursting periods median 0.07 Hz, iqr 0.04 – 0.19, $p=1.72 \times 10^{-10}$, Wilcoxon signed-rank test,

n=54 cells from 11 mice) (Fig 4E). We next assessed the coupling strength between firing

and oscillatory activity. Similar fractions of entorhinal neurons were phase-locked to RR

(75.93%, 41/54 units) and theta bursts (61.11%, 33/54 units, p=0.1, $\chi^2(1)$=2.7472). The

strength of coupling assessed by PPC was also stronger for RR (median: 0.21, iqr: 0.20-

0.22) and theta (median: 0.21, iqr: 0.20-0.21, p=2.98x10^{-4}, Wilcoxon rank-sum test, 4 outliers

removed, n=50 units), with most cells being locked to the trough of RR and theta oscillation

(Fig 4F).

Simultaneous recordings from OB and LEC (n=9) of neonatal mice gave first insights

into their dynamic coupling (Fig 5A). While both areas showed similar oscillatory activity, their

power significantly differed. Both RR power (OB: median 143.62 μV^2, iqr 78.03-247.78; LEC:

median 109.91, iqr: 26.72-110.51, p=0.0499, Wilcoxon signed-rank test, 1 outlier removed)

and theta power (OB: median 193.39 μV^2, iqr 97.47-262.01, LEC: median 112.37 μV^2, iqr

43.38-127.36, p= 0.0273) were higher in OB as compared to LEC (Fig 5B). Analysis of the

temporal correspondence of theta bursts in OB and LEC revealed that 48.70% of them co-

occurred with more than 60% temporal overlap. The coupling strength assessed by

imaginary spectral coherence, which excludes synchrony effects due to volume conductance

(47), revealed that the OB-LEC coupling is evident in both slow frequencies (i.e RR) and

theta band (i.e. theta bursts) (Fig 5C). In line with anatomical data, we detected no

differences in the coupling of dorsal and ventral OB with LEC. Both relative occurrence of co-

occurring events (dorsal: median 27.04 %, iqr 20.08–33.98 %; ventral: median 21.83 %, iqr

15.66-35.14 %; p=0.67, Wilcoxon rank-sum test) and mean imaginary coherence in both RR

(dorsal: median 0.11 Hz, iqr 0.09-0.14 Hz; ventral: median 0.08 Hz, iqr 0.05-0.10 Hz; p=0.13,

Wilcoxon rank-sum test) and theta frequency range (dorsal: median 0.07 Hz, iqr 0.06-0.11

Hz; ventral: median 0.06 Hz, iqr 0.06- 0.09 Hz; p=0.54, Wilcoxon rank-sum test) were similar

for dorsal and ventral OB in relationship to LEC (S1 Fig). These data are in line with

anatomical investigations in adult mice (48) as well as with our tracing data (Fig 1), showing

that, FG injections into neonatal LEC leads to homogenous MTC labeling throughout the OB.

To assess the influence of anesthesia on entorhinal activity patterns and coupling

between LEC and OB, we recorded both areas in mouse pups before and after urethane i.p.

injection (n=18). Urethane did not change the overall spectral distribution of activity patterns

in LEC. As in the non-anesthetized state, RR and theta bursts were the main patterns of

entorhinal activity, yet the RR power decreased and theta power augmented under urethane

action (S4A Fig, S1 Table). Urethane affected the duration of theta bursts and slightly

increased their occurrence (S4B Fig). The synchrony between OB and LEC varied in

magnitude, but not frequency distribution. The imaginary coherence peaked at 2-4 Hz and at

5 to 20 Hz, corresponding to RR and theta-beta frequencies, respectively. While mean RR

coherence did not differ between states (p=0.17, Wilcoxon rank-sum test, 2 outliers

removed), theta coherence was higher in the presence of urethane (p=0.0034).

These data indicate that, independent of brain state and anatomical subdivision, OB

and LEC couple tightly, both being synchronized in continuous RR and discontinuous theta

oscillations at neonatal age.

Since feed-forward projections from MTCs to LEC are dense, whereas feed-back

projections from LEC to OB are rather sparse, we asked whether the functional coupling

between the two areas is directed, and if so, whether directionality is frequency-specific. To

estimate the directionality of OB-LEC coupling, we used two approaches. First, we assessed

the phase lag between LFP in OB and LEC. While the phase lag for continuous RR was

centered to 0, it peaked in negative range for theta bursts, indicating that OB theta bursts

most likely drive LEC theta oscillations (Fig 5D). Second, we analyzed the temporal

relationship between spiking activity in one area and either LFP or spiking in the other area.

For RR, a similar number of clustered units in OB and LEC were phase-locked to RR in LEC

(31.48%, 17/54) and OB (25.27%, 23/91 p=0.54, χ^2(1)=0.38, χ^2 test of proportions),

respectively and their coupling strengths were comparable (p=0.35, Wilcoxon rank-sum test,

OB cells to LEC RR: median 0.21, iqr 0.19-0.23; LEC cells to OB RR: median 0.22, iqr 0.19-

0.28, Fig 5E). In contrast, a significantly higher fraction of LEC neurons were phase-locked to

theta bursts in OB (42.47%, 31/73) when compared to OB neurons timed by entorhinal theta

phase (10.20%, 5/49, p=1.28x10-4, χ^2(1)=14.67, χ^2 test of proportions, Fig 5E). The coupling

strengths of these neuronal populations, however, were comparable (p=0.44, Wilcoxon rank-

sum test, OB cells to LEC theta: median: 0.22, iqr: 0.19-0.24; LEC cells to OB theta: median:

0.23, iqr: 0.20-0.26, one outlier removed from OB theta-LEC units group). Notably, MTCs

preferentially fire during the trough of RR activity and theta bursts in OB, whereas LEC cells

preferentially fire on the rising phase after the trough of OB rhythms, indicating that MTC

firing precedes LEC cell firing by about a third of a cycle (Fig 5F, G).

Together, these data suggest that the continuous RR rhythm is not involved in

directed information flow within OB-LEC circuits, whereas theta bursts in OB drive the

oscillatory entrainment of LEC.

Pharmacological blockade of OB firing diminishes the slow and fast oscillatory

activity but not the coupling of OB-LEC circuits

To confirm the functional long-range coupling between OB and LEC, we pharmacologically

abolished the neuronal activity by unilateral pressure-injection of the voltage-dependent

sodium channel (hence action potential) blocker lidocaine (4% in sterile saline) into OB.

Extracellular recordings of LFP and MUA were performed simultaneously from OB and LEC

of mice (n=8) before and after lidocaine injection *in vivo* (Fig 6A). The injected lidocaine

volume of 4 µl was proven to not spread across the borders of OB (Fig 6B). Lidocaine

abolished OB firing within ten minutes of injection from a median baseline firing rate of 1.97

Hz (iqr: 0.77-2.80) to 0.00 Hz (iqr: 0.00-0.02). A partial recovery was observed after 30-40

min ($\chi^2(7)$=45.04, p=1.34x10^{-7}, Friedman test, with Wilcoxon signed-rank post hoc test with

Bonferroni correction) (Fig 6D). The firing of entorhinal neurons was also significantly

reduced after lidocaine treatment in OB from a median baseline firing rate of 2.4 Hz (iqr 1.46-

3.60) to 0.52 Hz (iqr 0.32-1.09) within the first 10 minutes after injection ($\chi^2(7)$=135.50,

p=4.45x10^{-26}, Friedman test, with Wilcoxon signed-rank post-hoc test with Bonferroni

correction). The decrease of firing rates in both areas was accompanied by changes of

oscillatory network activity. In OB, the power of RR (baseline: median 91.05 µV^2; iqr 70.66-

224.40; lidocaine: median 8.88, iqr 3.90-20.16; p=0.0078, Wilcoxon signed-rank test) as well

as the occurrence (baseline: median 4.76 bursts/min, iqr 3.58-5.93; lidocaine: 1.03

bursts/min, iqr 0.76-1.56, p=0.0234, Wilcoxon signed-rank test), duration (baseline: median

4.41 s, iqr 3.78-4.77; lidocaine: median 2.35, iqr 1.89-2.88, p=0.0078, Wilcoxon signed-rank

test) and power (baseline: median 202.07 µV^2, iqr 163.62-261.95; lidocaine: median 33.19,

iqr 24.14-109.00. p=0.0156, Wilcoxon signed-rank test) of theta bursts were reduced. In LEC,

the power of RR (baseline: median 55.88 µV^2, iqr 42.48-134.05; lidocaine: median 17.71, iqr

9.07-53.80, p=0.0391, Wilcoxon signed-rank test) as well as the duration (baseline: median

4.41 s, iqr 3.74-4.58; lidocaine: median 3.24, iqr 3.14-3.56, p=0.0156, Wilcoxon signed-rank

test, one outlier removed) and power (baseline: median 148.11 µV^2, iqr 115.76-191.62;

lidocaine: median 75.50, iqr 65.99-116.63, p=0.0313, Wilcoxon signed-rank test, 2 outliers

removed) of theta bursts were decreased after blockade of OB firing. While the coordinated

activity substantially diminished in OB and LEC, the coupling between both areas was not

affected by lidocaine. Coherence neither changed in slow frequencies (baseline: median

0.08, iqr 0.05-0.11; lidocaine: median 0.14, iqr 0.08-0.24, p=0.25, Wilcoxon signed-rank test)

nor in theta frequency band (baseline: median 0.11, iqr 0.07-0.16; lidocaine: median 0.14, iqr

0.10-0.18, p=0.54, Wilcoxon signed-rank test) after blocking OB firing (Fig 6E).

These data indicate that blocking of neuronal firing in OB causes massive

diminishment of coordinated activity in both OB and LEC but does not change the coupling

strength between the two regions.

Odors boost the oscillatory activity in neonatal OB and LEC and augment their fast

frequency coupling

In contrast to other sensory systems that lack peripheral sensitivity for environmental stimuli

during early postnatal development, the olfactory system is functional at birth. Therefore, the

characterized coordinated patterns of oscillatory activity, RR and theta bursts, might have a

dual origin, i.e. resulting from both spontaneous and/or stimulus-evoked activation of OB

neurons. To gain first insights into the relevance of environmental stimuli on oscillatory

activity and long-range entrainment of OB and LEC, we exposed P8-10 mouse pups to

different odors. Prominent oscillatory discharge with slow and fast frequencies as well as

MUA were induced in OB by olfactometer-controlled exposure to odors, such as octanal

(10%) (Fig 7A). Intriguingly, we observed odor-evoked responses also in LEC, albeit at lower

magnitude. Compared to theta bursts recorded in absence of stimuli (i.e baseline) and to

responses to saline, these octanal-evoked responses had a higher amplitude of slow

oscillations corresponding to RR both in OB (χ^2 (2)= 36.05, p=1.49x10^{-8}, Kruskal-Wallis test,

Wilcoxon rank-sum test with Bonferroni correction as post-hoc test) and LEC (χ^2 (2)= 13.80,

p=0.001, Kruskal-Wallis test, Wilcoxon rank-sum test with Bonferroni correction as post-hoc

test). Similarly, octanal augmented the amplitude of theta bursts in both regions (OB: χ^2(2)=

36.30, p=1.31x10^{-8}, Kruskal-Wallis test, Wilcoxon rank-sum test with Bonferroni correction as

post-hoc test, LEC: χ^2(2)= 20.52, p=0.000035, Kruskal-Wallis test, Wilcoxon rank-sum test

with Bonferroni correction as post-hoc test) (Fig 7B, C, Table 1). In contrast to coordinated

theta burst activity recorded in the absence of olfactory stimulation, evoked responses

included beta band (15-30 Hz) activity. The amplitude of beta activity was significantly higher

in the presence of octanal than during baseline or saline exposure both in OB (χ^2(2)= 56.52,

p=5.33x10^{-13}, Kruskal-Wallis test, Wilcoxon rank-sum test with Bonferroni correction as post-

hoc test) and LEC (χ^2(2)= 31.94, p=1.16x10^{-7}, Kruskal-Wallis test, Wilcoxon rank-sum test

with Bonferroni correction as post-hoc test) (Fig 7B, C, Table 1). The presence of odor-driven

OB activity confirms the maturity of receptor cells and odor-processing mechanisms in the

olfactory system at early postnatal age. Moreover, the presence of odor-driven LEC activity

indicates that coordinated activity from OB drives the oscillatory entrainment of LEC. To

determine which oscillatory patterns are mainly involved in these directed OB-LEC

interactions, we calculated the imaginary coherence between the two areas upon exposure

to either saline or octanal. While the coherence in the slow frequency band (i.e. RR) was

higher for odor-triggered events as compared to baseline events, it was similar for saline and

octanal (χ^2(2)= 23.22, p=9.06x10^{-6}, Kruskal-Wallis test, Wilcoxon rank-sum test with

Bonferroni correction as post-hoc test, Fig 7D, Table 1). In contrast, the coherence in fast

frequencies significantly augmented in the presence of octanal when compared to saline-

evoked or baseline events (theta: χ^2(2)= 43.99, p=2.81x10^{-10}, Kruskal-Wallis test, Wilcoxon

rank-sum test with Bonferroni correction as post-hoc test, beta: χ^2(2)= 48.48, p=2.98x10^{-11},

Kruskal-Wallis test, Wilcoxon rank-sum test with Bonferroni correction as post-hoc test) (Fig

7D, Table 1). These data suggest that discontinuous bursts, either spontaneous or odor-

induced, facilitate the long range OB-LEC coupling and boost local entrainment in beta band

of entorhinal circuits.

450
451 **Table 1 (related to Figure 7). Quantification of odor-responses in neonatal OB-LEC networks.**

	OB amplitude (relative change)				LEC amplitude (relative change)				OB-LEC coherence (relative change)			
	No odor	Saline	Octanal	p	No odor	Saline	Octanal	p	No odor	Saline	Octanal	p
RR	1.34 1.00-1.61	2.78 1.17 - 3.20	3.41 2.41 - 4.50	<0.001	1.59 1.17-3.31	2.27 1.29 -4.45	5.38 2.21 - 8.94	=0.001	0.69 0.50-1.0	1.18 0.76 - 1.49	1.38 0.90 - 1.73	<0.001
Theta	1.90 1.05-2.93	4.22 2.72 - 6.80	5.79 3.82-14.57	<0.001	2.01 1.48-4.43	1.19 1.48 - 2.71	4.87 2.64 - 7.18	<0.001	0.71 0.55-0.79	0.93 0.78 - 1.05	1.33 1.03 - 1.51	<0.001
Beta	1.37 1.14-1.68	2.10 1.62 - 3.31	5.61 4.50 - 9.79	<0.001	1.52 1.15-2.66	1.76 1.18 - 2.34	3.74 2.60 - 6.03	<0.001	0.61 0.42-0.76	1.03 0.91 - 1.19	1.32 1.19 - 1.44	<0.001

452
453
454 The values are given as median and inter-quartile ranges and p-values (Kruskal-Wallis H test) for odor-evoked changes in oscillatory activity in

455 RR, theta and beta band in OB and LEC, as well as OB-LEC coherence, when compared to baseline events are included.

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DISCUSSION

Assembling of neurons into functional networks during development is the pre-requisite for

behavioral performance in adults. Entrainment of neurons into coordinated oscillatory

rhythms represents a powerful assembling principle that has been initially identified to control

the topographic organization of sensory systems (6, 8, 49, 50). More recently, patterns of

coordinated activity have been characterized in developing limbic systems (9, 14, 15, 51, 52).

However, it is still unclear whether sensory and limbic circuits adhere to similar assembling

principles and how they interact during early development. In the present study, we tested

the hypothesis that coordinated activity patterns of neuronal assemblies in neonatal OB

contribute to the oscillatory entrainment of LEC, the gatekeeper of limbic circuits during

development. Combining *in vivo* electrophysiology, optogenetics and pharmacology with

anatomical tracing of projections, we demonstrate that (i) two major patterns of coordinated

activity entrain the neonatal OB: continuous slow frequency oscillations temporally related to

respiration and discontinuous theta band oscillations critically depending on MTC activity; (ii)

both rhythms temporally couple the neonatal OB and LEC via dense direct axonal

projections, with OB theta bursts boosting the oscillatory entrainment of entorhinal circuits;

and (iii) olfactory stimuli augment oscillatory power, induce activity in fast frequency bands,

and strengthen the coupling within OB-LEC circuits (Fig 8). These data reveal that

endogenously-generated and stimulus-driven activities in OB control the oscillatory

entrainment of LEC.

Brain development has been extensively investigated in rodents because they

enable insights into a time window that remains inaccessible in humans. As altricial species,

rodents are born at an immature stage of brain development. They are blind, deaf, do not

whisker and have limited motor abilities during the first postnatal days. Before the onset of

the ability to actively respond to sensory stimuli, coordinated activity patterns, typically

characterized by rhythmic burst discharge separated by periods of quiescence, emerge

endogenously. Such patterns have been described in developing somatosensory, visual and

auditory systems. Their onset, properties, and underlying mechanisms are relatively well

understood. For example, retinal waves emerge before the onset of light-sensitivity and

vision as local patterns of coordinated activity mediated by gap junctions, cholinergic and

glutamatergic circuits (53, 54). Retinal waves synaptically propagate along the visual tract to

primary visual cortex (35, 55, 56) and are mandatory for the refinement of visual maps (57).

Similarly, cochlear burst activity emerges before the onset of hearing as a result of

coordinated firing and propagates along auditory pathways (58, 59). These cochlear bursts

are crucial for the establishment of precise tonotopic maps (5, 49). The precision of whisker

maps in the primary somatosensory cortex seems to be equally controlled by coordinated

activity evolving during postnatal development (8, 60). In the absence of a sensory periphery

with bursting activity before the onset of whisking, passive activation of whiskers is replayed

within thalamo-cortical circuits and contributes to refinement of topographic maps (6).

At the same postnatal age, the olfactory system is considered to be fully mature, the

sense of smell being of particular relevance for pup survival. This early maturity poses the

question, whether the mechanisms of organization differ between developing olfactory

pathways and other sensory systems. The present data indicate that, similar to retina or

cochlea, OB generates discontinuous patterns of oscillatory activity peaking in theta

frequency range. MTCs are critical mediators of theta bursts. These bursts are

complemented by the continuous RR that is timed by respiration/air flow and largely

independent of neuronal firing in OB. While endogenously generated discontinuous theta

bursts are a common activity pattern in peripheral sensory structures, independent of system

maturity (OB *versus* retina, cochlea), the continuous RR was not reported for other sensory

systems. The frequency structure of network activity in neonatal OB was largely independent

of the brain state. Anesthesia augmented theta but not RR power, suggesting that MTC

activity is enhanced. Similarly, in adult OB, MTCs augmented odor-evoked activity, thus

broadening their odor tuning under anesthesia (45, 61).

Overall, bursting OB activity during development profoundly differs from the

oscillatory activity in adults. Supporting previous observations *in vitro* (62), we showed that

neonatal MTCs are not only preferentially attuned to theta band activity but also contribute to

its generation. In adult mice, network activity in theta band emerges from respiration-coupled

sensory input in the glomerular layer (63) and MTCs are mainly involved in the generation of

fast oscillatory activity in gamma band (64-67). Similarly, fast rhythms are absent in the

developing OB (68) and only in the presence of odors beta band activity was induced. The

protracted emergence of fast oscillations has been hypothesized to result from late

integration of OB interneurons into local circuits and from age-dependent intrinsic biophysical

properties of MTCs. As a consequence, it has been postulated that the developing OB

encodes only first-order (e.g. odor identity) but not second-order sensory information (e.g.

odor context) (45, 69).

The presence of both stimulus-related and endogenous network activity raises the

question, whether and, if so, how both activity types either concurrently or independently

shape the maturation of the olfactory system. Already the role of spontaneous activity

endogenously generated in the sensory periphery has been subject of debate. As shown for

network activity in the immature retina and cochlea, discontinuous OB bursts in neonatal

mice are likely to have a permissive role in the establishment of precise connectivity that is

inherent in an olfactory map (70). However, it remains unclear how the spontaneous and

stimulus-evoked activities create a coherent sensory representation lacking mutual

perturbations. This is a unique feature of the developing OB. Spontaneous retinal waves and

cochlear bursts diminish and disappear with the onset of light sensitivity and hearing.

Therefore, they do not interfere with stimulus-evoked activity. Understanding the

mechanisms of theta bursts and RR during early development, as initiated in the present

study, will enable us to disentangle their function(s) along the olfactory pathway.

In sharp contrast to most sensory pathways, the olfactory system bypasses a

thalamic relay and directly conveys information from OB to cortical areas. Much research

focused on the PIR, where the bulbar topography is largely discarded and dense inputs from

OB are integrated to form odor percepts (16, 71-73). MTC axons also target LEC and in fact

these axons represent the main input that rodent LEC receives (74). LEC neurons respond to

odors (75, 76) and have been proposed to act as a modulator of olfactory coding through

interactions with the PIR (24, 77, 78). The present results show that already at neonatal age

a tight coupling links the OB with LEC. MTC axons target layer I of neonatal LEC as

previously shown for adults (17, 79). These projections mediate the coupling by synchrony

between the two areas as well as the early drive from OB to LEC. Remarkably, the neonatal

LEC and OB show similar patterns of oscillatory activity, RR and theta bursts, albeit with

lower power in LEC. The coupling by synchrony between the two areas peaked within the

same frequency bands, 2-4 Hz and 4-10 Hz. Given the measures used for the assessment of

synchrony it is unlikely that similarities result from volume conduction. Reflecting the more

pronounced OB-to-LEC innervation as compared with *vice versa* projections, the entorhinal

firing was stronger timed by the phase of RR and theta bursts in the OB than the OB firing

was driven by the entorhinal activity. Interestingly, lidocaine blockade of MTC firing did not

abolish the coupling between OB and LEC, suggesting that alternative pathways might

contribute to the synchrony between the two areas.

While feedback projections from LEC to OB (and piriform cortex, not shown) emerge

early in life, their function seems to mature postnatally to reach the anticipatory top-down

modulation and optimal input discrimination that have been identified at adult stage (80).

Recent findings revealed that the cellular substrate of feedforward and feedback interactions

between OB, LEC and PIR of adult mice are highly complex (24). We hypothesize that,

under the influence of excitatory inputs from OB, the local entorhinal circuitry is activated.

The MTC target neurons in LEC are mainly glutamatergic, suggesting that coordinated OB

activity causes an overall excitation in LEC that might facilitate the formation and refinement

of local circuits.

Olfactory information reaches the adult HP (CA1 and dentate gyrus) via reelin-

positive neurons in LEC (24, 81). Along these axonal projections, the oscillatory activity is

synchronized and enables directed functional interactions between OB, LEC and HP. In turn,

HP unidirectionally projects to PFC. At the functional level, the communication across areas

involves oscillatory activity that temporally coordinates the neuronal assemblies. For

example, respiration-related slow activity, even if subject to debate regarding its relationship

to previously described slow rhythms (82), has recently been found to occur simultaneously

with theta oscillations (83) and moreover entrain faster beta and gamma oscillations in LEC,

HP, and PFC (46, 84, 85). Taking into account the role of HP and PFC for cognitive

processing (86), the OB activity that directly entrains the limbic circuit via LEC activation

might represent a powerful control mechanisms of memory and executive performance of

adult (87, 88).

It is tempting to speculate about the potential functions of OB-driven entrainment of

LEC during neonatal development, before the emergence of cognitive abilities. Our previous

results demonstrated that LEC acts as gatekeeper of prefrontal-hippocampal interactions

shortly after birth (11). Discontinuous theta bursts in LEC drive the oscillatory entrainment

and time the firing of both prelimbic subdivision of PFC and CA1 area of the

intermediate/ventral HP. Here we show that MTC-dependent theta activity of neonatal OB

boosts RR and theta bursts in LEC. On the other hand, olfactory stimuli elicit even faster

entrainment of OB-LEC circuitry, with beta band oscillations being only detectable in the

presence of odors, such as octanal. An important issue that remains to be elucidated is

whether specific scents that the pups naturally encounter during development, such as

maternal odors, shape the network function even stronger than "artificial" odors. The effects

of maternal odor on physical, neuroendocrine, and behavioral development of pups has been

extensively investigated (27, 89, 90), yet very little is known about the underlying cellular and

circuit mechanisms. We propose that endogenously generated and odor-evoked OB activity,

especially as a result of maternal odor, might increase the level of excitability within

entorhinal-prelimbic-hippocampal networks and strengthen their wiring. By these means, the

olfactory system could facilitate the postnatal maturation of limbic circuitry and, ultimately, the

emergence of cognitive abilities.

MATERIALS AND METHODS

Ethics statement. All experiments were performed in compliance with the German laws and

the guidelines of the European Union for the use of animals in research and were approved

by the local ethical committee (15/17).

Experimental model and subject details

Mice. Timed-pregnant C57Bl/6J and Tbet-cre mice from the animal facility of the University

Medical Center Hamburg-Eppendorf as well as B6.Cg-*Gt(ROSA)26Sor*$^{tm40.1(CAG-aop3/EGFP)Hze}$/J

mice (Ai40(RCL-ArchT-EGFP)-D, Jackson Laboratory, stock no: 02118), and Tbet-cre;ArchT-

EGFP mice (bred by the animal facility of the University Medical Center Hamburg-Eppendorf)

were housed individually in breeding cages at a 12 h light / 12 h dark cycle and fed *ad*

libitum.   Mouse   lines   used   for   CLARITY   experiments   (Tbet-cre   mice,   B6.Cg-

Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J (Ai9(RCL-tdT), Jackson Laboratory, stock no: 007909 and

Tbet-cre;tdT mice) were bred in the animal facility at RWTH Aachen University under similar

conditions. The day of vaginal plug detection was defined E0.5, while the day of birth was

assigned   as   P0.   Male   mice   underwent   sensory   manipulation,   light   stimulation,

pharmacological treatment and multi-site electrophysiological recordings at P8-10. For

CLARITY experiments, male and female mice were used. Genotypes were determined using

genomic DNA and following primer sequence (Metabion, Planegg/Steinkirchen, Germany: for

Cre   in   Ai40(RCL-ArchT-EGFP)-D   mice: PCR   forward   primer   5'-

ATCCGAAAAGAAAACGTTGA-3' and reverse primer 5'-ATCCAGGTTACGGATATAGT-3';

for ROSA26-wt PCR forward primer 5'-AAAGTCGCTCTGAGTTGTTAT-3' and reverse

primer 5'-GGAGCGGGAGAAATGGATATG-3'; for GFP-tg PCR forward primer 5'-

CTGGTCGAGCTGGACGGCGACG-3'   and   reverse   primer   5'-

GTAGGTCAGGGTGGTCACGAG-3'; for Cre in Ai9(RCL-tdT) mice: forward primer 5'

CATGTCCATCAGGTTCTTGC 3' and reverse primer 5' AGAGAAAGCCCAGGAGCAG 3'; for

tdTomato forward primer 5' GGCATTAAAGCAGCGTATCC 3' and reverse primer 5'

CTGTTCCTGTACGGCATGG 3'. The PCR reactions were as follows: 10 min at 95°C, 30

cycles of 45 s at 95°C, 90 s at 54°C, 90 s at 72°C, followed by a final extension step of 10

min at 72°C (Cre-tg and ROSA26-wt), 10 min at 95°C, 30 cycles of 45 s at 95°C, 90 s at

68°C, 90 s at 72°C, followed by a final extension step of 10 min at 72°C (GFP-tg). In addition

to genotyping, EGFP expression in OB prior to surgery was detected using a dual fluorescent

protein flashlight (Electron microscopy sciences, PA, US).

Surgical procedures

Surgical preparation for electrophysiology and light delivery in vitro. For patch-clamp

recordings, pups were decapitated and brains were sliced in 300 µm-thick coronal sections.

Slices were incubated in oxygenated ACSF containing (in mM) 119 NaCl, 2.5 KCl, 1

NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$ (320 mOsm) at 37 °C. Prior to recordings,

slices were maintained at room temperature and superfused with oxygenated ACSF.

Surgical preparation for electrophysiology and light delivery in vivo. For recordings in non-

anesthetized state, 0.5% bupivacain / 1% lidocaine was locally applied on the neck muscles.

For recordings under anesthesia, mice were injected i.p. with urethane (1 mg/g body weight;

Sigma-Aldrich, MO, USA) prior to surgery. For both groups, under isoflurane anesthesia

(induction: 5%, maintenance: 2.5%) the head of the pup was fixed into a stereotaxic

apparatus as previously reported (9).

The surgery protocols are described in detail in Supplemental Information.

Electrophysiology

Electrophysiological recordings in vivo. One-shank electrodes (NeuroNexus, MI, USA) with

16 recording sites were inserted into dorsal (depth 0.5-1.2 mm, angle 0°) or ventral OB (1.4-

1.8 mm, angle 0°) as well as in LEC (depth: 2 mm, angle: 10° from the vertical plane). Two-

shank optoelectrodes (Buzsaki16-OA16LP, NeuroNexus, MI, USA) with 8 recordings sites on

each shank aligned with an optical fiber ending 40 µm above the top recording site were

inserted into ventral OB. Extracellular signals were band-pass filtered (0.1 Hz – 9 kHz) and

digitized (32 kHz) by a multichannel amplifier (Digital Lynx SX; Neuralynx, Bozeman, MO;

USA) and Cheetah acquisition software (Neuralynx).

Electrophysiological recordings in vitro. Whole-cell patch-clamp recordings were performed

from MTCs identified by their location in the mitral cell layer and visualized by membrane-

bound EGFP. All recordings were performed at room temperature. Recording electrodes (4-9

MΩ) were filled with K-gluconate based solution containing (in mM): 130 K-gluconate, 10

HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 8 NaCl (285 mOsm, pH 7.4) and 0.5% biocytin

for post-hoc morphological identification of recorded cells. Recordings were controlled with

the Ephus software (91) in the MATLAB environment (The MathWorks, Inc., MA, USA).

Morphological investigation

CLARITY. Brains from neonatal mice of both sexes were sliced in 1 mm- (for LEC) and 500

µm-thick (for OB) coronal sections. To maintain the structural integrity, the tissue was fixed

overnight at 4°C in hydrogel fixation solution containing 4% acrylamide, 0.05% bis-

acrylamide, 0.25% VA-044 Initiator, 4% PFA in PBS-/-. After polymerization and embedding

the nuclear marker DRAQ5 (1:1000) was added to the samples. After washing steps, the

samples were incubated for 24 h in RIMS80 containing 80 g Nycodenz, 20 mM PS, 0.1%

Tween 20, and 0.01% sodium acid.

Retrograde tracing. For retrograde tracing, anesthetized P3-4 mice received unilateral

Fluorogold (FG) (Fluorochrome, LLC, USA) injections into OB (0.8 mm anterior from the

fronto-nasal suture, 0.8 mm from midline) or LEC (1 mm posterior to bregma, 5 mm from

midline). After 4-5 days, pups were deeply anesthetized and perfused at P8.

All staining protocols are described in detail in Supplemental Information.

Manipulations

Light stimulation in vitro. Whole-cell current-clamp recordings were performed from ArchT-

EGFP expressing mitral cells in coronal slices of the neonatal Tbet-cre;ArchT mice. Yellow

light pulses (595 nm) of different light intensities (0.2 – 2.6 mW) were applied to test the

effect on the membrane potential.

Light stimulation in vivo. Trapezoid light stimulation was applied using a diode pumped solid

state (DPSS) laser (Cobolt Mambo, 594 nm, Omicron, Austria), controlled by an Arduino Uno

(Arduino, Italy).

Naris occlusion. One naris was closed using silicon adhesive (Kwik-Sil, World Precision

Instruments). After a recovery period of five minutes, the recording was pursued while one

naris was sealed.

Pharmacological inactivation. To block the firing of OB neurons, lidocaine hydrochloride 4%

in 0.9% NaCl, pH 7.0 with NaOH) was slowly infused into the OB

Odor stimulation. An eight channel dilution olfactometer (Aurora Scientific) was used for

stimulus delivery.

All manipulation protocols are described in detail in Supplemental Information.

Quantification and statistical analysis

Immunohistochemistry quantification. Images were analyzed using ImageJ.

Detection of respiration frequency. Respiration was monitored using a piezo-electric sensor

placed under the pup's chest.

LFP analysis. Data were analyzed offline using custom-written scripts in the MATLAB

environment (Version 9, MathWorks, Natick, MA).

For details, see Supplemental Information.

Statistics. Statistical analysis was performed using SPSS Statistics 22 (IBM, NY) or

MATLAB. Gaussian distribution of the data was assessed using the Kolmogorov-Smirnov

test. None of the data sets were normally distributed. Therefore, data were tested for

significance using Wilcoxon signed-rank test (2 related samples), Wilcoxon rank-sum test (2

unrelated samples), Friedman test (>2 related samples; Wilcoxon signed-rank post hoc test

with Bonferroni correction) and Kruskal-Wallis H test (>2 unrelated samples; Wilcoxon rank-

sum test post hoc test with Bonferroni correction). Differences in proportions were tested

using χ^2 test. For classification of single unit responses to light stimulation, significant firing

rate changes were assessed statistically using Wilcoxon signed-rank test. Data are

represented as median and inter-quartile range.

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Author contributions

I.L.H.-O. designed the experiments, S.G, J.K.K., H.H., K.W., D.F., A.M.T carried out the

experiments, S.G., J.K.K., H.H., K.W. analyzed the data, I.L.H.-O. S.G., J.K.K. and M.S.

interpreted the data and wrote the paper. All authors discussed and commented on the

manuscript.

REFERENCES

1.   Hanganu-Opatz IL. Between molecules and experience: role of early patterns of coordinated activity for the development of cortical maps and sensory abilities. Brain research reviews. 2010;64(1):160-76.

2.   Blankenship AG, Feller MB. Mechanisms underlying spontaneous patterned activity in developing neural circuits. Nat Rev Neurosci. 2010;11(1):18-29.

3.   Luhmann HJ, Khazipov R. Neuronal activity patterns in the developing barrel cortex. Neuroscience. 2018;368:256-67.

4.   Colonnese MT, Kaminska A, Minlebaev M, Milh M, Bloem B, Lescure S, et al. A conserved switch in sensory processing prepares developing neocortex for vision. Neuron. 2010;67(3):480-98.

5.   Wang HC, Bergles DE. Spontaneous activity in the developing auditory system. Cell and tissue research. 2015;361(1):65-75.

6.   Minlebaev M, Colonnese M, Tsintsadze T, Sirota A, Khazipov R. Early gamma oscillations synchronize developing thalamus and cortex. Science. 2011;334(6053):226-9.

7.   Tiriac A, Uitermarkt BD, Fanning AS, Sokoloff G, Blumberg MS. Rapid whisker movements in sleeping newborn rats. Current biology : CB. 2012;22(21):2075-80.

8.   Dupont E, Hanganu IL, Kilb W, Hirsch S, Luhmann HJ. Rapid developmental switch in the mechanisms driving early cortical columnar networks. Nature. 2006;439(7072):79-83.

9.   Brockmann MD, Poschel B, Cichon N, Hanganu-Opatz IL. Coupled oscillations mediate directed interactions between prefrontal cortex and hippocampus of the neonatal rat. Neuron. 2011;71(2):332-47.

10.   Cichon NB, Denker M, Grün S, Hanganu-Opatz IL. Unsupervised classification of neocortical activity patterns in neonatal and pre-juvenile rodents. Frontiers in Neural Circuits. 2014;8.

11.   Hartung H, Brockmann MD, Poschel B, De Feo V, Hanganu-Opatz IL. Thalamic and Entorhinal Network Activity Differently Modulates the Functional Development of Prefrontal-Hippocampal Interactions. J Neurosci. 2016;36(13):3676-90.

12.   Marguet SL, Le-Schulte VT, Merseburg A, Neu A, Eichler R, Jakovcevski I, et al. Treatment during a vulnerable developmental period rescues a genetic epilepsy. Nature medicine. 2015;21(12):1436-44.

13.   Mohns EJ, Blumberg MS. Synchronous bursts of neuronal activity in the developing hippocampus: Modulation by active sleep, and association with emerging gamma and theta rhythms. J Neurosci. 2008;28(40):10134-44.

14.   Ahlbeck J, Song L, Chini M, Bitzenhofer SH, Hanganu-Opatz IL. Glutamatergic drive along the septo-temporal axis of hippocampus boosts prelimbic oscillations in the neonatal mouse. eLife. 2018;7.

15.   Bitzenhofer SH, Ahlbeck J, Wolff A, Wiegert JS, Gee CE, Oertner TG, et al. Layer-specific optogenetic activation of pyramidal neurons causes beta–gamma entrainment of neonatal networks. Nature Communications. 2017;8.

16.   Sosulski DL, Lissitsyna MV, Cutforth T, Axel R, Datta SR. Distinct Representations of Olfactory Information in Different Cortical Centers. Nature. 2011;472(7342):213-6.

17.   Witter MP, Doan TP, Jacobsen B, Nilssen ES, Ohara S. Architecture of the Entorhinal Cortex A Review of Entorhinal Anatomy in Rodents with Some Comparative Notes. Frontiers in Systems Neuroscience. 2017;11.

18.   Adam Y, Mizrahi A. Long-term imaging reveals dynamic changes in the neuronal composition of the glomerular layer. J Neurosci. 2011;31(22):7967-73.

19.   Wachowiak M, Shipley MT. Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb. Seminars in cell & developmental biology. 2006;17(4):411-23.

20.   Luskin MB, Price JL. The topographic organization of associational fibers of the olfactory system in the rat, including centrifugal fibers to the olfactory bulb. The Journal of comparative neurology. 1983;216(3):264-91.

21.   Agster KL, Burwell RD. Cortical efferents of the perirhinal, postrhinal, and entorhinal cortices of the rat. Hippocampus. 2009;19(12):1159-86.

22.   Witter MP. The perforant path: projections from the entorhinal cortex to the dentate gyrus. Progress in brain research. 2007;163:43-61.

23.   Chapuis J, Cohen Y, He X, Zhang Z, Jin S, Xu F, et al. Lateral entorhinal modulation of piriform cortical activity and fine odor discrimination. J Neurosci. 2013;33(33):13449-59.

24.   Leitner FC, Melzer S, Lutcke H, Pinna R, Seeburg PH, Helmchen F, et al. Spatially segregated feedforward and feedback neurons support differential odor processing in the lateral entorhinal cortex. Nature neuroscience. 2016;19(7):935-44.

25.   Eichenbaum H. A cortical-hippocampal system for declarative memory. Nat Rev Neurosci.
2000;1(1):41-50.
26.   Keene CS, Bladon J, McKenzie S, Liu CD, O'Keefe J, Eichenbaum H. Complementary
Functional Organization of Neuronal Activity Patterns in the Perirhinal, Lateral Entorhinal, and Medial
Entorhinal Cortices. J Neurosci. 2016;36(13):3660-75.
27.   Logan DW, Brunet LJ, Webb WR, Cutforth T, Ngai J, Stowers L. Learned recognition of
maternal signature odors mediates the first suckling episode in mice. Current biology : CB.
2012;22(21):1998-2007.
28.   Welker WI. Analysis of Sniffing of the Albino Rat 1)1964. 223-44 p.
29.   Whishaw I, Kolb B. The Behavior of the Laboratory Rat: A Handbook with Tests. New York,
NY, US: Oxford University Press; 2005. 1-520 p.
30.   Walz A, Omura M, Mombaerts P. Development and topography of the lateral olfactory tract in
the mouse: imaging by genetically encoded and injected fluorescent markers. Journal of neurobiology.
2006;66(8):835-46.
31.   Haddad R, Lanjuin A, Madisen L, Zeng H, Murthy VN, Uchida N. Olfactory cortical neurons
read out a relative time code in the olfactory bulb. Nature neuroscience. 2013;16(7):949-57.
32.   Wouterlood FG, Nederlof J. Terminations of olfactory afferents on layer II and III neurons in
the entorhinal area: degeneration-Golgi-electron microscopic study in the rat. Neuroscience letters.
1983;36(2):105-10.
33.   Mazo C. GABAergic Signaling in Cortical Feedback to the Olfactory Bulb. Paris: Université
Pierre and Marie Curie; 2017.
34.   Wilson DA, Sullivan RM. Cortical processing of odor objects. Neuron. 2011;72(4):506-19.
35.   Hanganu IL, Ben-Ari Y, Khazipov R. Retinal waves trigger spindle bursts in the neonatal rat
visual cortex. Journal of Neuroscience. 2006;26(25):6728-36.
36.   Vinck M, van Wingerden M, Womelsdorf T, Fries P, Pennartz CM. The pairwise phase
consistency: a bias-free measure of rhythmic neuronal synchronization. NeuroImage. 2010;51(1):112-
22.
37.   Miyamichi K, Amat F, Moussavi F, Wang C, Wickersham I, Wall NR, et al. Cortical
representations of olfactory input by trans-synaptic tracing. Nature. 2011;472(7342):191-6.
38.   Mori K, Sakano H. How is the olfactory map formed and interpreted in the mammalian brain?
Annu Rev Neurosci. 2011;34:467-99.
39.   Li A, Zhang L, Liu M, Gong L, Liu Q, Xu F. Effects of Different Anesthetics on Oscillations in
the Rat Olfactory Bulb. Journal of the American Association for Laboratory Animal Science : JAALAS.
2012;51(4):458-63.
40.   Mooney R, Penn AA, Gallego R, Shatz CJ. Thalamic Relay of Spontaneous Retinal Activity
Prior to Vision. Neuron. 1996;17(5):863-74.
41.   Minlebaev M, Ben-Ari Y, Khazipov R. NMDA Receptors Pattern Early Activity in the
Developing Barrel Cortex In Vivo. Cereb Cortex. 2009;19(3):688-96.
42.   Yang JW, Hanganu-Opatz IL, Sun JJ, Luhmann HJ. Three Patterns of Oscillatory Activity
Differentially Synchronize Developing Neocortical Networks In Vivo. Journal of Neuroscience.
2009;29(28):9011-25.
43.   Bitzenhofer SH, Sieben K, Siebert KD, Spehr M, Hanganu-Opatz IL. Oscillatory activity in
developing prefrontal networks results from theta-gamma-modulated synaptic inputs. Cell reports.
2015;11(3):486-97.
44.   Franks KM, Isaacson JS. Synapse-Specific Downregulation of NMDA Receptors by Early
Experience: A Critical Period for Plasticity of Sensory Input to Olfactory Cortex. Neuron.
2005;47(1):101-14.
45.   Kato Hiroyuki K, Chu Monica W, Isaacson Jeffry S, Komiyama T. Dynamic Sensory
Representations in the Olfactory Bulb: Modulation by Wakefulness and Experience. Neuron.
2012;76(5):962-75.
46.   Tort ABL, Brankačk J, Draguhn A. Respiration-Entrained Brain Rhythms Are Global but Often
Overlooked. Trends in Neurosciences. 2018;41(4):186-97.
47.   Nolte G, Bai O, Wheaton L, Mari Z, Vorbach S, Hallett M. Identifying true brain interaction from
EEG data using the imaginary part of coherency. Clinical Neurophysiology. 2004;115(10):2292-307.
48.   Hintiryan H, Gou L, Zingg B, Yamashita S, Lyden HM, Song MY, et al. Comprehensive
connectivity of the mouse main olfactory bulb: analysis and online digital atlas. Frontiers in
neuroanatomy. 2012;6:30.
49.   Clause A, Kim G, Sonntag M, Weisz Catherine JC, Vetter Douglas E, Rűbsamen R, et al. The
Precise Temporal Pattern of Prehearing Spontaneous Activity Is Necessary for Tonotopic Map
Refinement. Neuron. 2014;82(4):822-35.
50.   Huberman AD, Speer CM, Chapman B. Spontaneous retinal activity mediates development of
ocular dominance columns and binocular receptive fields in v1. Neuron. 2006;52(2):247-54.

51.   Del Rio-Bermudez C, Kim J, Sokoloff G, Blumberg MS. Theta Oscillations during Active Sleep
Synchronize the Developing Rubro-Hippocampal Sensorimotor Network. Current Biology.
2017;27(10):1413-24.e4.
52.   Picardo Michel A, Guigue P, Bonifazi P, Batista-Brito R, Allene C, Ribas A, et al. Pioneer
GABA Cells Comprise a Subpopulation of Hub Neurons in the Developing Hippocampus. Neuron.
2011;71(4):695-709.
53.   Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ. Requirement for cholinergic
synaptic transmission in the propagation of spontaneous retinal waves. Science.
1996;272(5265):1182-7.
54.   Wong WT, Myhr KL, Miller ED, Wong RO. Developmental changes in the neurotransmitter
regulation of correlated spontaneous retinal activity. J Neurosci. 2000;20(1):351-60.
55.   Ackman JB, Burbridge TJ, Crair MC. Retinal waves coordinate patterned activity throughout
the developing visual system. Nature. 2012;490(7419):219-25.
56.   Colonnese MT, Khazipov R. 'Slow activity transients' in infant rat visual cortex: a spreading
synchronous oscillation patterned by retinal waves. The Journal of neuroscience : the official journal of
the Society for Neuroscience. 2010;30(12):4325-37.
57.   Ackman JB, Crair MC. Role of emergent neural activity in visual map development. Curr Opin
Neurobiol. 2014;24:166-75.
58.   Tritsch NX, Bergles DE. Developmental regulation of spontaneous activity in the Mammalian
cochlea. J Neurosci. 2010;30(4):1539-50.
59.   Tritsch NX, Yi E, Gale JE, Glowatzki E, Bergles DE. The origin of spontaneous activity in the
developing auditory system. Nature. 2007;450(7166):50-5.
60.   Mitrukhina O, Suchkov D, Khazipov R, Minlebaev M. Imprecise Whisker Map in the Neonatal
Rat Barrel Cortex. Cereb Cortex. 2015;25(10):3458-67.
61.   Rinberg D, Koulakov A, Gelperin A. Sparse odor coding in awake behaving mice. J Neurosci.
2006;26(34):8857-65.
62.   Yu Y, Burton SD, Tripathy SJ, Urban NN. Postnatal development attunes olfactory bulb mitral
cells to high-frequency signaling. J Neurophysiol. 2015;114(5):2830-42.
63.   Wachowiak M. All in a sniff: olfaction as a model for active sensing. Neuron. 2011;71(6):962-
73.
64.   Bathellier B, Lagier S, Faure P, Lledo PM. Circuit properties generating gamma oscillations in
a network model of the olfactory bulb. J Neurophysiol. 2006;95(4):2678-91.
65.   Fukunaga I, Herb JT, Kollo M, Boyden ES, Schaefer AT. Independent control of gamma and
theta activity by distinct interneuron networks in the olfactory bulb. Nature neuroscience.
2014;17(9):1208-16.
66.   Kay LM. Circuit oscillations in odor perception and memory. Progress in brain research.
2014;208:223-51.
67.   Lepousez G, Lledo PM. Odor discrimination requires proper olfactory fast oscillations in awake
mice. Neuron. 2013;80(4):1010-24.
68.   Fletcher ML, Smith AM, Best AR, Wilson DA. High-Frequency Oscillations Are Not Necessary
for Simple Olfactory Discriminations in Young Rats. J Neurosci. 2005;25(4):792-8.
69.   Cury KM, Uchida N. Robust odor coding via inhalation-coupled transient activity in the
mammalian olfactory bulb. Neuron. 2010;68(3):570-85.
70.   Yu CR, Power J, Barnea G, O'Donnell S, Brown HEV, Osborne J, et al. Spontaneous Neural
Activity Is Required for the Establishment and Maintenance of the Olfactory Sensory Map. Neuron.
2004;42(4):553-66.
71.   Boyd AM, Sturgill JF, Poo C, Isaacson JS. Cortical feedback control of olfactory bulb circuits.
Neuron. 2012;76(6):1161-74.
72.   Haberly LB. Parallel-distributed Processing in Olfactory Cortex: New Insights from
Morphological and Physiological Analysis of Neuronal Circuitry. Chemical Senses. 2001;26(5):551-76.
73.   Kay L, Laurent G. Kay, L.M. & Laurent, G. Odor- and context-dependent modulation of mitral
cell activity in behaving rats. Nat. Neurosci. 2, 1003-10091999. 1003-9 p.
74.   Kerr KM, Agster KL, Furtak SC, Burwell RD. Functional neuroanatomy of the parahippocampal
region: the lateral and medial entorhinal areas. Hippocampus. 2007;17(9):697-708.
75.   Petrulis A, Alvarez P, Eichenbaum H. Neural correlates of social odor recognition and the
representation of individual distinctive social odors within entorhinal cortex and ventral subiculum.
Neuroscience. 2005;130(1):259-74.
76.   Young BJ, Otto T, Fox GD, Eichenbaum H. Memory representation within the
parahippocampal region. J Neurosci. 1997;17(13):5183-95.
77.   Chen CF, Barnes DC, Wilson DA. Generalized vs. stimulus-specific learned fear differentially
modifies stimulus encoding in primary sensory cortex of awake rats. J Neurophysiol.
2011;106(6):3136-44.

78.   Xu W, Wilson DA. Odor-evoked activity in the mouse lateral entorhinal cortex. Neuroscience.
2012;223:12-20.
79.   Burwell RD, Amaral DG. Cortical afferents of the perirhinal, postrhinal, and entorhinal cortices
of the rat. The Journal of comparative neurology. 1998;398(2):179-205.
80.   Kay LM, Freeman WJ. Bidirectional processing in the olfactory-limbic axis during olfactory
behavior. Behavioral neuroscience. 1998;112(3):541-53.
81.   Burwell RD, Witter MP, Amaral DG. Perirhinal and postrhinal cortices of the rat: a review of the
neuroanatomical literature and comparison with findings from the monkey brain. Hippocampus.
1995;5(5):390-408.
82.   Bagur S, Benchenane K. Taming the oscillatory zoo in the hippocampus and neo-cortex: a
review of the commentary of Lockmann and Tort on Roy et al. Brain structure & function.
2018;223(1):5-9.
83.   Tort ABL, Ponsel S, Jessberger J, Yanovsky Y, Brankack J, Draguhn A. Parallel detection of
theta and respiration-coupled oscillations throughout the mouse brain. Scientific reports.
2018;8(1):6432.
84.   Biskamp J, Bartos M, Sauer JF. Organization of prefrontal network activity by respiration-
related oscillations. Scientific reports. 2017;7:45508.
85.   Lockmann ALV, Laplagne DA, Tort ABL. Olfactory bulb drives respiration-coupled beta
oscillations in the rat hippocampus. Eur J Neurosci. 2017.
86.   Nadel L, O'Keefe J. The hippocampus as a cognitive map: Clarendon; 1978.
87.   Egorov AV, Hamam BN, Fransen E, Hasselmo ME, Alonso AA. Graded persistent activity in
entorhinal cortex neurons. Nature. 2002;420(6912):173-8.
88.   Eichenbaum H. Hippocampus: cognitive processes and neural representations that underlie
declarative memory. Neuron. 2004;44(1):109-20.
89.   Al Ain S, Perry RE, Nunez B, Kayser K, Hochman C, Brehman E, et al. Neurobehavioral
assessment of maternal odor in developing rat pups: implications for social buffering. Social
neuroscience. 2017;12(1):32-49.
90.   Sullivan RM, Stackenwalt G, Nasr F, Lemon C, Wilson DA. Association of an odor with
activation of olfactory bulb noradrenergic beta-receptors or locus coeruleus stimulation is sufficient to
produce learned approach responses to that odor in neonatal rats. Behavioral neuroscience.
2000;114(5):957-62.
91.   Suter BA, O'Connor T, Iyer V, Petreanu LT, Hooks BM, Kiritani T, et al. Ephus: Multipurpose
Data Acquisition Software for Neuroscience Experiments. Frontiers in Neural Circuits. 2010;4:100.

Figure legends

Fig 1. Patterns of axonal connectivity between OB and LEC in neonatal mice.

(A) Long-range projections of tdTomato fluorescently labeled OB M/T cells (left) when

superimposed on a bright-field image showing the ventral (middle) and lateral (right) view of

the whole brain of a P10 Tbet-cre;tdTomato mouse. **(B)** Unprocessed (left) and cleared

(right) brain of a P10 mouse. **(C)** Cleared 500 μm-thick coronal section containing the OB of

a Tbet-cre;tdTomato mouse showing MTCs (red) when counterstained with the nuclear

marker DRAQ5 (blue). Inset, tdTomato-stained MTCs displayed at larger magnification. **(D)**

MTC axons targeting LEC in a cleared 1 mm-thick coronal brain slice. Inset, axons of

tdTomato-expressing MTCs when counter-stained with DRAQ5 (blue) and displayed at larger

magnification. **(E)** Photographs of a 100 μm-thick coronal section from a P8 mouse depicting

retrogradely labeled neurons in the OB (right) after injection of FG into the LEC (left) at P3.

Inset, FG-labeled MTCs displayed at larger magnification. **(F)** Photographs of a 50 μm-thick

coronal section from a P8 mouse depicting retrogradely labeled neurons in LEC (right top)

after injection of FG into OB (100 μm-thick coronal section, left) at P4 (LEC, lateral entorhinal

cortex; PIR, piriform cortex; PMCo, posteromedial cortical amygdaloid nucleus; rf, rhinal

fissure; af, amygdaloid fissure). Counterstaining for GABA (red) shows no overlap between

GABA positive (red) and FG (white) labeled cell bodies (right bottom).

Fig 2. Continuous and discontinuous patterns of oscillatory activity in the neonatal

olfactory bulb.

(A) Digital photomontage reconstructing the track of the multi-site DiI-labeled recording

electrode (red) in a Nissl-stained (green) 100 μm-thick coronal section including the OB from

a P9 mouse. The dots (gray) show the position of the 16 recording sites of the silicon probe

and the recording channels (white) in the mitral cell layer (MCL) and external plexiform layer

(EPL) that were used for spike and LFP analysis, respectively. **(B)** LFP recording of the

oscillatory activity in the OB of a P10 mouse displayed band-pass filtered in different

frequency bands and accompanied by the wavelet spectrogram (white line represents time-

averaged power of the trace; white arrows point towards peak frequency values) as well as

simultaneously recorded MUA (high-pass filter >400 Hz) and respiration. **(C)** Characteristic

slow continuous oscillatory activity and theta bursts from the trace shown in b when

displayed at higher magnification. Insets, continuous oscillatory activity in relationship with

respiration (left, blue) and a discontinuous theta burst (right, red). **(D)** Power spectra (mean ±

SEM) of LFP in OB during non-burst activity (blue) and discontinuous bursts (red) as well as

of theta bursts normalized to non-bursting activity (purple). The respiration frequency was

depicted as horizontal bar and expanded at larger scale (top). **(E)** Temporal relationship

between neuronal firing and network oscillations in OB. Left, histogram showing the

percentage of spikes occurring during theta burst for all clustered units. Right, box plot

depicting the firing rates of OB units during non-burst periods and theta burst periods. Gray

dots and lines correspond to individual cells (Wilcoxon signed-rank test, ***$p < 0.001$). **(F)**

Histograms depicting the phase locking of OB cells to RR (left) and theta activity (right). Only

significantly locked cells were used for analysis.

Fig 3. Effects of optogenetic silencing of MTCs on the patterns of oscillatory activity in

the neonatal OB.

(A) Photograph of the brain of a P8 cre-positive Tbet-cre;ArchT-EGFP mouse (left) showing

EGFP-fluorescent MTCs cell bodies and their projections. **(B)** Left, photograph of a 100 μm-

thick coronal section including the OB from a P8 cre-positive Tbet-cre;ArchT-EGFP mouse.

The position of recording sites in MCL and EPL layers is marked by white squares. The light

guide ending just above the recording sites is shown in gray. The iso-contour lines of light

spreading calculated using Monte Carlo simulation are shown in yellow. Right, propagation of

light intensity in the brain as predicted by Monte Carlo simulation. Yellow lines correspond to

the iso-contour lines for light power of 1 and 10 mW/mm^2, respectively. **(C)** Neuronal firing

(SUA) and LFP band-pass filtered for different frequency bands (broad 1-100 Hz, RR 2-4 Hz,

theta 4-12 Hz) in response to light (yellow, 594 nm) stimulation of MTCs in a P8 cre-positive

Tbet-cre;ArchT-EGFP mouse. Traces are accompanied by the color-coded wavelet

spectrogram of LFP shown at identical timescale. **(D)** Raster plots and peri-stimulus time

histograms displaying the firing of MTCs in response to light stimulation. The color-coded bar

(bottom) displays the fraction of cells that responded with a firing decrease during stimulus

(red), constant firing during stimulus but a firing increase post-stimulus (blue) and unchanged

firing rate (white). **(E)** Box plots displaying the absolute power before and during light

stimulation in cre-positive pups (left) and the relative change of RR activity in neonatal OB of

cre-positive and cre-negative mice (right). Gray dots and lines correspond to individual

animals. **(F)** Same as E for discontinuous theta bursts (**p < 0.01, left: signed-rank test, right:

rank-sum test).

Fig 4. Continuous and discontinuous patterns of oscillatory activity in the neonatal

LEC.

(A) Digital photomontage reconstructing the track of the multi-site DiI-labeled recording

electrode (red) in a Nissl-stained (green) 100 µm-thick coronal section including LEC from a

P9 mouse. The gray dots show the position of the 16 recording sites. (PRh, perirhinal cortex;

LEC, lateral entorhinal cortex; PIR, piriform cortex; rf, rhinal fissure; af, amygdaloid fissure).

(B) LFP recording of the oscillatory activity in LEC of a P10 mouse displayed band-pass

filtered in different frequency bands and accompanied by the wavelet spectrogram (white line

represents time-averaged power of the trace) as well as simultaneously recorded MUA (high-

pass filter >400 Hz) and respiration. **(C)** Characteristic slow continuous oscillatory activity

and theta bursts from the trace shown in B when displayed at higher magnification. **(D)**

Power spectra (mean ± SEM) of LFP in LEC during non-burst activity (blue) and

discontinuous bursts (red) as well as of theta bursts normalized to non-bursting activity

(purple). The respiration frequency was depicted as horizontal bar and expanded at larger

scale (top). **(E)** Temporal relationship between neuronal firing and network oscillations in

LEC. Left, histogram showing the percentage of spikes occurring during theta burst for all

clustered units. Right, box plot depicting the firing rates of LEC units during non-burst periods

and theta burst periods. Gray dots and lines correspond to individual cells (Wilcoxon signed-

rank test, ***p < 0.001). **(F)** Histograms depicting the phase locking of LEC neurons to RR

(left) and theta activity (right). Only significantly locked cells were used for analysis.

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Fig 5. Frequency-dependent functional coupling between neonatal OB and LEC.

(A) Characteristic traces of band-pass filtered LFP recorded simultaneously in OB (top) and

LEC (bottom) of a P9 mouse, displayed together with wavelet spectrograms showing the

frequency content. Note the temporal correlation between discontinuous theta bursts in both

areas. **(B)** Boxplots displaying RR power (top, green) and theta burst power (bottom, purple)

in OB and LEC. Gray lines and dots correspond to individual pups. (*p<0.05, Wilcoxon

signed-rank test). **(C)** Plot of imaginary part of coherence between OB and LEC showing

prominent peaks in RR and theta band. The gray line corresponds to the significance

threshold as assessed by Monte Carlo simulation. **(D)** Histograms of phase differences

between RR (left, green) and theta (right, purple) activity recorded simultaneously in OB and

LEC. **(E)** Left, bar diagram displaying the percentage of OB units coupled to the RR (green)

and theta bursts (purple) in LEC and the percentage of LEC units coupled to the RR (green)

and theta bursts (purple) in OB. Right, box plot showing the coupling strength of OB cells

significantly locked to LEC oscillations (green: RR, purple: theta bursts) and of LEC cells

significantly locked to OB oscillations (green: RR, purple: theta bursts). Gray dots correspond

to individual cells (χ^2 test of proportions ***p < 0.001). **(F)** Histograms showing the distribution

of preferred phases of LEC cells significantly locked to RR (left) and OB theta bursts (right) in

neonatal OB. For comparison, histograms of OB cells locked to the respective OB rhythm are

plotted as white bars. **(G)** Histograms showing the distribution of preferred phases of OB

cells significantly locked to RR (left) and theta bursts (right) in neonatal LEC. For

comparison, histograms of LEC cells locked to the respective LEC rhythm are plotted as

white bars.

Fig 6. Effects of pharmacological blockade of neuronal firing in OB on patterns of

oscillatory activity in OB-LEC circuits.

(A) Schematic drawing of experimental protocol. **(B)** Photograph of the brain of a P10 mouse

showing the confinement of injections to one hemisphere of the OB. For visualization, the

same volume of methylene blue was used. **(C)** Characteristic LFP traces (black, filtered 1-

100 Hz) recorded in OB (top) and LEC (bottom) of a P9 mouse before (left) and after (right)

lidocaine infusion, displayed together with the wavelet spectrograms of the LFP and

simultaneously recorded MUA. **(D)** Top, mean MUA firing rate in OB (left) and LEC (right)

before and after lidocaine infusion. The time of infusion is considered 0. Bottom, box plots

displaying the mean MUA in OB (left) and LEC (right) before and after lidocaine infusion

(Friedmann test, Wilxocon signed-rank test with Bonferroni correction for post-hoc

comparison, *$p<0.0071$). **(E)** Box plots displaying the power of RR activity in OB and LEC as

well as the mean OB-LEC coherence in the RR band before and after lidocaine infusion.

Gray dots and lines correspond to individual animals (Wilcoxon signed-rank test, *$p < 0.05$;

$p < 0.01$). **(F) Same as E for the theta burst activity in neonatal OB and LEC.

Fig 7. Odor-triggered activity patterns in OB of neonatal mouse.

(A) Characteristic LFP traces (band-pass filtered 1-100 Hz) recorded in OB (top) and LEC

(bottom) of a P9 mouse before (baseline, left) and after application of odors (saline, middle;

octanal, right) displayed together with simultaneously recorded MUA. **(B)** Box plots showing

odor-evoked changes in the amplitude of RR (left), theta (middle) and beta (right) activity in

OB when normalized to baseline. **(C)** Same as B for LEC. **(D)** Odor-evoked relative changes

in OB-LEC coherence in RR (left), theta (middle) and beta (right) band when normalized to

baseline. Gray dots correspond to individual trials. (Kruskal-Wallis H test, Wilcoxon rank-sum

test with Bonferroni correction as post-hoc test, *$p<0.0167$).

Fig 8. Schematic diagram of structural and functional coupling within OB-LEC

networks of neonatal mice.

Mutual axonal projections (red) connect neonatal OB and LEC. In OB of neonatal mice,

continuous air flow-dependent RR and discontinuous MTC-driven theta bursts represent the

two major patterns of oscillatory activity. They are augmented by olfactory stimuli (blue) that

additionally evoke beta oscillations. OB activity boosts the oscillatory entrainment of neonatal

LEC that, in turn, might drive the limbic circuits during development.

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