Diversity and function of corticopetal and corticofugal GABAergic projection neurons

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Abstract | It is still widely thought that cortical projections to distant brain areas derive by and large from glutamatergic neurons. However, an increasing number of reports provide evidence that cortical GABAergic neurons comprise a smaller population of 'projection neurons' in addition to the well-known and much-studied interneurons. GABAergic long-range axons that derive from, or project to, cortical areas are thought to entrain distant brain areas for efficient information transfer and processing. Research conducted over the past 10 years has revealed that cortical GABAergic projection neurons are highly diverse in terms of molecular marker expression, synaptic targeting (identity of targeted cell types), activity pattern during distinct behavioural states and precise temporal recruitment relative to ongoing neuronal network oscillations. As GABAergic projection neurons connect many cortical areas unidirectionally or bidirectionally, it is safe to assume that they participate in the modulation of a whole series of behavioural and cognitive functions. We expect future research to examine how long-range GABAergic projections fine-tune activity in distinct distant networks and how their recruitment alters the behaviours that are supported by these networks.

Disinhibition
The release of inhibition from a neuron by the inhibition of inhibitory neurons; usually leads to increased excitability of the disinhibited neuron.

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GABAergic long-range projections were first described in 1966 (REF.[1]). The earliest studies of such projections pertained to GABAergic neurons with prominent projections in, for example, the basal ganglia and cerebellar output nuclei[1–3]. Many other GABAergic projections became accessible only recently with the development of tools, such as viral-based anterograde and retrograde labelling, that enable the detection of sparse projections. Indeed, many neocortical and archicortical GABAergic projections are relatively sparse and their investigation therefore requires highly sensitive and specific tools. Thus, the first cortical GABAergic projection was revealed as late as 13 years after the discovery of basal ganglia GABAergic outputs[4]. Studying cortical GABAergic projections remains a challenge, as reflected in the scarce literature on this topic: over the past 10 years, only about 5 papers per year have been published in this area.

There has been noticeable progress in this area over the past 8 years. The continuous addition of newly detected projections to the list of long-range GABAergic neurons can be attributed mainly to the increasing use of adeno-associated virus (AAV) as a vector to target GABAergic neurons for optogenetic manipulations. This tool has allowed researchers not only to reveal novel projections but also to characterize their postsynaptic effects in vitro and in vivo. In this Review, we focus on advances that have been made since the first use of AAVs to visualize and functionally study cortical GABAergic long-range projections in 2012 (REF.[5]) and highlight some pressing questions for future research. A review on cortical GABAergic projections discovered before 2012 is available elsewhere[6].

Based on anatomical and in vitro electrophysiological studies, certain connectivity patterns for GABAergic long-range projections have emerged and, based on these data, speculations as to the in vivo function of these projections have been made. Below, we discuss the in vivo studies that addressed the question of whether the frequently postulated function of synchronization and disinhibition at long distance holds true and, if so, under which behavioural conditions GABAergic long-range projection neurons get recruited.

Definition of GABAergic projections

In this article, we use the term 'GABAergic projection' to refer to long-range axons that span regions of different sensory modalities and/or executive and cognitive functions. Thus, we do not consider projections between subregions of a brain area (for instance, subfields of the hippocampus) or between primary and secondary cortical areas of the same modality. We do

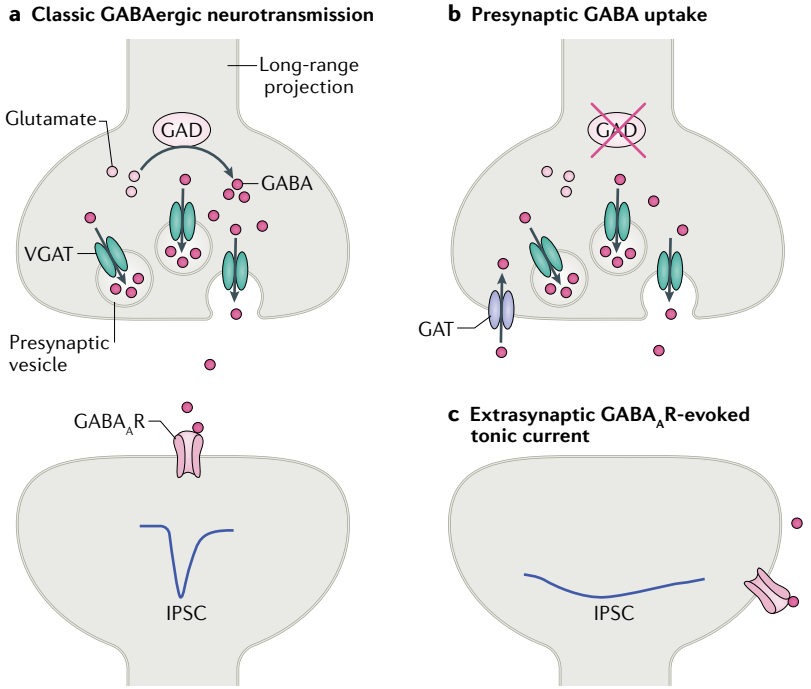


Fig. 1 | **GABAergic projection neurons form classical and atypical synapses.** **a** | Schematic drawing that depicts a classic GABAergic synapse. The presynaptic long-range axon terminal harbours the molecular machinery for GABA synthesis (glutamate decarboxylase (GAD)) and vesicular GABA transport (vesicular GABA transporter (VGAT)) and the postsynaptic site is equipped with synaptic $GABA_A$ receptors ($GABA_A$Rs) that mediate fast inhibitory postsynaptic currents (IPSCs) in response to GABA release. **b** | Alternatively, the presynaptic terminal contains GABA-containing, $VGAT^+$ vesicles as a result of GABA uptake mediated by the GABA transporter (GAT) in the absence of cell internal GABA synthesis. **c** | Activation of the long-range axonal terminal induces slow inhibitory postsynaptic currents that are mediated by extra-synaptic $GABA_A$Rs at the postsynaptic site. For most GABAergic projections, only one or a few of the features depicted in parts **a**–**c** have been investigated to determine their GABAergic nature.

include projections to the contralateral hemisphere that connect cortical areas of the same modality as well as projections connecting areas of different modalities within one hemisphere (for example, motor and somatosensory areas).

We use the term GABAergic projection neurons if the neurons under study met one of the following criteria: expression of GABA-synthetizing proteins; expression of vesicular GABA transporter (VGAT); presence of GABA in synaptic vesicles; expression of neurochemical markers known to be present in cortical GABAergic neuron subtypes such as parvalbumin (PV; a caveat is that PV is expressed in a few glutamatergic cells in the retrosplenial and the somatosensory cortices[7]), somatostatin (SOM; a caveat is that at least one SOM Cre-driver line that is often used to identify and target SOM neurons is associated with erroneous Cre expression in some PV^+ and PV^- SOM^- neurons[8–10]) and vasoactive intestinal peptide (VIP); and induction of GABA receptor-dependent postsynaptic currents in targeted neurons.

Most GABAergic projections presumably form classic GABAergic synapses (FIG. 1) but some probably deviate from this pattern. For instance, dopaminergic ventral tegmental area–substantia nigra neurons projecting to the striatum do not express any type of glutamate decarboxylase (GAD) and thus do not synthetize GABA, but they take up GABA from the extracellular space through membrane-bound GABA transporters[11,12]. Another 'atypical GABAergic projection' is the one from the tuberomammillary nucleus to the cortex. Here, GABA is synthesized and released and acts via extrasynaptic $GABA_A$ receptors, resulting in slow tonic inhibition[13]. This type of GABAergic connection has also been found in neurons of the developing hippocampus, in which its activation induces tonic currents that, in turn, cause depolarization owing to a high intracellular chloride concentration in the postsynaptic cell[14].

Heterogeneity of GABAergic projections

The ever-increasing use of AAV-based tracing methods has led to the identification of an unexpectedly high number of GABAergic projections that were unknown before 2012 (TABLE 1, FIG. 2) and it is safe to assume that more projections will be discovered in the coming years. In parallel, previously identified pathways have been studied in more detail and an unexpected diversity in these projections has been found. Based on anatomical investigations, it initially appeared that all GABAergic projections from the medial septum (MS) to the hippocampus were PV^+ (REF.[15]). However, subsequent electrophysiological studies identified fast-firing and burst-firing GABAergic projection cells in the MS, pointing towards the presence of at least two physiologically distinct subtypes. In fact, we now know that, in rodents, MS GABAergic projections to the hippocampus and the medial entorhinal cortex (MEC) originate from several cell types, namely calbindin $(CB)^+$, PV^+ and choline acetyltransferase $(ChAT)^+$ neurons[16–20]. There is still no consensus regarding the co-expression of some of these markers (for example, CB and ChAT[16,20]). Importantly, CB^+, PV^+ and $ChAT^+$ projections from the MS to the entorhinal cortex innervate layer 1/2 (L1/2) cells in the MEC with different cell-type specificity[17,18]. However, other studies demonstrated that PV^+ septal projection neurons that express DNA-binding protein SATB1 differ according to metabotropic glutamate receptor 1A (mGluR1A) expression, activity patterns and axonal targeting[21,22]. Thus, neurons with no detectable mGluR1A expression innervate PV^+ axo-axonic and cholecystokinin-expressing cells in the hippocampal CA3 region selectively[22], whereas mGluR1A-expressing neurons innervate GABAergic neurons mainly in the MEC and presubiculum[21]. The latter findings have been facilitated by the use of single-unit recordings and the juxtacellular labelling of single neurons, a technique that provides unparalleled detailed information about the functional, chemical and morphological diversity of projection neurons. Studies of this kind — that is, studies that provide information at multiple levels of analysis — will be of extreme importance in furthering our understanding of projection neurons.

In addition to the neurons described above, there is a plethora of cortical GABAergic projection neurons, many of which were discovered using AAV-based tracing. Previous retrograde tracing studies had indicated the presence of cortico-cortical projections originating mainly from neurons expressing SOM and neuronal

Tonic
This refers here to currents that are relatively long lasting and emerge through the opening of extrasynaptic receptors.

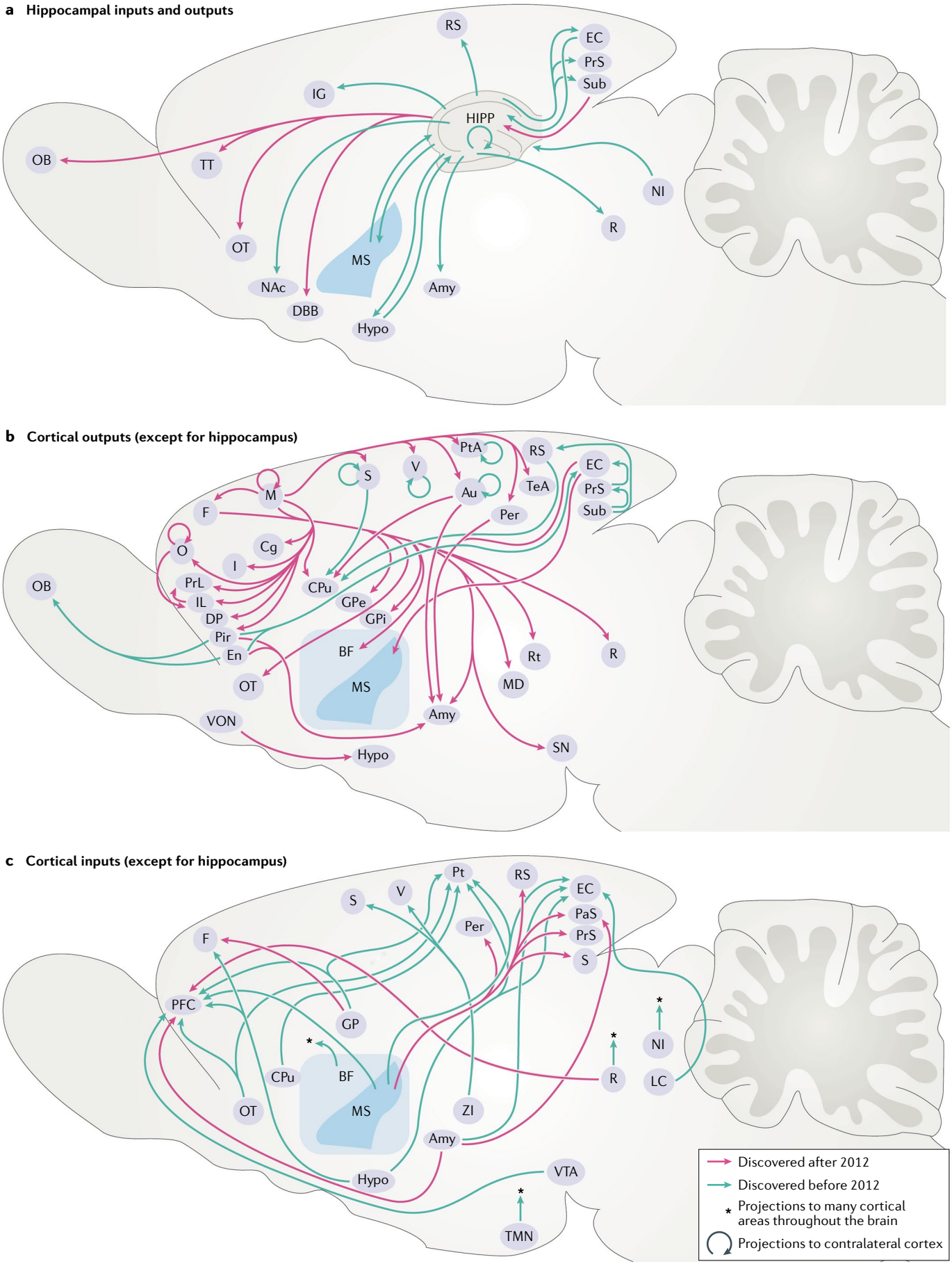
nitric oxide synthase (nNOS)[23] and of corticofugal PV$^+$ neurons projecting to the striatum[7]. Using AAV-based tracing, three recent publications revealed cortical SOM$^+$ neurons (mainly in deep layers) that project to several subcortical brain areas[24–26]. Moreover, PV$^+$ and VIP$^+$ GABAergic corticofugal projections have been discovered[24,26,27]. Several studies[24,28–30] provide increasing evidence that several, if not all, cortical areas harbour GABAergic neurons that project at a long distance to other cortical and subcortical regions (TABLE 1).

Recent functional studies that differentiate between GABAergic projection neurons based on SOM, PV

Table 1 | **Newly discovered projections to and from the neocortex and archicortex**

Source area	Target area	Tracing technique	Refs
Hippocampal projections			
CA1 stratum oriens (VIP$^+$)	Subiculum	Retrobeads, patch clamping with biocytin labelling	87
CA1 stratum oriens (nNOS$^+$)	Tenia tecta, diagonal band, medial septum, subiculum, entorhinal cortex, mammillary nuclei, lateral hypothalamus, olfactory tubercle, olfactory bulb, ipsilateral dentate gyrus and contralateral hippocampus	Cre and Flp-dependent AAV expression, fluorogold	101
Cortical projections			
Neocortex (orbitofrontal and motor cortices)	Mediodorsal thalamic nucleus (ipsilateral and contralateral), reticular thalamic nucleus, amygdala, dorsal raphe, cerebral cortex (ipsilateral and contralateral), CPu (ipsilateral and contralateral), LGP, MGP, nucleus accumbens, olfactory tubercle, substantia innominata, ventral pallidum (ipsilateral and contralateral) and substantia nigra	AAV, Fast Blue	24
MEC	MS	CTB	17
Motor and auditory cortices	Striatum	AAV	25
Subiculum	CA1	Pseudotyped rabies tracing	138
Endopiriform nuclei, piriform cortex, entorhinal cortex and perirhinal cortex	Amygdala	Fluorogold (iontophoretic)	29
Motor cortex	Striatum, insular cortex, frontal association area, somatosensory cortex, dorso-lateral orbital cortex, prelimbic cortex, anterior cingulate cortex, perirhinal cortex, auditory cortex, temporal association area, contralateral motor cortex, parietal cortex, orbital cortex, dorsal peduncular cortex, infralimbic cortex, piriform cortex and visual cortex	CTB, AAV, pseudotyped rabies tracing	26
Infralimbic cortex	Prelimbic cortex	Electrophysiology	30
Ventral olfactory nucleus	Lateral hypothalamus	CTB, pseudotyped rabies tracing	141
Auditory cortex	Lateral amygdala	AAV, CTB, Retrobeads	104
Medial septal projections			
MS (PV$^+$)	Parasubiculum, presubiculum and subiculum, LEC, MEC, retrosplenial cortex and perirhinal cortex	Retrobeads, PHAL, AAV	16,17
MS (CB$^+$)	MEC and hippocampus	AAV, PHAL, Retrobeads	16,17
MS	Double projection to the MEC and presubiculum	Juxtacellular labelling, AAV, retrograde rabies virus tracing	21
Subcortical projections to cortex			
GPe	Cortex	Fluorogold, AAV, biotinylated dextran amine, Retrobeads	103,142
Amygdala	Parasubiculum	Fluorogold (iontophoretic)	143
Amygdala (CeMA)	Ventromedial prefrontal cortex	AAV	144
Raphe nucleus	Medial prefrontal cortex	Fluorogold in GAD67–EGFP mice	145

AAV, adeno-associated virus; CB, calbindin; CeMA, centromedial nucleus of the amygdala; CPu, caudate putamen; CTB, cholera toxin subunit B; EGFP, enhanced green fluorescent protein; GPe, external globus pallidus; LEC, lateral entorhinal cortex; LGP, lateral globus pallidus; MEC, medial entorhinal cortex; MGP, medial globus pallidus; MS, medial septum; nNOS, neuronal nitric oxide synthase; PHAL, phaseolus vulgaris leucoagglutinin; PV, parvalbumin; VIP, vasoactive intestinal peptide.


a Hippocampal inputs and outputs
RS
EC
PrS
Sub
IG
HIPP
OB
TT
NI
R
OT
MS
NAc
Amy
DBB
Hypo
b Cortical outputs (except for hippocampus)
PtA
RS
EC
S
V
M
TeA
PrS
Au
Sub
F
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Hypo
c Cortical inputs (except for hippocampus)
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Hypo
VTA
*
TMN
Discovered after 2012
Discovered before 2012
* Projections to many cortical areas throughout the brain
Projections to contralateral cortex

◀ Fig. 2 | **Cortical GABAergic projections.** Cortical GABAergic projections form a widely distributed neuronal network spanning many cortical and subcortical brain areas. The schematics depict published GABAergic projections from and to the hippocampus (part **a**), from the cortex (part **b**) and to the cortex (part **c**). The discovery of cortical GABAergic projections has been strongly facilitated by the use of viral-tracing techniques. The first study in which these techniques were used in this context was published in 2012 and projections described in papers published from 2012 onwards are highlighted in pink. Projections that express different markers and that are from or to different sub-nuclei are not separately shown. Branching points in our schematic do not necessarily indicate the existence of double-projecting neurons but only serve the purpose of simplifying the image. Amy, amygdala; Au, auditory cortex; BF, basal forebrain (comprising the MS, ventral pallidum, diagonal band nuclei, substantia innominata–extended amygdala and peripallidal regions); Cg, cingulate cortex; CPu, caudate putamen; DBB, diagonal band of Broca; DP, dorsal peduncular cortex; EC, entorhinal cortex; En, endopiriform nucleus; F, frontal cortex; GP, globus pallidus; GPe, external globus pallidus; GPi, internal globus pallidus; HIPP, hippocampus; Hypo, hypothalamus; I, insula; IG, indusium griseum; IL, infralimbic cortex; LC, locus coeruleus; M, motor cortex; MD, mediodorsal thalamus; MS, medial septum; NAc, nucleus accumbens; NI, nucleus incertus; O, orbitofrontal cortex; OB, olfactory bulb; OT, olfactory tubercle; PaS, parasubiculum; Per, perirhinal cortex; PFC, prefrontal cortex; Pir, piriform cortex; PrL, prelimbic cortex; PrS, presubiculum; PtA, parietal association area; R, raphe; RS, retrosplenial cortex; Rt, reticular nucleus; S, somatosensory cortex; SN, substantia nigra; Sub, subiculum; TeA, temporal association area; TMN, tuberomammillary nucleus; TT, tenia tecta; V, visual cortex; VON, ventral olfactory nucleus; VTA, ventral tegmental area; ZI, zona incerta.

and/or SAT1B, and mGluR1A expression[21,22,26] suggest that a detailed classification of projection neurons is relevant to dissect their diverse complex postsynaptic effects and functions in fine-tuning behaviour. A classification beyond marker expression including other criteria, such as the identity of postsynaptic targets and state-dependent neuronal activity, as performed by Unal et al.[31] and Joshi et al.[22] for septo–hippocampal projections, is also a desirable goal in future studies of other GABAergic projection neurons. Thus, it is likely that SOM$^+$ cortical projection neurons are differentially recruited during the various cognitive or behavioural events: we know, for example, that in the somatosensory cortex, whisker stimulation results in decreased SOM$^+$ neuron activity in L2/3 and L5a but increased activity in L4 and L5b to L6 (REF.[32]). Whether such functional differences apply also to SOM$^+$ projection neurons in these cortical layers remains to be established.

Functions of GABAergic projections

Many ascending GABAergic projections from the brainstem, thalamus and basal forebrain have been implicated in wakefulness or arousal (for a review, see REF.[33]). In addition, several recent studies have revealed that certain GABAergic projections connecting subcortical brain areas support functions related to sleep states[34] and feeding behaviour[35]. However, subcortical projections, including GABAergic projection neurons in the basal ganglia, are beyond the scope of this Review.

For cortical GABAergic projection neurons, one major function that has been repeatedly proposed pertains to the synchronization of distant brain areas[6,23,36–38]. A number of considerations support this conjecture. First, GABAergic projection neurons often target GABAergic neurons, which in turn regulate local network oscillations (see the review by Caputi et al.[6] and Supplementary Table 1 for newly discovered targets). Second, GABAergic projections often exhibit extensive arborisation in the target area and there is evidence for individual GABAergic projection neurons that target more than one downstream area. These features probably facilitate the synchronization of several target cells or areas[21,39–41]. For example, individual PV$^+$ and/or CB$^+$ GABAergic neurons in the MS project to both the entorhinal cortex and hippocampus[17], the hippocampus and subiculum[31], or the entorhinal cortex and presubiculum[21]. Third, MS and hippocampal long-range axons are heavily myelinated, which might subserve fast action potential propagation velocity and temporal accuracy[15,41–43]. Single-cell sequencing revealed the specific expression of the myelin marker pleiotrophin in nNOS$^+$ putative cortical GABAergic projection neurons[44]. Fourth, several hippocampal and septal GABAergic projection neurons are coupled to network oscillations[21,22,31,41,43,45,46] (FIG. 3). Fifth, GABAergic projection neurons facilitate oscillations at resonance frequency as revealed by optogenetic activation and inhibition experiments[5,19,47–49].

It is thought that temporally coordinated activity in brain areas at long distance is necessary for the precise timing of incoming and outgoing signals to facilitate cognitive processes[50,51]. Indeed, synchronized neuronal activity has been detected between many cortical and subcortical brain areas and its functional significance has been discussed in the context of several disorders, such as schizophrenia, autism[52] and depression[53], and in processes such as decision-making[54] and memory formation[55]. However, studies addressing the cognitive and behavioural effects following the manipulation of long-range GABAergic activity have remained scarce. Moreover, it is not clear whether any of the reported cognitive and behavioural effects are linked to altered synchronization between distant brain areas via oscillatory coupling. Below, we take a closer look at projections from and to the cortex whose function and oscillatory activity have been investigated in more depth.

Septal projections

To date, the septo–hippocampal pathway is the best-studied of all corticopetal and corticofugal GABAergic projections in terms of diversity and functionality. This pathway comprises PV$^+$ and CB$^+$ GABAergic projections[56] as well as cholinergic–GABAergic projections (some of which also express CB[16,19,57]) that target distinct populations of neurons in CA1, CA3 and the dentate gyrus (DG) (FIG. 4).

The MS is crucial for the generation of theta oscillations in the hippocampus and has a role in spatial learning and memory[58–64]. Lesions of the MS lead to decreased theta rhythmicity in the hippocampus and to impaired spatial working memory[65]. Of note, inactivation of GABAergic septal neurons alone is sufficient to impair spatial learning[66,67]. Finally, pharmacological inactivation of MS neurons disrupts theta oscillations and the firing of spatially tuned neurons (grid cells) in the MEC[68–70].

Do septo–hippocampal projections modulate network oscillations? It has long been hypothesized that cholinergic fibres drive theta oscillations whereas GABAergic fibres support their precise timing[36,38]. Such a simplified dichotomy is difficult to maintain in view of the increasing evidence that a marked number of ChAT$^+$ terminals

Theta oscillations
Oscillations of extracellularly recorded currents in the hippocampus at frequencies between 5 and 12 Hz; this rhythmic activity is most prominent during exploratory behaviour.

Grid cells
Neurons in the medial entorhinal cortex that are spatially tuned and whose hexagonal firing pattern accounts for the naming of this cell type; they support spatial memory and navigation.

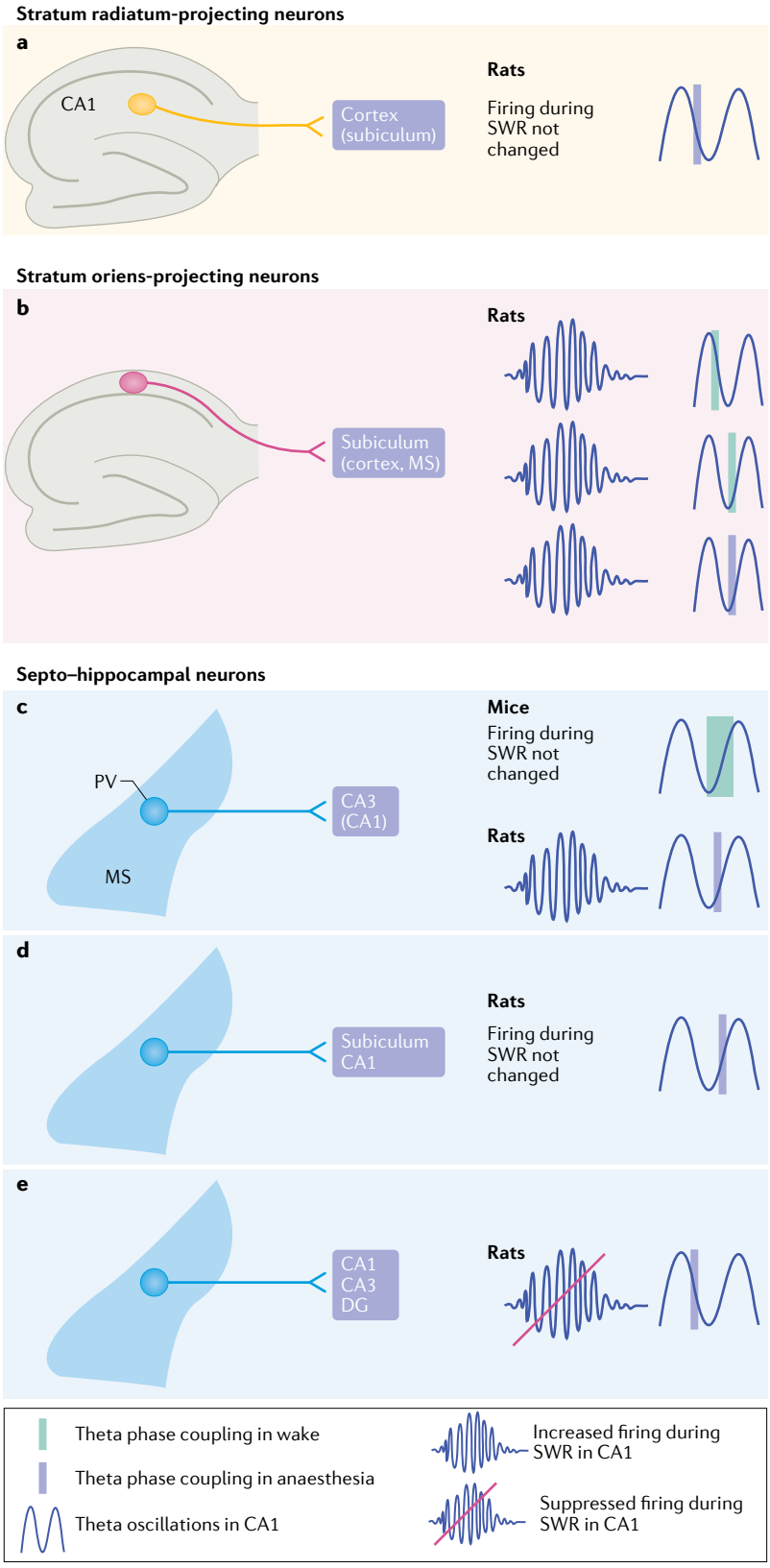

Fig. 3 | **Oscillatory coupling of rhythmically firing hippocampal and septal GABAergic projection neurons.** Schematic depicts projections with known activity patterns during theta oscillations and sharp wave–ripples (SWRs). **a** | A study found that projection neurons in stratum radiatum ($n = 2$ cells) exhibited preferred firing at the descending phase of CA1 theta oscillations[41]. Both cells projected to the cortex, with one of them projecting additionally to the subiculum. **b** | Projection neurons in stratum oriens fired preferentially at the early ascending phase of theta oscillations and increased their firing rate during SWRs. The target areas for the two cell types in the first two rows in part **b** are unknown but, considering a previous publication[41], the subiculum can be thought of as a putative target area. The depicted characteristics of the first and second types are based on 4 and 2 cells, respectively, with a theta phase coupling of 226–305 degrees (descending phase) and 0.6–2.7 degrees (early ascending phase), respectively[46]. Neurons of the third type depicted in the bottom row ($n = 6$ cells) projected to the subiculum. Of these 6 cells, 4 also projected to the medial septum (MS) and 3 also projected to the cortex[41]. **c** | Septal parvalbumin-expressing (PV^+) neurons projecting to CA3 fired preferentially during or around the trough of theta[22]. They included the so-called 'Teevra cells' ($n = 8$ cells with identified projections in CA3 and PV immunoreactivity) that fired shortly before the trough or during the ascending phase of theta (342–357 and 5.7–138.6 degrees, respectively). SWRs were detected only in a few cases ($n = 4$ Teevra cells) and, although not conclusive, it appears that the firing frequency during SWRs did not change in this cell type. Of the 8 cells, 3 had axonal branches in CA1 (REF.[22]) (see also TABLE 2, cell type 1). Unal et al. found a similar cell in anaesthetized rats (second row, $n = 1$; see also TABLE 2, cell type 2) without CA1 projections and with increased SWR-associated firing[31]. **d** | A septal PV^+ neuron projecting to CA1 and subiculum preferentially fired during the late ascending phase of CA1 theta oscillations ($n = 1$, see also TABLE 2, cell type 3)[31]. **e** | A septal PV^+ neuron projecting to the dentate gyrus (DG), CA1 and CA3 exhibited preferential firing during the descending phase of theta and a decreased firing rate during SWRs ($n = 1$, see also TABLE 2, cell type 4)[31].

co-transmit GABA[19] (see below). The first evidence that septal GABAergic projections affect hippocampal oscillations was obtained in acute slice preparations in which septo–hippocampal connections remained intact[71]. In this study, electrical stimulation of septal fibres at theta frequency entrained the firing of CA1 pyramidal cells, which was thought to result from the rebound firing of rhythmically inhibited GABAergic interneurons. Subsequently, recordings with extracellular electrodes in the MS and hippocampus in rats during sleep revealed that most cells in the MS are coupled to hippocampal theta oscillations but are less active during hippocampal sharp wave–ripples (SWRs)[45], further substantiating the idea that septo–hippocampal projections specifically drive hippocampal theta oscillations. Borhegyi et al. provided the first evidence for differential activation of GABAergic PV^+ projection neurons in 2004 (REF.[43]). The authors hypothesized that septal neurons firing at the peak and trough of hippocampal theta respectively target distal dendrite-targeting and somata-targeting interneurons in the hippocampus — an innervation pattern that would support the precise pyramidal cell firing at the trough of theta oscillations. More than 13 years later, using single-cell recordings and juxtacellular labelling, Joshi et al. and Unal et al. were able to characterize innervation patterns and in vivo activity patterns of

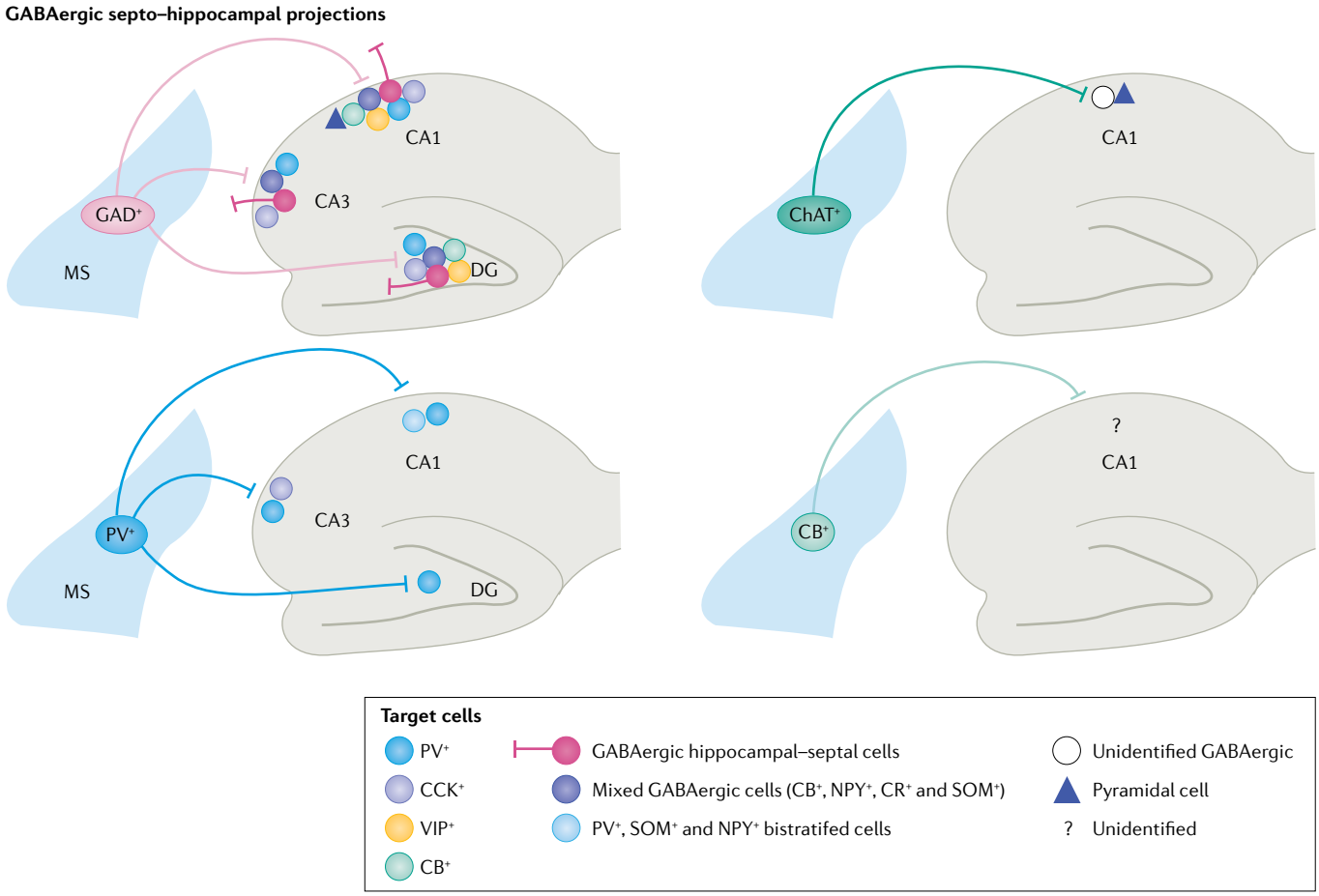

Fig. 4 | **Target specificity of septo–hippocampal GABAergic neurons.** Molecularly defined septo–hippocampal GABAergic neurons innervate distinct subtypes of hippocampal neurons. Schematic drawing of projections and target cells. GABAergic septo–hippocampal projections target a heterogeneous population of interneurons and pyramidal cells. Parvalbumin-expressing (PV^+) GABAergic projection neurons exhibit specific targeting of subsets of hippocampal interneurons (mainly PV^+). The target cells of choline acetyltransferase-expressing ($ChAT^+$) and calbindin-expressing (CB^+) projection neurons remain to be fully determined. CCK, cholecystokinin; CR, calretinin; DG, dentate gyrus; GAD, glutamate decarboxylase; MS, medial septum; NPY, neuropeptide Y; SOM, somatostatin; VIP, vasoactive intestinal peptide.

several PV^+ projection neurons with such properties[22,31] (TABLE 2). However, the latter study also pointed towards an unexpected diversity of PV^+ septo–hippocampal neurons. Thus, at least in rats, four PV^+ septo–hippocampal neuron types were discernible when considering their temporal spike-coupling to ongoing CA1 theta oscillations and their cellular targeting in the hippocampus[31] (TABLE 2, FIG. 3).

Interestingly, Unal et al. found that projection neurons also innervated local neurons, suggesting that these cells are ideally suited to synchronize local as well as distant networks[31]. It is noteworthy that, in one of the first publications on cortico-cortical GABAergic projection neurons, published in 1989, Germroth et al. already pointed out that individual GABAergic projection neurons from the MEC to the hippocampus exerted simultaneous local (MEC) and remote (hippocampus) inhibition[72]. In the context of these considerations, the most interesting question is whether connectivity rules in the two target areas diverge. One furthermore wonders whether local and distant axon branches derived from the same source neuron differ functionally (for example, axon thickness, myelination, release probability).

It should be noted here that not all septo–hippocampal neurons fire with high rhythmicity. A recent study demonstrated the existence of low-rhythmic-firing neurons with less reliable phase coupling to theta oscillations and frequent skipping of theta cycles[73]. Their molecular, morphological and target cell profile was distinct from the PV^+ projection neurons described above, thus adding to the diversity of septo–hippocampal projection neurons (TABLE 2).

Consistent with a scenario according to which GABAergic septo-hippocampal neurons are involved in the generation of hippocampal theta oscillations, a study found that, during locomotion, the overall activity of GABAergic septo–hippocampal axon terminals measured by GCaMP imaging in CA1 correlated with the power of theta oscillations (type I theta; the precise timing could not be revealed in this study owing to the low temporal resolution of GCaMP imaging)[74]. The identity of the GABAergic terminals was not further

Sharp wave–ripples (SWRs). SWRs are extracellularly recorded, high frequency (150–250 Hz) synaptic currents that emerge through the highly synchronous firing of neurons in the hippocampus during immobility and slow wave sleep.

Phase coupling Temporal alignment of the phases of two oscillators such that the first oscillator coincides with a fixed phase of the second oscillator.

Table 2 | **Characteristics of PV$^+$ septo–hippocampal neurons**

Target cells in the hippocampus	Coupling to CA1 theta phase[a]	Firing rate during theta versus non-theta	Spike rates during CA1 SWR events	Anaesthesia[b]	Organism	Number of cells detected	Refs
CA3 (2 out of 2 tested neurons innervated PV$^+$ axo-axonic cells, and 1 out of 2 tested neurons innervated CCK$^+$ cells); partly CA1 (3 out of 8 cells)	Trough and ascending phase (during run and rest)[c]	Unchanged	Constant	Non-anaesthetized, head fixed	Mice	9	22
CA3 (few somata in stratum lucidum and radiatum)	Trough or early ascending phase	Increased	Increased	Urethane	Rat	1	31
CA1 (PV$^+$, SOM$^+$, NPY$^+$ bistratified cells); subiculum	Late ascending phase	Unknown	Constant	Urethane	Rat	1	31
CA3 (PV$^+$ axo-axonic cells); DG (PV$^+$ cells and a single nNOS$^+$ cell); CA1 (PV$^+$ axo-axonic cells)	Descending phase	Unchanged	Suppressed	Urethane	Rat	1	31
CA3 PV$^+$ basket cells; a single CA3 nNOS$^+$ cell; a single CA3 SOM$^+$ cell	Unknown	Unknown	Unknown	Urethane	Rat	1	31

CCK, cholecystokinin; DG, dentate gyrus; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; PV, parvalbumin; SOM, somatostatin; SWR, sharp wave–ripple. [a]Theta oscillations were recorded in CA1 stratum oriens or pyramidale. [b]Urethane anaesthesia results in type II theta. [c]Theta during running is defined as type I theta whereas theta during rest can be defined either as type I (during rapid eye movement sleep) or type II (mainly upon salient stimuli).

analysed in this study. Hence, it is not clear whether any of the above-mentioned GABAergic terminals in CA1 (TABLE 2) were analysed. Interestingly, theta oscillations induced by salient events (putative type II theta, as described in REF.[75]) were not accompanied by increased axon terminal activity, suggesting that this population of CA1-projecting GABAergic neurons was implicated in type I theta specifically.

Although these findings strongly suggest that GABAergic septo–hippocampal neurons are implicated in hippocampal oscillatory activity, they do not provide causal evidence. One recent study addressed this association. It showed that optogenetic stimulation and inhibition of PV$^+$ septo–hippocampal projections in urethane-anaesthetized mice respectively enhanced (but did not initiate) and decreased type II theta yet both manipulations had no effect on type I theta in the hippocampus[48]. This finding is surprising considering the studies suggesting that the firing of PV$^+$ septo–hippocampal projections is phase-coupled to type I hippocampal theta[22,31]. Considering the naturally occurring diversity of PV$^+$ projection neuron firing with respect to theta phase[22,31] and the existence of additional PV$^-$ GABAergic projection neurons with unknown theta coupling, it remains an open question as to how these cells contribute to hippocampal type I and type II theta oscillations.

Do septo–hippocampal neurons modulate cognition and behaviour? GCaMP imaging of septo–hippocampal terminals in CA1 revealed surprisingly general, non-specific activation upon locomotion and presentation of stimuli of several sensory modalities, including air puffs, auditory stimuli and light flashes (but not appetitive water rewards)[74], raising the question of whether distinct projection neurons[31] exhibit functional specialization (in this study, GCaMP was expressed in septal GABAergic cells, thus comprising several types of GABAergic projection neurons). Indeed, responses in boutons from a given axon were more similar to each other than to those in boutons from other axons, suggesting functional diversity among the projection neurons[74]. It will be a challenging task to correlate those functional features with the chemical, morphological and electrophysiological properties described in other studies[16,17,22,31]. Of note, distinct boutons that were activated by stimuli of several modalities (for example, locomotion and air puffs) seemed to innervate the same postsynaptic CA1 interneuron. Interestingly, the population activity correlated with the intensity or salience of the cue. Thus, bouton activity correlated with the intensity of air puffs. Notably, the most salient stimulus (that is, an air puff) evoked the largest response, with more than 70% of imaged boutons being activated[74]. GABAergic septo–hippocampal projections could thus serve to encode the intensity or salience rather than the modality of the signal and detect the coincidence of multimodal inputs. The increased activation upon exposure to an intense or a salient cue could facilitate the routing of salient signals impinging onto hippocampal circuits from other neuronal sources.

The behavioural effects of the preferred saliency-dependent recruitment of septo–hippocampal neurons can be inferred by the findings from yet another study in which the authors demonstrated that optogenetic activation of PV$^+$ septo–hippocampal neurons enhances exploration in the presence of novel objects but not in an open field without salient cues[48].

Entorhino–hippocampal projections

GABAergic entorhino–hippocampal projection neurons are heterogeneous and include PV$^+$ and PV$^-$ neurons[5]. GABAergic projection neurons have been found both in the MEC[5] and lateral entorhinal cortex (LEC)[76]. It is widely held that excitatory spatially tuned cells in the MEC support spatial representations for episodic memory, whereas excitatory LEC neurons exhibit weaker spatial tuning[77] and convey non-spatial, object-related information[78]. Thus, it is probable that GABAergic projections from the MEC and LEC also contribute

differentially to distinct representations. Further evidence for differential information processing in the MEC and LEC is that, in the MEC, neuronal activity exhibits pronounced theta and space modulation (that is, 'phase precession')[79]. Consistent with this finding, GABAergic inputs from the MS that are thought to have a theta pacemaker function[80] are more abundant in the MEC than in the LEC[16]. These findings indicate that GABAergic projection neurons in the MEC are subject to stronger theta modulation than those in the LEC.

Consistently, we showed that optogenetic stimulation of PV^+ GABAergic MEC axon terminals in the CA1 enhances CA1 theta oscillations in vitro[5]. A recent study provided indirect evidence that PV^+ projections from the MEC might also be implicated in hippocampal theta oscillations in vivo[81]. In this study, 83% of hippocampus-projecting, fast-spiking neurons (putative PV^+ neurons) in the MEC were speed modulated, that is, their firing rates correlated with the running speed of mice. Since theta oscillation frequency and power correlate with locomotion speed[82–84], this study strongly supports the notion that the activity of PV^+ projection neurons in the MEC increases with the occurrence of hippocampal theta in vivo. It remains to be established whether these projections causally contribute to the occurrence of theta oscillations and whether precise theta phase coupling of PV^+ projection neuron firing is required.

GABAergic axon terminals in the CA1 that derive from LEC neurons[76] showed many similarities to those of the septo–hippocampal pathway[74]. First, GCaMP imaging of axon terminals revealed responses to stimuli of several modalities (visual and auditory sensory inputs as well as air puffs, water rewards, spontaneous running and licking). Second, the most salient stimulus (aversive air puffs) elicited the strongest response (22.9% of boutons were recruited). Third, responses to distinct stimuli were more similar across boutons from one axon than across neighbouring boutons from different axons, suggesting functional diversity within this pathway. In fact, individual boutons responded to stimuli of two modalities at most[76]. All this information prompts the still open question of whether boutons with different activity patterns exhibit molecular and morphological diversity.

The documented function of this GABAergic projection is the disinhibition of CA1 pyramidal cells via the inhibition of hippocampal cholecystokinin-expressing interneurons. Thus, the activation of LEC GABAergic projections amplified CA3 to CA1 pyramidal cell input[76]. Long-term potentiation (LTP) at this synapse is crucial for learning[85]. Interestingly, pharmacogenetic inhibition of GABAergic projections from the LEC to the hippocampus during learning increased freezing and overgeneralization in a contextual (but not cued) fear-conditioning task and impaired novel object recognition. This suggests that LEC–hippocampal projections support the specificity or accuracy of memories for objects and contexts[76]. Further classification of GABAergic LEC–hippocampal projections might help to dissect distinct projections that support either contextual or object memories. In addition, it remains to be established whether and to what extent GABAergic projections from the MEC to the hippocampus modulate the formation of hippocampus-dependent memories.

Long-term potentiation (LTP). Long-term potentiation is a form of plasticity reflecting long-lasting increases in synaptic strength.

Hippocampal outputs

It has been long known that hippocampal GABAergic projection neurons have diverse molecular identities, morphologies and firing activities[41,86]. By and large, CA1 stratum oriens neurons projecting to the subiculum, other cortices and the MS increase their firing during SWRs, whereas stratum radiatum neurons projecting to subiculum, presubiculum, retrosplenial cortex and indusium griseum fire strongly during theta oscillations and do not increase their firing rate during SWRs[41] (FIG. 3). A recent study by Katona et al.[46], comprising six projection neurons in stratum oriens of CA1 in non-anaesthetized rats, confirmed that all these neurons exhibited increased activity during SWRs (FIG. 3). The neurons were strongly phase-coupled to the descending phase (226–305 degrees) or trough (0.6–2.7 degrees) of type I theta (locomotion-associated theta)[46]. However, the study also revealed that the neurons were highly diverse with respect to state-dependent firing rates and oscillatory phase coupling. The mean firing rates of the six projection neurons ranged from 4.1 to 25.3 Hz during movement and 19.1 to 144.8 Hz during SWRs. In addition, neurons fired at different gamma frequencies and had different burst firing probabilities. In general, burstiness was more prevalent during slow-wave sleep than during movement, and two out of seven cells increased in burst probability from <1% to >88% during SWRs. In summary, projection neurons in this study exhibited multiple activity patterns during wakefulness and sleep, locomotion and rest, theta oscillations and SWRs. Each projection neuron had a unique combination of properties that did not match the main profiles of locally recorded (putatively non-projecting) GABAergic neurons. Most projection neurons were characterized by increased SWR-associated firing rates and coupling to the descending phase of theta. Notably, when considering local interneurons, these two properties typically did not co-occur in the same cell, but were reported for bistratified cells and PV^+ cells, respectively[46], suggesting that GABAergic projection neurons are physiologically distinct from local neurons.

Adding to this diversity, a recent study by Francavilla et al. demonstrated the existence of subiculum-projecting stratum oriens neurons whose activity did not increase during SWRs[87], thus contrasting to most neurons described by Katona et al.[46]. The neurons found by Francavilla et al.[87] increased their activity during immobility (independent of SWR activity) and decreased their activity during locomotion and theta oscillations. This cell type was marked by the expression of VIP and, depending on the mouse line, also of the muscarinic M2 receptor (M2R); two other VIP^+ cell types with local axon arbors lacked M2R. This study was performed based on two-photon imaging of GCaMP fluorescence after cortex aspiration in head-fixed *VIP-Cre* mice. It thus remains to be clarified to what extent the differences in activity in the studies by Katona et al.[46] and Francavilla et al.[87] can be attributed to species and methodological differences.

The first functional inactivation study of hippocampal projection neurons involved DREADD-mediated inactivation of GABAergic projections from the CA1 to the retrosplenial cortex. Inactivation of these neurons increased the duration of freezing in a contextual fear-conditioning task[88]. Further characterization of these cells revealed molecular features of neurogliaform cells. Interestingly, however, the dendritic and local axonal arbors were markedly different from those described for neurogliaform interneurons.

To conclude, similar to septal and entorhinal GABAergic projection neurons, hippocampal projection neurons comprise functionally diverse subclasses that await further differentiation based on molecular, morphological and physiological criteria to understand the full repertoire of these neurons in fine-tuning cognition and behaviour. It is remarkable that, in the hippocampus, electrophysiological recordings from as few as six neurons revealed such large diversity[46] and even more diversification is to be expected when more cells and additional criteria are considered. It appears to be a common theme that projection neurons in the hippocampus are molecularly, morphologically and functionally distinct from GABAergic interneurons. More detailed studies are needed to reveal whether this also pertains to neocortical and subcortical GABAergic projection neurons.

Neocortical outputs

Oscillations in distinct frequency bands in the neocortex have been implicated in perception, motor control and cognitive behaviours[89–97]. SOM$^+$ and PV$^+$ cortical interneurons were demonstrated to be crucial in the generation and/or maintenance of synchronous and rhythmic neuronal activity[98–100]. However, it is not clear whether GABAergic projection neurons support local and/or distant oscillatory activity and synchronization.

Two studies revealed putative behavioural functions of cortico-striatal GABAergic projections. The first study[27] demonstrated that optogenetic stimulation of projections from the medial prefrontal cortex to the nucleus accumbens induced avoidance behaviour in a real-time place preference task but had no effect on locomotion, anxiety-like behaviour, and social or novel object exploration. Since this pathway comprises at least two types of cells, namely PV$^+$ and VIP$^+$ neurons, it will be important to dissect the contribution of each cell type to the observed behavioural effect.

In a recent study, we compared PV$^+$ and SOM$^+$ projections from the primary and secondary motor cortex to the striatum and found region-specific and cell type-specific connectivity as well as differential effects on spontaneous locomotion upon optogenetic stimulation of axon fibre terminals[26]. Thus, this study substantiated the requirement for further differentiation of GABAergic long-range projections in future functional studies.

Common functions

In summary, GABAergic neurons connecting the MS, the hippocampus and parahippocampal areas constitute a neuronal network that comprises heterogeneous cell populations. As discussed above, strong evidence exists that the MS synchronizes the hippocampus and parahippocampal areas through precise theta-coupled firing. In turn, inhibition from the MEC to the hippocampus and from the hippocampus to the MEC and other parahippocampal areas probably supports synchronization, thereby enabling temporally precise information exchange to support coordinated processing of neuronal activity and spatial memory formation. We predict that further research will reveal additional cortical GABAergic projection neurons in these areas and will hopefully provide evidence as to their causal role in the initiation or maintenance of oscillations and in fine-tuning cognition and behaviour.

We expect that state-dependent and sensory input-specific neuronal codes will become apparent for distinct GABAergic projection neurons once the precise timing of action potential firing of individual projection neurons can be assessed in awake behaving animals engaged in specific tasks. Thus, it is probable that a neuron undergoes task-dependent shifts in firing frequency, rhythmicity and theta phase coupling, resulting in task-dependent synchronization of distant brain areas and thus in an increased signal transmission efficiency during precise time windows.

In our view, one of the most interesting questions is whether GABAergic projection neurons simultaneously control local and remote brain areas. For instance, the morphological characterization of hippocampal nNOS$^+$ GABAergic projection neurons[101] led the authors to conclude that the axons of these neurons arborize both locally within the CA1 region and in several distant brain areas, including the tenia tecta, diagonal band, MS, subiculum, entorhinal cortex, mammillary nuclei, lateral hypothalamus, olfactory tubercle, olfactory bulb, ipsilateral dentate gyrus and contralateral hippocampus. Locally, these GABAergic projection neurons target both pyramidal neurons and interneurons. The identity of the target cells in the remote brain areas has remained unknown.

Interestingly, the limited number of studies considered in this Review already allows us to conclude that local and distant connectivity rules for a given GABAergic projection neuron may diverge. For instance, a particular class of GABAergic projection neurons, namely VIP$^+$ neurons in the CA1, targets interneurons locally but interneurons and pyramidal cells distantly (that is, in the subiculum)[87]. When considering only remote targeting, the following scenarios have emerged: in the remote target area, GABAergic projection neurons inhibit inhibitory neurons (for example, GABAergic projections from the MEC to the CA1 (REF.[5]), the LEC to the CA1 (REF.[76]), the MS to the CA3 (REF.[31]), the MS to the MEC[17], the nucleus incertus to the CA1 (REF.[102]), and the globus pallidus to the cortex[103]), excitatory neurons (for example, GABAergic projections from the auditory cortex to the lateral amygdala[104]), or excitatory and inhibitory neurons (for example, GABAergic projections from the hippocampus to the MEC[5] and the hypothalamus to the CA1 (REF.[105])); more examples of remote targeting specificity are provided in Supplementary Tables 1, 2 and FIG. 4. When considering the different scenarios,

one pressing question arises, namely how effective is the recruitment of defined GABAergic projections in directing information flow between the different brain areas? For instance, Basu et al. demonstrated that axonal activation of LEC GABAergic projection neurons inhibited inhibitory neurons in the target area, thereby disinhibiting pyramidal cells and supporting the enhanced neurotransmission of LEC–hippocampal information flow within a defined time window following the recruitment of GABAergic long-range projections[76]. We suspect that the efficacy of GABAergic projection neurons in directing information flow depends largely on the network state of the source and target area, the connectivity pattern of the GABAergic projection neuron in the target area and the synaptic properties (for example, synaptic strength and the localization of the synapses along the somato–dendritic axis). For instance, axons derived from GABAergic projection neurons in the LEC cover a much larger territory in the CA1 than those from GABAergic projection neurons in the MEC. In addition, current amplitudes in the postsynaptic interneurons in the CA1 target area are four times larger upon axonal activation of LEC GABAergic projection neurons than of MEC GABAergic projection neurons[76]. Such pathway-specific functional characterization and the computations that the individual properties support remain to be elucidated for an ever-increasing number of novel GABAergic projection neurons.

Role in development and neurogenesis

During development, GABAergic interneurons modulate a number of processes, including cell proliferation, migration, synaptic wiring, synchronization of neuronal networks and plasticity, by which they govern the maturation of the cortex[106,107]. This, of course, prompts the question of whether GABAergic projection neurons support, in part or even entirely, any of these processes. Only a few studies have considered GABAergic projection neurons in the context of development. This is not surprising, given the lack of specific markers that help distinguish GABAergic interneurons and projection neurons.

Picardo et al. identified early-generated (that is, born before embryonic day 10.5 (E10.5)) GABAergic cells in the hippocampus with extensive axonal arborisation at postnatal day 7 (P7) that develop into long-range neurons[108]. These 'hub cells', as termed in an earlier study[39], can efficiently alter the hippocampal synchronized activity, which has been hypothesized to contribute to synaptic strengthening and structural refinement within the hippocampus[109,110].

Although the study by Picardo et al.[108] indicated that the peak of hub cell generation occurs well before that of GABAergic interneuron generation, this does not appear to hold true for long-range GABAergic projection neurons in general[101]. Indeed, Wick et al. described a class of hippocampal GABAergic projection neurons that are nNOS$^+$ and that are born between E10.5 and E11.5; that is, at a time that coincides with the peak of interneuron generation[101].

Two studies have described a GABAergic projection from the zona incerta to L1 of the somatosensory cortex[111,112]. This projection is densest during cortical development and becomes weaker after the second postnatal week, suggesting that it has a developmental function. Indeed, the ablation of synaptic vesicle release from zona incerta neurons during the first postnatal week decreased the number of spines and L5 pyramidal dendrite branches as well as the postsynaptic input frequency, suggesting that these projections promote neuronal development[113]. Consistent with earlier studies in the hippocampus[114,115], GABA released from these zona incerta neurons had depolarizing effects onto cortical pyramidal cells before P21 (REFS[113,116]). Intriguingly, GABAergic long-range input was also found in cortical L1 in human embryos at gestational week 24 (REF.[113]), but the source of the axonal plexus and whether it exerts a possible developmental function is not known.

GABAergic projection neurons have also been found in the subplate–L6b of neonatal mice[117]. At P2, these neurons constitute only 4–8.4% of all projection neurons with axons in the corpus callosum (presumably targeting the contralateral cortex), internal capsule (presumably targeting subcortical areas) and to other cortical areas. Interestingly, the GABAergic component of long-range projecting neurons decreases with development (measured at P7) when considering the subcortical and ipsilateral cortical projections but increases when considering the contralateral cortical projections. It is not clear whether these changes can be accounted for by the changes in the number of glutamatergic or GABAergic projections. The functional impact of these GABAergic projections on neuronal and circuit development has remained enigmatic so far. However, the relative decrease of ipsilateral cortical and subcortical projections during the first week of life suggests that these projections play a role in early postnatal development of the brain.

GABAergic neurotransmission in the olfactory bulb and DG is important for the integration of newly born neurons into the local circuitry in adult mice[118,119]. GABAergic signalling promotes neuronal differentiation in adult hippocampal progenitor cells[120]. Bao et al. provide evidence that GABA release from septo–hippocampal projections regulates adult neurogenesis in the DG[14]. Thus, PV$^+$ and PV$^-$ SOM$^-$ MS GABAergic neurons induce tonic depolarization of PV$^+$ cells in the DG via extrasynaptic GABA[14]. Bao et al. suggest that these inputs are necessary to maintain neuronal stem cell quiescence and to suppress the proliferation of progenitors, activated astrocytes, immature neurons and branches of newly born neurons in the DG[14].

Interestingly, the shift from depolarizing GABA to hyperpolarizing GABA during late development seems to go along with a functional shift from promoting neuronal connectivity to dampening evoked epileptiform activity. Indeed, activation of the incertocortical[113] and septo–hippocampal GABAergic projections[19] in adult mice inhibited epileptiform activity.

GABA and co-transmitters

The release of GABA and other neurotransmitters from the same neuron at certain terminals was postulated in 1976 (REF.[121]). Several studies and reviews have reported

Progenitor cells
Descendants from stem cells that have the ability to divide and differentiate but with a more limited differentiation potential than stem cells.

Stem cell quiescence
The state of a stem cell in which it does not divide but can be re-activated by external cues.

Table 3 | **GABAergic projections co-expressing neurotransmitters and/or secreted proteins**

Source area	Target area	Putatively co-transmitted neuropeptide	Putatively co-transmitted neurotransmitter and/or secreted protein	Refs
Hippocampal projections				
DG	DG	SOM, NPY	–	146–148
DG	Hippocampus	SOM	–	149
Hippocampus	Subiculum	Enkephalin	–	41
Hippocampus	MEC	SOM	–	5
Hippocampus	Retrohippocampus, septum	SOM, NPY	–	41
Hippocampus	Septum	Partly NPY, 90–100% SOM	59% NGF	150–153
Hippocampus, subiculum	Lateral septum, stria terminalis, anteroventral thalamic area	CCK	–	154
Ventral hippocampus	Amygdala	NPY, CCK, SOM	–	155
Hippocampus	Tenia tecta, diagonal band, medial septum, subiculum, entorhinal cortex, mammillary nuclei, lateral hypothalamus, olfactory tubercle, olfactory bulb, ipsilateral DG and contralateral hippocampus	–	nNOS[a]	101
Other cortical projections				
Cortex	Cortex	91% SOM; 82% NPY	71% nNOS[a]	123,156,157
Infralimbic cortex	Prelimbic cortex	NPY	–	30
Piriform cortex, perirhinal cortex, entorhinal cortex, endopiriform nucleus, lateral amygdala, locus coeruleus	Entorhinal cortex	NPY	–	158
Auditory cortex, motor cortex	Striatum	SOM	–	25,26
Medial prefrontal cortex	Nucleus accumbens	VIP	–	27
Auditory cortex	Lateral amygdala	SOM	–	104
Medial septal projections				
Medial septum	Hippocampus	–	ACh	19
Medial septum	MEC and LEC	–	ACh	18
Subcortical projections to cortex				
GPe	Cortex	–	ACh	103
Basal forebrain	Cortex	SOM	–	139
Basal forebrain	Cortex layer 1	–	ACh	124
Supra-mammillary body	Dorsal hippocampus, entorhinal cortex	CCK, VIP	Glutamate	105,159,160
Tuberomammillary nucleus	Cortex	Galanin	Histamine	13,126
Nucleus incertus	Hippocampus, neocortex	Relaxin 3	–	102,128,129
Dorsal raphe	Medial prefrontal cortex	–	Serotonin	145

A dash indicates either not studied or not detected. ACh, acetylcholine; CCK, cholecystokinin; DG, dentate gyrus; GPe, external globus pallidus; LEC, lateral entorhinal cortex; MEC, medial entorhinal cortex; NGF, nerve growth factor; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; SOM, somatostatin; VIP, vasoactive intestinal peptide. [a]nNOS is a bona fide marker for NO release.

the co-transmission (release from different vesicles) and co-release (release from the same vesicles) of several neurotransmitters[121,122]. However, co-transmission and/or co-release from corticofugal and corticopetal GABAergic projections has rarely been addressed, although many GABAergic projection neurons co-express acetylcholine (ACh) and neuropeptides like SOM and VIP along with GABAergic markers (TABLE 3).

Expression of multiple co-transmitters in GABAergic neurons. Recent single-cell RNA sequencing studies revealed the specific expression of several neuropeptides and secreted signalling proteins in a subclass of cortical GABAergic projection neurons[44]. Indeed, most long-distance (1.5 mm) cortico-cortical GABAergic neurons co-express SOM, nNOS, and neuropeptide Y and most nNOS-expressing neurons are cortico-cortical

projection neurons[123]. One study found that this cell-type co-expresses pleiotrophin, WNT family member 2, relaxin 1, neuropeptide Y, cortistatin and proenkephalin[44], raising the question as to whether these proteins and peptides are released in distant target areas. Cortical PV^+ cells also express tachykinin precursor 1, adrenomedullin and R-spondin 2 precursor[44]. Cortical SOM^+ calretinin-expressing cells express naturally processed peptides[44]. It is possible that a subpopulation of these PV^+ and SOM^+ neurons project to the striatum and other brain areas[24,25,61,104]. Only a few studies have started to dissect the effects of co-transmitters, which we highlight below.

Cholinergic co-transmission. A subpopulation of basalo-cortical and septal GABAergic projection neurons co-express ACh. Notably, a study reported that a large fraction of $ChAT^+$ MS–diagonal band of Broca neurons (>80%) expressed VGAT and GAD65 (but not GAD67) and 31% of L1 interneurons responded to brain-wide $ChAT^+$ terminal stimulation with GABAergic responses[124]. Neurons in other layers were not investigated in this study. Furthermore, more than 80% of $VGAT^+$ terminals from cortically projecting globus pallidus neurons co-expressed the vesicular ACh transporter (VAChT) and 58% of $VAChT^+$ terminals also expressed VGAT[103]. Cortical GABAergic neurons in L1, L2, L3 and L6 were the exclusive targets for fast transmission from $ChAT^+$ globus pallidus projection neurons. Interestingly, most responding interneurons (>70%) exhibited either nicotinic or GABAergic responses, with nicotinic responses confined to L1 and L6 interneurons. The functional results paralleled the observed packaging of ACh and GABA into separate vesicles[103].

Takács et al. suggested that all cholinergic septo–hippocampal neurons co-transmit GABA[19]. Similar to the basalo-cortical projection, ACh and GABA were packaged into different vesicles. Interestingly, the release of the two transmitters was differentially regulated by different voltage-dependent calcium channels and short-term plasticity (GABAergic but not cholinergic responses underwent short-term plasticity following optogenetic stimulation of septo–hippocampal projections with five light pulses at 2–20 Hz). Importantly, the effects that were previously attributed to ACh release from these fibres could now be attributed to GABA release. Thus, epileptiform activity and SWRs were dampened by GABA release from cholinergic septo–hippocampal neurons alone[19]. Adding to the complexity, ACh can act on muscarinic and nicotinic receptors and the contribution of these receptors to LTP and long-term depression in CA1 pyramidal cells depends on the exact timing of cholinergic fibre stimulation relative to the stimulation of Schaffer collateral input to CA1 pyramidal cells[125]. Thus, LTP and long-term depression might be regulated by a complex interplay of GABA and ACh that derive from the same neuronal population.

A similar scenario was reported for septal projections to the MEC and LEC; 89% of cholinergic projection neurons co-expressed mRNA for GAD65 (REF.[18]). Most excitatory responding cells in superficial layers of the MEC and LEC exhibited only muscarinic cholinergic responses. By contrast, around half of the responding interneurons in the MEC exhibited muscarinic and/or nicotinic cholinergic and GABAergic responses, with the rest showing either GABAergic or cholinergic responses[18]. It will be a challenge to understand how this complex interplay of GABA and ACh modulates theta oscillations and memory formation.

Together, these studies also prompt the question of whether released co-transmitters can act presynaptically to differentially regulate the release of different co-transmitters. However, this does not seem to be the case for GABA and ACh release from septo–hippocampal projections. In these projections, GABA and ACh act on presynaptic $GABA_B$ and muscarinic receptors, respectively, and both suppress the release of GABA and ACh[19].

Histamine co-transmission. The only source of histamine in the brain is the tuberomammillary nucleus. Most histaminergic projections from the tuberomammillary nucleus to the cortex express GABA, histamine and galanin[126]. Although nothing is known about galanin release from these projections in the cortex, one study has examined GABA–histamine co-transmission. Yu et al. found that optogenetic 5 Hz stimulation of histaminergic tuberomammillary–cortical fibres for 3 mins induced direct inhibitory GABAergic tonic currents in pyramidal cells as well as histamine release-mediated excitation of inhibitory neurons via histamine receptors 1 and 2, which in turn led to an increase in inhibitory postsynaptic input frequency in pyramidal neurons[13]. Consistent with this divergence in the effects and target cells of histamine and GABA, Kukko-Lukjanov and Panula[127] found that histamine and GABA are stored in different vesicles. Functionally, histamine and GABA seem to have opposing effects: histaminergic cell activation is behaviourally stimulating (increasing wakefulness and locomotion), whereas GABA release from histaminergic projections seems to counteract this effect[13].

Relaxin 3 co-transmission. An interesting projection to the cortex, hippocampus and several other brain areas derives from the nucleus incertus and contains relaxin 3 and GABA[128,129]. The presence of relaxin 3 in terminals reaching the cortex and hippocampus and the expression of relaxin 3 receptors in these areas[129] have been viewed as evidence for relaxin 3 release. Haidar et al.[130] suggested that relaxin 3 release from GABAergic nucleus incertus terminals in the hippocampus enhances reference and working memory; this conclusion was based on results from relaxin 3 receptor knockout mice. However, it was not shown whether relaxin 3 was released specifically during the memory task or whether task-independent homeostatic release of relaxin 3 is necessary for normal hippocampal function. Notably, a recent study provided evidence for GABA release from relaxin 3-expressing nucleus incertus terminals, which acted preferentially on SOM^+ neurons in hippocampal slices[102]. In vivo manipulation indicated that these projections were involved in the formation of associative fear memories[102]. However, this elegant study did not provide evidence for the

Long-term depression
Long-term depression is a form of plasticity reflecting long-lasting decreases in synaptic strength.

Schaffer collateral
An excitatory pathway from the CA3 area to the CA1 area of the hippocampus that undergoes plasticity and is thought to underlie certain forms of memory formation in the hippocampus.

AAV2-retro
A designed variant of recombinant adeno-associated viruses that allows the virus to be efficiently retrogradely transported from the axon terminal to the neuronal cell body.

relative contributions of relaxin 3 release versus GABA release in vivo.

SOM co-transmission. SOM is one of the most prevalent markers of cortical and hippocampal GABAergic projection neurons[5,24–26,104]. Nevertheless, so far, the function of SOM co-transmission in distant target areas has not been addressed. Interestingly, neuropeptide release has been suggested to be facilitated by burst firing[131]. Notably, the majority of SOM$^+$ GABAergic CA1 stratum oriens projection neurons exhibited increased burst firing during SWRs[46]. SWRs have been postulated to support memory consolidation and retrieval[132]. Thus, it stands to reason to investigate whether SOM release is enhanced during SWR events and whether it regulates memory consolidation. The effects of SOM on cell firing, epileptic activity, and learning and memory have been reviewed elsewhere[133].

Conclusion. In summary, most of the above cited studies indicate that co-transmitters at GABAergic long-range terminals are sorted to different vesicles[19,103,127]. This adds to the complexity regarding the function of GABAergic long-range projections as it allows for the differential regulation of acute and long-term neurotransmitter release and the targeting of segregated postsynaptic neuronal populations, not only through differential postsynaptic receptor expression but also through the presynaptic differential segregation and regulation of vesicles. A major challenge will be to differentiate the functional effects resulting from GABA itself and those induced by other co-transmitted or co-released neurotransmitters and neuromodulators. Increasing numbers of Cre-dependent knockout mice as well as the use of CRISPR/Cas9-mediated knockout or siRNA-mediated knockdown combined with local infusion of specific antagonists will facilitate the teasing apart of opposing, additive or complex actions and interactions of co-transmitters. Understanding these differential effects will be of high relevance to better understand the aetiology of neuropsychiatric disorders. For instance, specific downregulation of GABA at GABA/glutamate co-releasing synapses in the habenula has been found in an animal model of depression and has been suggested to underlie some of the phenotypic changes in this mouse model[134].

Future questions and challenges

The sparsity of GABAergic long-range neurons in cortical brain areas places high demands on the specificity and efficiency of research tools. Although several old tracing techniques, including retrobeads, fluorogold and cholera toxin subunit B, are still considered efficient and reliable tracers, new viral expression systems can be exploited to inhibit or activate neurons to thereby gain insights into the function of selected subpopulations.

The diversity of the thus far identified subclasses of GABAergic long-range projections hints at how important it will be to reveal functions in a subtype-specific manner. Joshi et al.[22] and Unal et al.[31] convincingly showed that a classification based only on the chemical markers known thus far is not sufficient to explain functional diversity. Single-cell RNA sequencing of retrogradely labelled GABAergic neurons might help to identify better markers.

Investigating the endogenous activity of subtypes of projection neurons during different behavioural states will be required to better understand the effects of optogenetic manipulations. The cellular as well as the network effects of optogenetic stimulation may depend on the physiological state of the cells. For instance, Mamad et al. demonstrated that optogenetic stimulation of ChAT$^+$ cells in the MS differentially affected the number of evoked spikes depending on whether the stimulation occurred during a low-firing or a fast-firing state[135]. Furthermore, optogenetically inhibiting the baseline firing rate of neurons and hence altering their contribution to the excitatory–inhibitory balance in the network might have a strong effect even if these neurons do not exhibit task-specific firing activity (for example, see the discussion by Otchy et al.[136]). The identification of markers or the exploitation of new retrograde tracing tools, such as AAV2-retro[137], to selectively visualize or record the activity from those few GABAergic neurons that are long-range projecting will be essential to understand their function.

A major challenge will be to disambiguate the impact of precise oscillatory activity from that of general synaptic drive or inhibition in the context of behavioural performance. For example, it has been shown that electrical stimulation of combined GABAergic and non-GABAergic septo–hippocampal projections at a precise fixed frequency, but not irregular stimulation with the same average frequency, promotes spatial learning in rats[64]. Considering the strong rhythmicity and phase coupling of most identified septo–hippocampal GABAergic cells, it is conceivable that the exact timing of their firing is a major determinant of their behavioural impact.

It can be assumed that the total population of GABAergic projection neurons is larger than the low numbers that were found in most retrograde tracing studies. Several studies suggested that segregated subpopulations of GABAergic cortical and hippocampal neurons project to different target areas[16,24]. So far, four reports indicate the presence of GABAergic neurons that project to more than one target area[17,21,31,41]. For example, Fuchs et al. found single neurons that projected to the MEC and hippocampus when employing fluorogold[17]. Unal et al. did not find dual targeting of MS neurons in a study using retrobeads[16]. Whether the uptake and transport efficiency of the tracers only or whether other factors, such as injection volume, injection site and signal to noise ratio, may also account for the differential results in the two studies is not clear; however, it is clear that conventional retrograde tracers are suitable for qualitative but not quantitative evaluations.

Sun et al. used a technique that circumvented some of these problems, enabling them to perform a quantitative analysis: using rabies tracing, they calculated a connection strength index from the number of presynaptic retrogradely labelled neurons normalized to the number of starter cells[138]. Similarly, Do et al. normalized the number of rabies-mediated, retrogradely labelled cells in each brain area to the total number of labelled neurons

in the whole brain, which allowed them to calculate the relative numbers of the connection strength distribution in the whole brain[139].

Conclusions

Research during the past decade has revealed that cortical GABAergic projection neurons do not constitute a rare cell population. Similar to GABAergic interneurons, they populate many brain areas connecting cortico-cortical and cortico-subcortical brain regions and they come in different types that can be distinguished based on molecular marker expression and connectivity. It is only the advent of virus-mediated tracing and optogenetics that rendered functional electrophysiological and behavioural studies of GABAergic projection neurons possible. A direct comparison with GABAergic interneurons reveals the glaring lack of knowledge regarding the properties and functions of GABAergic projection neurons. The most pressing questions include the following: what are the genetic programmes that determine whether a GABA neuron becomes an interneuron or a projection neuron? How do connectivity rules for defined GABAergic projection neurons compare to those for GABAergic interneurons? (This question pertains to both cell type, cell compartment of the targeted neuron and release probability of the GABAergic projection neuron.) To what extent can GABAergic projection neurons be classified into defined subtypes? Do criteria that have been considered for the classification of GABAergic interneurons[140] hold true for GABAergic projection neurons? Investigations at the cellular level that aim to answer these questions will be paramount to understand the functions of GABAergic projection neurons. The scarce literature regarding GABAergic projection neurons does not only reflect a lack of knowledge in this field but also points to the great potential that research of this *terra incognita* offers.

Considering the diversity of GABAergic projection neurons that has been revealed so far, it will be of utmost importance to find tools that allow researchers to specifically target and manipulate the subclasses of GABAergic projection neurons based on their molecular identity, postsynaptic targeting specificity, and in vivo firing patterns and that, at the same time, allow the differentiation between long-range effects and those that are exerted through local branches.

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