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TetTag technology and the RAM system: activity-dependent neuronal marking from a circadian perspective

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Nothing great was ever achieved
without enthusiasm.

R. W. Emerson

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Riassunto

Introduzione

Tutte le piante e gli animali terrestri adattano la loro fisiologia e il loro comportamento al ciclo notte-giorno della durata di 24 ore sotto il controllo di un orologio biologico interno. Sonno, immunità, variazioni della temperatura corporea, attività locomotoria, performance cognitiva, rilascio di specifici ormoni e funzioni digestive sono alcuni esempi di fenomeni fisiologici fondamentali caratterizzati da una periodicità funzionale definita "circadiana" (da *circa diem*, "circa un giorno"). Presumibilmente, questi orologi interni si sono evoluti per mantenere l'organismo in grado di adattarsi alle variazioni ambientali giornaliere e stagionali in punti differenti del pianeta. Per una corretta e continua sincronizzazione dei processi fisiologici con il ciclo luce-buio, o fotoperiodo, l'orologio biologico dei mammiferi è in grado di rilevare cambiamenti in livello di luminosità nell'ambiente esterno attraverso uno specifico pigmento, la melanopsina, contenuto nelle cellule gangliari intrinsecamente fotorecettive della retina (Fig. 1 A). L'informazione luminosa, una volta captata da questi fotorecettori, viene trasmessa via tratto retinoipotalamico al nucleo suprachiasmatico dell'ipotalamo (s*uprachiasmatic nucleus,* o SCN), il sito "pacemaker" dove avviene la regolazione circadiana. Nei mammiferi, l'SCN è formato da circa 20 000 neuroni, suddivisi in sottopopolazioni distinguibili sulla base della loro localizzazione, dei neuropeptidi sintetizzati e rilasciati dalle sinapsi, dalle connessioni e dalla loro funzione (Fig. 1 B). Il meccanismo molecolare fondamentale in grado di garantire questa periodicità è conservato nelle diverse specie ed è basato su loop a retroazione negativa tra loro interconnessi (Fig. 1 C). Un complesso sistema formato da fattori positivi e negativi, da regolazione trascrizionale, traduzionale, post-traduzionale ed epigenetica conferisce simultaneamente robustezza e flessibilità nei confronti degli input esterni, o *zeitgebers*, tra i quali la luce è il più rilevante (Fig. 1 C). La distruzione di questa armonia temporale tra ambiente interno ed esterno, ma anche tra i diversi orologi periferici localizzati nei vari organi, può quindi favorire l'insorgenza di alterazioni della funzionalità di organi o sistemi e talvolta predisporre allo sviluppo di patologie neurodegenerative e metaboliche (Panda, 2016).

Nonostante il core fondamentale alla base dell'omeostasi circadiana sia ormai noto, molto rimane ancora da chiarire sulla struttura dei circuiti neuronali interni all'SCN e le sue efferenze. Sempre più spesso, infatti, emergono nuove funzioni attribuibili a specifiche popolazioni di neuroni orologio e relazioni causa-effetto tra circuiti neuronali e fenomeni fisiologici e comportamentali (Collins, sottomesso per pubblicazione; Gizowski, Zaelzer & Bourque, 2016). In particolare, è plausibile che le diverse attività circadiane siano generate dall'attività di differenti popolazioni neuronali attive in diversi momenti durante il giorno, tuttavia molto rimane da chiarire al riguardo. Non è da escludere la possibilità che si tratti dello stesso set di neuroni orologio ad essere attivo ripetutamente, capace di presentare diversi pattern

di firing con effetti diversi. La ragione di queste fluttuazioni periodiche potrebbe essere da ricercare anche a livello di circuito, in diverse vie di comunicazione interneuronale innescate in modo periodico con conseguente generazione di risposte diversificate.

Per aggiungere complessità a questo scenario, è noto che anche i neuroni della corteccia presentano un firing caratteristico a seconda dello stato di vigilanza (Vyazovskiy & Faraguna, 2015). Misurazioni elettroencefalografiche permettono infatti di distinguere il sonno dalla veglia e le diverse fasi del sonno stesso sulla base di caratteristiche oscillazioni del potenziale di membrana neuronale (Fig. 2). Durante il sonno profondo, queste oscillazioni presentano una frequenza minore di 4 Hz (Steriade, 2000), e vengono chiamate "Slow Wave Activity (SWA)" o "onde delta". È stato dimostrato che esse aumentano in funzione della durata dello stato di veglia, per tornare al livello basale durante il sonno (Borbély, Daan, Wirz-Justice & Deboer, 2016). Per questa caratteristica, le SWA sono considerati indicatori del "bisogno di sonno" (Borbély et al., 2016). Inoltre, è stata osservata una componente localizzata del fenomeno delle SWA, in altre parole, l'intensità di queste oscillazioni varia a seconda delle regioni neuronali, suggerendo una regolazione spazio-specifica dipendente dall'intensità di utilizzo precedente (Mukhametov, Supin & Polykova, 1977; Rodriguez et al., 2016).

In questo scenario affascinante ed enigmatico, lo sviluppo di tecnologie capaci di rilevare l'attività neurale con elevata risoluzione temporale è essenziale per districare i meccanismi alla base di queste sincronie (e asincronie). Diversi approcci molecolari sono correntemente utilizzati a questo scopo; in particolare, le strategie basate sui geni precoci immediati (Immediate Early Genes, IEG) permettono di identificare i neuroni elettricamente attivi in un determinato momento (Sagar, Sharp, & Curran, 1988). Esse sono basate sui promotori di geni come *FOS*, *ARC* e *ZIF268*, la cui espressione genica viene innescata rapidamente in seguito ad attività di firing rilevante (Guenthner, Miyamichi, Yang, Heller, & Luo, 2013). Nelle ultime decadi, si è tratto vantaggio da questa caratteristica, ideando svariati tool genetici per la visualizzazione e la manipolazione diretta di specifiche popolazioni neuronali in modo temporalmente e spazialmente controllato. In questi costrutti, il promotore dell'IEG può essere inserito a monte di un gene reporter, come la Green Fluorescent Protein (GFP), di componenti del sistema di controllo di espressione tetracicline-dipendente (Gossen & Bujard, 1992; Dogbevia, Marticorena, Alvarez, Bausen, Sprengel, & Hasan, 2015), o anche di proteine optogenetiche (Canalrodopsina o Alorodopsina), capaci di innescare o inibire l'attività elettrica neuronale in seguito a stimolo luminoso (X. Liu et al., 2012). Elevati livelli di specificità, sensibilità e versatilità sono già stati raggiunti e sistemi sempre più sofisticati vengono sviluppati incessantemente.

In questo studio, due tecnologie per il tagging e la manipolazione neuronale basate sul promotore dell'IEG *FOS* sono state prese in esame, al fine di comprenderne la precisa dinamica temporale, in prospettiva di un potenziale utilizzo in ambito circadiano. Entrambi gli approcci sono infatti basati sul sistema tetracicline-

dipendente Tet-Off, grazie al quale l'espressione di potenzialmente qualsiasi gene reporter dipende dalla presenza/assenza del composto, somministrato in modo esogeno all'animale esprimente il costrutto transgenico (Gossen & Bujard, 1992; Dogbevia, Marticorena-Alvarez, Bausen, Sprengel, & Hasan, 2015). In conseguenza, la concentrazione e il tempo necessario per il clearing dell'antibiotico dai tessuti costituiscono due variabili fondamentali influenzanti la dinamica di attivazione del sistema genetico. Questi parametri, tuttavia, non sono mai stati testati in modo dettagliato e un'elevata ambiguità in termini di precisione temporale tuttora permane. Pertanto, queste tecnologie non sono ancora state sfruttate per lo studio dei fenomeni circadiani, caratterizzati da variazioni che si realizzano nell'arco di poche ore o addirittura minuti.
L'obiettivo primario di questo studio è stato quindi l'identificazione della finestra temporale minima di attivazione del sistema di tagging neuronale di queste due strategie molecolari. Tenendo in considerazione i risultati ottenuti, si è quindi tentato di utilizzare le due tecniche per osservare differenze nel pattern di firing dei neuroni corticali in funzione di diversi stati di vigilanza.

Il sistema TetTag

Il sistema TetTag, sviluppato da Reijmers et al. nel 2007, è basato sulla co-presenza di due costrutti (indicati in Figura 3) nel genoma del topo, detto *topo TetTag*, in grado di marcare indefinitamente i neuroni che si attivano all'interno di una determinata finestra temporale. Gli aspetti di maggior rilevanza di questa tecnica comprendono: (1) la selettività della marcatura riguardante soltanto cellule neuronali attive elettricamente in assenza dell'antibiotico (Doxiciclina o Dox) e (2) la persistenza di questa marcatura anche in seguito alla risomministrazione del composto. Ciò che si ottiene è una mappatura a livello di intere strutture cerebrali dei circuiti attivati in un determinato momento o in seguito a uno stimolo d'interesse.
In questo studio, topi bi-transgenici TetTag sono stati utilizzati in un esperimento con design time-course, durante il quale 5 diverse finestre temporali (4, 10, 16, 22 e 28 ore) di assenza di antibiotico sono state create tramite iniezione di Doxiciclina diluita in soluzione salina e mediante sostituzione della dieta trattata con la dieta non trattata (design sperimentale e risultati in Fig. 8). Al termine del periodo off-Dox, l'antibiotico è stato risomministrato per 24 ore, dopodiché tutti gli animali sono stati sacrificati. I cervelli sono stati quindi processati e sottoposti alla procedura di Immunostaining, utile all' identificazione delle cellule marcate, ovvero esprimenti il gene reporter tau-LacZ. Come controlli sono stati utilizzati topi bi-transgenici mantenuti in costante trattamento o totale assenza di trattamento, insieme a un gruppo di topi per i quali è stata creata una finestra temporale permissiva al tagging di ampia durata (48 ore). I risultati ottenuti sono mostrati in Figura 8 B-E. Innanzitutto, marcatura neuronale è stata osservata soprattutto in due aree corticali, la corteccia somatosensoriale primaria e la corteccia piriforme. In

queste regioni è stata quindi effettuata la quantificazione delle cellule marcate. La percentuale di neuroni taggati in tutti i gruppi testati è risultata significativamente maggiore rispetto alla percentuale nel gruppo di controllo on-Dox in entrambe le regioni analizzate. Questi dati sembrano pertanto suggerire che, dopo soltanto 4 ore, i livelli di Doxiciclina nella corteccia cerebrale siano tali da permettere l'attivazione del sistema di tagging genetico. La rilevanza di quanto osservato consiste nel fatto che una finestra temporale di soltanto poche ore potrebbe essere efficace nel confrontare neuroni spontaneamente attivi in momenti diversi nell'arco della stessa giornata, ovvero per osservare variazioni circadiane nel firing neuronale in varie regioni cerebrali. Quest'ipotesi è stata quindi successivamente testata creando tre finestre temporali permissive al tagging in tre gruppi di topi TetTag durante tre diverse fasi di vigilanza: sonno, veglia e deprivazione del sonno, per una durata di 6 ore (Fig. 9, A). 24 ore dopo la risomministrazione di Dox, gli animali sono stato sacrificati e le analisi svolte come in precedenza. In questo caso, i risultati si sono rivelati difficilmente interpretabili a causa di un'elevata variabilità all'interno degli stessi gruppi in termini di percentuale di marcatura. I dati ottenuti dalla quantificazione sono inoltre risultati fortemente discordanti rispetto all'atteso, con una marcatura consistente durante la fase di sonno e inaspettatamente scarsa durante la fase di veglia (Fig. 9, B-E). In seguito a tali valutazioni, è stato ritenuto opportuno procedere con ulteriori esperimenti (qui non presentati) per ottenere un maggior numero di dati da discutere.

Da entrambi i test è stato possibile ricavare ulteriori informazioni: tra le più evidenti, l'assenza di marcatura dell'SCN e la presenza di background di espressione del gene reporter nonostante la presenza di antibiotico. In merito la prima osservazione, è stata avanzata l'ipotesi di un'elevata soglia di attivazione del sistema TetTag, ovvero soltanto neuroni capaci di firing ad alta frequenza, come quelli corticali, sarebbero in grado di innescare il meccanismo di marcatura neuronale. In aggiunta, una ridotta penetranza del transgene attraverso le varie regioni cerebrali potrebbe essere una causa ulteriore della variabilità osservata nell'efficienza di tagging.

Per quanto concerne la presenza di background, ricerche precedenti suggeriscono che si tratti di una limitazione intrinseca degli approcci basati sui sistemi tetracicline-dipendenti (Zhou, Vink, Klaver, Berkhout, & Das, 2006; Loew, Heinz, Hampf, Bujard, & Gossen, 2010; Dogbevia, Roßmanith, Sprengel, & Hasan, 2016). Inoltre, non è da escludere che il contesto genomico nel quale è casualmente inserito il transgene possa influenzare l'espressione del gene reporter in modo difficilmente controllabile e prevedibile.

Infine, il sistema TetTag è risultato in grado di catturare soltanto una minima percentuale dei neuroni presumibilmente attivi durante la finestra temporale. Pertanto, questo approccio è stato valutato come potenzialmente utile per lo studio del firing a livello corticale utilizzando ridotte finestre temporali, tenendo presente tuttavia che dovrebbe essere utilizzato in combinazione con sistemi più sensibili per una corretta stima dei livelli di marcatura.

Il sistema RAM

Il secondo metodo preso in esame il questo studio, il sistema Robust Activity Marking o RAM, è stato sviluppato dal gruppo di Sørensen nel 2016 come tool per la manipolazione neuronale attività-dipendente. Come il metodo precedente, è stato validato in ricerche sulla memoria e sull'apprendimento, tuttavia l'imprecisa conoscenza della dinamica temporale ne ha finora impedito l'applicazione per lo studio di processi con ritmicità circadiana. Il sistema RAM è basato sulle stesse componenti fondamentali del sistema TetTag, il promotore di Fos e il sistema tetracicline-dipendente, ma presenta delle componenti aggiuntive che conferiscono elevata sensibilità, specificità e robustezza alla marcatura neuronale (Fig. 4). A monte del promotore di Fos, 4 ripetizioni tandem di una sequenza enhancer sono responsabili dell'elevato rate trascrizionale dei geni che costituiscono il sitema e della stretta dipendenza da firing neuronale rilevante. A valle del risultante promotore (chiamato P_{RAM}), inoltre, è stata introdotta una versione perfezionata del transattivatore tetracicline-dipendente (d2TTA), caratterizzata da un'elevata sensibilità alla presenza di Doxiciclina. Infine, oltre al gene reporter per la marcatura neuronale (EYFP), è stato inserito il gene codificante la canalrodopsina (ChR2), permettendo la riattivazione selettiva in un secondo momento soltanto i neuroni precedentemente marcati durante il periodo off-Dox.
A differenza del sistema TetTag, basato su animali esprimenti uno o entrambi i transgeni teoricamente nell'intera area cerebrale, il sistema RAM è stato ideato per essere contenuto interamente in un unico costrutto, il quale può essere diretto in specifiche aree cerebrali tramite iniezione di virus adeno-associati (AAV). Un ulteriore aspetto che distingue il sistema RAM dal sistema TetTag è l'assenza del meccanismo a feedback loop per la permanenza della marcatura, con conseguente arresto dell'espressione dei geni reporter 5-10 giorni dopo la risomministrazione dell'antibiotico.

Come nel precedente caso, il sistema RAM è stato testato al fine di identificare la durata temporale minima del periodo off-Dox per permettere un'efficace marcatura neuronale, con un possibile utilizzo di questa tecnologia entro una prospettiva circadiana.
In seguito all'iniezione stereotattica del costrutto virale nel cortex di 22 topi wildtype e la somministrazione di una dieta trattata con Doxiciclina, è stato condotto un esperimento time-course aprendo tre finestre temporali permissive al tagging di durata 4, 10 e 16 ore. Le modalità utilizzate hanno ricalcato quelle precedentemente presentate per il sistema TetTag (Fig. 10 e 11), inclusi i gruppi di controllo. In questo caso, tuttavia, gli animali sono stati sacrificati al termine del periodo off-Dox, per evitare la perdita della marcatura a causa della mancanza del feedback loop di autosostenimento.
Dai risultati di questo esperimento sono emerse chiaramente le caratteristiche vantaggiose del sistema RAM: la marcatura neuronale è risultata robusta ed

efficace, capace di evidenziare non soltanto il soma dei neuroni attivati, ma anche gran parte delle proiezioni dendritiche (Fig. 10). Il labelling neuronale è risultato inoltre circoscritto all'area di iniezione, comprendente tutti gli strati corticali sottostanti e le strutture ippocampali situate nell'emisfero interessato. Per quanto concerne il tempo necessario per il clearing della Doxiciclina, dalla quantificazione dei neuroni CA1 ippocampali marcati è emerso che sembrano essere sufficienti tra le 4 e le 10 ore dall'ultima somministrazione per ottenere l'attivazione del sistema RAM. Similmente alla tecnologia TetTag, è stata notata la presenza di cellule esprimenti EYFP anche in presenza costante di antibiotico, nonostante la percentuale fosse significativamente minore rispetto ai gruppi testati.

Il successivo esperimento è stato condotto su due gruppi di topi iniettati con lo stesso costrutto virale, sostituendo la dieta trattata con dieta normale per una durata di 12 ore, durante la fase di sonno (ZT0 – ZT12) o durante la fase di veglia (ZT12 – ZT24), come mostrato in Figura 12. Lo scopo dell'esperimento era quello di individuare differenze tra i due stati in termini di numero e neuroni elettricamente attivi. Tuttavia, la percentuale di marcatura ottenuta con il sistema RAM nelle due condizioni non ha differito in modo statisticamente significativo. Si ritiene che il motivo di tale risultato sia da ricercare nel ridotto numero di repliche biologiche e tecniche, per cui sarebbero necessarie ulteriori osservazioni per poter discutere ulteriormente i dati ottenuti. Anche in questo caso, soltanto una parte di neuroni attivati nelle ore precedenti il sacrificio è risultato marcato tramite il sistema RAM. Pertanto, nonostante l'efficienza di labelling di questo sistema si sia rivelata superiore a quella del sistema TetTag, anche per il sistema RAM si ritiene necessario valutare con accortezza i livelli effettivi di marking neuronale, possibilmente comparando i risultati con i dati ottenuti con altre tecniche.

Considerazioni finali e conclusione

In questo studio vengono messi in luce i vantaggi e le limitazioni dei metodi TetTag e RAM per la manipolazione attività-dipendente dei neuroni, le caratteristiche comuni e gli aspetti complementari (riassunti in Tabella 1). Entrambi gli approcci sembrano avere una dinamica di attivazione più rapida rispetto a quanto assunto finora; infatti, poche ore sono risultate essere sufficienti per ottenere una significativa marcatura neuronale. In conseguenza, la ricerca cronobiologica potrebbe beneficiare di queste tecnologie nell'identificazione di relazioni causa-effetto tra variazioni nei pattern di firing neuronale e le diverse fasi del ritmo sonno-veglia o di altri processi fisiologici con periodicità circadiana.
Mentre il sistema TetTag permette soltanto l'osservazione di tali fenomeni neuronali, perlopiù con diversa efficacia in differenti strutture cerebrali; l'approccio RAM possiede chiaramente un maggior potenziale applicativo. Idealmente, il successivo esperimento prevederebbe l'iniezione del costrutto virale a livello dell'SCN o di specifiche aree cerebrali e permettendo il tagging durante il naturale

periodo di sonno dell'animale. A ciò seguirebbe la riattivazione artificiale selettiva dei neuroni precedentemente marcati tramite l'optogenetica, in un arco di tempo nel quale questi neuroni non sono spontaneamente attivi, per esempio durante la fase di veglia. Quali effetti si avrebbero a livello fisiologico e comportamentale? In altre parole, sarebbe sufficiente la riattivazione di questi neuroni per far cadere il topo in un "sonno innaturale" o per provocare durante la veglia fenomeni tipici del sonno? Quali specifiche sottopopolazioni di neuroni orologio sarebbero coinvolte e quali circuiti cerebrali si attiverebbero? I metodi presi in esame in questo studio potrebbero essere affiancati a quelli correntemente in uso per poter rispondere a queste domande e indagare i diversi fenomeni circadiani, ponendo particolare attenzione a diversi aspetti a seconda della tecnica utilizzata e in modo sempre più approfondito. In un futuro prossimo, l'utilizzo sapiente di queste strategie potrebbe portare alla comprensione dei meccanismi secondo i quali oscillazioni a livello molecolare e neuronale possano influenzare aspetti della vita umana quali l'umore, la routine, la performance fisica e cognitiva e la salute.

Abstract

Circadian clocks orchestrate metabolism, physiology and behaviour with Earth's diurnal cycle. Different populations of clock neurons are thought to become active at a specific time of the day, generating these circadian rhythms synchronized throughout the body. Several molecular tools have been developed to overcome the barrier between the activity of relevant encoding circuits and daily oscillations in sleep patterns; however, the underlying neural mechanisms remain poorly understood. The TetTag technology and the RAM system are two strategies for genetic alteration of neural ensembles activated upon a relevant stimulus, allowing selective tagging and manipulation of neurons with time and space-specificity. Nonetheless, they have never been applied in circadian research due to their unclear activation dynamics, intrinsic in their nature of drug-based approaches. Here, the temporal resolution of these two activity-based tools is clarified, showing that a time window of about 4 hours is sufficient to enable an effective neuronal tagging after antibiotic withdrawal. The TetTag and the RAM systems were then applied to investigate alterations in cortical neuronal firing during different vigilance states. Taken together, our results suggest that these genetic tools are suitable to visualize and obtain direct molecular control over the neurocircuits regulating sleep-wake cycles and circadian activity.

Introduction

Biological clocks and the sleep-wake cycle

Every creature on earth possesses a clock that ticks away in its body. Since the first traces of life, all the organisms, from the unicellular proteobacterium to the mammal, share an internal biological clock that evolved throughout time to keep in synchrony with the rise and fall of the sun, the seasonal changes, the phases of the moon (Dunlap & Loros, 2004). Even plants open and close their blossoms at a precise time and raise their leaves during the day and lower them at night. Every day, we are awake for approximately sixteen hours during which we move, work, feed, interact with others until we return to an altered state of consciousness, that we call *sleep,* this sequence of actions is periodic in fashion. These clocks confer survival advantage by enabling to anticipate daily environmental changes and thus adapt the behaviour and physiology to the appropriate time of the day.

Since the length of the body clock's day was only close but not exactly the normal twenty-four-hour day length, these rhythms produced by the body clock were called *circadian rhythms* (from the Latin *circa diem,* "about one day"). However, every individual possesses his own timing type, or *chronotype.* These differences, even representing the same twenty-four-hour day of our planet, can indeed differ markedly with the extremes being up to twelve hours apart. "Morning types" or "larks" have better performances in the first half of the day, while "evening types" or "owls" operate better in the evening. Not only performance and cognitive functions vary according to time of day, many other aspects of human life, oscillations of body temperature, locomotor activity, hormonal release, many traits of digestion and, naturally, sleep habits. Because these circadian rhythms reflect daily changes, they must be susceptible to seasonal changes in day length (photoperiod), maintaining at the same time their coupling with the other oscillators throughout the body. Unsurprisingly, disorders of circadian timekeeping affect sleep efficiency and cognitive performance, and, in the most severe cases, the disruption of circadian program is implicated in various psychiatric, neurological and metabolic diseases (Panda, 2016).

But how can this natural physiologic synchrony be modified and even disrupted? It is well known that light is the most potent signal able to reset the body clocks of plants and animals, including humans, to the twenty-four hours of the earth's rotation. In addition, other environmental cues, also termed *zeitgebers,* can influence it, such as external temperature and feeding time. Clearly, differences in the intensity and timing of these zeitgebers can shift the period of the biological clock, and perhaps cause internal discrepancies between the timing of physiological functions.

The suprachiasmatic nucleus and the circadian molecular machinery

In mammals, the internal time was found to be controlled by a small group of approximately twenty thousand neurons located directly above the optic chiasm, for this reason called *suprachiasmatic nucleus* (SCN, Figure 1 A and B). Every single neuron of the SCN can perform autonomously in maintaining the clock mechanism through an interlocked network of transcriptional feedback loops, whose underlying core mechanisms is conserved even between species relatively distant in the phylogenetic tree, like flies and rodents (Figure 1 C). This core circadian machinery is based on gene products that act positively (transcriptional factors CLOCK and BMAL1 in mammals) and negatively (CRY1-2, PER1-3, REV-ERBα), binding to *cis*-acting elements (E-boxes and ROR elements) and undergo dynamic protein-protein, protein-nuclear receptor interactions, and post-translational modifications that lead to controlled and rapid protein degradation and renewal (Koike et al., 2012). Furthermore, redox regulation and chromatin remodelling also play a fundamental role at the base of the core circadian machinery. But the circadian system is not simply a neuronal center that ticks away in the brain, it involves an entire pathway with inputs and outputs. This pathway starts in the eyes, where light or darkness are detected by the intrinsically photosensitive retinal ganglion cells (ipRGCs) through special photoreceptors, the melanopsins, and this information is sent via retinohypothalamic tract (RHT) to the SCN (Lucas, Freedman, Muñoz, Garcia-Fernández, & Foster, 1999; Figure 1 A). This structure, in turn, acts as a "master circadian pacemaker" by releasing entrainment signals to the other parts of the brain and the peripheral oscillators in the cells of other tissues and organs. The resulting hierarchical organization of the clock system ensures the coupling of these periodic oscillations through the whole body and flexibility in the synchronization with both photic (the light-dark cycle) and non-photic zeitgebers. At the base of this robust pacemaker activity there is neuropeptidergic signalling across different subpopulations of SCN neurons (A. C. Liu et al., 2007). According to data obtained by selective genetic manipulation (Herzog, Hermanstyne, Smyllie, & Hastings, 2017), the ventral SCN neurons is dominated by gastrin-releasing peptide (GRP) and vasoactive intestinal polypeptide (VIP) expressing neurons (Antle, Kriegsfeld, & Silver, 2005). Together they constitute the "core" region of the SCN, which is entrained by the photic input and whose role is to preserve the internal synchronization of the SCN. Moreover, another function of the VIP neurons would be the timekeeping between the core and the "shell" region (Kriegsfeld & Silver, 2006), dominated instead by dorsal SCN neurons expressing the neuropeptide arginine vasopressin (AVP), responsible for maintaining the rhythmicity and capable to impose their intrinsic periodicity to mouse behaviour. Although the molecular machinery by which the central master clock controls timekeeping is becoming increasingly clear, knowledge of how this timing information is distributed to regulate physiology and behaviour is only just emerging.

Are the circadian activities caused by different subpopulations of neurons that fire at different time of the day? Or is there only one set of SCN activated neurons, and the difference lies on the firing rates and the synchrony between specific neuronal ensembles? In other words, which neuronal circuits are responsible for the different phases of the sleep-wake cycle? For instance, recent discoveries suggest that a specific population of VIP neurons in the SCN in rodent species drives the phenomena of the "siesta", a period of reduced alertness or sleep between two bursts of high activity during the wake period (Collins, submitted for publication). The group of Gizowski, instead, found that AVP neurons become electrically active during the increase in water intake that typically precedes sleep period in mice (Gizowski, Zaelzer, & Bourque, 2016). Taken together, these researches show how several aspects of the precise intra- and extra-SCN neurocircuitry that determines circadian regulation of bodily functions remains to be elucidated.

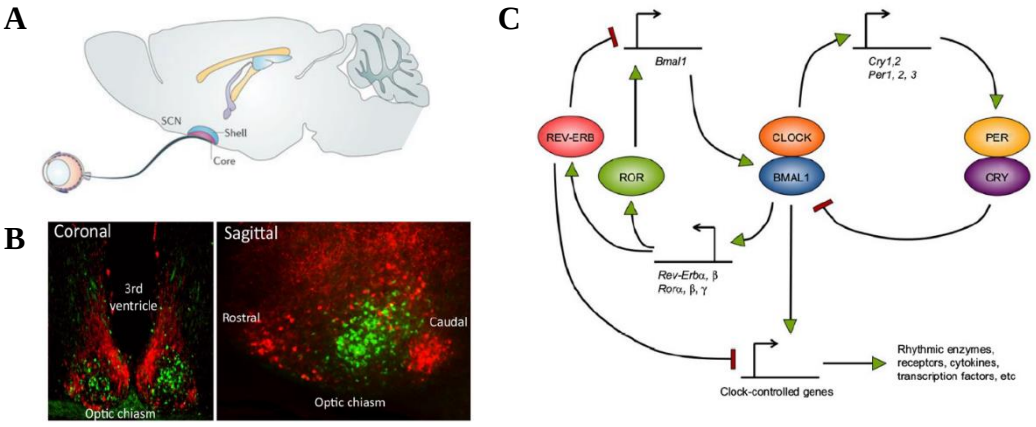


Figure 1. The mammalian circadian clock. (**A**) The SCN is located in the hypothalamus, just above the optic chiasm. The photic input reaches the core of the SCN via the RHT, synchronizing the internal clock with the external light-dark cycle. Figure taken from Colwell, 2011. (**B**) The SCN (coronal and sagittal views) can be distinguished in "core", identified by VIP and GRP expressing neurons (green), and "shell", constituted by AVP-containing cells (red). Figure taken from Pauls et al., 2016. (**C**) The transcriptional-translational negative feedback loop underlying the circadian machinery drives rhythms in gene expression. At the beginning of the cycle, the heterodimer of CLOCK and BMAL1 proteins binds to the E-box in period (*Per1, Per2* and *Per3*) and cryptochrome (*Cry1* and *Cry2*) gene promoters, driving their transcription and translation. The levels of transcripts for *Per* and *Cry* genes reach their peak between midday and early night, whereas the PER and CRY proteins peak in the early night. PER and CRY heterodimerize and translocate to the nucleus where they turn off the transcriptional activity driven by CLOCK-BMAL1. The proteins are degraded by ubiquitylation, allowing the cycle to begin again. In a second feedback loop, Rev-erbα gene transcription is also triggered by the binding of CLOCK and BMAL1; once synthetized, REV-ERBα competes with RORα to cyclically repress the transcription of *Bmal1*. Thus, in its simplest form, many cells contain this molecular feedback loop that

regulates the rhythmic transcription of a number of genes. Figure taken from Cermakian et al., 2016.

Sleep homeostasis and cortical neuronal firing

Sleep timing is the most prominent expression of the body clock in humans and other species. Nevertheless, there are many other aspects of sleep that are still poorly understood and make this complex phenomenon highly fascinating, such as the function of sleep, sleep structures, sleep pathologies, or the relationship between sleep and the immune system, to name just a few.

The alternations between sleep and wakefulness can be seen as reflections of daily fluctuations of bodily functions, fluctuations that include turning genes on and off, changes in the hormonal cocktails and transmitters in the tissues, and constant variations in the neural activity inside the brain. When sleep-wake cycles and all bodily functions do not oscillate in synchrony, health is affected and, if temporal disharmony becomes chronic and severe, obesity, cardiovascular disease, stroke, asthma, psychiatric disorders, cancer and other pathologies are more likely to arise.

It has been established that neuronal firing and transmitter release at SCN axon terminals mediate output signals that confer the periodicity to the other oscillators throughout the brain and peripheral tissues (Gachon, Nagoshi, Brown, Ripperger, & Schibler, 2004). To add in complexity, other brain regions have shown diurnal fluctuations in their activity, to which the basic questions valid for the master circadian pacemaker can also be addressed. For instance, neuronal firing rate in the barrel cortex of the mouse, measured extracellularly using microelectrode arrays, changes dramatically between sleep and wake (Vyazovskiy & Faraguna, 2015).

During much of sleep, the membrane potential of cortical neurons presents characteristic oscillations, appearing in electroencephalograms as slow wave activity (SWA) of less than 4 Hz (Steriade, 2000), also termed “delta waves” (Fig. 2). It has been shown that these SWA increase in function of previous wakefulness and return to baseline in the course of sleep (Borbély, Daan, Wirz-Justice, & Deboer, 2016). An increase in SWA have been measured in mice kept in extended wakefulness, or “sleep deprived”, in several studies (Hanlon et al., 2011). Because of this feature, SWA is considered an index of the homeostatic process, reflecting the increased need for sleep rising with wake duration (called process “S”) in combination with the circadian factor (named factor “C”), which influences sleep timing (Borbély et al., 2016). A well-established hypothesis for this phenomenon suggests that SWA homeostasis may reflect synaptic changes underlying a cellular need for sleep (Tononi & Cirelli, 2003). In other words, sleep may have a restorative function, causing the downscaling of synapses that underwent potentiation triggered by the learning tasks during the preceding waking period.

Another intriguing feature of these slow oscillations is that they seem to occur in phase across most brain regions, as global events, but also in a minority of areas independently of the others, as local phenomena. In fact, the intensity of these SWA

has been observed to vary across cortical regions, meaning that they could be regionally regulated as a function of prior use and plastic processes (Rodriguez et al., 2016). Other evidence, such as natural sleep being restricted to only one hemisphere at a time in some animals (Mukhametov, Supin, & Polyakova, 1977), support this concept of local sleep. According to these findings, sleep should not be considered a unitary phenomenon, but a complex process during which patterns of activity typical of sleep and wake simultaneously coexist.
Despite the high number of studies conducted so far, it is still unclear whether this local sleep is generated and controlled by a neuronal network, possibly connected with the master clock, or if it is governed by cell-autonomous components and local circuitry. In this enigmatic scenario, tools able to detect active ensembles of neurons with a high temporal resolution may be helpful to unravel the mechanisms underlying these spatiotemporal asynchronies.

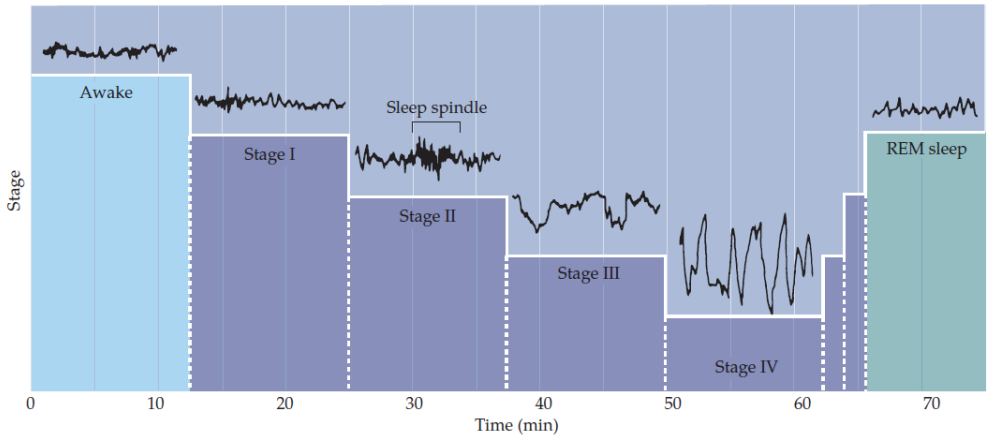


Figure 2. Different stages of sleep are characterized by different brain activity. The successive stages of sleep are recognizable by characteristic firing frequencies measured through EEG recordings. Non-REM sleep can be divided into several stages with distinct EEG frequency: stage I (4-8 Hz, called "theta waves"), stage II (10-12 Hz, named "spindles"), stage III (2 -4 Hz). Slow waves, or "delta waves", are typical of the Stage IV and have the lowest frequency (0.5-2 Hz). Follows the REM sleep, with high-frequency activity, similar to the EEG activity recorded during wakefulness (15-60 Hz, called "beta activity"). All these stages occur in this characteristic repeated sequence that lasts about one hour. Figure taken from Purves et al, 2004.

Activity-based genetics to examine neural circuits

Since the dawn of neurobiology, observational techniques, such as single unit-recording and functional magnetic resonance imaging (fMRI), have been enormously successful for the investigation of the patterns of neural activity and the understanding of information processing in the brain. Calcium imaging has also been widely used to document neuronal ensembles activated by an experience or a complex physiological phenomenon such as sleep (C. Hanlon, V. Vyazovskiy, Faraguna, Tononi, & Cirelli, 2012; Cox, Pinto, & Dan, 2016). However, dissecting

neural circuits into relevant neuronal populations still constitutes a major challenge in neurobiology.

Over the last few decades, the understanding of neural circuits has been greatly facilitated by genetically encoded tools that allow not only to visualize the neuronal structure and activity, like the techniques previously described, but they are also capable of manipulating cellular function and highlighting synaptic connections. Thanks to these novel tools, new types of questions previously inconceivable have become concretely accessible: which would be the behavioural effect of artificially stimulating a specific population of neurons at a particular time of the day? Which molecular and physiological alterations would follow this unnatural activation? Which other brain circuits would be activated with what kind of consequences? Currently applied technologies allow to address these types of questions by both watching and manipulating neurons within the context of a defined circuit in a freely-moving animal. The goal of watching brain activity has been achieved thanks to a class of immediate early genes (or IEGs), genes whose expression is induced rapidly and transiently in response to high-level neural firing (Sagar, Sharp, & Curran, 1988). The rapid transcription initiation pivots on pre-existing transcription factors, so that de novo protein synthesis is not required, together with the binding of RNA polymerase II to the promoter region under resting condition but ready to be released upon electrical stimulation (Lemaire et al., 2011). Consequently, they provide a connection between gene expression and neuronal electrical and/or synaptic activation, or in other words, the expression pattern of IEGs in animal brain section represents a record of the firing activity from few hours before the sacrifice. *FOS*, *ARC* and *ZIF268* are the IEGs most widely used as reliable markers for neural activity. The expression of *FOS* (FBJ osteosarcoma oncogene) has been reported to peak after 1 hr from the induction and to return to baseline in approximately 3 hr, providing a picture of the brain activity within a 3-hr time window (Guenthner, Miyamichi, Yang, Heller, & Luo, 2013). Strikingly, the promoter regulatory elements of these genes, responsible for the neural activity dependency, can be linked to any heterologous transgene, such as a fluorescent protein, to drive its expression in an activity-dependent fashion. By genetically modifying animals to drive the expression of protein markers or optogenetic proteins with this mechanism, it is possible not only to visualize, but also to control neurons activated in response to a specific stimulus. Hence, these genetic tools can be adopted to determine whether the same or different neuronal populations are activated and express the IEGs in different contexts or behaviours, and also to manipulate the activated neurons to investigate their causal functions.

Different IEG-based methods have been used so far that allow the expression of transgenes upon neuronal firing, and transgenic mouse lines have been created carrying these transgenes in the whole brain (X. Liu et al., 2012; Reijmers, Perkins, Matsuo, & Mayford, 2007; Guenthner et al., 2013). In the current study, we used two of these IEG-based systems, described in detail separately. In these IEG-based

transgenic lines, the effector molecule is expressed in neurons that show activity at a specific time point, to fulfil the aim of probing the function of active neural ensembles. Ideally, to provide access to recently active neuronal populations efficiently, these strategies should be characterized by the following critical features: first, they should be temporally precise to be able to link the pattern of Fos^+ neurons with a behaviour or environmental stimulus; second, they must be highly sensitive and specific, with a low background labelling. Thirdly, these mechanisms should drive a robust effector gene expression for a prolonged period, for an effective alteration of the activated neurons, allowing their visualization and/or manipulation. Furthermore, IEGs-based strategies should have a modular design, so that the promoter and effector genes can be substituted depending on the experimental question. Finally, they should be versatile and functional in preferably all the brain regions and in several species, as valuable tools for the broad neuroscientific research. However, IEG-based systems may not be ideal; in fact, they can have high background, becoming active upon non-relevant neuronal activity, or have high activation thresholds, responding only to strong neuronal activation. Moreover, some of these rely on the use of exogenous antibiotic, accordingly, their precise activation timing can be ambiguous, depending on the antibiotic concentration during the administration and the time required for the brain clearing after the antibiotic withdrawal.

These approaches have been extensively used to examine neural circuits mediating learning and memory paradigms, like the retrieval of contextual fear conditioning, in brain areas known to be necessary and active during these phenomena, such as hippocampus, amygdala and prefrontal cortex (Reijmers et al., 2007; Knapska & Maren, 2009). Other applications of IEG-liked reporter genes include the projections tracing of specific active neural populations by linking the c-Fos promoter with an axonally targeted β-galactosidase (Wilson et al., 2002); and the local stimulation of the hippocampus by optogenetic activation of the channelrodopsin (ChR2), which gene was placed under the regulation of the c-Fos promoter (X. Liu et al., 2012).

Beyond studying learning and memory, these approaches could be useful for the investigation of many different functions within mammalian brains, including the circadian regulation by the SCN of the sleep-wake cycle and other periodic phenomena. To be applicable and relevant for the circadian research, a technique should be able to detect variations within a given phenomenon with a time scale of few hours. IEG-based methods, with their relatively fast on- and off-dynamics, have been hardly applied in this research area. The reason may derive from the dependency of most of these genetic tools on the presence of a chemical compound to be switched on and off, hence, the uncertain timing required for the brain clearing may render these systems inappropriate for the discrimination of events occurring closely in time.

In the current study, two of these IEG-dependent tools are tested in order to clarify their temporal resolution. Both the techniques rely on the combination of the c-Fos promoter with the tetracycline-controlled Tet-Off gene expression system, thus coupling the electrical activity dependency with the modulation of the expression of a gene of interest by administration or withdrawal of tetracyclines (Gossen & Bujard, 1992; Dogbevia, Marticorena-Alvarez, Bausen, Sprengel, & Hasan, 2015). The first, termed TetTag, was developed by the group of Reijmers and consists of a transgenic mouse in which neurons spontaneously activated in a given time window express the tau-LacZ neuronal marker indefinitely. The second, the Robust Activity Marking (RAM) system, is a relatively novel IEG-based system designed by the group of Sørensen in 2016 for tagging and manipulating recently activated neurons with high levels of specificity and sensitivity (Sørensen et al., 2016).

The TetTag technology

The Tetracycline transactivator controlled genetic Tagging of active neural circuits, or TetTag technology, is a genetic approach that enables control of the expression of a transgene in a neuronal activity-dependent fashion. Based on the combination of the tetracycline system and the *FOS* promoter, it is functionally active in a bi-transgenic mouse, the TetTag mouse, where two separate transgenes are both present (Figure 3). In the first transgene (*Fos-tTA:shEGFP*), the IEG promoter drives the expression of the tetracycline-controlled transactivator (tTA) and two hour half-life Green Fluorescent Protein (shEGFP) only upon high-level electrical stimulation. The tTA is a transcription factor whose activity can be controlled both reversibly and quantitatively by exposing the transgenic animals to varying concentration of tetracycline or doxycycline (Dox), a more stable analogue of tetracyclines. The tTA protein can regulate the expression of a target gene that is under transcriptional control of a tetracycline-responsive promoter element (TRE), underlying the regulatory mechanism named Tet-Off expression system (Kistnert et al., 1996; Walters & Zuo, 2015). When Dox is present, neuronal firing results in the expression of tTA through the c-Fos promoter, but the transcription factor is blocked by the antibiotic, preventing the binding to its binding site in the tetO promoter, incorporated in the second transgene: *tetO-tTA*:tau-LacZ*. Here, the tetO-promoter sequence is linked to the somato-axonal marker tau-LacZ, the structural gene for β-galactosidase typically present in the *lac* operon of *E.coli,* and a version of tTA (tTA*) made Dox-insensitive by introducing the point mutation H100Y in the Tet binding domain. Accordingly, the Dox administration to the bi-transgenic mouse inhibits the expression of tau-LacZ in recently activated neurons, preventing their labelling. However, if Dox is removed, tTA can bind to tetO-promoter, which in turn triggers the expression of both the tau-LacZ reporter gene and the tTA*. The presence of the latter gives life to a transcriptional self-perpetuating feedback loop that, once activated, allows for the sustained expression of the tetO-linked genes even upon Dox re-administration.

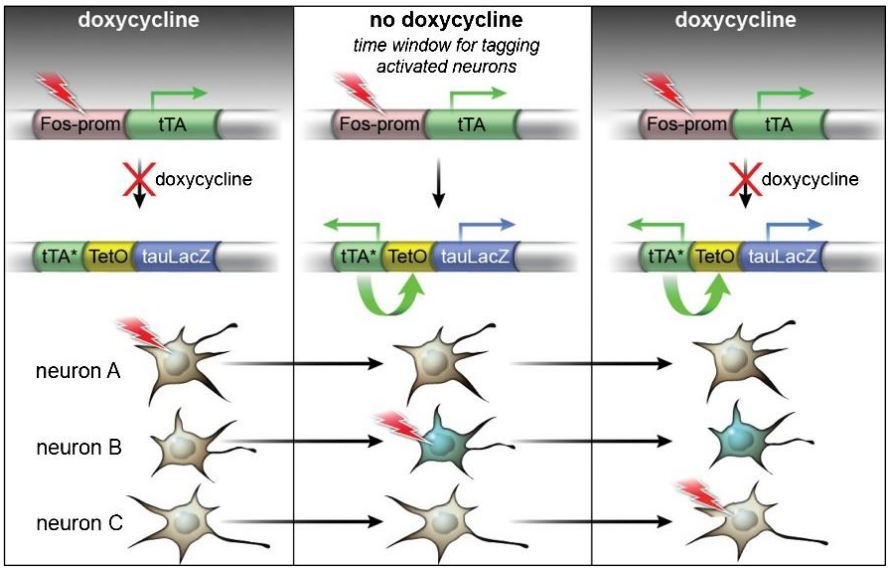


Figure 3. The TetTag system. Only the presence of both transgenes in the same animals allows the functionality of the TetTag approach: one expressing tTA under c-Fos promoter control, the other expresses Dox-insensitive tTA* and tau-LacZ downstream of the tetO promoter. Electrical stimulation results in the expression of tTA through c-Fos promoter activation. The presence of Dox (left panel) prevents the binding of tTA to tetO, with no expression of the effector genes. When Dox administration is ceased (middle panel), tTA can instead turn on the expression of the tau-LacZ marker gene and tTA*. As a result, firing neurons are "tagged" and detectable even after that mice are put back on Dox treatment (right panel). Re-administration of Dox coincides with the closure of the permissive time window for the marking of activated neurons with tau-LacZ. Figure taken from Deng et al., 2016.

The relevance of the TetTag approach lies in the fact that (1) the expression of the transgene of interest is triggered by neural activity only within an experimenter-controlled time window, and (2) the transgene expression is maintained in the active neurons indefinitely, but no further labelling occurs following the closure of the permissive time window. Strikingly, by opening and closing discrete time windows through Dox removal and administration, a persistent record is generated of those neurons that were active during the off-Dox period, for instance when a behavioural task is executed. The TetTag mouse has been used by Reijmers and colleagues to investigate hippocampal neural circuits mediating fear memory and learning, examining which neurons activated during a first fear-conditioning paradigm conducted in absence of Dox were subsequently reactivated during retrieval of the memory. The hippocampus, as key structure for episodic memory, receives information from the cortex through multiple parallel pathways to each of its main subregions, including the dentate gyrus (DG), CA3 and CA1, forming the classic tri-synaptic pathway DG → CA3 → CA1. Despite its potential, the TetTag technology has never been used in the circadian field. Indeed, the high temporal precision required in circadian studies may be hampered by the uncertain timespan

ranging between the Dox removal and the effective activation of the system and neuronal tagging. All previous researches based on this tool report indeed time windows of a minimum of 24 hr (Reijmers et al., 2007; Deng, Mayford, & Gage, 2013; Davis, Zaki, Maguire, & Reijmers, 2017) to ensure enough time for Dox to clear from the brain. This amount of time is clearly excessive if the aim is capturing daily oscillations in neuronal electrical activity. Nevertheless, the minimum time window allowing the effective neuronal labelling in vivo after Dox clearing has not been identified yet. In this study, the TetTag mice were used to test whether an off-Dox period lasting less than 24 hr could be enough for an effective tagging of recently-activated neurons. Afterwards, it was attempted to apply this technique to detect variations in cortical neuronal firing between three different vigilance states, sleep, sleep-deprivation and wakefulness.

The RAM system

The Robust Activity Marking (RAM) system was developed as a genetic tool to label and manipulate active ensembles of neurons associated with sensory and behavioural experiences (Sørensen et al., 2016). As the previously described IEG-based approaches, this mechanism consists of a designed DNA sequence switched on by neural activity through the c-Fos promoter, in addition, it contains additional elements that confer high sensitivity, selectivity and versatility (Figure 4 A). Upstream the classic human c-Fos minimal promoter, indeed, were placed four tandem repeats of a 24 bp enhancer module, previously assembled by combining the Activator Protein 1 (AP-1) site, a consensus sequence for the FOS/JUN family transcription factors, with the binding motif of the neuronal-specific activity-dependent gene *NPAS4* (NRE). This core was subsequently inserted into the transcriptional regulatory sequence Central Midline Element (CME), which secondary structure fosters the transcription activation. The resulting 199 bp synthetic promoter was named P_{RAM} and was reported to show strong activity-dependent induction profile (Figure 4 B). The P_{RAM} is incorporated into the Tet-Off system, driving the expression of a destabilized version of tTA, d2tTA, deriving by the fusion of the N-terminus with the degradation domain of Mouse Ornithine Decarboxylase (MODC). The resulting protein has been reported increasing the performance of the RAM mechanisms thanks to its significantly lower basal expression, tighter Dox regulation and highly improved fold induction compared with the conventional tTA. Several versions of this system were generated with different effector genes placed under the control of the tTA-responsive element (TRE) promoter, and the resulting plasmids were inserted as expression cassettes into backbone Adeno-Associated Virus (AAV) vector V032 (pFB-AAV-CMV-WPRE-SV40pA) for constructing AAV-RAM vectors. In this study, the AAV-RAM-ChR2:EYFP version was used, where the sequence encoding for the opsin Channelrhodopsin 2 (ChR2) is fused with the sequence of the Enhanced Yellow Fluorescent Protein (EYFP) as effector genes. Accordingly, after electrical activity the P_{RAM} promoter leads to the expression of the d2tTA, which in turn in absence

of Dox allows the neuronal labelling by switching on the expression of ChR2 and EYFP (Fig 4 C). As a result, recently activated neurons can be both visualized through the fluorescence protein and manipulated by optogenetically stimulating ChR2. In wider terms, the modular design has been shown to confer to the RAM system remarkable features: (1) robust labelling of neural ensembles active during an experience, given by the optimized synthetic neuronal activity-dependent promoter; (2) improved temporal control due to the modified Tet-Off system; (3) use of a single AAV containing both the transcription components and the effector genes thanks to the small size of the construct; (4) high versatility allowed by the possibility of using specific promoters and effector genes according to the experimental question; (5) transferability to several species for an extensive use in the neuroscientific community. Still unknown parameters remain to be evaluated, such as the minimal threshold of neuronal activity able to activate RAM and whether this system is appropriate for different behavioural paradigms and brain areas. This system has been tested to label active ensembles in the hippocampus and in the amygdala following contextual- and tone- fear conditioning paradigms, it was also applied in model organisms other than the mouse (rats and *Drosophila melanogaster*), however it has yet to be used to capture circadian variations in brain activity.

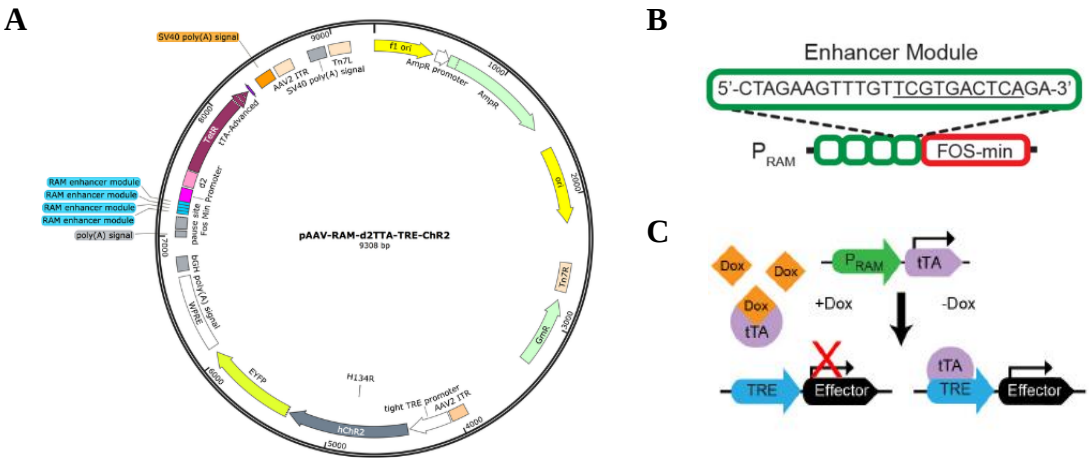


Figure 4. The RAM system. (**A**) In addition to the basic components for the AAV-based delivery, the AAV-RAM-ChR2:EYFP is composed of the P_{RAM} promoter, containing four RAM enhanced modules and the c-Fos minimal promoter, the tTA-Advanced version of the tetracycline transactivator d2TTA and its binding site in the tight TRE promoter, placed upstream the genes encoding hChR2(H134R) and EYFP. The figure of the plasmid was taken from www.addgene.org. (**B**) The four tandem repeats of the 24 bp enhancer module upstream the c-Fos minimal promoter provides high transcriptional strength, tight activity-dependency and sensitivity. The underline sequence corresponds to the motifs of the NRE and AP-1 sites, their partial overlap was shown to confer strong activity-dependent induction profile. (**C**) Schematic outline of the Tet-Off system underlying the RAM approach: Dox

administration (+Dox) prevents the binding of tTA to the TRE promoter after neuronal firing, while withdrawal of Dox (-Dox) allows the transcription of the effector genes placed downstream, with consequent marking of active neurons. Figures taken by Lin *et al.*, 2016.

Similarly to the TetTag technology, the purpose of this study was to understand whether the RAM system could be a valuable tool to investigate the sleep-wake dependent firing of cortical neurons, by identifying the minimum timespan from Dox removal required for an efficient neuronal tagging. The following experiment was conducted with the aim of observe whether this reduced time window was able to capture variations in cortical neuronal activity occurring in different time of the day. Ideally, in the next step we would take advantage of the presence of the optogenetic protein for selectively and artificially reactivated tagged neurons when they are not spontaneously firing, following with the observation of the effects in terms of physiology and behaviour.

Aims of the current study

In this study, two IEG-based genetic tools for neuronal tagging were tested with the purpose of understanding their activation dynamics upon antibiotic removal, under the prospect of applying them in circadian studies. For both these approaches, the temporal resolution is still poorly understood, due to the relatively slow metabolism and clearing of Dox from the brain. Consequently, the main question addressed in the research was to find the minimum time required for an effective neuronal labelling following the antibiotic withdrawal. Ideally, our aim is to capitalize on the relatively fast activation dynamics of neuronal marking to investigate the network properties of the different neuronal populations in the SCN and the local aspects of sleep regulation in the cortical area.

Experimental Procedures

Animals and treatments

The TetTag bi-transgenic mice were obtained from The Jackson Laboratory (stock No. 008344) and re-derived into a background of C57BL/6J. The mice were then bred by intercrossing the hemizygous *Fos-tTA:shEGFP* line with the hemizygous *tetO-tTA*:tau-LacZ* line. To confirm animal genotypes, PCR was conducted using amplification of DNA taken by ear biopsies. LacZ and EGFP alleles were amplified using PCR (Fig 5). All the mice were socially housed with food and water available ad libitum. The breeding pairs were treated with 40 mg/kg dox diet, and the doubled transgenic TetTag mice were raised on the same food after weaning. For the experiment with the RAM technology wild-type and *tetO-tTA*:tau-LacZ* mice were used. They were kept under the same dox diet of TetTag mice since the stereotactic injection of the virus until the experiment. The treated food was obtained by mixing 400 mg Doxycycline Hyclate (HY-N0565B, MedChemExpress) with 10 kg pellet M/R Haltung Extrudat Cat.#3436, the production was made by KLIBA NAFAG, Switzerland, and then was stored at 4°C. Mice were kept in 12:12 light-dark photoperiod (lights on at 08:00), zeitgeber time (ZT) was used to indicate time of day, with ZT0 (or ZT24) marking light onset and ZT12 dark onset. All the animals were at least 11 weeks old at the start of the experiments. All experimental procedures were conducted in accordance with applicable veterinary law of the Zürich cantonal veterinary office and were approved by the Zürich cantonal veterinary office.

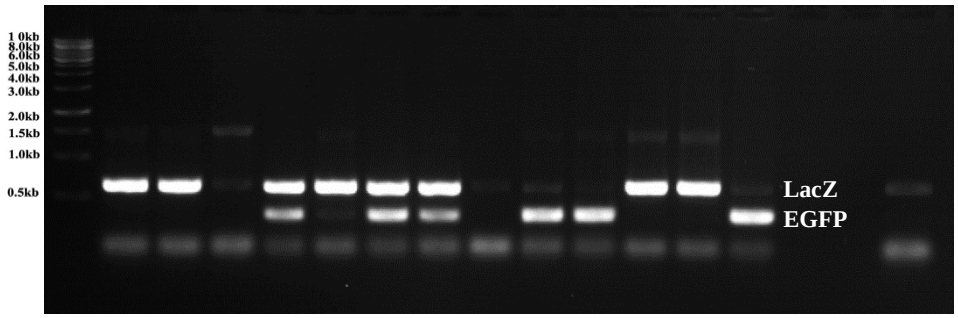


Figure 5. Example of a gel electrophoresis revealing the mouse genotype. The PCR products, visible as bands in the gel electrophoresis, reveal the presence of the transgenes at the base of the TetTag system. The upper bands correspond to the amplicon of the tau-LacZ marker gene, the lower bands represent instead the amplification of the shEGFP gene, linked to the tTA under the Fos promoter in the first construct. The presence of both the bands reveals the genotype of the TetTag mouse.

The TetTag system - Time course experiment

To assess the time required for the LacZ gene to be expressed after Dox withdrawal, a preliminary experiment was firstly set up to test whether the Dox concentration administered was enough to prevent the neuronal labelling by raising one TetTag mouse on Dox until sacrifice. As positive control, a TetTag mouse never treated with Dox was used, while a single transgenic *tetO-tTA*:tau-LacZ* mouse and a wildtype mouse represented the negative controls for immunostainings. Afterwards, to verify that a period of 48 hr in absence of Dox was sufficient to allow for the expression of β-gal marker in the recently-activated neurons, one bi-transgenic animal was kept on Dox for at least 15 days, then it was intraperitoneally injected with 1µg/g body weight Dox to set the precise time of the last antibiotic administration, at the same time Dox food was removed for 48 hr. After this time, the mouse was i.p. injected with a higher Dox concentration of 10 µg/g body weight to block further neuronal labelling, placed back on Dox diet and sacrificed after 24 hr. Another TetTag mouse was kept under Dox for the whole experiment to be used as negative control. After verifying that the Dox concentration was effectively preventing neuronal labelling, but 48 hr from its removal were sufficient to switch on the genetic system, the time course experiment was set up to test the minimum timespan required for the Dox clearing and the reporter gene expression and detection. 21 TetTag mice were raised on Dox diet for at least one month, then they were divided into seven groups of 3 mice each, 5 test groups and 2 control groups. Each group was constituted by two males and one female. Five time windows of Dox withdrawal were created of 28, 22, 16, 10 and 4 hr as previously described, by removing treated food and i.p. injecting with Dox at five different times of day (ZT4, -10, -16, -22 and -4 of the following day). The remaining two groups were used as controls, with a 48 hr off-Dox period for the positive group (ZT8 – ZT8) and constant Dox administration for the negative group. To close the time windows of tau-LacZ gene expression, all the mice were i.p. injected with 10 µg/g body weight Dox at ZT8 and they were put back under treated food. Exactly 24 hr later, all the animals were sacrificed and their brains were processed for immunohistochemical analysis.

The TetTag system - Sleep deprivation vs Sleep

To try to compare the activation of cortical neuronal populations of different sleeping patterns, 12 TetTag mice under Dox diet were equally distributed within three groups. For all the animals a 6-hr off-Dox window was created as previously described; for two groups, Dox was removed at ZT0 and readministered at ZT6, but the mice of one group were sleep deprived by "gentle handling" for the entire time off-Dox, while the second group was allowed to sleep. For the third group, the time-window was opened during the night phase, between ZT12 and ZT18, when mice are normally active, as positive control. 24 hr after Dox diet re-administration, all the animals were transcardially perfused and the brains analysed with immunohistochemistry.

The RAM system - Time course experiment

To validate the RAM system, a preliminary experiment was performed to verify the Dox-dependency of this approach, the efficacy of the AAV stereotactic injection and whether an off-Dox period of 48 hr was sufficient to allow a substantial RAM labelling of recently-activated neurons. 13 wildtype mice were stereotactically injected with AAV-RAM-ChR:EYFP in the cortex or in the hippocampus, 4 were then placed under constant Dox diet administration (40 mg/kg Dox food) as negative control, three were fed with normal food as positive control, while the remaining 6 were kept under Dox treatment for 26 days, to allow the viral infection and the integration of the construct in the mouse genome. Other three mice were instead injected in the same region but only with 1X PBS, as negative controls for the stereotactic injection and the staining. At day 27, the mice were i.p. injected at ZT4.5 with 1 µg/g body weight to precisely record the opening time of the permissive window for the neuronal labelling and Dox food was replaced with normal food. 48 hr later, all the animals were sacrificed and the brains processed for immunohistochemical analysis.
Following this preliminary experiment, a time course experiment was set up using 9 wildtype and *tetO-tTA*:tau-LacZ* mice to assess the minimum time window allowing the expression of the ChR2 and the EYFP. All the animals were injected in the right parietal cortex. After 26 days on Dox diet, three time windows of Dox removal were created by i.p. injecting 1 µg/g body weight Dox and switching to normal diet for 4, 10 and 16 hr during the active phase (ZT2, ZT8 and ZT14 respectively). At the end of this time (ZT18), all the mice were transcardially perfused and the brain fixed and collected for the immunohistochemical analysis. Brain sections of the mice used in the preliminary test were used as controls for this experiment.

The RAM system – Active wakefulness vs Sleep

As for the TetTag technology, also the application of the RAM system was attempted in order to examine cortical firing alterations in different phases of the day. 6 wildtype mice were injected in the cortex, delivering 500 nl of AAV-RAM-ChR:EYFP and placing them on Dox diet, as previously. After at least 26 days of recovery, three mice were i.p. injected with 1µg/g body weight Dox at ZT0, the beginning of the sleep phase, and treated food was replaced with normal chow. The same procedure was applied for the other three mice but at ZT12, to create the permissive time window for the neuronal labelling during the active phase. After exactly 12 hr from the Dox removal, mice were deeply anaesthetized and perfused; the brains were isolated and processed for immunohistochemical analysis.

Virus injection and stereotactic surgery

For the experiments performed using the RAM system, AAV-RAM-ChR:EYFP (AAV-RAM-d2TTA-pA::TRE-ChR2:EYFP-WPRE-pA, serotype 5/2, 6.8E12 vg/ml) virus was kindly provided by the Viral Vector Facility at the ETH Zürich.

This was diluted to 5E12 vg/ml by adding 3.7 µl 1X PBS to optimize the viral titer according to previous experiments. On the day of surgery, mice were anaesthetized using isoflurane (3% induction, 1.5% maintenance during surgery), i.p. injected with 20 µl/30g body weight of Temgesic (Buprenorphinum, Invider Schweiz AG) for analgesia and secured to a stereotactic frame (KOPF Stereotaxic Instruments) on a heating pad to avoid a decrease in body temperature. Following the exposure of the skull by a midline incision, a small craniotomy was made unilaterally overlying the right parietal hemisphere. AAVs (500 nl, 80 nl/min) were delivered by using a glass capillary connected to a glass syringe (10 µl, Hamilton Company, Reno, Nevada), injecting vertically (90° to the skull) with a Ultra Injector (Harvard Apparatus 70-3005 PhD Ultra Injector) and allowed to diffuse 10 min from the capillary tip before withdrawing the capillary. The coordinates of the target brain area in reference to bregma were as follows: AP: -1.8, ML: +1.2, DV: -0.8. The skin was stitched with polyglactin 910 braided suture (Novosyn® Quick, Braun). After surgery, mice were housed in their home cages collectively under dox diet, Baytril 2.5% was administered in water (200µl/50ml) for 4 days after surgery to prevent infections. The animals were monitored for two weeks and allowed to recovery for at least 20 days following surgery.

Immunofluorescence staining

Mice were deeply anaesthetised by inhalation of isoflurane and i.p. injection of pentobarbital (0.1 ml, 50 mg/ml solution), and they were transcardially perfused with 1X Phosphate-Buffered Saline (PBS) followed by 50 ml 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4) at steady flow rate. Brains were isolated and post-fixed in 4% paraformaldehyde overnight at 4°C, the day after they were transferred in 30% sucrose in PBS at 4°C for 48-72 hr until they sank. Subsequently, coronal sections of 40 µm thickness were sliced using a cryostat and stored at -20°C in anti-freeze solution (15% glucose, 30% ethylene glycol, 0.02% sodium azide in 50 mM phosphate buffer, pH 7.4) until use. For the immunostaining of the TetTag mice brains, six to seven brain sections were selected with the following anterior-posterior coordinates from bregma: around 0.10 mm, approximately -0.46 mm for the analysis of the SCN and about -1.96 mm to analyse the hippocampus. Only sections of the injected area were instead selected for the RAM experiments, and typically the staining was performed in 3-4 of these slices. Sections were washed three times for 10 min with 0.05% Tris-Triton (0.05% Triton X-100 in Tris-saline (50 mM Tris, 150 mM NaCl), pH 7.4) at 60 rpm and room temperature, followed by primary antibody incubation (in 2% normal goat serum, 0.2% Triton X-100 in Tris-saline, pH 7.4) in a wet chamber at 4°C overnight in continuous agitation (60 rpm). The sections were either double stained with anti-Fos and anti-β galactosidase antibodies, for the experiment with TetTag mice, or double stained with anti-Fos and anti-EGFP antibodies for the AAV-RAM-ChR::EYFP injected mice. After other 3 washes in 0.05% Tris-Triton for 10 min (60 rpm, room temperature), secondary antibodies were diluted in blocking solution containing 0.05% Tris-

Triton and 2% normal goat serum and applied to the sections for 1 hr at room temperature. Sections were washed with DAPI (1 µg/ml DAPI in Tris-Triton 0.05%) for 5 min to visualize cell nuclei and, followed two additional washing steps, they were mounted onto gelatine-coated glass-slides, air dried, sealed with Mounting medium (Dako Ltd., Denmark) and stored at 4°C for at least 24 hr before imaging. The following primary antibodies were used: mouse anti-c-Fos [2H2] (1:500, Abcam, ab208942), chicken anti-beta galactosidase (1:500, Abcam, ab9361), chicken anti-GFP (1:500, Cat#N-GFP-1020, Aves).
All secondary antibodies were used in 1:1000 dilutions: Cy3 goat pAb to chicken IgY (ab97145, Abcam), Alexa Fluor647 goat anti-mouse IgG (H+L) (115-605-003, Lucerna Chem).
By comparing the antibody-mediated Fos visualization with the direct detection of EGFP fluorescence in the recently activated neurons, the anti-Fos primary antibody presented higher detection sensitivity and efficiency (Fig 6), hence the former was preferred for the analysis of neuronal activity in all the following experiments. Ideally, to verify the specificity of the antibody a mouse knock-out for Fos would have provide optimum negative control, however, the product was ordered by a quite trustable source and it was decided no to further test the specificity of the antibody.

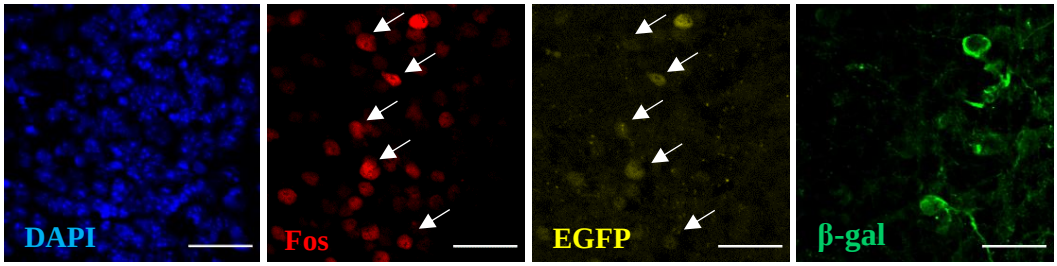


Figure 6. the Fos staining (red) colocalizes with the EGFP fluorescence (yellow), revealing the presence of both the transgenes in one of the two constructs present in the TetTag mouse. Arrows highlights the colocalization. The Fos staining showed higher intensity than the EGFP fluorescence detection alone, thus it was performed in all the following experiments. The presence of the second transgene in the same mouse is verified by the β-galactosidase staining (green), labelling recently-activated neurons when the TetTag mouse in without Dox. Cell nuclei are highlighted by DAPI staining (blue). Scale bars indicates 30 µm.

Image acquisition

For mouse brain sections, low magnification images were acquired with a LSM800 Airy Scan (Carl Zeiss) using the ZEN software (blue edition). For the TetTag experiment, the absolute number of β-gal marked cells in each slice was counted manually using a 25X objective. Channels used were DAPI for the total number of neurons, A647 for the Fos expressing neurons and Cy3 for the β-gal labelled neurons. Counts were performed in 6-7 separate sections from 3 animals per condition. Images were collected in the brain areas which showed the highest

number of β-gal$^+$ cells, the primary somatosensory area, the piriform area and the hippocampus. Moreover, the SCN was imaged to assess the efficiency of TetTag labelling in this area. Typically, a minimum of three 25X Z-stacks images were acquired for each animal in each region. Acquisition settings were optimized and were identical across regions and groups. Regions including damage from brain isolation were excluded. All the images for the quantification were acquired using identical pinhole, gain and laser settings.
For the brains injected with AAV-RAM vectors, image acquisition for the quantification in CA1 was performed with the same confocal system using 40X and 63X. In addition to the DAPI channel for the visualization cell nuclei, the Cy3 channel was used to detect the EYFP positive cells. Confocal images showing the overview of the injected area were collected with a Zeiss ApoTome 2.0, on one z-focal plane using 10X with 8 x 4 tiling, and they were acquired using identical pinhole, gain and laser settings. Typically, three to four images were analysed for each animal. The same instrument was used to take the whole brain images, using one z-focal plane with 10X objective and 15 x 19 tiling, again using the same settings for the different conditions.

Quantification of labelled cells

ImageJ (ImageJ, Wayne Rasband, USA) was used to select and perform cell quantification for the experiment with TetTag mice. The field of view of the analysed images was 511.12 x 511.12 μm. Four type of cells were quantified in each image: DAPI$^+$ cells, Fos$^+$ cells, β-gal $^+$ cells and Fos β-gal double-positive cells. The first three populations were counted using the Cluster Analysis Plugin, developed by PhD student David Colameo (https://github.com/dcolam/Cluster-Analysis-Plugin). The last two populations were instead counted manually due to the relatively small number of labelled neurons, the channels were set in composite option to validate the co-localization. From these quantifications, the percentages of β-gal$^+$ neurons were calculated from the ratio

$$\frac{number\ of\ \beta-gal\ positive\ cells}{number\ of\ DAPI\ positive\ cells},$$

where the number of DAPI positive cells was assumed to represent the total number of cells in the analysed area.

The activation rate for each area was calculated according to the following formula:

$$\frac{number\ of\ Fos\ positive\ cells}{number\ of\ DAPI\ positive\ cells}$$

The ImageJ software and the Cluster Analysis Plugin were used also to quantify the number of RAM labelled neurons in the CA1 hippocampal subregion and in the cortical area surrounding the injection site. For each image of the injected area, the total number of cells was estimated by DAPI positive cells counting, while the

quantification of Cy3 labelled cells provided the number of $EYFP^+$ neurons. The percentage of EYFP expressing neurons was calculated from the ratio

$$\frac{number\ of\ EYFP\ positive\ cells}{number\ of\ DAPI\ positive\ cells}$$

The measurements of the RAM labelled areas were performed on ImageJ by selecting the marked area as region of interest (ROI) from tile pictures and measuring the width and height. Typically, 2-3 pictures per condition were used to calculate the average.

Statistics

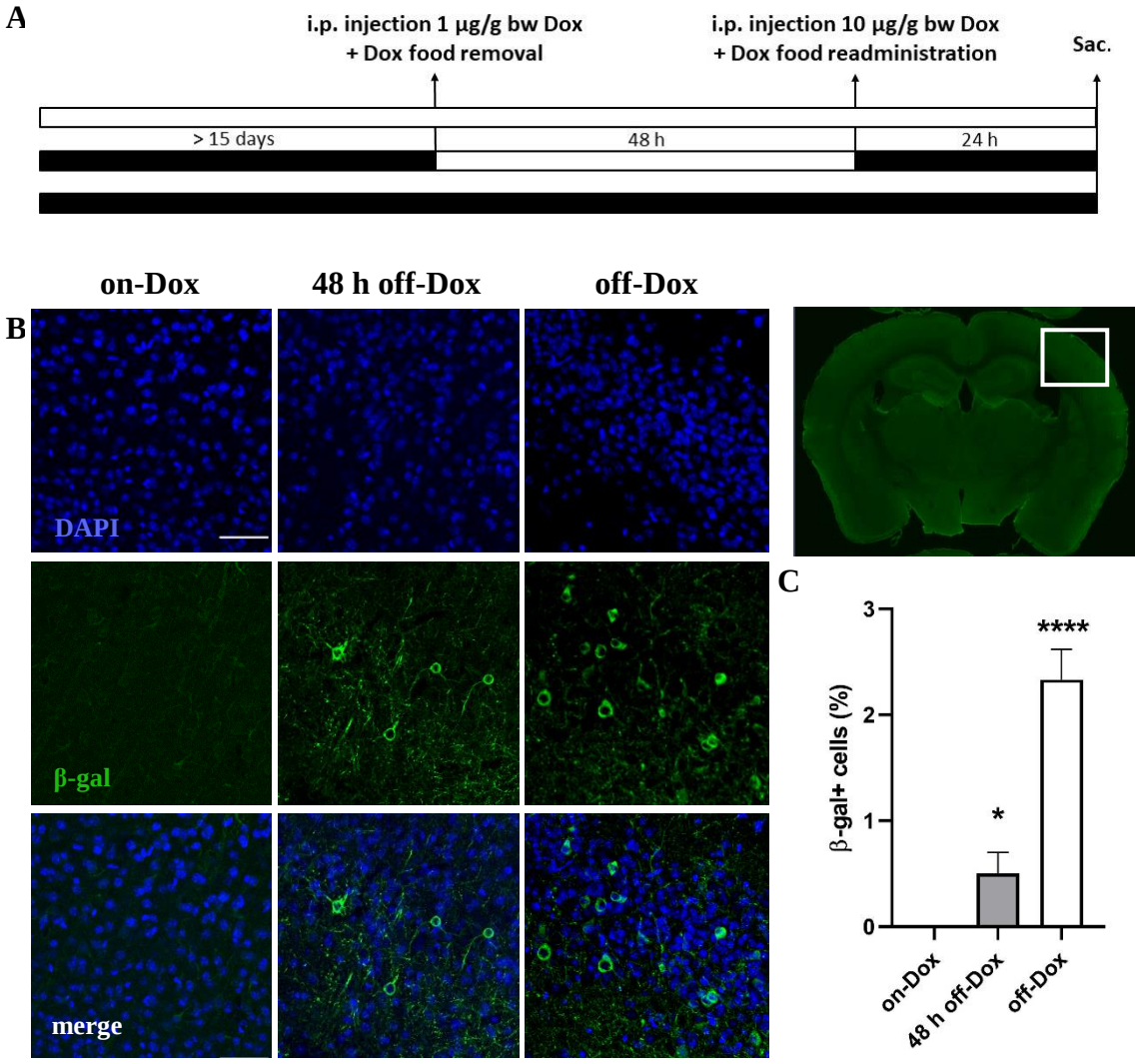
Statistical analysis were conducted with R version 3.5.2 (www.r-project.org) and RStudio version 1.1.463 (RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, URL www.rstudio.com) using the package ggpubr and the libraries dplyr, ggplot2, car and ggpubr. The graphs were instead designed with Prism (GraphPad Software, Inc., La Jolla, CA, USA).
For each experiment, the values of the technical replicates taken by the same mouse were averaged because we assumed that the expression within a given mouse was constant.
The appropriate statistical test was selected after using Shapiro-Wilk and Levene's tests to evaluate the normality of the data and the homogeneity of variances, respectively. Subsequently, either the parametric analysis of variance (ANOVA) or the One-way nonparametric ANOVA (Kruskal-Wallis ranked sum test) were used to detect significant differences between the groups. After the ANOVA, only the pairwise comparisons relevant for the aim of the study were performed in the time course experiments; typically, the negative control groups with the groups with the different time-window length. The Welch unpaired Two-Sample t-test was used when the variances of the groups were significantly unequal, while the nonparametric one-tailed Wilcoxon ranked sum test with continuity correction was used when data were not normally distributed. In the other cases, the standard Unpaired Two-Sample t-test was used. Differences with $P \leq 0.05$ were considered to be significant. All errors on the data are reported as mean ± SEM.

Results

The TetTag system – A 4 hr time window seems to be sufficient for neuronal labelling

In this study, the TetTag bi-transgenic mice were used to identify the minimum timespan of Dox withdrawal enabling the persistent labelling of neuronal activity. Firstly, the effect of Dox in preventing the tTA binding to tetO promoter and subsequent tau-LacZ expression was verified by keeping mice constantly on Dox diet (Fig 7 A). Conversely, in the mice never placed under Dox treatment and those kept on-Dox except for 48 hr off-Dox, neuronal tagging was clearly visible and regarded not only the neuronal soma, but in the whole somatodentritic compartment. As a general pattern, three brain regions were mainly characterized by the TetTag tagging, in other words, they presented neurons marked by the β-gal expression. Firstly, high intensity of tagging was observed in the piriform cortex, the largest cortical recipient of direct olfactory bulb projections, typically highly prominent in the mouse brain, accounting up to 10% of cortical volume in some rodent species (Fig 7 D). Secondly, a considerable number of TetTagged cells was found in the layers 2, 3 and 4 of the primary somatosensory area, located in the postcentral gyrus in the parietal lobe and responsible for the processing of somatic sensations (Fig 7 B). Since the hippocampus is a key structure for episodic memory and learning, receiving information from the cortex, the quantification of the labelled cells within this vital region was attempted. However, the number of biological replicates showing labelling in the hippocampus was not sufficient to perform cell quantifications with statistical relevance. Concerning the primary somatosensory area, 2.33 ± 0.28% and 0.5 ± 0.20% of neurons were found positive for the tau-LacZ expression in the permanently off-Dox condition and in the 48 hr off-Dox condition, respectively (Fig 7 C), surprisingly small values considering the large permissive time windows for the tagging. However, in this cortical area of the negative controls no β-gal^{+} neurons were found, confirming the effectiveness of the Dox treatment. In the positive control and the 48 hr off-Dox group, approximately the 29.63% and 15.75% of β-gal^{+} neurons were expressing also Fos at the time of the fixation, revealing that these neurons were reactivated shortly before the sacrifice, after at least one previous firing experience during the permissive time window that resulted in the neuronal labelling. Regarding the piriform cortex, the average percentages for the TetTagged neurons were 1.97 ± 0.11% and 2.43 ± 0.31% for the off-Dox and 48 hr off-Dox condition, respectively, results that were significantly different compared with the 0.22 ± 0.04% of β-gal expressing neurons found in the brain under constant antibiotic exposure (one-tailed Welch Two Sample t-Test after significant ANOVA, $p < 0.01$, Fig 7 E). The amounts of reactivated cells in this area for the three conditions were 73.33% for the positive control raised without antibiotic treatment, 14.75% for the condition “48 hr off-Dox”, while in the negative control, kept constantly on Dox administration, the

26.67% of neurons were expressing both Fos and β-gal. The staining of the brain sections from the wildtype mouse did not reveal any β-gal^{+} cell, confirming the efficiency of the immunostaining procedure and the antibodies used. Following our expectations, also in the brain sections of the single transgenic *tetO-tTA*:tau-LacZ* mouse no tagged cells were found, suggesting the lack of a significant basal expression of the reporter gene. The main surprising outcome of this preliminary experiment on the TetTag technology was the absolute absence of labelled neurons in the SCN for all the experimental conditions (Fig 7 F). Moreover, approximately 5% of TetTag mice did not show any β-gal marked neuron in the whole brain; these subjects were excluded by the subsequent analysis.



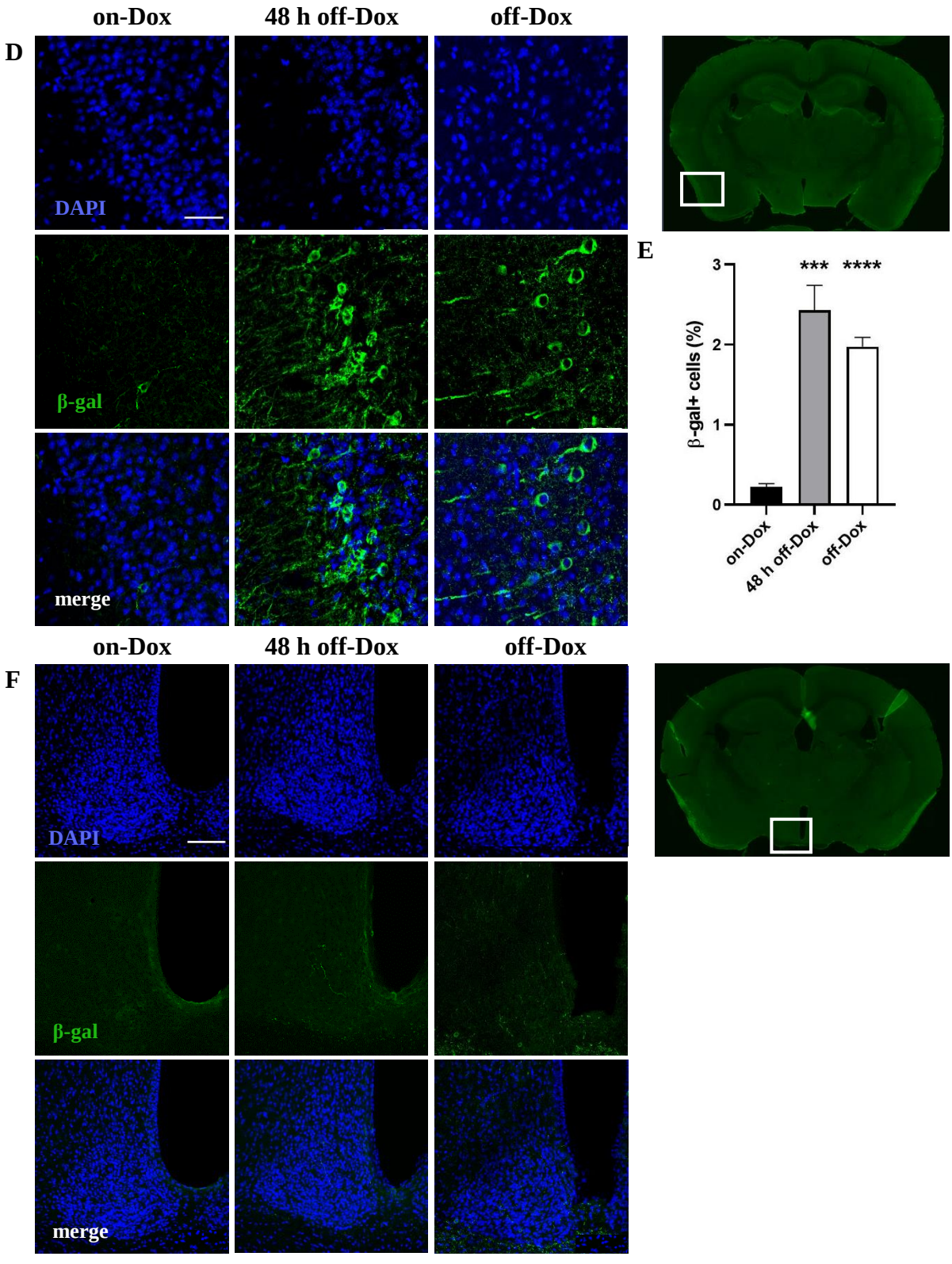


Figure 7. A small number of neurons were labelled in the cortex with the TetTag system, while no labelling was observed of SCN neurons. (**A**) Experimental design. One mouse was kept constantly under Dox treatment (black bars) to verify the effect on the antibiotic on the TetTag system, the positive control, in contrast, was raised under normal

food (white bars), resulting in a clear neuronal labelling. A third mouse was treated with Dox, then the antibiotic was removed for 48 hr, following by re-administration for one day before sacrifice. **(B**) Expression of β-gal marker (green) in the primary somatosensory area and (**D**) in the piriform cortex. Cell nuclei are oulined by DAPI (blue). The scale bar represents 50 μm. Quantifications showed that the percentages of β-gal$^+$ neurons are significantly higher in the off-Dox and 48 hr off-Dox condition compared with the on-Dox condition both in the (**C**) Primary somatosensory area (ANOVA, group x %labelled cells, $F = 30.29$, $p < 0.001$, one-tailed Unpaired t test between off-Dox or 48 hr off-Dox and on-Dox: $p < 0.0001$ and $p < 0.05$) and (**E**) Piriform area (ANOVA, %labelled cells x group, $F = 18.7$, $p = 0.00478$, one-tailed unpaired t test between off-Dox or 48 hr off-Dox and on-Dox: $p < 0.0001$ and $p = 0.0010$). (F) For all the groups, no labelling was found in the SCN. The scale bar represents 80 μm and is applied to all the images. Asterisk indicates statistically significant difference between groups. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. Data are shown as mean ± SEM.

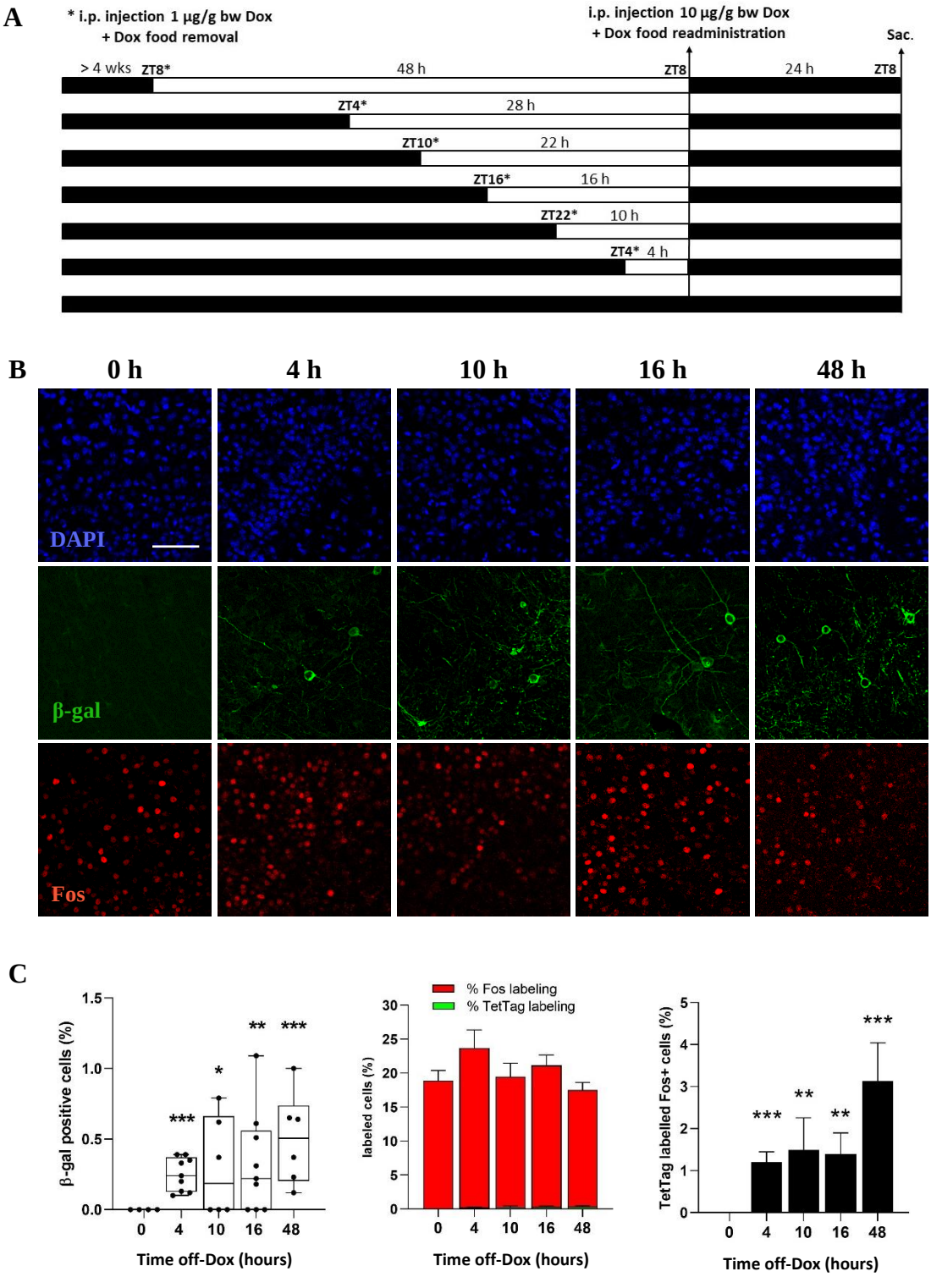
Considering these results, the time course experiment was set up by removing Dox-treated food from the cages of five groups of mice at five different time points at the distance of 6 hr each, recording the precise time of last Dox uptake by i.p. Dox injection (Fig 8 A). At the end of the off-Dox period, treated food was re-administered to prevent further neuronal labelling and after 24 hr mice were euthanized to examine the expression of β-gal protein. The quantification of the β-gal$^+$ neurons was performed for all the groups, but between the 28 hr, 22 hr and 16 hr off-Dox conditions there was no significance difference in the number of labelled neurons (Kruskal-Wallis rank sum test: % β-gal$^+$ cells x group, $p = 0.9419$ for the primary somatosensory area, $p = 0.1397$ for the piriform cortex). Consequently, the analysis was conducted only between the groups with the three shortest permissive time windows (16 hr, 10 hr and 4 hr) and the negative control. The average percentages of TetTag labelled neurons for the primary somatosensory area were 0.25 ± 0.04%, 0.30 ± 0.14%, 0.32 ± 0.12%, 0.14 ± 0.09% and 0.06 ± 0.04% for the 4, 10, 16, 22 and 28 hr off-Dox conditions, respectively (Fig 8 B and C). The positive control showed 0.5 ± 0.13% of neuronal labelling in contrast to the negative control, where no labelled cells were found in this region. In the piriform cortex the results were, in the same order, 0.51 ± 0.11%, 0.45 ± 0.09%, 0.78 ± 0.16%, 0.58 ± 0.29% and 0.78 ± 0.23%, 1.32 ± 0.64% and 0.17 ± 0.08% for the positive and the negative control, respectively (Fig 8 D and E). Despite the overall relatively low number of tagged cells, statistical analysis were performed in order to test the hypothesis whether 4 hr, 10 hr or 16 hr of Dox withdrawal were sufficient to allow the neuronal labelling. The result of the Kruskal Wallis rank sum test for the three shortest off-Dox periods and the permanent on-Dox treatment condition was significant for both the analysed brain regions (% β-gal$^+$ cells x group, $p = 0.019$ for the primary somatosensory area and $p = 0.038$ for the piriform cortex). Subsequently, each condition was compared with the negative control. According to this analysis, all the three permissive time windows seemed to be long enough to

allow an effective neuronal labelling through the TetTag technology for both the cortical areas (one-tailed Student T-Test and one-tailed Wilcoxon rank sum test, results and p-values are indicated in Fig. 8).

Because of the overall relatively low amount of marked cells, in order to test the labelling efficiency of the TetTag system, the percentages of β-gal expressing cells were compared with the neuronal activation rate at the time of the sacrifice (ZT8), represented by the percentage of Fos^+ cells. In fact, the labelling of the Fos protein reveals which neurons were activated in the last 1-2 hr (Flavell & Greenberg, 2008). Consequently, being the TetTag technology an IEG-based approach, the amount of cells marked by the TetTag system was expected to be at least approximately equal to amount of the recently-activated cells, or higher for the largest permissive time windows. Surprisingly, the percentage of the β-gal labelling resulted dramatically lower than the Fos labelling (Fig. 8 C): the neuronal activation rate at the time of the closure of the permissive time window and the sacrifice (ZT 8) oscillated around the average of 18.87 ± 1.5% (group constantly under Dox) and 23.68 ± 2.67% (group 4 hr off-Dox) in the primary somatosensory area, where the neuronal TetTagging reached a maximum average value of 0.32%. Similarly, in the piriform cortex between 11.44 ± 0.67% (16 hr off-Dox condition) and 13.40 ± 2.11% (on-Dox condition) of neurons were marked by the IEG at ZT8, where only between 0.51% and 0.85% of cells were TetTagged in the previous several hours of permissive time window (Fig. 8 E). From another perspective, the percentages of firing cells that resulted effectively labelled by the TetTag system in the primary somatosensory area were 0.00%, 1.2 ± 0.24%, 1.49 ± 0.76%, 1.39 ± 0.50% and 3.13 ± 0.91% for the 0, 4, 10, 16 and 48 hr off-Dox conditions, respectively. The same calculations for the piriform cortex gave as average percentages 1.70 ± 0.49%, 5.47 ± 1.6%, 6.86 ± 2.9%, 7.73 ± 2.02% and 20.81 ± 4.35% for the same groups in the same order. The Kruskal-Wallis rank sum test conducted on the activation rate did not reveal any significant difference between the groups for both the analysed regions (Kruskal-Wallis rank sum test, activation rate x group interaction, p-value = 0.39 for the primary somatosensory cortex, p-value = 0.46 for the piriform cortex). Thus, the different percentages derive exclusively by the different off-Dox time duration allowing the neuronal marking.

As previously mentioned, the analysis of the hippocampus, a key structure for memory and learning, turned out to be more complex to analyse than the other two brain regions previously considered. Indeed, $\beta\text{-gal}^+$ neurons were detected only in 9 mouse brains out of 21 for the CA1 hippocampal subregion, while 12 cases out of 21 showed TetTagged neurons in the Dentate Girus. Furthermore, the rate of activation of these two regions was highly variable between the groups the few hours before the sacrifice (ANOVA, group x activation rate, $F = 3.958$, $p < 0.05$). Hence, a significative difference in the hippocampal activation also during the off-Dox period was a possibility that could not be excluded, creating a bias in the number of tagged cells. In other words, a different number of $\beta\text{-gal}^+$ neurons could

have represented the result of a different rate of hippocampal activation, instead of a diverse length of permissive time window. Consequently, it was decided not to further proceed with the analysis of this area.



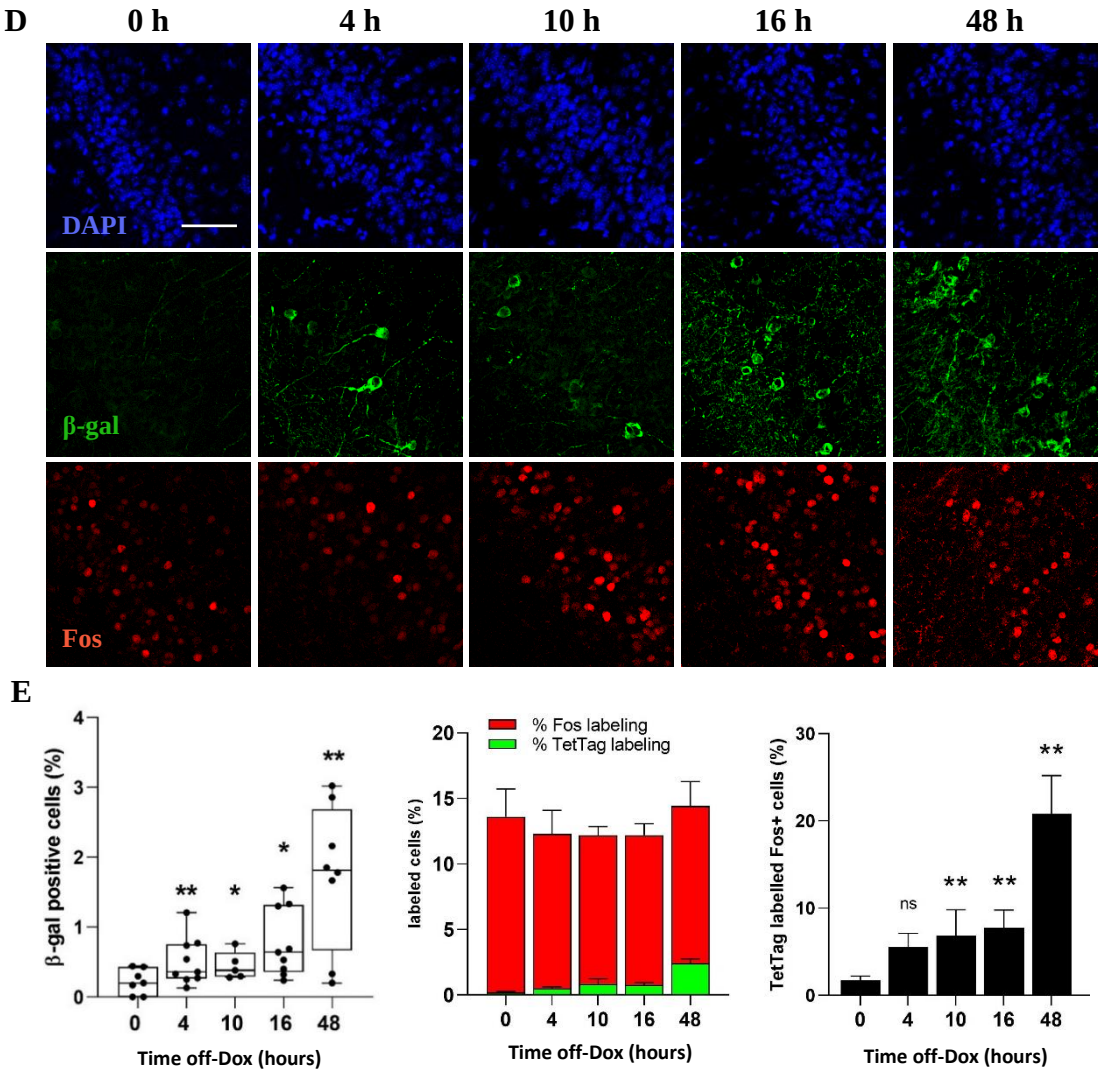
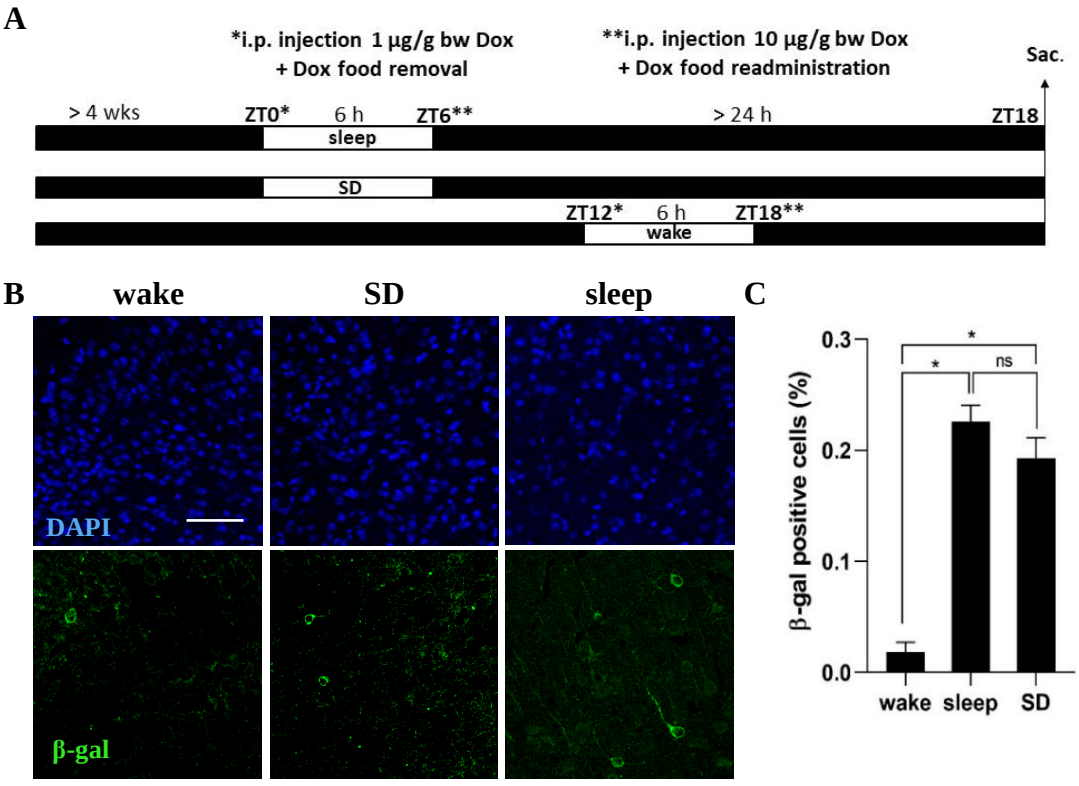


Figure 8. Time course experiment with the TetTag technology. (**A**) Experimental design of the time course experiment. Black bars indicate Dox administration, while white bars correspond to Dox withdrawal. Representative images of the primary somatosensory area (**B**) and the piriform cortex (**C**) from the data quantified in **D** and **E**, respectively. The scale bar is 80 µm and is applied to all the images. (**C**) Quantification for the primary somatosensory area: 4 hr off-Dox seems to be sufficient to enable a significant rate of neuronal labelling with the TetTag system (Wilcoxon rank sum test with continuity correction for pairwise comparisons). However, this rate is extremely low if compared with the percentage of Fos+ cells in the same area (red, percentages of Fos+ cells). In the right graph the percentage of Fos+ cells expressing β-gal is shown. (**E**) Percentages of β-gal expressing cells among total DAPI positive cells in the piriform area. In the middle, the percentages of β-gal expressing cells are compared with the percentages of Fos expressing cells. On the right, the percentages of Fos+ cells which were also β-gal positives is shown. The amount of tagged cells resulted dramatically lower than the Fos expressing neurons (red bars), accounting less than 20% of the recently-activated neurons (Wilcoxon rank sum test with continuity correction for pairwise comparisons). Data are shown in mean ± SEM. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. n = 3 animals per group.

TetTag system - Sleep deprivation vs Sleep

Ascertained that a time window of approximately 6 hr off-Dox was sufficient to switch on the TetTag labeling system, it was attempted to apply the TetTag approach to compare the rate of cortical neuronal firing in different vigilance states, sleep, wakefulness and extended wakefulness, also named *sleep deprivation* (SD, Fig. 9 A). As a general pattern, the rate of tagging was dramatically low, following the results of the previous experiment. Concerning the primary somatosensory area, the results of the cell quantification were not what we expected: the highest average number of β-gal expressing cells was found in the sleep group (0.22 ± 0.05%), closely followed by the sleep-deprived group (0.19 ± 0.04%). By contrast, the awake group revealed an extremely low percentage of labelled neurons compared to the other two conditions (0.02 ± 0.013%, Fig. 9 B and C). The piriform area was also analysed, here, the highest percentage of β-gal expressing cells was found in the mouse brain that underwent sleep-deprivation (1.22 ± 0.17%, Fig. 9 D and E). However, no significant difference resulted from the ANOVA when the three groups were compared. In addition, the levels of tagging in the hippocampal region were observed, however, each condition displayed elevated internal variability: high numbers of marked neurons were found in 2 sleep-deprived mouse brains and in one mouse brain of the "sleep group" (0.78 ± 0.16% labelled neurons). In contrast, no considerable level of TetTag labeling was found in the brain sections of the "awake" group (0.68 ± 0.16%).



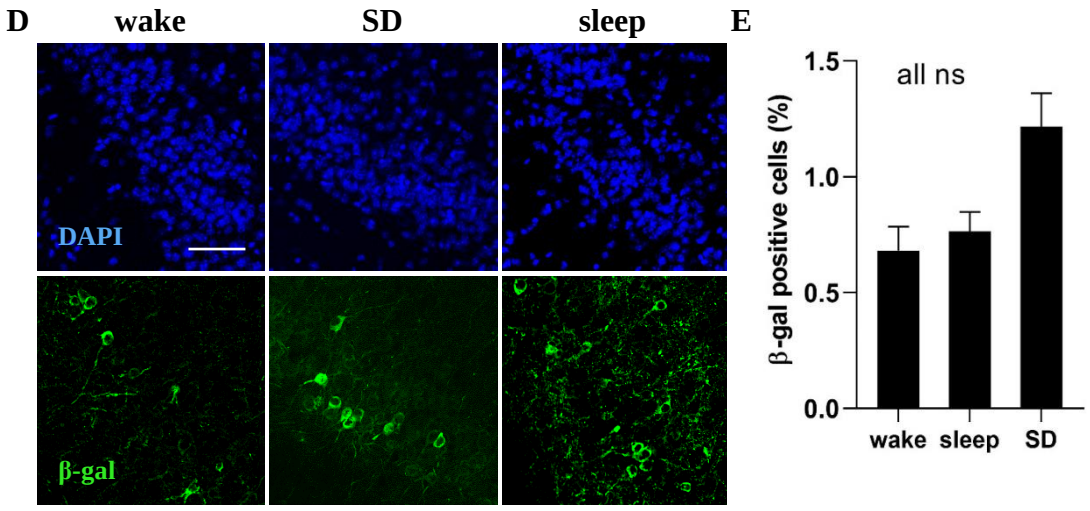


Figure 9. Comparison of three conditions of vigilance states in terms of cortical neuronal firing with the TetTag system. (**A**) Experimental design. A short time window off-Dox was applied during three different condition of vigilance states: sleep, sleep deprivation and wake. Black bars correspond to periods of Dox food administration, while white bars indicate antibiotic removal. (**B**) Representative images of the primary somatosensory area for the three groups. The scale bar represents 80 µm and is applied to all the images. (**C**) Quantification of the TetTag labelled cells for the Primary somatosensory area (Kruskal Wallis test, group x %labelled cells, $p < 0.001$, Wilcoxon rank sum test for pairwise comparisons). (**D**) Representative images for the analysis in the piriform cortex, with relative quantification of TetTagged neurons in (**E**). No significant difference resulted from the ANOVA (ANOVA, group x %labelled cells, $F = 1.7$, $p = 0.236$). Data are shown in mean ± SEM.
*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. n = 4 mice per group.

The RAM technology - Time course experiment

The preliminary evaluation of the RAM system efficiency was conducted by injecting the AAV-RAM-ChR2::EYFP in the right parietal cortex of wildtype animals and then feeding them until sacrifice with either treated food or normal food (Fig. 10 A and B). A group of mice under antibiotic were switched to normal diet for 48 hr, then sacrificed, to verify the activation of the RAM marking system during this time window. As a result, clear marking of the neurons was detected around the injection site for all the mice kept in complete absence of Dox, with robust labelling of the neuronal soma and projections (Fig. 10 C). The average area of neuronal marking covered approximately 1560 µm x 1330 µm underlying the injection site, including all the cortical layers and the hippocampus. From the analysis of the other two groups, the brain area containing $EYFP^+$ cells, the number of labelled neurons and the intensity of the neuronal marking displayed high visible variability among the mice of the same condition. Consequently, the small number of replicates available prevented the possibility to quantify reliably the number of labelled cells in this experiment. As general pattern for the 48 hr off-Dox group,

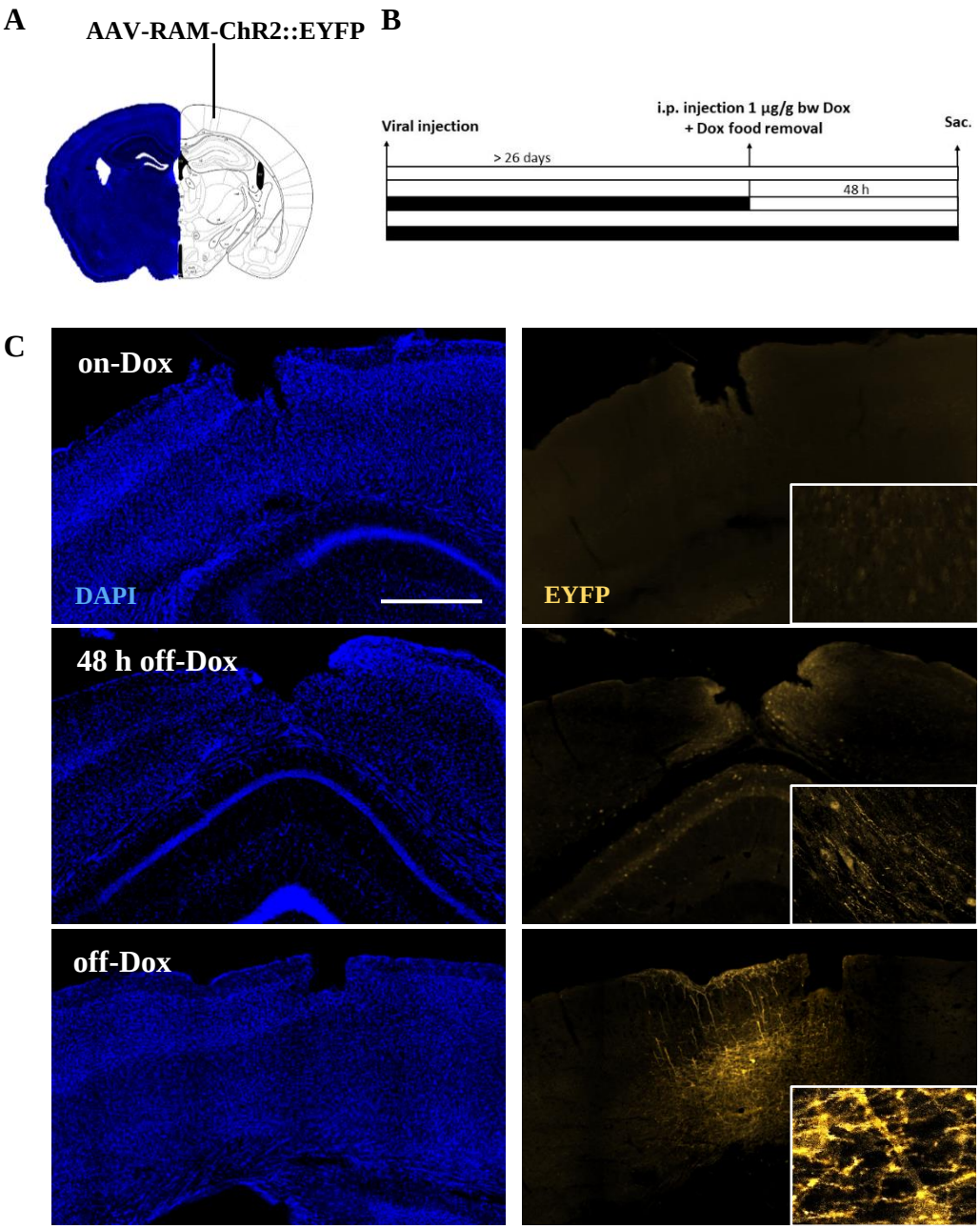


Figure 10. Visualization of the cortical labeled neurons with the RAM system. (**A**) Strategy of injection of the AAV-RAM-ChR2::EYFP in the brain cortex. (**B**) Experimental scheme. Animals were infected with AAV-RAM-ChR2::EYFP and kept on Dox diet (black bars) or on normal diet (white bars). A group of animals was taken off Dox diet 48 hr before sacrifice. (**C**) Induction of EYFP marker (yellow) in the cortical area around the injection site. There are no or few neurons if the mice are kept on Dox diet, while after 48 hr from antibiotic withdrawal some neurons are marked with the RAM system. Clear labelling in the soma and in the dendritic arborization characterize the neurons in the injected area when Dox is not present to prevent the RAM labelling system. Cell nuclei are oulined by DAPI (blue). Scale bar: 500 µm, applied to all the images. Inset: 150 µm. n = 3-5 mice per group.

inconstant amounts of marked neurons were found in the region surrounding the injection site and/or in the hippocampal CA1 in 4 out of 6 injected mice. No labelled cells were observed in 2 cases, likely due to an unsuccessful stereotactic injection. Unexpectedly, a small number of $EYFP^+$ cells was found around the injection area and in the hippocampus of one of the three mice kept constantly under Dox treatment. However, this amount was visibly lower than that of the other two conditions; moreover, only the neuronal soma and a short portion of dendrites were interested by the marking. In all cases, no marked cells were found in brain areas underlying the hippocampus and in the contralateral hemisphere. Furthermore, the sections obtained from the brains injected only with phosphate buffer did not show any RAM tagging, confirming the identity of the identified positive cells as products of the viral construct.

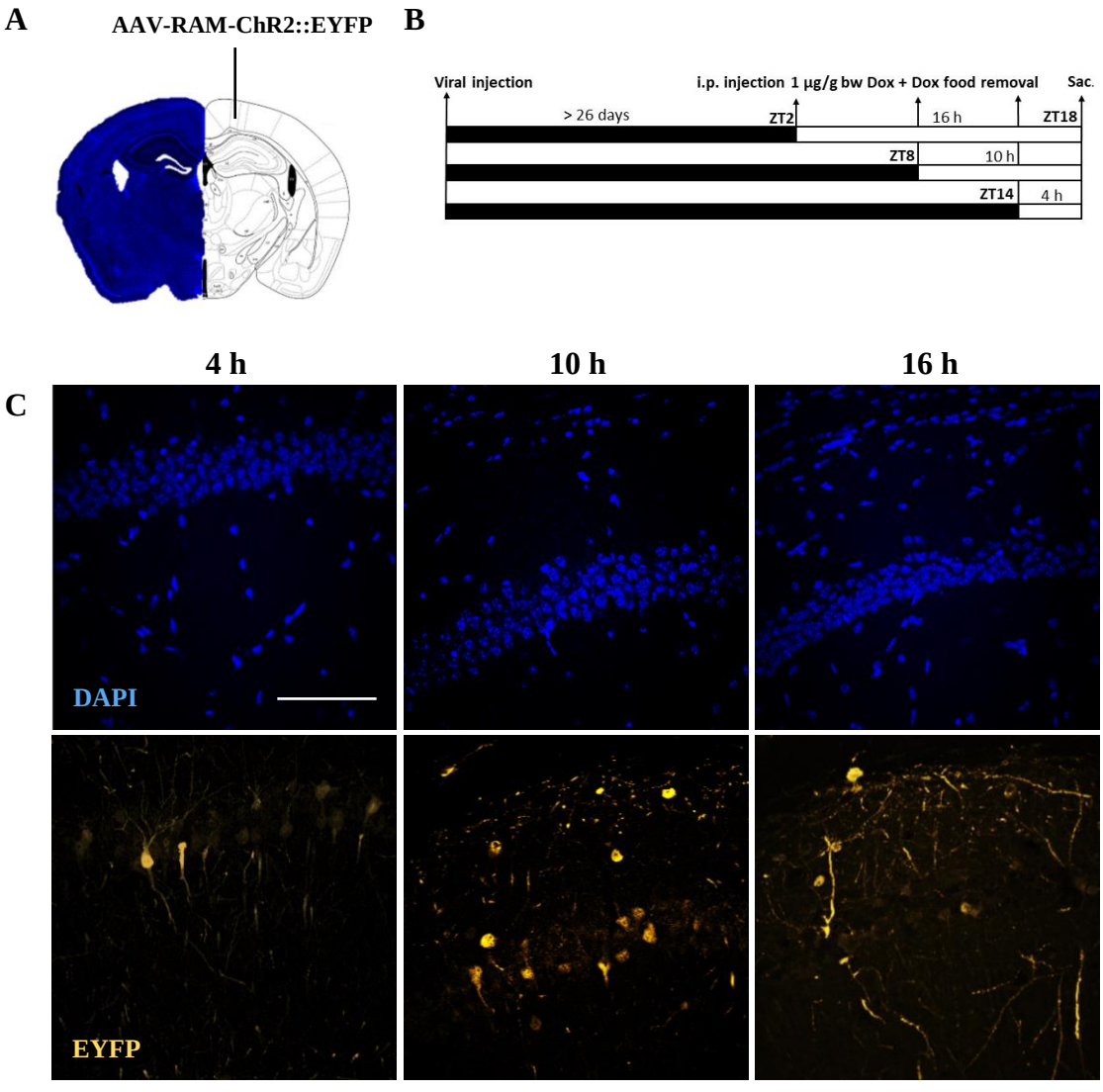
Because a duration of 48 hr of Dox removal was enough to trigger a visible neuronal marking with the RAM system; a time course experiment was set up to find the minimum off-Dox period allowing the specific labelling of recently activated neurons. Treated food was serially removed, as previously described, every 6 hr for three groups during the active phase, when cortical neurons should display highest rate of activity (Fig. 11 A and B). The longest time window was 16 hr (ZT 2 – ZT 18), while the shortest lasted 4 hr (ZT 14 – ZT 18). As a result, all the three conditions displayed RAM labelling, but with different patterns in different animals: 1/3 of the mouse brains resulted marked in the whole hippocampus, 4 out of 9 showed $EYFP^+$ neurons only in the CA1 hippocampal subregion and a small amount in the cortical area, just below the injection site. In one case the DG of the hippocampus was highly labelled, but almost no $EYFP^+$ neurons were found in the CA1 subregion, an oddity that could derive by a differential propagation or integration of the virus. Finally, in one brain the number of RAM marked cells was relatively very small and spread in the whole hippocampal area. The number of RAM labelled neurons resulted also visibly variable, likely due to the different rate of electrical activity during the permissive time window among the animals. Even in this case, no $EYFP^+$ neurons were found in the contralateral hemisphere and in other brain regions.

Because the CA1 hippocampal subregion was the only brain structure were RAM marked cells were detected consistently, the quantifications and comparisons between the three off-Dox conditions and the controls were performed from the images taken in this region (Fig. 11 C). The average percentages of ChR2-EYFP expressing cells were 6.01 ± 0.71%, 6.97 ± 1.04% and 5.82 ± 0.50% for the 4 hr, 10 hr and 16 hr off-Dox time windows, respectively, while the on-Dox condition displayed 2.72 ± 1.10% of positive cells in the analysed area (Fig. 11 D). The ANOVA analysis revealed that there was significant interaction between groups and marking rates in the CA1 (ANOVA: group x % $EYFP^+$ cells, $F=3.116$, $p < 0.05$). The pairwise comparisons were statistically significant for each of the three test groups with the negative control (one tailed Welch two sample t-test, $p < 0.05$

for the group 4 hr and 16 hr off-Dox with on-Dox group, $p < 0.01$ between 10 hr group and on-Dox group).

The IEG labelling was used to approximately estimate the efficiency of the RAM technology, as previously for the TetTag system. The evaluation was conducted only in the CA1 region, for the above-mentioned reasons. The average percentages of Fos labelling were 23.67 ± 5.34%, 24.01 ± 1.69% and 14.46 ± 6.88% for the 4, 10 and 16 hr off-Dox time windows and 24.37 ± 2.38% for the negative control (Fig. 11 D). ANOVA analysis of the activity rates did not reveal any significant difference between the groups (ANOVA: group x activation rate interaction, $F = 1.083$, $p = 0.41$).

The percentage of recently activated neurons effectively labelled through the RAM technology was calculated by normalizing the average percentage of RAM^+ cells on the percentage of Fos^+ cells for each group. As a result, 25.41 ± 1.8%, 29.03 ± 4.79% and 40.24 ± 3.43% of recently activated neurons in the CA1 were RAM labelled after 4, 10 and 16 hr from antibiotic removal, respectively. In case of constant Dox treatment, the percentage was 11.16 ± 4.60% (Fig. 11 D).



D

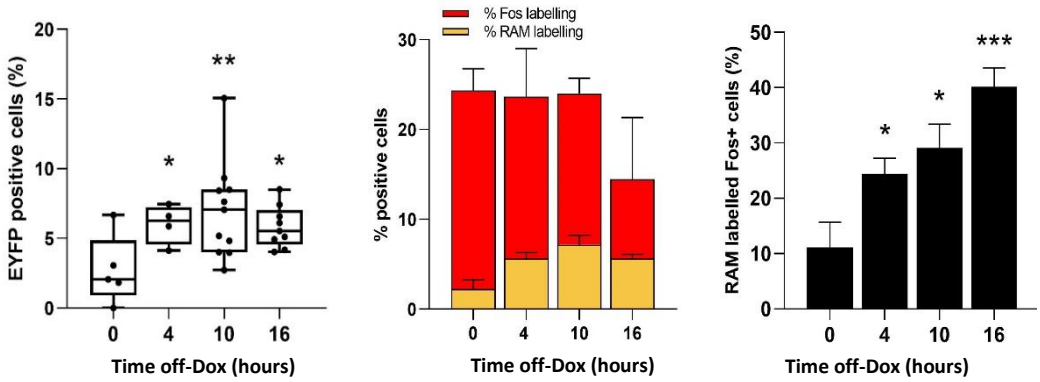


Figure 11. Time course experiment with the RAM labelling system. (**A**) Strategy of injection of the AAV-RAM-ChR2::EYFP in the brain cortex. (**B**) Experimental design. Three groups of mice were injected in the cortex with the AAV-RAM-ChR2::EYFP and kept on Dox diet for at least 26 days (black bars). Afterwards, three permissive time windows for neuronal RAM labelling were created by removing Dox (white bars) serially every six hours (ZT2, ZT8, ZT14). At ZT18 all the animals were sacrificed. (**C**) Representative confocal images of CA1 labelling for the three conditions. The EYFP allows the clear visualization of the some and the projections of recently-activated neurons in the surroundings of the injection area. Scale bar represents 100 µm and is applied to all the images. (**D**) Quantification of the RAM marked neurons in CA1 under the injection site after 4 hr, 10 hr and 16 hr after Dox removal, data were compared with the results of the quantification for the group kept constantly under Dox treatment. The results were compared with the percentages of Fox labelled cells (red bars, middle graph) and normalized on the percentages of recently activated neurons in the same area (graph on the right). ANOVA: group x percentage EYFP+ cells, $F=3.116$, $p < 0.05$; one tailed Welch two sample t-test for the pairwise comparisons. One tailed Unpaired t test for pairwise comparisons, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. Data are shown as mean ± SEM. n = 3 mice per group.

The RAM technology – Active wakefulness vs Sleep

To test whether the RAM labelling system could be used to detect daily changes in cortical neuronal firing, the Dox administration was removed by two groups of mice previously injected with the AAV-RAM-ChR2:EYFP during the active phase or during the sleep phase, respectively (Fig. 12 A). The selected off-Dox period was 12 hours. As a result, one-third of the mice tagged during the awake period showed cortical neurons labelled in surrounding the injection site, while two-thirds of the same group were predominantly tagged in the CA1 and DG hippocampal subregions (Fig. 12 C). Analysing the cortical area of the group marked during the sleep phase, a relatively small number of EYFP expressing cells was detected in one case, both in the cortical layers underlying the injection site and in the hippocampus. In another brain, $EYFP^+$ were observed prevalently in the CA1, likely

due to a reduced spreading of the virus. Finally, in one mouse brain no RAM tagged cells were observed, therefore this was excluded from the analysis. The resulting data for the sleep group were concentrated around the mean of 2.57 ± 0.17% (Fig. 12 D). In contrast, although in the awake group were observed higher levels of labelling with a maximum of 5.07% EYFP expressing neurons, overall the range of values was considerably wider, resulting in an average percentage of 1.78 ± 1.24% of marking. Consequently, no significant difference emerged from the comparison of the two groups (Welch two sample t-test, p = 0.59).

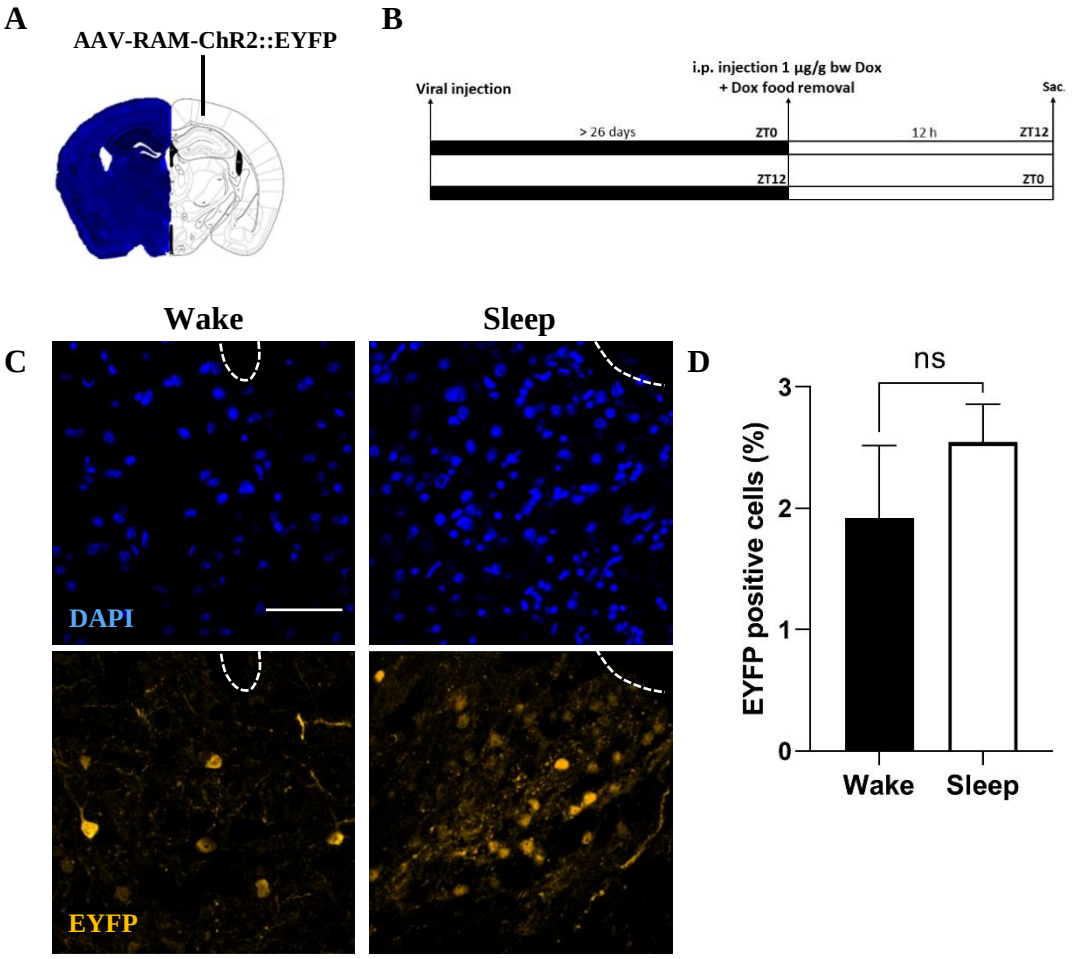


Figure 12. No significant variations were observed in the cortical RAM labeling between sleep and wake phase. (A) Strategy of injection of the AAV-RAM-ChR2::EYFP in the brain cortex. (B) Experimental design. Two groups of mice, previously injected with AAV-RAM-ChR2::EYFP and kept on Dox diet for at least 4 weeks (black bars), were shifted to normal diet for 12 hr either during the natural sleep phase (ZT0-ZT12) or during the active phase (ZT12-ZT24), to allow the RAM labeling of neurons firing during this time (white bars). (C) representative images from the quantification shown in D. Scale bar represents 50 µm and is applied to all the images. The dotted line indicates the injection site. (D) No significant difference was observed between the cortical RAM labelling during the wake phase and the sleep phase (Wilcoxon rank sum test, p = 0.8). n = 3 mice per group.

Discussion

In this study, two IEG-based technologies for selective marking and manipulation of active neurons were investigated in terms of temporal resolution of the activation dynamics. The timespan required for the antibiotic clearing and effective expression of the reporter gene has been shown to be less than the previously assumed for both the approaches. From these results, a suitable time window of Doxycycline withdrawal was selected and applied to observe possible changes in cortical neuronal firing in different conditions of vigilance states.

Short time window of few hours of Dox removal enable robust neuronal labelling with the TetTag system

The TetTag technology is a tool that has been commonly used to examine neural circuits that mediates fear memory and learning paradigms. However, a considerable level of uncertainty still remains on its dynamic of activation following the antibiotic removal. In this study, we show that the "switch-on" dynamics of the activity-dependent neuronal tagging after Doxycyline withdrawal requires less time than that so far hypothesized. Whether a period of only 4 hours of antibiotic removal may constitute a "border condition", with results not reproducible in all the experimental paradigms, a timespan between 6 and 10 hr off-Dox seems to be a reliable choice for different experimental designs. In all previous studies based on this approach, the latency period off-Dox preceding the event of interest (e.g. the fear conditioning) lasted at least one or two days, in order to ensure enough time for Dox clearing from the brain and expression of the transgene. This large time window preceding the event of interest is likely to cause the tagging of non-relevant neurons, increasing the uncertainty on the causal functions of different ensembles of neurons activated closely in time. Consequently, a period of antibiotic withdrawal in the range of only few hours would allow minimal tagging of neurons non-specifically activated during the permissive time window, improving the temporal resolution of this method. Secondly, it would be possible to compare the activities of the same neuronal populations in response to two events at sequential time points, possibly also in the same day. Under this perspective, the TetTag technology could be applied in the circadian research to investigate daily fluctuations in neuronal firing in the multiple damped circadian oscillators outside the SCN, like the pineal and paraventricular nucleus of the hypothalamus or the arcuate nucleus (Abe et al., 2002). Moreover, it may allow to examine the phenomenon of cortical local sleep by permanently tagging ensembles of neurons specifically active during particular phases of the sleep-wake cycle.

Despite the potential of the TetTag technology, the limitations of this approach were clearly revealed by this study, starting from the lack of labelling in the SCN structure in all experimental conditions. Several speculations can arise from this outcome: the firing rate could play a role in discriminating which neurons can or

cannot be labelled. Indeed, SCN neurons fire at sustained rates that rarely exceeds 15 Hz (Mazuski et al., 2018). In contrast, the cortical region hosts neuronal populations with firing frequency of hundreds of Hz, for instance, cortical fast spiking neurons in mice can reach firing frequencies of 500-600 Hz, or ever higher (Wang et al., 2016). Consequently, the activation threshold of the TetTag system could be too elevated to trigger the labelling of low-frequency firing neurons. As previously mentioned, this parameter has not been assessed yet for this technique, and in general for the other IEG-based methods it remains unclear. For this reason, the possibility that only some specific populations of activated neurons (i.e. the population with the highest activities) could be tagged cannot be formally ruled out. Another hypothesis proposed for this variable induction efficiency across brain regions is the low penetrance and inconstant expressivity of the transgenes, a problem already encountered with approaches based on transgenic mice. Specifically, the low efficiency of tagging observed using the TetTag approach is consistent with previous studies conducted in the CA1, CA3 and in basolateral amygdala (Deng et al., 2013; Reijmers et al., 2007). Transgenic mouse models have been extensively employed in neurobiology and the development of transgenic and targeted mutant mouse strains allowed researchers to broadly investigate gene functions in the context of a whole mammalian organism. However, they suffer several intrinsic limitations: the most significant for this study is the possible influence on the expression of a TRE-regulated target transgene by its chromosomal insertion site. In other words, the surrounding genetic background could play a role inducing overexpression or silencing of the transgene. Regardless of the phenomenon or concurrent phenomena causing the absence of labelling in the SCN, this study seems to show that the TetTag mouse is not suitable for the study of SCN clock neurons.

Another evident finding of the time-course experiment conducted with the TetTag mice is that this method is affected by a relatively high background expression. Indeed, the administered Dox concentration was not able to fully prevent the tau-LacZ expression in the negative control group, which showed a significantly lower but not unimportant rate of tagged neurons. Increasing the Dox concentration to minimize this leakage may not represent a feasible strategy to perceive, since this "leakage" is likely deriving by the random integration at the base of transgenic models, meaning that the desired gene could integrate anywhere in the host genome with poor control by the researchers. Moreover, a higher dose of antibiotic would prolong the time required for its clearing from the tissues, with consequent enlargement of the time window needed for the activation of the tagging system. Additionally, the presence of labelled cells in the brain of the negative control may come from a small food intake of the mice during the rest period, with a consequent reduction in Doxycycline concentration in the tissues and in the brain and labelling of activated neurons. Periodic measurements of Dox concentration in the blood could have been performed to test this hypothesis.

The impact of epigenetic factor cannot be omitted when discussing transgenic approaches. In this study, 2 out of 36 TetTag mice used did not show any tau-LacZ expressing cell in the whole brain, meaning approximately 5.56% of the analysed mice. The same percentage was already reported by the groups of Bejar and Reijmers in their studies with the bi-transgenic animals (Bejar, Yasuda, Krugers, Hood, & Mayford, 2002). In this regard, they advanced the hypothesis that epigenetic silencing of the feedback loop tetO-driven transgene may occur during brain development when mice were bred and raised in presence of Dox. A possible preventive measure that could have been taken before the experiment was to raise the animals with normal food and start the treated diet after weaning, and afterwards waiting until the developmentally expressed β-galactosidase protein was completely degraded. However, further evaluations should have been conducted in this case, with the purpose of determining the time required for the self-perpetuating feedback loop to be switched-off by the presence of Doxycycline, without ruling out the possibility of a basal background of permanently tagged neurons.

Among the main features of the TetTag approach emerged by this study there is certainly the partial recapitulation of the endogenous Fos expression by the Fos-tTA transgene. The time course experiment showed that the TetTag system was able to capture approximately between 1.2% and 6.8% of the Fos expressing neurons in the two analysed cortical areas, in other words, the neurons that were firing in the last 1-2 hr before the closure of the permissive time window. This percentages agree with the results of previous studies, where in the hippocampus of mice that underwent learning tasks were quantified between 1.5 ± 0.5% and 5.1 ± 0.5% of LacZ expressing cells for the CA1, while in the DG the range was between 1.9 ± 0.7% and 6.9 ± 1.0% (Deng et al., 2013). In Tayler, Tanaka, Reijmers, & Wiltgen, 2013, the amount of neurons tagged upon contextual fear conditioning with the TetTag system never reached the 10% of total cells, while in 2007 Reijmers and colleagues found that when animals were exposed to the same paradigm the basolateral amygdala showed between 0.5% and 0.95% of the total number of neurons. However, according to previous researches, approximately 15% of neurons in layer 2 and 3 of the somatosensory cortex should be c-Fos – shEGFP positive, even in the absence of any specific environmental stimuli (Lemaire et al., 2011; Bejar et al., 2002). Therefore, we conclude that the TetTag system is not able to activate the labelling of all the neurons that were firing during the permissive time window. This weak point of the technique was already revealed by a recent study, in which a novel technology, named Capturing and Manipulating Activated Neuronal Ensembles or CANE, was able to mark a moderately higher number of neurons compared with the Fos-tTA system in the same experimental paradigms (Sakurai et al., 2016). From this consideration new questions arise: what caused the labelling of these specific activated neurons instead of others? In other words, which special feature has this subpopulation of neurons to allow the tagging? Possible speculations may be that these cells were firing at higher frequencies compared to the others during the period of antibiotic withdrawal. An alternative

hypothesis could be that the observed TetTag labelling would represent an exclusive record of the neurons that were electrically activated repetitively or persistently during the permissive time window. If true, the neurons with these features may have a preeminent role in the circuit where they are involved. In this perspective, the TetTag system would constitute a potential useful approach to identify the function of single neurons inside their network, possibly in conjunction with other more developed genetic tools.

A final consideration about the TetTag technology: it represents a certain useful tool for probing and visualizing neurons naturally active at a specific time, but it does not allow their subsequent functional manipulation, task that has been instead accomplished by relatively recent techniques already applied successfully in neurobiology. Among these, the most sophisticated tools for artificial neuronal reactivation include certainly the Designer Receptors Exclusively Activated by Designer Drugs, also referred as DREADD approach, and the use of light-sensitive opsins for optogenetic stimulation. The first consists of a chemogenetic tool based on the exclusive activation of the G Protein-Coupled Receptor (GPCRs) hM_3D_q by a synthetic ligand (clozapine-N-oxide), providing selective and remote control of neural activity with a high degree of spatial resolution (Dobrzanski & Kossut, 2017; Gomez et al., 2017). This strategy was successfully applied in sleep research in combination with the TetTag approach by the group of Zhang (Zhang et al., 2015). In this study, the researchers capitalized on the TetTag pharmacogenetics in mice to functionally mark neurons activated in the preoptic hypothalamus during drug-induced sedation or recovery sleep. The tagged ensembles were then selectively reactivated using the hM_3D_q receptor and its ligand CNO, leading to the recapitulation of both NREM sleep and the typical accompanying drop in body temperature. Noticeably, in this study is proved that the TetTag strategy can be effectively combined with other novel approaches and applied to investigate the biochemical mechanisms regulating sleep patterns. The second strategy above mentioned, the optogenetics, relies on the artificially-driven expression of opsins, such as Channelrhodopsin (ChR2), or Halorhodopsine (HR), light-sensitive ion channels capable to change the cell membrane voltage and altering the electric state of the neuron upon illumination (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005; X. Liu et al., 2012). Control of the neuronal activity is restricted to genetically modified cells and firing stimulation or silencing can be performed in a spatiotemporal-specific fashion by directly applying light pulses. The possibility to apply the optogenetics in vivo allows not only to analyse the molecular and physiological effects of the artificial activation or silencing of particular ensembles, but also to observe the response of the behaviour in freely-moving animals. The RAM system discussed in this study is just an example of use of this potent approach, which has already emerged as extremely powerful and versatile tool and is constantly in improvement.

In this landscape, the TetTag technology still represents a useful method for the investigation of neuronal circuitry, especially to study reactivation of ensembles

following repetitive stimuli or paradigms as done so far. However, the use of different and complementary approaches could constitute the better strategy when the aim is to achieve a deeper knowledge on the biochemical and molecular mechanisms underlying a specific behaviour or physiological phenomenon.

No clear results emerged by comparing different vigilance states with the TetTag system

The results of the experiment conducted by labelling cortical neurons in different vigilance states displayed high levels of variability and revealed to be hardly interpretable. As previously mentioned, it is known that cortical neuronal firing increases during the wake state and sustained wakefulness (i.e. sleep deprivation), while during sleep the firing rates decreased progressively (Vyazovskiy & Faraguna, 2015; Rodriguez et al., 2016), visible as delta waves or SWA. Thus, the expected was a significative major number of labelled neurons in the wakefulness and especially in the sleep-deprivation conditions, in contrast, a low rate of tagging was presumed in the brain tagged during the natural sleep phase. However, the data obtained did not follow these patterns. A possible explanation for the dramatically low percentage of TetTagged neurons in the awake animals compared to the sleep and sleep deprived conditions may be the insufficient number of biological and technical replicates to obtain relevant results. Furthermore, the possibility that the occurrence of REM sleep, during which it has been observed an increased neocortical firing (Watson, Levenstein, Greene, Gelinas, & Buzsáki, 2016), could influence the results cannot be excluded. EEG recording may be performed in the future on these mice in order to monitor electrical activity in the brain during sleep and analyse the effects on neuronal labelling. Another possibility to consider is that the majority of the active cells were located in cortical areas other than the selected one for the quantification. Moreover, we cannot exclude in absolute terms that an accidental event prevented mice to fall asleep, or, more in general, that during the 6 hr-time window mice behaved differently from the assumed. Consequently, it was not possible to analyse deeply the results in order to compare potential differences in the cortical firing activity among the three vigilance states. Therefore, it was decided to proceed with additional experiments to obtain reliable results.

The RAM system can capture active neurons after few hours from antibiotic removal

The RAM system was developed as a tool to overcome the limitations of the previous IEG-based systems. In fact, this technology combines neural activity-driven production of protein markers with optogenetics, conferring both the ability of visualizing and control of neurons activated in response to a stimulus.
From the results of this study, the RAM technology seems to be able to capture active neuronal ensembles after only 4 hr from Doxycycline removal. Therefore, only few hours may be required for the clearing of the antibiotic from the brain and

the expression of the reporter genes. Nonetheless, since the statistical significance between the 4 hr off-Dox group and the negative control was present but not high, we speculated that a duration of 10 hr of Dox withdrawal would likely give more reproducible results under different experimental designs, in agreement with the previous results with the TetTag system. Another clear outcome shared with the method above discussed is the minimal but clearly present background expression of the reporter genes even in the presence of the antibiotic. According to the literature, this leakiness is an unavoidable drawback when using the tetracycline inducible systems (Zhou, Vink, Klaver, Berkhout, & Das, 2006; Loew, Heinz, Hampf, Bujard, & Gossen, 2010; Dogbevia, Roßmanith, Sprengel, & Hasan, 2016).
In spite of the problem of background labelling, the experiments performed in this study revealed also several remarkable features of the RAM system. First, the strong induction of the effector gene expression achieved by the improved activity-dependent P_{RAM} promoter, resulting in a clear and robust ensemble labelling. The presence of the EYFP in the whole somatodendritic compartment allowed the probing of the neuronal projections across the cortical layers and the hippocampal substructures. The intensity of labelling was higher and more robust than the one observed for the TetTag system, permitting a clear visualization of the whole dendritic arborization in the positive cells of all the experimental groups. Remarkably, this expression of the opsins in the dendrites may open to the perspective of anatomically-targeted optogenetic manipulation of RAM marked neurons, increasing the range of possible biological questions that could be addressed with this technique.
Importantly, the percentage of RAM labelling observed in this study is consistent with the data collected by the group of Lin, who developed this approach: contextual fear conditioning resulted in the RAM labelling of 11.4% of infected CA3 pyramidal neurons and 4.4% of infected DG granule cells, percentages that are in the same range of that observed in this study (between 5.82% and 6.97%) for the CA1. Therefore, the outcomes of this study represent an additional validation of this neuronal marking approach.
Compared with the other method analysed here, the RAM system showcases a higher sensitivity of labelling active ensembles of neurons, capturing more than 30% of recently activated neurons. Despite these relative percentages constitutes only approximate estimations, they can be read as an evidence of the better tagging performance of this technique compared with the TetTag approach.
To further discuss the main strengths of the RAM system highlighted by our experiments, the use of a single adeno-associated virus (AAV) with all the components required for the neuronal marking was considered extremely straightforward. With this strategy, the transgenes can be targeted specifically in theoretically any region of the brain through stereotactic injection, overcoming the issue of the variable efficiency across the brain previously encountered with the TetTag technology, and in general with the transgenic lines. Additionally, the viral delivery would permit to improve the specificity of the genetic modifications, since

only neurons active in one brain region would be tagged. Furthermore, the efforts in terms of time were incomparable: the sufficient number of bi-transgenic mice to perform the experiments was obtained after approximately six months of breeding and crossing of the two transgenic mouse lines. In contrast, the set of mice used for the experiment with the RAM system was created in few weeks, including two to four days for the stereotactic injection and at least three weeks for the integration of the viral construct in the mouse genome. Finally, common wildtype mice can be used without requirement of multiple transgenic mouse lines.

No significant difference in the cortical neuronal marking of sleep or wake condition was captured with the RAM system

We could not detect significant differences between the conditions "awake" and "sleep" in terms of number, type and location of the cortical neurons activated during the antibiotic withdrawal. The scarce amount of data collected for the experiment or a variable diffusion of the AAV in the mouse brain may constitute possible reasons for this finding. In addition, potential inaccuracies occurred during the selection of the region for the cell quantification cannot be formally ruled out. As a consequence, the data available are not sufficient to further discuss this experiment and to elaborate conclusions.

In this study, considerable variability was observed across the mice in terms of brain region and area showing EYFP expressing cells. This fact was attributed to unintentional differences in the dorsal-ventral coordinates, causing a higher number of marked neurons in the first layers of the cortex in some cases, while in other brains were observed more RAM labelled cells in the deep cortex and in the hippocampus. Clearly, the ability of the experimenter in targeting the region of interest and injecting the optimized virus titer is critical for the success of the experiment. Likely, a more efficient and standardized stereotactic injection technique would have generated less variability in the results, allowing proper quantifications and reliable comparisons between the different experimental groups.
From the experiments performed here, another difference emerges between the two tools that is worthy of attention. While the TetTag system was specifically designed for a permanent neuronal tagging through the presence of the tetracycline-insensitive for of transactivator (tTA*), the robust marking achieved with the RAM approach has reduced persistence after block of the tagging by Dox re-administration. Specifically, after 2 weeks under antibiotic, Lin and colleagues observed the decay of the RAM marking. Therefore, this tool may not be appropriate the neuronal activity underlying two events separated in time by more than 8-10 days.
Two further considerations should be comprised in the discussion of the data obtained. Firstly, the results of the time course experiments are highly dependent

on the concentration of Dox used. Indeed, the antibiotic concentration in the tissues at the last administration before the withdrawal is a critical variable when using drug-based tools. Higher doses of antibiotic will require more time to be metabolized, with a delayed activation of the neuronal tagging compared to the time recorded in this study. Oppositely, lower doses than the used in our procedure may decrease the time needed for the activation, with a consequent potential rise in background expression of the reporter genes even under antibiotic administration. Moreover, as all the drug-based approaches, the TetTag and RAM systems are exposed to the potential collateral physiological effects of the chemical used. Doxycycline has been extensively used in genetic research with the tetracycline-dependent system primarily and significant consequences and interactions with other physiological and metabolic processes were never observed in rodents. Nevertheless, a recent study suggests that Doxycycline may impact on memory acquisition in mammals (Bach, Tzovara, & Vunder, 2018), bringing into question the efficiency that these approaches have had so far in the learning and memory research. However, these results are still preliminary and further investigations and evaluations are needed.

A further critical parameter to discuss concerns the processing and quantifications of the biological samples performed in the procedure of this study. The percentages of the reporter genes-expressing cells were calculated by normalizing the number of positive neurons on the number of DAPI positive cells. DAPI is a widely used counterstain for nuclei of all the types of cells; thus, not exclusively neurons. It is well known that glial cells represent a conspicuous component of the cellular population of the Central Nervous System, comprising prevalently astrocytes, oligodendrocytes and microglial cells (Dimou & Götz, 2014). Moreover, the possibility that a bias was introduced in the estimation of the total and relative numbers of labelled neurons cannot be ruled out. To achieve higher precision in the quantification of the neuronal tagging, markers specific for neuronal cells, such as NeuN or MAP2, should be considered more appropriate.

Overall, the experiments conducted in this study using these two approaches for the tagging of recently-activated neurons brought to light strengths and weak aspects that are shared or complement one another. On one side, the TetTag system constitutes a tool more suitable for long-lasting neuronal tagging, with high activation thresholds but rapid and high expression of the transgene. The RAM system, on the other hand, represents a useful strategy for both visualization and control of neuronal firing, characterized by high sensitivity and specificity but short persistence of the gene expression after readministration of the antibiotic. In Table 1 the main features of the two activity-dependent tagging methods are summarized, for an immediate evaluation of their advantages and limitations.

Table 1. Comparison of the two tested activity-dependent methods for neuronal tagging.

Method	TetTag technology	RAM system
Utilized IEG	*FOS*	*FOS*
Mouse line	Bi-transgenic mouse containing both the *Fos-tTA:shEGFP* cassette and the *tetO-tTA*:tau-LacZ* construct	Wildtype
Vehicle used to capture activated cells	Doxycycline administration, removal, then readministration	Doxycycline administration and removal
Effector genes	tTA-shEGFP, tetO-tau-LacX, tetO-tTA*	D2TTA, TRE promoter-ChR2-EYFP
Time for the activation	Between 4 and 6 hr	Approximately 6 hr
Duration of the capturing window	From removal of Dox until a few hours after readministration of Dox	From removal of Dox after a few hours after readinistration of Dox
Advantages	Rapid and high expression of the transgene Persistent labeling through the feedback loop	Modular design Rapid and high expression of the transgene Tagging and manipulation High sensitivity and specificity Viral delivery in potentially all the brain regions
Limitations	Requirement of breeding and crossing to obtain bi-transgenic mice High background expression Low sensitivity Variable efficiency across the brain regions (no labeling in the SCN) Partial recapitulation of endogenous Fos expression Only tagging, no subsequent manipulation of tagged neurons	Transient expression of the reporter genes (absence of feedback loop) Background expression (low)
References	Reijmers et al., 2007 Matsuo et al., 2008 Liu et al., 2012 Gamer et al., 2012 Ramirez et al., 2013 Redondo et al., 2014 Cowansage et al., 2014 Ramirez et al., 2015	Lin et al., 2016

Conclusions and future perspectives

In this study, two IEG-based approaches for activity-dependent neuronal tagging have been tested in order to elucidate the time course of gene activation in the mammalian brain. We found that for both the TetTag and the RAM systems the Dox clearing from the cortical regions and the reporter genes expression in firing neurons require less time than previously assumed, allowing genetic access to neurons that are active during a time window of less than 10 hr. In addition, some brain regions, including the SCN, resulted less prone to the genetic tagging through the TetTag pharmacogenetics for still undefined reasons. These findings highlight the strengths and also the challenges and potential pitfalls in the use of transgenic mice. Indeed, we clearly show here that certain transgenic lines are more suitable for the study of specific neuronal populations and not others.

Remarkably, the RAM system emerged as an efficient and versatile tool to obtain a robust activity-driven neuronal marking, characterized by high temporal and spatial resolution, improved sensitivity and versatility.

These outcomes in the future may certainly benefit the investigation of neuronal circuitry underlying learning and memory, that represent the traditional field of application of these techniques. A reduced permissive time window, in fact, could contribute to an increase in temporal precision of these drug-based tools and a reduction in the amount of non-relevant neuronal labelling.

Our attempts to use the two technologies to investigate the local sleep component did not lead to significant results; notwithstanding, the features of the TetTag and the RAM systems emerged here allow to rethink their potential utility in the sleep research. Concerning the TetTag system, we suggest the possibility to capitalize on the reduced time window of potential tagging to identify possible changes in cortical neuronal activity between different circadian phases. Ideally, this technique may also be helpful to unravel the circuitry underlying the local component of SWA.

The potential of the RAM technology is possibly even wider due to its capability of expressing the optogenetic proteins in activity-driven manner. After allowing the robust neuronal marking in the targeted area during the desired time point, it would be possible to stimulate exclusively the labelled neurons in a second time, when they are not normally active, observing the physiological and behavioural reactions. For instance, by artificially stimulating in the natural wake period specific SCN neuronal populations previously activated and tagged during the sleep phase, would it be sufficient to induce changes in the sleep timing? Would the mouse sleep when it usually is awake? Vice versa, would the optogenetic stimulation of selective wake neurons during sleep lead to a prolong wakefulness? With what fidelity does the artificial stimulation of the clock neurons in one brain region recapitulate brain activity pattern produced by the natural firing itself? The list of possible interesting questions that can be addressed with these approaches may proceed.

Ideally, we would like to investigate the neuronal function and activity pattern with a unique powerful and versatile approach, which is able to label, trace, record and manipulated neurons in different brain regions at specific timing. Nevertheless, different genetically encoded technologies usually present particular features optimized to address specific questions, which is another clear outcome emerged from this study. For this reason, the combination of several complementary tools was frequently revealed as the best strategy to dissect relevant neural circuits, and, from a wider perspective, to study relevant phenomena. Here, only two of the existing possible methods capable of targeting the desired changes to relevant ensembles have been discussed. The already mentioned DREADD approach, for the artificial reactivation of neurons, together with other recently developed methods for time and space-specific alteration of neural ensembles, such as the Targeted Recombination in Active Populations or TRAP (Guenthner et al., 2013) the CANE systems, constitute approaches whose potential has not be fully discovered yet. In addition, newer and even more striking methods will probably enter in the neurobiology scenario in the next future.

These tools, used in combination or complementarity, constitute an enormous potential to dissect the intricate circuitry regulating complex and still enigmatic phenomena such sleep-wake cycles, providing the opportunity to examine deeply and from different perspectives the intricate responsible networks. In the science of sleep, these strategies will make possible to address the hypothesis that specific populations of clock neurons, active at different times of the day, acutely drive different behaviours. If true, this hypothesis would explain how a biological clock of twenty-four-hour length can in fact determine human's health and performance, characterize individuals as "owls" or "larks" and separately anticipate dawn and dusk.

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