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GENETIC TECHNIQUES AND CIRCUIT ANALYSIS

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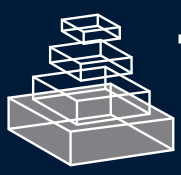

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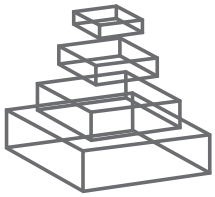

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GENETIC TECHNIQUES AND CIRCUIT ANALYSIS

Hosted By
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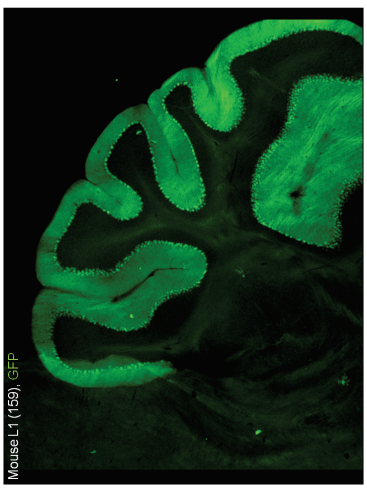

How new genetic techniques are revolutionizing the study of neural circuits for both invertebrate and vertebrate systems.

Understanding how specific types of neurons contribute to behaviour is an ambitious goal. For invertebrate model systems (e.g. worms, flies), neurons in the brain are often too small to be studied routinely by electrophysiological approaches. For vertebrates, large ensembles of cells have to be studied, and these cells are often distributed over considerable volumes e.g. GABAergic interneurons in neocortex. Cell type-selective manipulations may be a way forward for treating illness. Before such aims can be realized, or even appreciated as feasible, the brain circuitry in experimental animals has to be known by both establishing the connections between cell types and reversibly manipulating the activity of the cells subtype-selectively. Methods that have all appeared in just the last couple of years to tackle this include: retrograde tracing of circuitry using viruses, ligand-receptor combinations that make subtypes of neurons uniquely sensitive to a drug (e.g. zolpidem, allatostatin, serotonin ligands or ivermectin), and light-activated channels and pumps for stimulation and inhibition. This collection of methods promises much, forming the new subdisciplines of "pharmacogenetics" and "opticogenetics". These methods are revolutionizing the study of brain circuitry for both invertebrates and vertebrate systems.
Image: "Pharmacogenetics: cerebellar Purkinje cells engineered to be uniquely sensitive to zolpidem, a GABA-A receptor modulator (see the article by Wisden et al., in this book)"

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EDITORIAL
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Genetic techniques and circuit analysis

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Reaching an understanding of how neuronal circuits work and what they compute is a fundamental aim of neuroscience, perhaps even the most fundamental. We have to both establish the connections between cell types and reversibly manipulate their activity cell-type-selectively. Such work sounds in principle straight-forward, but it has been difficult to achieve. This has now all changed. There has been a quite remarkable development of genetic techniques published in the last years, so that the topic of "Genetic techniques and circuit analysis" covered by the articles in this Special Issue is truly flourishing. The extremely easy applicability of the channelrhodopsin-2 system (ChR2) in diverse animals and circuit settings has been a phenomenal breakthrough and captured the imagination of the neuroscience community (see, for example, Adamantidis et al., 2009; Han et al., 2009). A major advantage of ChR2 is that precise patterns of activation can be delivered cell-type selectively, to probe, for example, the requirements for rhythm generation in a network. This has been termed "informational lesioning" (Han et al., 2009). Yet in spite of its undoubted importance and significance, ChR2 is just one of many possibilities that could be deployed. Indeed, one of the most enjoyable aspects of researching brain circuits is that so many methods and approaches exist. There is no "one size fits all" solution for the challenge of selecting particular genetic technique(s). In this collection of articles for the themed Special Issue, the broad nature of this area is apparent. The articles cover cell type-selective manipulations of neuronal activity using light (Alilain and Silver, 2009; Adamantidis et al., 2009; Han et al., 2009), unique ligand-receptor combinations (Nichols and Roth, 2009; Wisden et al., 2009), other manipulations of ion channels to control neuronal activity (Hodge, 2009; Holford et al., 2009; White and Peabody, 2009) or, in *Drosophila*, the deployment of reversible blockade of neurotransmission using the *shibire*^{ts} system or tetanus toxin (also in mice) (Kasuya et al., 2009; Tessier and Broadie, 2009; White and Peabody, 2009). Indeed, in *Drosophila*, the ease of applicability of genetic screens for behavioural phenotypes means that combined enhancer trapping and manipulation of neuronal activity is allowing advances in establishing what, or how, particular types of neurons contribute to behaviour, a process of discovery termed by White and Peabody as "neurotrapping". Reijmers and Mayford (2009) provide an elegant extension of circuit mapping using genetic techniques; here, the TetTag mouse line permits exploration of how activity in small ensembles of neurons contributes to memory formation. Finally, while we are considerably far from any therapies based on the methods described in this Special Issue, ChR2 activation with light has succeeded in partially repairing breathing defects produced by spinal cord lesions in experimental animals (Alilain and Silver, 2009).

Although there is much emphasis on manipulating reversibly the activity of particular cell types in circuits, other techniques covered in this Special Issue remain vital. The 2008 Nobel Prize in Chemistry went to O. Shimomura, M. Chalfie and R.Y. Tsien for the discovery and development of green fluorescent protein (GFP). GFP and it variants (e.g. YFP) are now such "household" items in our molecular toolboxes that it is difficult to imagine doing any molecular biology research without them. In our Special Issue, Bregestovski et al. (2009) appraise YFP-based Cl^- indicators for measuring Cl^- concentrations; Perron et al. (2009) review voltage-sensitive fluorescent proteins (VSFPs). All of these indicators can be used in specific cell types or cellular compartments to reveal the ionic milieu of *in vivo* circuitry. Another recent Nobel Prize (2007) in Physiology or Medicine went to M.R. Capecchi, M.J. Evans and O. Smithies for their development, using mouse embryonic stem cells, of a general method for manipulating the mouse genome. The technology has been so successful that knockouts are now high throughput; indeed, the Knockout Mouse Project (KOMP) plans to knockout every mouse gene, and make conditional alleles for many genes. Resources such as GENSAT will soon offer Cre driver mouse lines for region-selective and cell-type-selective knockouts. There is, however, a feeling among some that knockouts, because of compensations that emerge as a consequence of losing the protein, are not always worth making. In our view this is wrong. Loss of function gene mutations, whether spontaneous (i.e. classical genetics) or engineered, have given, and continue to give, essential information about the roles of a protein in the organism (see Heldt and Ressler, 2009; Tessier and Broadie, 2009; Weber et al., 2009; Wisden et al., 2009).

We warmly thank all the Contributors for their articles.

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Optogenetic deconstruction of sleep–wake circuitry in the brain

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How does the brain regulate the sleep–wake cycle? What are the temporal codes of sleep- and wake-promoting neural circuits? How do these circuits interact with each other across the light/dark cycle? Over the past few decades, many studies from a variety of disciplines have made substantial progress in answering these fundamental questions. For example, neurobiologists have identified multiple, redundant wake-promoting circuits in the brainstem, hypothalamus, and basal forebrain. Sleep-promoting circuits have been found in the preoptic area and hypothalamus. One of the greatest challenges in recent years has been to selectively record and manipulate these sleep–wake centers *in vivo* with high spatial and temporal resolution. Recent developments in microbial opsin-based neuromodulation tools, collectively referred to as "optogenetics," have provided a novel method to demonstrate causal links between neural activity and specific behaviors. Here, we propose to use optogenetics as a fundamental tool to probe the necessity, sufficiency, and connectivity of defined neural circuits in the regulation of sleep and wakefulness.

Keywords: hypothalamus, sleep, wakefulness, optogenetics, hypocretins/orexins

INTRODUCTION

Sleep is commonly defined as "*a rapidly reversible state of (behavioral) immobility and greatly reduced sensory responsiveness to environmental stimuli*" (Siegel, 2008). Although the parameters of the sleep–wake cycle vary within and between species, all animals show rest-activity patterns or sleep-like states. In addition to mammalian, reptilian, and avian species, recent studies report the existence of "sleep-like" states in lower organisms including the fish, fly, and worm (Shaw et al., 2000; Yokogawa et al., 2007; Allada and Siegel, 2008; Raizen et al., 2008; Zimmerman et al., 2008).

In mammals, there are three states of vigilance: wakefulness, slow-wave sleep (also called non-rapid eye movement "NREM" sleep), and rapid eye movement (REM) sleep, defined by electroencephalographic (EEG) and electromyographic criteria. Wakefulness consists of fast EEG oscillations (5–12 Hz) of low amplitude with frequent and sustained motor activity. NREM sleep is characterized by slow oscillations (1–4 Hz) of high amplitude and can be divided into four stages in human (two in rats, one in mice), corresponding to increasing depth of sleep. REM sleep is characterized by relatively fast oscillations (6–12 Hz) of low amplitude and persistent muscle atonia. REM sleep has also been coined "paradoxical sleep" due to the simultaneous occurrence of cortical activity similar to wakefulness and a lack of a muscle tone (Jouvet, 1965).

Sleep naturally follows a period of wakefulness. Prolonging the wake period increases the propensity to sleep, revealing a homeostatically regulated process (Borbely, 1982, 2001). In addition to homeostatic drive, sleep is regulated by circadian rhythms, which determine the timing of sleep, as well as other physiological variables including core body temperature and production of hormones (such as cortisol or melatonin). Thus, sleep homeostasis and circadian rhythms both influence the ultradian processes that governs the architecture of the sleep–wake cycle (Borbely, 2001; Achermann and Borbely, 2003; Saper et al., 2005). These homeostatic and circadian factors govern a complex, yet partially defined, balance between sub-cortical excitatory and inhibitory neural populations in the brain (Pace-Schott and Hobson, 2002; Fort et al., 2009). Although these wake- and sleep-active neurons are distributed throughout the brain, most of the wake-promoting neurons are found in restricted brain areas including the brainstem, the hypothalamus and the basal forebrain. Interestingly, relatively few sleep-active nuclei have been discovered, mostly restricted to the lateral hypothalamus and preoptic area.

Brain arousal and sleep centers contain overlapping neuronal and non-neuronal cell types of different chemical composition and electrical properties (Gerashchenko and Shiromani, 2004), that may influence the sleep–wake cycle and sleep-dependent processes (Halassa et al., 2009). This heterogeneity has severely hampered our ability to record from and manipulate the neural dynamics of sleep and wake circuitry. For example, using a microelectrode to record or stimulate neural activity in brain regions known to promote sleep or wakefulness is incredibly difficult or even impossible given the 3D architecture of some neural populations spread out over millimeters, even in small rodents. Furthermore, the presence of multiple cell types that overlap within the same volume of tissue prevents an electrode from selectively stimulating specific cells of interest. New technology is clearly needed to establish causal, functional relationships between identified sleep- and wake-promoting neural circuits and how these circuits interact to govern the sleep/wake cycle.

We have recently used a new technology called optogenetics to selectively stimulate or inhibit specific neural circuits in the brain. Optogenetic tools combine the temporal precision of a microelectrode with the spatial precision of a genetically encodable probe, allowing the stimulation or inhibition of complete populations of

specific neuronal subtypes. This technology has allowed us to gain insight into a population of wake-promoting neurons, hypocretin neurons, and has the potential to answer multiple questions about many other neural populations, as well as the sleep/wake cycle as a whole.

Here, we review the neural substrates of sleep and wakefulness in small and large mammals. These neural circuits have been identified and studied throughout the past few decades using a combination of histological, pharmacological, genetic, and *in vitro* and *in vivo* electrophysiology techniques. We focus on the use of these tools to study the hypocretin system, demonstrating the strengths and limitations of traditional electrophysiological and transgenic technologies. Then, we describe the use of optogenetic technology to probe the function of the hypocretin system in sleep regulation and how it has allowed us to make progress in our understanding of the role of these neurons *in vivo*. Finally, we propose to use optogenetics as a fundamental tool to investigate the necessity, sufficiency, and connectivity of defined neural circuits in the regulation of sleep and wakefulness.

SLEEP–WAKE CIRCUITS OF THE BRAIN

The neural substrates of sleep-to-wake transitions are governed by distinct neural populations in the brain. Activity in these nuclei is correlated with wakefulness: not only does their activity increase when an animal is awake compared to asleep, but this activity also increases during states of enhanced arousal, such as moments of high alertness or stress. These arousal systems include:

- The noradrenergic locus coeruleus (LC), located in the pontine brainstem (Aston-Jones and Bloom, 1981)
- The neuropeptide S cells, located in the pontine brainstem (Xu et al., 2004)
- The serotinergic raphe neurons, located in the medial brainstem (McGinty and Harper, 1976; Guzman-Marin et al., 2000; Sakai and Crochet, 2000)
- The dopaminergic neurons in the ventral mesencephalic midbrain (Dahan et al., 2007) and the brainstem (Lu et al., 2006)
- The histaminergic tuberomammillary nucleus, located in the posterior hypothalamus (Parmentier et al., 2002; Takahashi et al., 2006)
- The hypocretin neurons, located in the lateral hypothalamus (Peyron et al., 2000; Lee et al., 2005; Mileykovskiy et al., 2005)
- The cholinergic neurons, located in the basal forebrain (Hassani et al., 2009a) and pontine brainstem.

These arousal centers each send widespread ascending projections to the cerebral cortex, stimulating cortical desynchronization characterized by high frequency gamma and low frequency theta rhythmic activity (Steriade et al., 1993).

In addition, these systems have descending projections that enhance or modulate muscle tone, sensory-motor responsiveness, and physiological activity. These multiple arousal systems show obvious redundancy, since triple lesions of the main arousal centers in the brain (basal forebrain, tuberomammillary nucleus, LC) have no major effect of the architecture of the sleep–wake cycle (Blanco-Centurion et al., 2007), yet they can be differentiated based on their unique activity patterns throughout the sleep–wake cycle, as well as their contribution to other complex behaviors (such as food and drug seeking behaviors, attention, decision making, and the response to stress). During sleep, they are under an inhibitory tone from sleep-active neurons.

In contrast to the multiple, redundant arousal systems, there are relatively few identified sleep-promoting neural populations. These nuclei were identified on the basis of immunoreactivity to the immediate early gene c-Fos – a biomarker of neuronal activity –, single unit recordings, neurotoxic lesions, neurochemical, and thermal stimulation studies. Sleep-promoting nuclei include: the ventrolateral preoptic area (VLPO), located in the preoptic area and the median preoptic nucleus (MnPN), located in the preoptic area (Alam et al., 1995; Gallopin et al., 2000; Lu et al., 2002; McGinty et al., 2004).

Both the VLPO and MnPN neuronal populations express GABA, and send descending projections to arousal-promoting cell groups. Thus, they may inhibit noradrenergic, serotonergic, cholinergic, histaminergic and hypocretinergic neurons, as suggested by the "reciprocal inhibitory" model of the sleep–wake switch (Pace-Schott and Hobson, 2002; McGinty and Szymusiak, 2003) as recently suggested by Suntsova et al. (2007). The role of these neurons in promoting or maintaining sleep is not well understood and will require future investigation.

Brain structures responsible for REM sleep onset and maintenance were first identified in the brainstem in the pericoeruleus region in cats using a physical lesion approach (Jouvet and Michel, 1959; Fort et al., 2009) and many neurons in this brain area are selectively active during REM sleep (Sakai et al., 2001). According to the "reciprocal-interaction model", noradrenergic and serotonergic, histaminergic and hypocretin (Hcrt) neurons cease firing during REM sleep (Aston-Jones and Bloom, 1981; Lee et al., 2005; Mileykovskiy et al., 2005; Takahashi et al., 2006). Finally, pericoeruleus cholinergic and GABAergic neurons located in the dorsal paragigantocellular reticular nucleus (DPGi) and the ventrolateral periaqueductal gray have recently been identified as additional neuronal populations regulating the onset and maintenance of REM sleep events (Jones, 2004); Pace-Schott, 2002 #17; Fort, 2009 #3122.

Recent studies identified additional sleep modulating neuronal populations in the brain (Verret et al., 2003; Gerashchenko et al., 2008; Hassani et al., 2009b). One of those includes the hypothalamic neurons expressing the melanin-concentrating hormone (MCH) that are active mainly during REM sleep (Verret et al., 2003; Hassani et al., 2009b). However, contrasting results from experimental approaches using different techniques (immunodetection of c-Fos, pharmacology, knockout mouse models) are found in the literature (Verret et al., 2003; Modirrousta et al., 2005; Adamantidis et al., 2008; Willie et al., 2008).

In addition to classical neurotransmitters and neuropeptides described above, numerous endogenous factors modulate sleep, including prostaglandin D2 (Ueno et al., 1982; Scammell et al., 1998), adenosine (Virus et al., 1983; Porkka-Heiskanen et al., 1997), growth hormone-releasing hormone and interleukins (Krueger et al., 1999; Kushikata et al., 1999), all of which also have other physiological functions.

Now that we have examined the key components of the neural circuitry modulating sleep and wake states, we focus on the hypocretin system as an example of how sleep nuclei are studied and manipulated during experiments.

THE HYPOCRETIN SYSTEM AS A MODULATOR OF THE SLEEP–WAKE CYCLE

In 1998, two groups independently reported a novel pair of neuropeptides called the Hcrts (also known as "orexins") exclusively expressed in a population of glutamatergic neurons in the lateral hypothalamus. These neuropeptides are cleaved from the same genetic precursor (preprohypocretin) (de Lecea et al., 1998; Sakurai et al., 1998). There are two hypocretin receptors (Hcrtrs) distributed throughout multiple brain areas that match the afferent fiber projections from Hcrt neurons. The projection pattern of afferent projections and receptor expression suggests that the Hcrt system is involved in multiple brain function (Sakurai, 2007).

Hypocretin neurons are activated by neurotransmitters that promote arousal including glutamate (Li et al., 2002; Yamanaka et al., 2003), CRF (Winsky-Sommerer et al., 2004), ATP (Wollmann et al., 2005), noradrenaline and charbachol (acethycholine agonist) (Bayer et al., 2005), A subpopulation of Hcrt neurons are activated by Ach (Sakurai et al., 2005). Importantly, sleep-promoting neurotransmitters inhibit Hcrt neurons, including GABA (through $GABA_{a,b}$; Li et al., 2002; Yamanaka et al., 2003; Xie et al., 2006), and adenosine (A1) (Liu and Gao, 2007).

In the 10 years since their discovery, much has been learned about the Hcrt system by correlating neural activity with behavioral output, as well as gain-of-function and loss-of-function studies. Experiments that manipulate Hcrt function typically involve pharmacology, in which an agonist or antagonist (or Hcrt peptides themselves) are injected into the ventricular system or discrete brain regions. Alternatively, many studies employ genetic manipulation of the Hcrt system using transgenic or knockout technologies in the mouse. We briefly summarize these studies below.

CORRELATION BETWEEN HCRT NEURAL ACTIVITIES AND BEHAVIORAL STATES

Recently, technically challenging *in vivo* single unit recordings of identified Hcrt neurons confirmed their high discharge activity during arousal, including behavior accompanied with a strong locomotor activity (Lee et al., 2005; Mileykovskiy et al., 2005; Takahashi et al., 2008). In contrast to their oscillatory activity in brain slices (Eggermann et al., 2003), Hcrt neurons are completely silent during quiet wakefulness, NREM and REM sleep and are reactivated during REM sleep-to-wake transitions (Lee et al., 2005; Mileykovskiy et al., 2005).

GAIN-OF-FUNCTION STUDIES

Many gain-of-function studies have been applied to the lateral hypothalamus as a whole. In addition to promoting food intake (Anand and Brobeck, 1951; Bernardis and Bellinger, 1996), electrical stimulation of lateral hypothalamic region decreased REM sleep duration in rats and cats (Suntsova et al., 2000), possibly through a Hcrt-mediated inhibition of neurons in the oral nucleus of the pons (Dergacheva et al., 2005; Nunez et al., 2006), which is an important structure in the generation and maintenance of REM sleep. Disinhibition of LH cells by GABAA antagonists (bicuculline or gabazine) injections into the LH area induced a continuous quiet waking state associated with a robust muscle tone in head-restrained rats (Goutagny et al., 2005), partly via activation of Hcrt neurons (Lu et al., 2007). However, none of these approaches have selectively targeted the Hcrt system.

Other studies used icv infusion of hcrt peptides or hcrt agonists (Akanmu and Honda, 2005), as well as local injection of the peptide in the LC (Bourgin et al., 2000), LH (Methippara et al., 2000), laterodorsal tegmental nucleus (Xi et al., 2001), basal forebrain structures (Espana et al., 2001), in rodents and cats enhanced wakefulness and locomotor activity which was accompanied by a marked reduction in REM and non-REM sleep. Local Hcrt1 injections in cholinergic nuclei of the pons (nucleus pontis oralis) have promoted wakefulness, suppressed SWS and "defacilitated" REM sleep, whereas it directly inhibited REM sleep when injected in the ventral part of the NPO vs RPO (Xi et al., 2002; Moreno-Balandran et al., 2008; Watson et al., 2008). Interestingly, Hcrt administration can reverse behavioral attacks in narcoleptic dogs (see below) (John et al., 2000; Fujiki et al., 2003). More recently, genetic disinhibition of Hcrt neurons using a selective GABAB receptor gene deletion only in Hcrt neurons in mice were found to induce severe fragmentation of sleep/wake states during both the light and dark periods, without showing an abnormality in total sleep time or signs of cataplexy (Matsuki et al., 2009).

LOSS-OF-FUNCTION STUDIES

Although electrical or chemical anatomical lesions of the LH (the "lateral hypothalamic syndrome") are not specific to the arousal-promoting Hcrt neurons, such lesions were reported to induced aphagia and adipsia for several days during which they showed a disorganized EEG characterized by rapid low voltage activity and high voltage low frequency waves (Danguir and Nicolaidis, 1980). Sleep increased gradually and normal amounts of both NREM and REM sleep was observed during "stage 4" of recovery, when rats eat and drink as normal.

Other lesional studies found that extensive LH lesions caused either insomnia (Trojniar et al., 1990; Jurkowlaniec et al., 1996) or transient hypersomnia (Denoyer et al., 1991) with disturbed hippocampal theta activity both during waking and paradoxical sleep (Jurkowlaniec et al., 1989) in rodents and cats. In human, patients with cataplexy (Schwartz et al., 1984), disrupted temporal patterning of the sleep–wake cycle (Cohen and Albers, 1991) and increased total sleep time (Eisensehr et al., 2003) have been reported secondary to a surgical lesion that involved the perichiasmal, the rostral hypothalamus, or bilateral posterior hypothalamus, respectively.

To selectively target Hcrt-responsive neurons, saporin-coupled Hcrt molecules were used to suppress Hcrt neurons *in vivo*. Saporin is a ribosome inactivating protein that kills target cells once internalized. Thus, the use of saporin toxin conjugated to the Hcrtr binding ligand, $Hcrt_2$, lesions Hcrtr-expressing neurons. This has the effect of inducing sleep when injected into the LH (Gerashchenko et al., 2001, 2003) independently of adenosine levels in the basal forebrain (Murillo-Rodriguez et al., 2008) and insomnia when injected in the VTA and substantia nigra (Gerashchenko et al., 2006). Unfortunately, saporin alone has toxic properties on Hcrt neurons themselves, thus constituting weak control conditions.

Hypocretin receptor antagonists have been used to target the Hcrt system with receptor selectivity. Blockade of Hcrt-2R is sufficient to initiate and prolong sleep and co-administration of Hcrt-1R with Hcrt-2R antagonists greatly attenuated the sleep-promoting effects of the Hcrt2R antagonist (Dugovic et al., 2009). Hcrt-R1 antagonist delays emergence from anesthesia, without changing

anesthetic induction (Kelz et al., 2008). Dual Hcrt receptor antagonists increased both non-REM and REM sleep in rats (Whitman et al., 2009), somnolence and increased surrogate markers of REM sleep in dogs, and electrophysiological signs of sleep in human (Brisbare-Roch et al., 2007). However, these antagonists differ in their affinities for HcrtR-1 and HcrtR-2. Additionally, they may, at very low binding concentrations, modulate other receptors when administered *in vivo*.

The most significant loss-of-function studies demonstrate that deficiency of the Hcrt system is linked to narcolepsy in humans (Peyron et al., 2000), dogs (Lin et al., 1999) and mice (Chemelli et al., 1999; Blumberg et al., 2007). Narcoleptic patients with cataplexy have non- or barely-detectable levels of Hcrt in the cerebro-spinal fluid, in addition to the absence of *preproHcrt* gene transcripts in the hypothalamus (Peyron et al., 2000). Doberman narcoleptic dogs bear a mutation in Hcrt-R2, and all genetically engineered rodents with either a deletion of the Hcrt (Chemelli et al., 1999; Hunsley et al., 2006; Blumberg et al., 2007), HcrtR-2 gene (Willie et al., 2003) or Hcrt cells (Beuckmann et al., 2004; Zhang et al., 2007b; Fujiki et al., 2009) present behavioral arrests that resemble cataplexy, the hallmark of narcolepsy. However, HcrtR-2 KO mice are less affected with cataplexy-like attacks of REM sleep compared to Hcrt KO mice that are more severely affected (Willie et al., 2003), suggesting that the altered REM sleep control in narcolepsy-cataplexy syndrome emerges from loss of signaling through both HcrtR-2-dependent and Hcrt-R2-independent pathways (Willie et al., 2003). Finally, alternative approaches to genetic technologies include the use of short interfering RNAs (siRNA) targeting prepro-orexin mRNA. Once injected into the rat LH, animals exhibited a transient increase in REM sleep (few days) compared to scrambled siRNA-treated animals (Chen et al., 2006).

Collectively, these studies support a role for the Hcrt system in "*lowering the arousal treshold*" (Sutcliffe and de Lecea, 2002), resulting in a facilitation of wakefulness when animal are asleep. Furthermore, the ability of Hcrts to modulate the reward system of the brain (Boutrel and de Lecea, 2008) also suggest that it could promote hyperarousal (defined as a transient, hyper-alertness state triggered by salient stress or reward) when the animal is awake (DiLeone et al., 2003; Winsky-Sommerer et al., 2004; Boutrel et al., 2005).

NOVEL METHODS OF MANIPULATING Hcrt NEURONS

Selectively stimulating or inhibiting Hcrt function represents the next step in understanding Hcrt cell function. Although the studies mentioned above provide substantial evidence for the role of Hcrt neurons in promoting wakefulness and arousal, the 3D architecture of the Hcrt field, as well as the heterogeniety of cell types found in the lateral hypothalamus, have imposed limitations using traditional microstimulation or pharmacological techniques to perform loss-of-function or gain-of function studies. Indeed, in addition to non-neuronal cells, lateral hypothalamic cells include neurons expressing MCH, CRF, TRH, substance P or Neurotensin (Gerashchenko and Shiromani, 2004). The most common way of inducing electrical activity in neurons for the past century has been injecting current through a microelectrode (**Figure 1A**). Although temporally precise, a microelectrode cannot distinguish between cell types in the stimulated area. Furthermore, it may inadvertently stimulate other, unintended regions that are adjacent to the electrode. Pharmacological techniques lack both spatial and temporal precision, as psychoactive substances can spread throughout the brain and require minutes to hours to clear the system (Bittencourt and Sawchenko, 2000) (**Figure 1A**). Modern biochemical techniques, such as the delivery of photosensitive "caged glutamate" to neurons, improve upon the spatial and temporal properties of pharmacological methods. However, restriction of the caged glutamate to specific neurons of interest cannot be guaranteed, and the time-course of photostimulation is on the order of seconds to minutes. It is possible to deliver genetically encodable probes to neurons that can be activated to depolarize discrete subsets of neurons. For example, specific targeting of a ligand-gated ion channel that is not normally expressed in the brain may help to stimulate neural activity by just adding its selective ligand. Unfortunately, this strategy has a poor temporal resolution, as endogenous ligand must be added and then eventually cleared from the local area of interest. Finally, the crudest method of silencing Hcrt neural activity in the brain has been to ablate these neurons, either physically with a sharp instrument, pharmacologically with a toxin, or genetically with a genetically encodable toxin. Of course, these non-reversible lesions cause a total loss of neural function, with no chance for recovery.

Recently, our lab has employed optogenetic technology (Deisseroth et al., 2006) to selectively manipulate the Hcrt system *in vivo* (**Figure 1B**). Optogenetics can be thought of as a perfect combination of an electrode, which has high temporal precision, with a genetically encodable probe, which has high spatial resolution. Genetic delivery of a light-sensitive ion channel called channelrhodopsin-2 (ChR2) (Nagel et al., 2003) into specific populations of neurons can be used to stimulate neural activity upon illumination in several animal species including rodents and non-human primates (Boyden et al., 2005; Li et al., 2005; Lima and Miesenbock, 2005; Nagel et al., 2005; Ishizuka et al., 2006; Schroll et al., 2006; Adamantidis et al., 2007; Arenkiel et al., 2007; Petreanu et al., 2007; Wang et al., 2007; Huber et al., 2008; Gradinaru et al., 2009; Han et al., 2009a; Tsai et al., 2009). This channel is naturally expressed in green algae and normally absent in animals. Because ChR2 is genetically encoded, specific promoter and enhancer elements can be used to express the channel in discrete populations of neurons in the brain. Then, ChR2-expressing neurons can be activated by delivering blue light using thin fiber-optic cables, implanted into the brain for long-term experiments over days or weeks (Adamantidis et al., 2007; Aravanis et al., 2007).

Alternatively, a separate light-sensitive protein, Halorhodopsin (NpHR), naturally expressed by archaebacteria and normally absent in animals, has been recently used to inhibit neural activity. NpHR is a chloride pump that hyperpolarizes neurons (and thus inhibits neural activity) upon stimulation with yellow light (Han and Boyden, 2007; Zhang et al., 2007a; Gradinaru et al., 2008; Han et al., 2009b). Importantly, ChR2 and NpHR both have a temporal resolution of milliseconds, allowing stimulation or silencing of single action potentials. Thus, optogenetic technology allows for temporally precise manipulation of specific populations of neurons in an awake, behaving animal.

In an attempt to better understand the role of Hcrt neurons on arousal, we used optogenetics to study the effect of Hcrt neuronal activation on SWS and REM sleep transitions to wakefulness. First, we genetically targeted the light-activated channel ChR2 to Hcrt

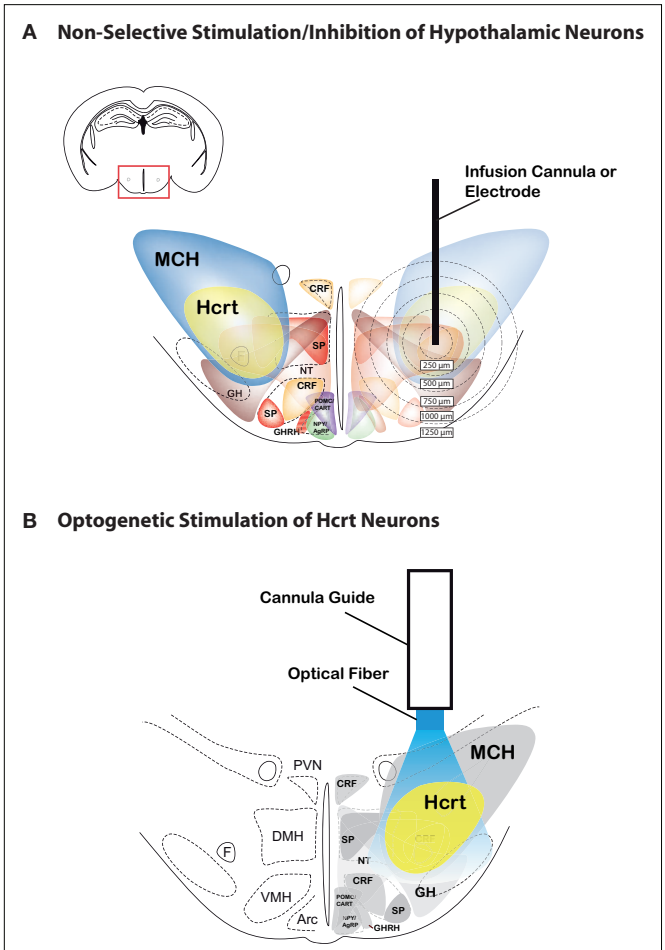

FIGURE 1 | Comparison between electrical/pharmacological activation or inhibition and optogenetic activation of Hcrt neurons in the lateral hypothalamus. 2D architecture of the Hcrt field showing the heterogeniety of cell types found in the lateral hypothalamus, including MCH, Hcrt, corticotropin-releasing factor (CRF), NPY/AgRP, POMC/CART, growth hormone-releasing hormone (GHRH), growth hormone (GH), neurotensin (NT), and substance P. Note that, in addition to these defined cell types, the hypothalamus contain glutamatergic and GABAergic neurons. For clarity, Nesfatin-1, thyrotropin-releasing hormone (TRH), vasopressin and oxytocin are not represented. **(A)** Limitations of traditional microstimulation or pharmacological techniques to perform loss-of-function or gain-of function studies include confounding effect on neuronal and non-neuronal cells surrounding the tarted cells. For instance, infusion of Hcrt peptide or non-peptide agonists or antagonists can spread up to 1000 µm away from the infusion site, therefore activating or inhibiting several neuronal populations in addition to the Hcrt neurons. Alternatively, temporally precise microelectrode stimulation cannot distinguish between cell types in the stimulated area, and thus may inadvertently stimulate other, unintended regions that are adjacent to the electrode. Concentric circles represent distance (in µm) from possible injection, stimulation or lesion site. **(B)** Optogenetic technology allow selective stimulation (using ChR2) or inhbition (using NpHR) of genetically targeted Hcrt neurons, with no confounding modulation of surrounding cells that may regulate the same brain function.

neurons using a well-characterized Hcrt specific promoter in a lentiviral delivery system (Adamantidis et al., 2007). Although the lentivirus infected multiple hypothalamic cells types, only the cells with the endogenous cellular machinery to express Hcrt were found to express ChR2. Therefore, deep brain optical stimulation activated only the Hcrt neurons and not the surrounding cells. Second, the fast ON–OFF kinetics (i.e., millisecond timescale) of ChR2 were found to induce single action potentials in brain slices in ChR2-expressing Hcrt neurons. Deep brain delivery of high frequency light pulse trains were found to activate Hcrt neurons (as measured by c-Fos immunohistochemistry) *in vivo*. Importantly, the millisecond timescale temporal resolution mimicked the physiological range of neuronal spiking rate, overcoming the limitations of previous techniques (e.g., uncontrolled persistence of Hcrt peptide in the brain after local infusion).

We found that direct, deep brain optical stimulation of hypocretin neurons in the hypothalamus increased the probability of transitions to wakefulness from either NREM or REM sleep (Adamantidis et al., 2007). Interestingly, photostimulation using 5–30 Hz light pulse trains reduced latency to wakefulness, whereas 1 Hz trains did not. We also asked whether Hcrt-mediated sleep-to-wake transitions are affected by light/dark period and sleep pressure. We found that stimulation of Hcrt neurons increased the probability of an awakening event throughout the entire light/dark period but that this effect was diminished with sleep pressure induced by 2 or 4 h of sleep deprivation (Carter et al., 2009). These results suggest that the Hcrt system promotes wakefulness throughout the light/dark period by activating multiple downstream targets, which themselves are inhibited with increased sleep pressure. Finally, stimulation of Hcrt neurons was still sufficient to increase the probability of an awakening event in histidine decarboxylase-deficient knockout animals, suggesting that histamine neurons of the TMN are not the main downstream target of Hcrt-mediated increase of arousal, as suggested by other studies (Carter et al., 2009). These studies demonstrate that we now have the right tools to establish causal links between frequency-dependent activation of restricted neuronal population and specific behavioral state transitions.

PERSPECTIVES

A series of recent findings has begun to identify the important neuroanatomical substrates of sleep and wakefulness. However, important questions remain unanswered about how the brain integrates homeostatic sleep pressure and circadian rhythms at the neuronal circuit level. For example,

- Are known neural populations (e.g., catecholaminergic, noradrenergic neurons) sufficient or permissive to promote either sleep, wakefulness or state transitions?
- What are the kinetics of neurotransmission between sleep- and wake-promoting circuits in the brain in an unrestrained, behaving animal?
- What are the consequences of neurotransmitter vs neuropeptide release from synapses of sleep- or wake-promoting circuits on the sleep–wake cycle and associated brain functions (e.g., metabolism, cognition, brain plasticity)?

Addressing these questions or probing other neural circuits will require genetic targeting of optogenetic tools to specific neuronal populations. Current targeting technologies include the identification of minimal promoters compatible with viral-based approaches

(Adamantidis et al., 2007) and Cre-assisted transgenic tools (Atasoy et al., 2008; Tsai et al., 2009). Over the next several years, it will be important to use these different targeting strategies to apply optogenetic studies to neural populations implicated in sleep/wake circuitry. It will also be interesting to apply optogenetic manipulation to multiple sleep–wake populations to investigate circuit interactions and stimulate, for example, Hcrt neurons while simultaneously inhibiting other cell populations in order to determine their necessity in Hcrt-mediated sleep-to-wake transitions.

Current light delivery methods to activate optogenetic tools include optical fibers coupled to pulsed lasers or light emitted diodes. These methods of light delivery should be customized for the specific experiment (e.g. cortical or deep brainstimulation, unilateral or bilateral stimulation, etc.). It is currently difficult to target diffuse structures, such as the hippocampus or cerebral cortex. However, future tools may allow light delivery to large-scale neuronal structures.

Novel, synergistic combinations of recently-developed approaches (Sjulson and Miesenbock, 2008), including *in vivo* optogenetics, genetic tagging of neural circuits (e.g., brainbow technology) (Livet et al., 2007), optogenetic assisted neural circuit mapping (Petreanu et al., 2007, 2009; Atasoy et al., 2008) and *in vivo* state-of-the-art imaging techniques now can address the questions raised above. This will expand the experimental possibilities available and allow *in vivo* deconstruction of previously inaccessible genetically defined sleep–wake neuronal circuits located in the hypothalamus, basal forebrain and brainstem with temporal and spatial resolutions relevant to physiological dynamics. These approaches will help to understand the function of sleep and identify new therapeutical targets to cure sleep disorders and sleep-associated neuropsychiatric disorders, including metabolic imbalance, mood-related pathology and cognitive impairment.

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Informational lesions: optical perturbation of spike timing and neural synchrony via microbial opsin gene fusions

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Synchronous neural activity occurs throughout the brain in association with normal and pathological brain functions. Despite theoretical work exploring how such neural coordination might facilitate neural computation and be corrupted in disease states, it has proven difficult to test experimentally the causal role of synchrony in such phenomena. Attempts to manipulate neural synchrony often alter other features of neural activity such as firing rate. Here we evaluate a single gene which encodes for the blue-light gated cation channel channelrhodopsin-2 and the yellow-light driven chloride pump halorhodopsin from *Natronobacterium pharaonis*, linked by a 'self-cleaving' 2A peptide. This fusion enables proportional expression of both opsins, sensitizing neurons to being bi-directionally controlled with blue and yellow light, facilitating proportional optical spike insertion and deletion upon delivery of trains of precisely-timed blue and yellow light pulses. Such approaches may enable more detailed explorations of the causal role of specific features of the neural code.

Keywords: optogenetics, channelrhodopsin-2, halorhodopsin, fusion protein, synchrony

INTRODUCTION

It has long been debated to what extent synchronous or precisely-timed neural activity contributes to neural computation and behavior. Synchronous neural activity within or between brain regions has been observed during, or associated with, many brain functions including timing-dependent plasticity, global stimulus feature processing, visuomotor integration, emotion, working memory, motor planning, and attention (e.g., Gray et al., 1989; Roelfsema et al., 1997; Brivanlou et al., 1998; Donoghue et al., 1998; Steinmetz et al., 2000; Fries et al., 2001; Tallon-Baudry et al., 2001; Froemke and Dan, 2002; Perez-Orive et al., 2002; Seidenbecher et al., 2003; Courtemanche and Lamarre, 2004; Buschman and Miller, 2007), as measured with multielectrode recording, electroencephalography (EEG), magnetoencephalography (MEG), and local field potential (LFP) analysis. Furthermore, abnormal patterns of neural synchrony have been associated with a variety of neurological and psychiatric disorders such as Parkinson's disease, epilepsy, autism, and schizophrenia (e.g., Bragin et al., 2002; Uhlhaas and Singer, 2006; Berendse and Stam, 2007; Brown, 2007; Huguenard and McCormick, 2007; Orekhova et al., 2007; Gonzalez-Burgos and Lewis, 2008; Roopun et al., 2009). Computationally, synchrony has been implicated in multiple processes, including grouping neurons into 'cell assemblies' that can more effectively represent information to downstream neural networks, acting as a clock for gating or multiplexing information, coordinating information flow within small neural networks, selecting stimuli for attention, and performing pattern recognition (e.g., Hopfield and Brody, 2001; Friedrich et al., 2004; Tiesinga and Sejnowski, 2004; Borgers et al., 2005, 2008; Sohal et al., 2009). However, determining the causal role of neural synchrony to neural computation and behavior has remained elusive, in part because selective perturbation of spike timing is difficult. In some specific systems, in which pharmacological or genetic strategies for selectively disrupting synchrony happened to be compatible with local cellular and network properties, pioneering attempts have been made to perturb spike timing without altering other aspects of neural coding such as spike rate (MacLeod and Laurent, 1996; Bao et al., 2002; Robbe et al., 2006), but no generalized method for doing so exists.

Recently we and others have developed strategies for optically sensitizing neurons to being activated and silenced with different colors of light, by delivering the gene encoding for the blue light-gated cation channel channelrhodopsin-2 (ChR2) or the gene for the yellow-light driven chloride pump halorhodopsin from *N. pharaonis* (Halo/NpHR) to neurons, and illuminating them with pulses of light (Boyden et al., 2005; Han and Boyden, 2007; Zhang et al., 2007). New neural activators and silencers continue to be developed, and these molecules have already begun to be applied to the study of neural dynamics, as investigators optically drive the activity of excitatory and inhibitory neurons and electrophysiologically characterize the resultant patterns (e.g., Boyden et al., 2009; Cardin et al., 2009; Han et al., 2009; Sohal et al., 2009; Talei Franzesi et al., 2009). However, none of these experiments have attempted to isolate the distinct properties of synchrony and spike rate, both of which are modulated when neurons are activated or silenced by using either ChR2 or Halo by itself. Previously, we presented a strategy for perturbing spike timing without altering spike rate (Han and Boyden, 2007), taking advantage of the fact that the spectral peaks for ChR2 activation

and Halo activation are separated by over 100 nm (**Figure 1A**), making it possible to express both molecules in the same neuron (**Figure 1B**), thus conferring bi-directional control of the voltage of that neuron by blue and yellow light (**Figures 1C,D**) (Han and Boyden, 2007). As a consequence of this two-color bidirectional voltage control, precisely-timed blue and yellow light pulse trains, delivered to a neuron expressing both ChR2 and Halo, can be used to insert spikes into, and delete spikes from, the ongoing activity experienced by that neuron (**Figure 1E**), resulting in precise perturbations of spike timing without changing overall spike rate (**Figures 1F,G**). However, the delivery of two separate genes to precisely the same cell population can be a daunting proposition. Furthermore, it is important that ChR2 and Halo be expressed in a predictable proportion to one another, so that the insertion and deletion of spikes can be balanced; separate delivery of the two genes, or independent expression of the two genes, would not accomplish this. Recently we and others have presented gene fusions between ChR2 and Halo that enable them to be expressed in the same expression cassette, bridging ChR2 and Halo with an optimized form of the 'self-cleaving' 2A peptide sequence from foot-and-mouth disease virus or other picornaviruses (Han et al., 2008; Tang et al., 2009). The 2A sequence encourages a ribosomal skip between a glycine and proline within the 2A sequence, resulting in separate proteins being formed from the sequences on either side (Ryan et al., 1991; Ryan and Drew, 1994). In the Tang et al. paper, the first peer-reviewed presentation of opsin fusions using a 2A peptide bridge, the authors showed functional expression of both opsins in the same cell, *in vivo*, after delivery via AAV. We here quantitate the proportional expression of ChR2 and Halo in the same cell when they are expressed separated by a 2A bridge, and discuss the consequences for the specific difficult experimental goal of perturbing synchrony and precise spike timing. Such proteins will also be useful for exploring a large set of systems neuroscience experiments in which bi-directional control of the activity of a given neuron population is desired.

MATERIALS AND METHODS

CELL CULTURE

All procedures involving animals were conducted in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Massachusetts Institute of Technology Animal Care and Use Committee. Hippocampal regions CA3-CA1 of postnatal day 0 Swiss Webster or C57 mice from Taconic or Jackson Labs were isolated and treated with trypsin (1 mg/ml) for ~12 min. Digestion was stopped by Hanks solution supplemented with 20% fetal bovine serum and trypsin inhibitor (Sigma). Tissue was dissociated with Pasteur pipettes and centrifuged at 1000 rpm at 4°C for 10 min. Dissociated neurons were plated on glass coverslips pre-coated with Matrigel (BD Biosciences) at a rough density of approximately two to four hippocampi per 20 coverslips. Neurons were transfected using a commercially available calcium phosphate transfection kit (Invitrogen), at 3–5 days *in vitro*. GFP fluorescence was used to identify successfully-transfected neurons, indicating a net transfection efficiency of 1–10%. All images and electrophysiological recordings were made on 9–15 day-*in-vitro* neurons (approximately 6–10 days after transfection).

MOLECULAR BIOLOGY

The ChR2GFP-2A-HaloYFP-N1 plasmid ('ChR2-2A-Halo') was constructed by first inserting the extended N-terminus 2A sequence (DNA sequence AAGAAACAGAAAATTGTGGCACC AGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCG GGAGACGTCGAGTCCAACCCTGGGCCC, translated peptide sequence KKQKIVAPVKQTLNFDLLKLAGDVESNPGP) into the BsrGI and NotI sites in the pEGFP-N1 vector, followed by inserting ChR2-GFP without stop codon into the KpNI and BsrGI sites in front of the 2A sequence, and then by inserting Halo-YFP without start codon into the XbaI and EcoRI sites downstream of the 2A sequence. The pEGFP-N1 vector drives gene expression from the CMV promoter.

ELECTROPHYSIOLOGY

Whole cell patch clamp recording was made on 9–15 day-*in-vitro* neurons using a Multiclamp 700B amplifier, connected to a Digidata 1440 digitizer (Molecular Devices) attached to a PC running pClamp 10. During recording, neurons were bathed in Tyrode's solution containing (in mM) 150 NaCl, 2.4 KCl, 2 CaCl, 2 MgCl, 10 HEPES, 10 Glucose, 10 μM NBQX, 10 μM gabazine and 50 μM D-APV. Borosilicate glass (Warner) pipettes were filled with a solution containing (in mM) 130 K-Gluconate, 7 KCl, 2 NaCl, 1 MgCl2, 0.4 EGTA, 10 HEPES, 2 ATP-Mg, 0.3 GTP-Tris and 20 sucrose. Pipette resistance was ~5 MΩ and the access resistance was 10–25 MΩ, which was monitored throughout the voltage-clamp recording.

Light-induced membrane photocurrents were induced by brief light pulses separated by periods in the dark, in neurons current-clamped or voltage-clamped, respectively. Light pulse trains were either input directly into pClamp software (Molecular Devices) or synthesized by custom scripts written in MATLAB (Mathworks), then played back through a Sutter DG-4 light source via a Digidata 1440 (Molecular Devices). Light was reflected into the sample off of a 700DCXRU (Chroma) dichroic in a Leica DMI6000B inverted microscope. In the DG-4, a yellow filter (Chroma, bandpass 575 ± 25 nm) was used to deliver light to activate Halo, and a GFP excitation filter (bandpass 470 ± 20 nm) was used to activate ChR2. Powers out the 40× objective were approximately 10 mW/mm^2 in irradiance.

DATA ANALYSIS

Amplitude and timing data were analyzed using Clampfit 10 (Molecular Devices) and custom analysis scripts written in MATLAB.

RESULTS

We recently presented a demonstration of how one could use co-expression of separate ChR2 and Halo genes to support optical disruption of the timing of spikes, without significant alteration of spike rate (**Figure 1**, adapted from Han and Boyden, 2007). In review of this previously-published work (see Han and Boyden, 2007 for detailed results and methods): we cultured hippocampal neurons and then caused them to fire precisely-timed spike trains by patch clamping them in current-clamp mode, and repeatedly delivering to each neuron the same filtered Gaussian white noise current trace (see **Figure 1Ei**, top, for a fragment thereof). Such noisy currents had been previously found to induce reliable spike

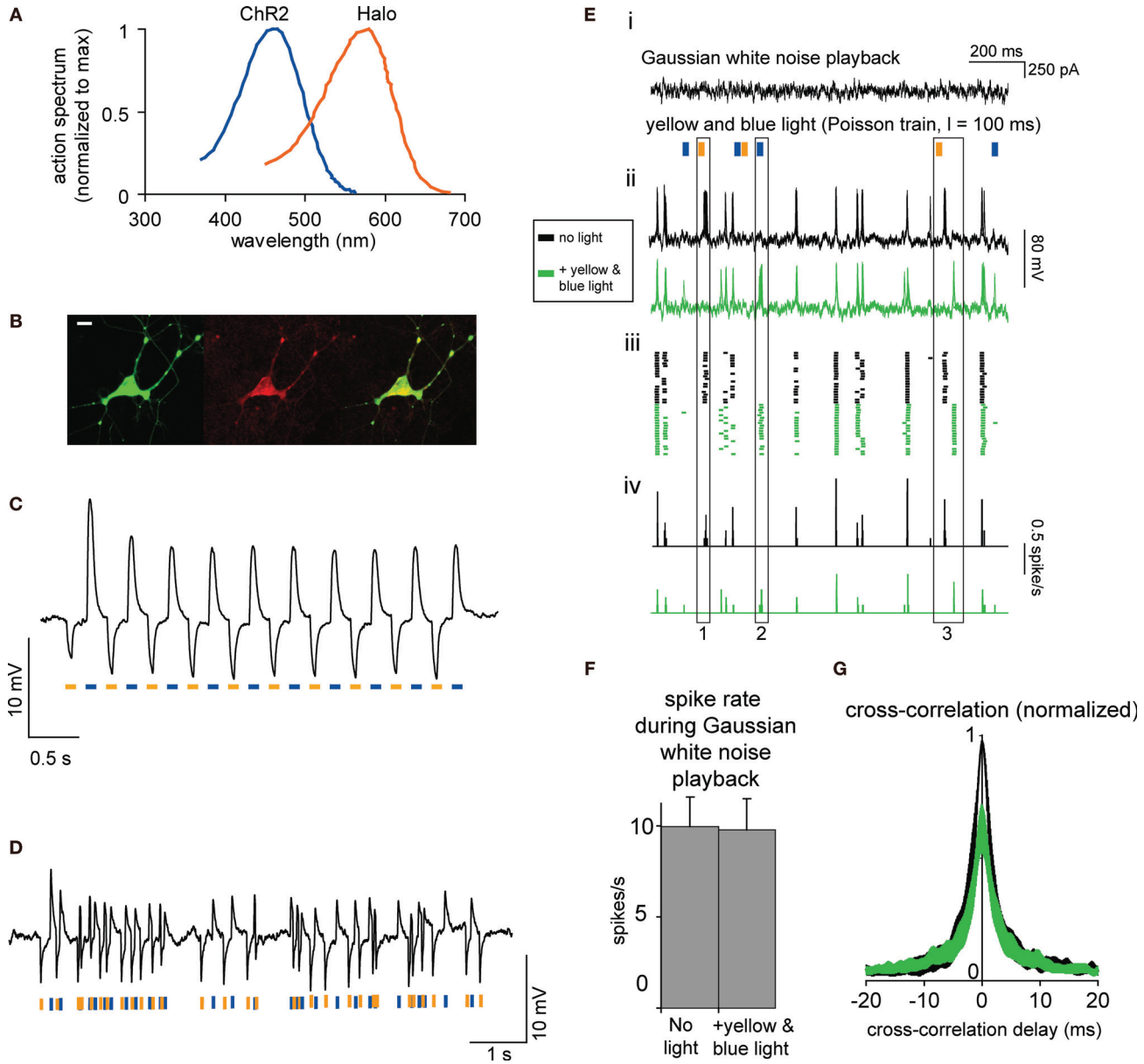

FIGURE 1 | Schema for how to alter neural spike timing, using two-color illumination of neurons co-transfected with both ChR2 and Halo in a 1:1 ratio. Adapted from (Han and Boyden, 2007). **(A)** Action spectrum for ChR2 (blue, adapted from Nagel et al., 2003), overlaid with absorption spectrum for Halo (orange, adapted from Duschl et al., 1990). Each spectrum is normalized to its own peak, for ease of comparison. **(B)** Co-expression of Halo-GFP (*left*) and ChR2-mCherry (*middle*) in a single neuron expressing both (*right*, overlay). Scale bar, 20 μm. **(C)** Hyperpolarization and depolarization events elicited in a single representative neuron, by two interleaved 2.5 Hz trains of yellow and blue light pulses (50 ms duration each), denoted by bars of respective coloration below the trace. **(D)** Hyperpolarization and depolarization events induced in a representative neuron by a Poisson train (mean inter-pulse interval λ = 100 ms) of alternating pulses of yellow and blue light (10 ms duration), denoted by bars of respective coloration below the trace. **(E)** Optical disruption of spike timing, without alteration of spike rate, for a representative current-clamped hippocampal neuron transfected simultaneously with ChR2 and Halo *in vitro*. Vertical boxes (labeled at bottom 1, 2, 3) highlight features referred to in the text of the Results. **(i)** Stimulus trace showing a subsegment of the somatically injected filtered Gaussian white noise current used in all these experiments (top), as well as of the Poisson train (mean inter-pulse interval λ = 100 ms) of alternating yellow and blue light pulses (bottom). **(ii)** 20-trace overlays of voltage responses to the somatically injected white noise current, either with no light (top, black traces) or with delivery of a Poisson train of yellow and blue light pulses (bottom, green traces). **(iii)** Spike raster plots for the traces shown in **(Eii)**. **(iv)** Spike-timing histograms (bin size: 500 μs) for the rasters shown in **(Eiii)**. **(F)** Spike rates of current-clamped hippocampal neurons transfected simultaneously with ChR2 and Halo *in vitro* ($n = 7$), injected with filtered Gaussian white noise current, either with no light (left) or with concurrent delivery of a Poisson train of yellow and blue light pulses (right). Plotted is mean ± standard error. **(G)** Cross-correlation between spike trains elicited by the same filtered Gaussian white noise current injection, played twice, when either both current injections were performed in the dark (black curve), or when one of the current injections was performed with concurrent delivery of a Poisson train of yellow and blue light pulses (green trace). Data is plotted as mean ± standard error (averaged across $n = 7$ neurons).

trains in current-clamped neurons in acute cortical brain slices (Mainen and Sejnowski, 1995). On some trials, we also illuminated the neuron with the Poisson train of alternating yellow and blue light pulses shown in **Figure 1D** (see **Figure 1Ei**, bottom). We found that when delivered alone, the filtered Gaussian white noise current trace indeed induced reliably-timed spike trains, across repeated trials (see **Figure 1Eii**, top, for 20 overlaid traces, and **Figure 1Eiii**, top, for corresponding spike rasters). When an optically-sensitized neuron was additionally driven by the Poisson train of yellow and blue light pulses, the neuron fired spikes with different timings than occurred in darkness, but the resultant spikes were nevertheless still similar across repeated trials of current injection + light illumination (see **Figure 1Eii**, bottom, and **Figure 1Eiii**, bottom, for overlaid traces and spike rasters respectively, for 20 trials; **Figure 1Eiv** shows spike histograms). Inspection of this data shows that relative to the spike train elicited by current injection alone, the Poisson train of light pulses could sometimes abolish spikes that were previously reliable (vertical box 1 spanning **Figures 1Ei–iv**), create new spikes which were not previously present (vertical box 2 spanning **Figures 1Ei–iv**), or advance or delay the timing of specific spikes relative to their original timing in the dark (vertical box 3 spanning **Figures 1Ei–iv**). We compared mean spike rates for neurons receiving the filtered Gaussian white noise current injection alone vs. with the additional Poisson light pulse train, and found no difference in spike rates for these two conditions ($p > 0.90$, *t*-test; $n = 7$ neurons; **Figure 1F**), indicating that our optical intervention preserved spike rate. However, precise spike timing was altered significantly: cross-correlations of the spike trains elicited from the filtered Gaussian white noise current injection alone vs. with the additional Poisson light pulse train resulted in zero-lag cross-correlations that were on average 37% smaller than cross-correlations of pairs of spike trains elicited from the filtered Gaussian white noise current injection alone ($p < 0.005$, $n = 7$ neurons). This indicates that precise spike timing was indeed disrupted by the activation of Halo and ChR2, even while spike rate was preserved.

The strategy illustrated in **Figure 1** centers around the idea of using blue light to push the voltage of a cell over spike threshold, and using yellow light to push the voltage of a cell under spike threshold. Critical to this strategy is the proportional expression of ChR2 and Halo in a predictable ratio to one another. Ideally, for every spike inserted, another will be deleted. A neuron that expresses high levels of both ChR2 and Halo will have a high probability of generating a spike in response to a blue light pulse, but will also have a high probability of losing a spike in response to a yellow light pulse. On the other hand, a neuron that expresses low levels of both ChR2 and Halo will have a low probability of generating a spike in response to a blue light pulse, but will also have a lower probability of losing a spike in response to a yellow light pulse. Thus to a first approximation, the strategy in **Figure 1** should work to disrupt spike timing but not spike rate, independent of the net expression levels of ChR2 and Halo, as long as their proportion is kept constant. Of course, this approximation will break down if the scenario approaches an extreme: for example, a high-firing rate neuron may not be responsive to blue light pulses because of a ceiling effect on firing rate, but may be greatly affected by yellow pulses; on the other hand, a low-firing rate neuron may respond to blue light pulses with increased spiking, but may have few spikes to lose when yellow light pulses arrive. Thus it will be important to choose light pulse trains that deliver light pulses at appropriate rates given the neural population under investigation; the light pulse rate should scale with the firing rate of the neural population (and in principle, could even be adapted in real time during a physiology or behavior experiment). Two further comments should be made: given that ChR2 and Halo generate intrinsically different scales of currents (ChR2's peak currents are an order of magnitude greater than Halo's), the ratio of blue to yellow light powers may need to be adjusted to balance the current magnitudes against one another. Finally, in the intact brain *in vivo*, light powers will fall off with distance from the light source; however, the difference between blue and yellow light power attenuation in brain tissue is not very large over the short distances (~1 mm) utilized for *in vivo* optical control scenarios, which are significantly less than the absorption length constant of visible light in tissue (Bernstein et al., 2008b). Tiling the brain with arrays of LED- or laser-coupled optical fibers may allow for more even light distribution in the brain, than possible with just one inserted fiber (Bernstein et al., 2008a; Zorzos et al., 2009).

To express both ChR2 and Halo in a stoichiometric ratio, we evaluated a number of candidate strategies. Using two viruses to deliver the two genes could lead to significant variation of the ChR2/Halo ratio across cells. Placing an internal ribosome entry site (IRES) – a long sequence of several hundred base pairs, large compared to the space accorded in many kinds of viral vector – in between two genes often results in much lower expression of the second gene (levels 0–20% those of the first gene) (Mizuguchi et al., 2000; Hennecke et al., 2001; Yu et al., 2003; Osti et al., 2006). We decided upon a methodology using the 'self-cleaving' 2A sequence (**Figure 2A**) from foot-and-mouth disease virus (Ryan et al., 1991; Ryan and Drew, 1994). When an mRNA encoding for ChR2-2A-Halo is being translated, a specific Gly-Pro bond in the 2A sequence would not be enduringly formed by the ribosome (a 'ribosomal skip'), resulting in the separate translation of ChR2 and Halo into separate proteins. We utilized a variant of 2A with additional amino acids appended to the N-terminus which enhances cleavage from 90% to 96–99% (as assessed by an *in vitro* translation assay) (Donnelly et al., 2001a,b) (**Figure 2A**, blue and green colored residues). The use of a different 2A peptide to co-express channelrhodopsin-2 and halorhodopsin within a single gene was recently published, including proof-of-principle electrophysiological data resulting from *in vivo* infection of neurons with the resulting virus (Tang et al., 2009). Here, expression of the ChR2-2A-Halo gene by transfection in cultured hippocampal neurons resulted in excitatory and inhibitory photocurrents in response to blue and yellow light respectively. The photocurrents were smaller than would result from expression of either gene alone (**Figure 2C**), in part because of overlap in the two molecules' action spectra (**Figure 1A**). Blue-light elicited photocurrents, measured in voltage clamp mode, were 157 ± 63 pA ($n = 22$ neurons; mean ± standard deviation), ~30% of the value previously reported for raw ChR2 expression in cultured hippocampal neurons (Boyden et al., 2005), and yellow-light elicited photocurrents were 40 ± 24 pA, ~45% of the value previously reported for raw Halo expression in cultured hippocampal neurons (Han and Boyden, 2007). These currents, nevertheless,

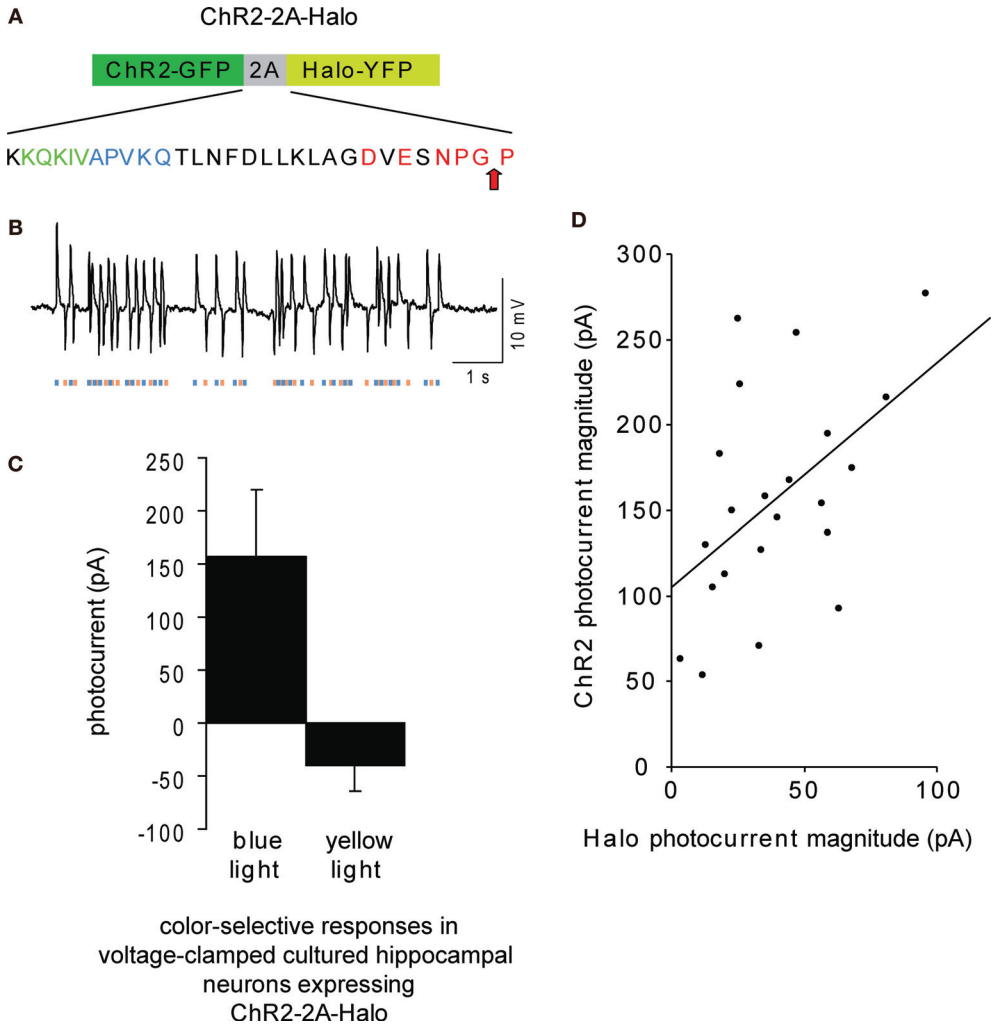

FIGURE 2 | Gene fusion enabling proportional co-expression of ChR2 and Halo under a single promoter. (A) Schematic of the gene fusion ChR2-2A-Halo, highlighting the sequence of the 2A peptide here used. Red letters indicate consensus sequence for 2A-like ribosomal skip sequence (Donnelly et al., 2001a; de Felipe et al., 2006); blue letters indicate amino acids from the 1D peptide sequence that, when appended to the N-terminus of the 2A sequence, increase *in vitro*-translated protein cleavage from 90% to 96%, and green letters are part of a sequence from the 1D peptide sequence that boosts *in vitro*-translated protein cleavage to 99–100% levels (Donnelly et al., 2001a,b). **(B)** Hyperpolarization and depolarization events induced in a representative current-clamped hippocampal neuron *in vitro*, transfected with ChR2-2A-Halo, by a Poisson train (mean inter-pulse interval λ = 100 ms) of alternating pulses of yellow and blue light (10 ms duration), denoted by bars of respective coloration below the trace. **(C)** Peak photocurrents measured in voltage clamped ChR2-2A-Halo-expressing cultured hippocampal neurons under 1-s blue (left) or yellow (right) exposure (n = 22 neurons; bars represent mean ± standard deviation). **(D)** Plot of magnitude of blue light-elicited photocurrents vs. magnitude of yellow light-elicited photocurrents. Line shows linear regression fit to the plotted data.

are sufficient to cause a neuron near the threshold of spiking to fire an action potential or to be momentarily silent, and routinely resulted, in current-clamped neurons, in effective perturbations of membrane voltage by 5–10 mV (**Figure 2B**). Plotting, for each recorded neuron, the blue-light elicited photocurrents vs. the yellow-light elicited photocurrents yielded a significant linear relationship with correlation coefficient $r = 0.51$ ($R^2 = 0.26$, $p < 0.02$; **Figure 2D**). Thus, the 2A expression vector here utilized was able to mediate proportional, functional expression of ChR2 and Halo in the same cell.

What kinds of experiment are enabled by the use of linked microbial opsins for bi-directional control of the voltage of individual neurons? Many 'obvious' but much-desired experiments may be facilitated with use of this reagent. For example, by appending to ChR2 a myosin-binding domain (MBD) that preferentially targets it to cell bodies and dendrites (Lewis et al., 2009), the resultant ChR2-MBD-2A-Halo could enable activation of a population of neurons when their cell bodies are illuminated with blue light, as well as silencing of specific projections when their axonal terminals are illuminated with yellow light. The ability to guarantee that a single population of neurons can be both activated and silenced is itself valuable, enabling testing of necessity and sufficiency of the same population of neurons, to a specific neural computation or behavior. However, it is useful to explore conceptually how this technology might be useful for probing complex questions concerning synchrony. We have, in **Figure 3**,

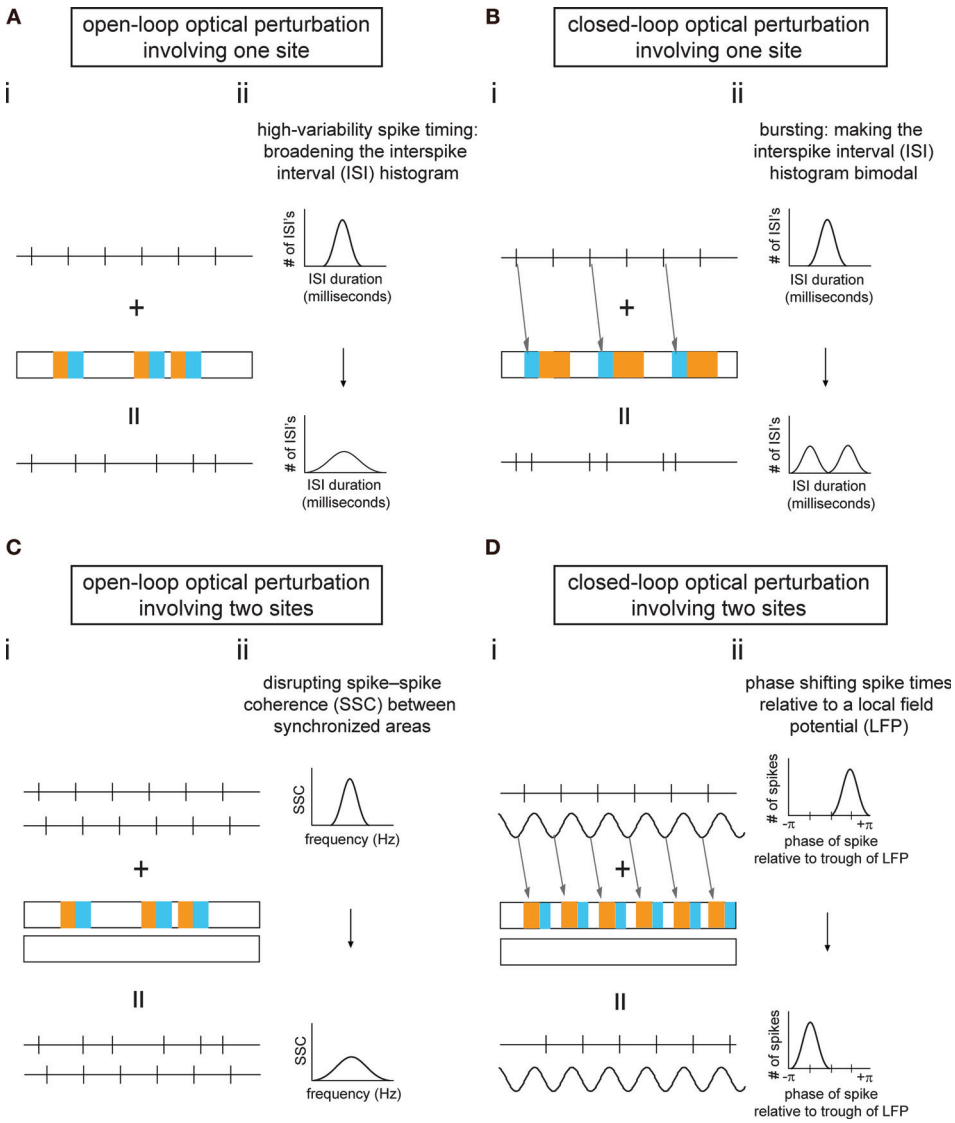

FIGURE 3 | Synchrony- and spike timing-perturbation protocols, enabled by use of ChR2-2A-Halo. Each of the four panels **(A–D)** displays a schematic experimental protocol **(i)** along with the anticipated effect of the protocol on neural circuit dynamics **(ii)**. An example is given for each of four kinds of experiment – **(A)** open-loop/one site, **(B)** closed-loop/one site, **(C)** open-loop/two sites, **(D)** closed-loop/two sites. Detailed descriptions of each panel follow. **(A)** Shows the use of Poisson train light pulse delivery to a site in which rhythmically-firing neurons express ChR2-2A-Halo. This protocol results in the interspike interval (ISI) becoming more variable than in the unilluminated state. **(i)** From top to bottom: spike train trace (for one example neuron) in dark; timeline of when yellow or blue light is delivered; spike train trace (for the example neuron) in light (e.g., the result of combining the original case, shown at top, with the delivery of light, shown in the middle). **(ii)** From top to bottom: ISI histogram in dark; ISI histogram in light. **(B)** Shows the use of optical perturbation in a closed-loop fashion, triggering blue-yellow pairs of light pulses off of particular recorded spikes in order to make the spike train more bursty. **(i)** From top to bottom: spike train trace in dark; timeline of when yellow or blue light is delivered (in this case, each blue-yellow pair is triggered by a spike; each trigger is indicated by an arrow); spike train trace under the closed-loop protocol. **(ii)** From top to bottom: ISI histogram in dark; ISI histogram under the closed-loop protocol. **(C)** Shows the use of optical perturbation in the style of **(Ai)**, with light delivered to one site to disrupt the timing of activity at that site, relative to another site, which is left unilluminated. **(i)** From top to bottom: spike train traces recorded at two sites in the dark; timeline of when yellow or blue light is delivered to one of the sites while the other is kept in the dark; spike train traces in light. **(ii)** From top to bottom: spike-spike coherence (see Fries et al., 2008 for experimental examples of this measure) across the two areas plotted vs. spike frequency, measured in dark; spike-spike coherence across the two areas plotted vs. spike frequency, measured when one site is illuminated. **(D)** Shows the use of optical perturbation in a closed-loop fashion, triggering yellow-blue pairs of light pulses at one site, off of a feature of the local field potential (LFP) recorded at another site, which is left unilluminated. In this case, the trough of the LFP (bandpass filtered to yield a defined set of frequencies) at the second site serves as the trigger for delaying spikes at the first site. **(i)** From top to bottom: spike train trace and local field potential trace recorded at two separate sites; timeline of when yellow or blue light is delivered to the site from which spikes are recorded (in this case, each yellow-blue pair is triggered by the trough of the LFP at the second site; each trigger is indicated by an arrow); spike train trace and local field potential trace under the closed-loop protocol. **(ii)** From top to bottom: phase relationship between spikes recorded at the first site and local field potential troughs recorded at the second site, in the dark (e.g., see Pesaran et al., 2002; Gregoriou et al., 2009) for examples of this kind of data); phase relationship between spikes recorded at the first site and local field potential troughs recorded at the second site, under the closed-loop protocol.

outlined in schematic form four protocols enabling perturbation of spike timing or synchrony, in order to study their contribution to circuit output. In all four cases, appropriately-timed blue and yellow light pulses are used to disrupt the timing of spikes in a region containing neurons sensitized to light with a 2A construct, thus altering the spike statistics. Because light will be delivered to all the neurons within a defined region, one caveat may apply: the optically-sensitized neurons in the region will be exposed to the same temporal pattern of blue and yellow light, and this common input may alter (e.g., increase) the synchrony between optically-sensitized neurons within the illuminated region; this may potentially complicate experiments where altered synchrony within a region is undesired, although again the use of multiple light sources to deliver multiple light patterns to a single region may ameliorate this issue.

One protocol is a direct generalization of the strategy outlined in **Figure 1**, the delivery of a light pulse train to a brain region to increase interspike interval (ISI) variability, turning rhythmic neurons into irregularly-firing ones (**Figure 3A**). This 'informational lesion' might enable the deletion of the information encoded by the precision of spiking of those cells, without eliminating all activity generated by those cells, and thereby causing a gross disruption of connected circuits (as might occur with a conventional lesion of the cells of interest). A slightly more complex version of this idea is illustrated in **Figure 3B**, in which light pulses are precisely triggered, in a closed-loop fashion, upon the occurrence of particular recorded spikes, so that the neuronal firing rate becomes more bursty (but, once again, without changing overall spike rates). The ability to test the role of bursting in spike signaling may help resolve how the temporal integration properties of neural circuit elements contribute to neural computations. The 2A strategy can also be used to disrupt correlations between multiple regions that exhibit synchrony (**Figure 3C**), making spike timing more variable in one region so that its activity correlation with activity in a second region decreases, or making spike timing more variable in one region to see if such activity causally influences the activity within the second region. In this way it might be possible to understand how regions communicate and process information together in a coordinated fashion. As a final example, the multi-site experiment can also be performed in a closed-loop fashion (**Figure 3D**), triggering the delivery of a specific light pulse train to one region, upon a particular phase of the local field potential (LFP) recorded in a second region, and thus phase shifting spike times in the first region with respect to the LFP of the second. Many other possible protocols exist; the key advance enabled by the 2A construct in such protocols is the ability to lesion information or alter information in the brain, without grossly disrupting the spike rate, as happens with traditional lesions, some forms of pharmacology, or normal activation or silencing of neurons.

DISCUSSION

We here discuss the implications of gene fusions of ChR2 and Halo, linked by the 'self-cleaving' 2A peptide, capable of supporting cellular synthesis of both opsins in proportion to one another, towards enabling a set of neurons to be simultaneously sensitized to blue light activation and yellow-light silencing. As an example of computational neuroscience-driven molecular engineering, such reagents may enable novel kinds of perturbation, such as the disruption of spike timing in the absence of altering spike rate. The construct presented here is freely available from the nonprofit service Addgene (reagent 'ChR2-2A-Halo' at http://www.addgene.org/Edward_Boyden) and is ready for use for transfection, gene gunning (Zhang et al., 2008), transgenic implementation (Wang et al., 2007), or electroporation (Petreanu et al., 2007; Lagali et al., 2008). The current construct, ChR2-2A-Halo yielded, when transfected into neurons, blue- and yellow-light elicited currents that were 30–40% of those obtained when ChR2 or Halo were expressed alone, respectively. Another paper that recently also disclosed the use of a 2A bridge to co-express ChR2 and Halo also showed currents that were smaller than those resulting from expression of individual opsins alone (Tang et al., 2009). These are still useful currents, especially if the goal is to dither the activity of a neuron above and below spike threshold in order to add and subtract spikes (as opposed to strongly driving or inhibiting neurons); however, a question for any technology is whether the efficacy can be improved further. One solution would be to use stronger opsins to begin with, such as novel molecules like ChIEF (Lin et al., 2009) and Arch (Chow et al., 2009a,b), which can mediate functionally higher currents than ChR2 and Halo, respectively. Other methods for expressing two genes also exist: for example, bi-directional promoters can lead to higher expression levels than can 2A, but at the expense of significantly more variability in the ratio of the protein levels resulting from each gene (e.g., see Fig. 3A in Amendola et al., 2005), due to the greater chance for noise to creep in downstream of the stoichiometry-determining event.

The prospects for using optical control technologies for ultraprecise neuromodulation therapies in clinical settings are exciting, since precise activation and silencing of specific cell types may increase efficacy and reduce side effects of treatments relative to purely electrical methods. However, it is important to consider not just straightforward optical activation and silencing, but also complex perturbations such as desynchronization as here described, which could enable correction of neural dynamics abnormalities such as those found in neurological and psychiatric disorders like Parkinson's and epilepsy. As pre-clinical studies of the safety and efficacy of optical control technology begin (Han et al., 2009), it will be increasingly important to derive principles of neural control, prototyping novel therapies that take full advantage of the computational power of these new technologies.

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Shedding light on restoring respiratory function after spinal cord injury

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Loss of respiratory function is one of the leading causes of death following spinal cord injury. Because of this, much work has been done in studying ways to restore respiratory function following spinal cord injury (SCI) – including pharmacological and regeneration strategies. With the emergence of new and powerful tools from molecular neuroscience, new therapeutically relevant alternatives to these approaches have become available, including expression of light sensitive proteins called channelrhodopsins. In this article we briefly review the history of various attempts to restore breathing after C2 hemisection, and focus on our recent work using the activation of light sensitive channels to restore respiratory function after experimental SCI. We also discuss how such light-induced activity can help shed light on the inner workings of the central nervous system respiratory circuitry that controls diaphragmatic function.

Keywords: C2 hemisection, spinal cord injury, plasticity, regeneration, respiration, phrenic nucleus, channelrhodopsin, optogenetics

Trauma at the cervical level is one of the most common types of spinal cord injury (SCI) (National Spinal Cord Injury Statistical Center, 2009). Injuries at this level are particularly devastating since this results in disruption of the bulbospinal projections to the phrenic nucleus (PN), which is composed of motor neurons that directly innervate the diaphragm. Unfortunately, high cervical lesions of the cord oftentimes lead to paralysis of the diaphragm and dependence on mechanical ventilation for survival.

In the laboratory, the model of choice for investigating cervical SCI and its resulting respiratory deficits has been the C2 hemisection. In this model the cervical SC is exposed and hemisected from the midline all the way to the lateral most aspect of the cord. This results in unilateral transection of the descending respiratory pathways and paralysis of the ipsilateral hemidiaphragm (**Figure 1**) (Moreno et al., 1992). The animal is still able to survive without use of a ventilator since the contralateral hemidiaphragm is still active. From this point, injury-induced physiological and neuroanatomical changes in the animal can be observed and methods to restore hemidiaphragmatic function can be investigated.

CNS RESPIRATORY CIRCUITRY AND THE CROSSED PHRENIC PHENOMENON

The PN is located at the caudal end of C3 to the rostral most part of the C6 level of the spinal cord in the rat (Goshgarian and Rafols, 1984). Phrenic motor neurons (PMNs) are organized tightly in a cylindrically shaped nucleus and for the most part, the dendrites travel in a rostral/caudal direction (Furicchia and Goshgarian, 1987). A variety of different inputs, which include those from glutamatergic, GABAergic, serotonergic and norepinephrine neurons innervate the PMNs (Zhan et al., 1989; Liu et al., 1990; McCrimmon et al., 1989; Chitravanshi and Sapru, 1996).

Generation of the frequency and rhythm of breathing comes from supraspinal centers, in particular, the pre-Botzinger complex; and the source of the glutamatergic inspiratory drive is from the rostral ventral respiratory group (RVRG) located in the medulla (**Figure 1**) (Smith et al., 1991; Moreno et al., 1992; Chitravanshi and Sapru, 1996). The left and right RVRG have axons that decussate in the medulla, travel primarily in the ventrolateral funiculi, and project to the ipsilateral and contralateral phrenic nuclei (**Figure 1**) (Ellenberger and Feldman, 1988). These are the respiratory pathways which can be disrupted following cervical injury.

As early as 1895 Porter showed that a hemisection of these pathways would lead to paralysis of the ipsilateral hemidiaphragm. However, he also demonstrated that if the contralateral phrenic nerve was also transected, the initially paralyzed hemidiaphragm would become rapidly active again. But now the hemidiaphragm ipsilateral to the phrenic nerve transection became paralyzed. This was termed the "crossed phrenic phenomenon". Porter later determined that the anatomical substrate behind this recovery was a previously unknown respiratory pathway that crosses the midline at the level of the phrenic nuclei – essentially bypassing the lesion (**Figure 1**) (Porter, 1895, for an extensive review please read Goshgarian, 2003). This pathway was deemed latent or ineffective because although the pathway to the PN ipsilateral to the hemisection remained intact it was not active or being physiologically expressed in the absence of phrenicotomy. Since then, much work has been done in trying to activate this latent pathway to restore function without having to transect the contralateral phrenic nerve.

RESTORING FUNCTION AFTER EXPERIMENTAL CERVICAL INJURY

The physiological mechanism behind the crossed phrenic phenomenon and expression of the latent crossed phrenic pathway (CPP) is an increase in central drive due to the resulting asphyxiation produced by the contralateral phrenicotomy (Lewis and Brookheart, 1951). It was further demonstrated that there is a direct relationship between the amount of central respiratory drive and the expression of the CPP and hemidiaphragmatic recovery (Lewis

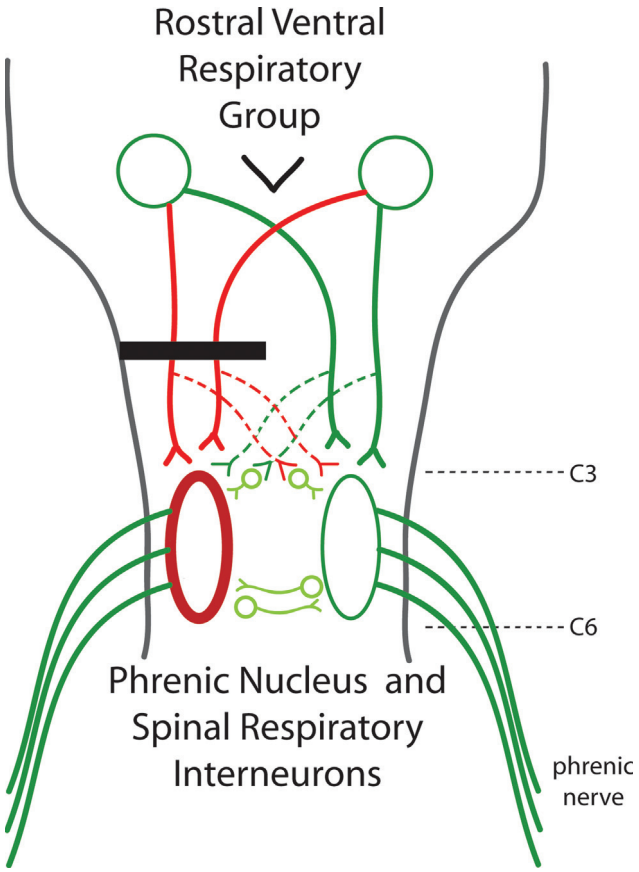

FIGURE 1 | A diagram of the respiratory pathways to the spinal cord. The phrenic nuclei are located bilaterally in the caudal C3 to rostral C6 spinal cord. The excitatory inspiratory drive comes from the rostral ventral respiratory group located in the medulla. Axons from the RVRG can decussate in the medulla to bilaterally innervate the phrenic nuclei. C2 hemisection (black bar) results in unilateral disruption of RVRG-spinal pathways (red lines) and quiescence of the ipsilateral phrenic nerve leading to paralysis of the ipsilateral hemidiaphragm. The crossed phrenic pathway (dashed lines) can circumvent the C2 hemisection by descending contralateral to the lesion and crossing over near the level of the phrenic nuclei. Normally inactive, activation of this spared pathway can restore function. It has been recently shown that spinal interneurons may play a role in this pathway.

and Brookheart, 1951). With this in mind, it was hypothesized that pharmacological intervention which can increase central respiratory drive might be a potential way to induce recovery of the ipsilateral hemidiaphragm following C2 hemisection – without contralateral phrenicotomy. One such possible treatment comes from the family of CNS stimulants, the methylxanthines. Methylxanthines, and in particular, theophylline, have been extensively used for treatment in respiratory related diseases, including asthma (Riegelman and Jenne, 1980). Indeed, in a series of elegant experiments, Nantwi from the Goshgarian group showed that theophylline administration could induce recovery of the paralyzed hemidiaphragm acutely following C2 hemisection (Nantwi et al., 1996, 2003; Nantwi and Goshgarian, 1998a,b). This was attributed to antagonistic properties of theophylline to the adenosine receptor (Nantwi and Goshgarian, 2002). In addition, theophylline's inhibitory affect on phosphodiesterase activity, resulting in an increase of cAMP levels, plays an important role in the restoration of hemidiaphragmatic activity (Kajana and Goshgarian, 2008).

Instead of increasing respiratory drive pharmacologically, altering oxygen content in the environment to induce physiological changes in the injured animals has also proven to restore or improve recovery following C2 hemisection. Alternating bouts of hypoxia separated by episodes of normoxia can increase or augment the spared synaptic inputs to the PN restoring function (Fuller et al., 2003; Golder and Mitchell, 2005; Doperalski and Fuller, 2006). The mechanism behind this recovery is derived from a form of respiratory plasticity called long term facilitation (LTF) which both acute and chronic intermittent hypoxia can induce. In short, LTF is a persistent increase in respiratory output after the last bout of hypoxia, lasting a long period of time (>1 h). This plasticity can manifest as an augmentation in the synaptic pathways to PMNs resulting in increased phrenic nerve activity and ventilation (Millhorn et al., 1980; Fuller et al., 2000; Mitchell et al., 2001; McGuire et al., 2003). Some of the mechanisms behind this plasticity include the activation of serotonin (5HT) and the glutamatergic NMDA receptors (Ling et al., 2001; McGuire et al., 2005; Ling, 2008).

In fact, it has been shown that 5HT and glutamate neurotransmission play important roles in respiratory plasticity and in restoring hemidiaphragmatic function following C2 hemisection. Depletion of 5HT by adding the 5HT synthesis inhibitor parachlorophenylalanine (p-CPA) can abolish morphological changes following C2 hemisection associated with the crossed phrenic phenomenon, as well as, attenuate respiratory recovery (Hadley et al., 1999a,b). Administration of 5HT receptor agonists can also induce the expression of the CPP (Ling et al., 1994; Zhou et al., 2001; Zimmer and Goshgarian, 2006). Similarly, in other models of SCI which results in respiratory deficits, administration of select 5HT-R agonists can also result in improved function (Teng et al., 2003 and Choi et al., 2005). In the case of glutamate, the NMDA receptor, and in particular the 2A subunit, has been implicated as a mediator of the recovery following chronic SCI including C2 hemisection. Upregulating the 2A subunit (NR2A) with the NMDA receptor antagonist MK-801 possibly through a process of "disuse hypersensitization" can restore function acutely (McDonald et al., 1990; Wilson et al., 1998; Alilain and Goshgarian, 2007). Chronically, the NR2A subunit is increased at the level of the PN and is correlated with the spontaneous onset of recovery (Alilain and Goshgarian, 2008). Also in these more chronic time points there is an increase of 5HT immunoreactive terminals and increased lengthening of glutamatergic terminals on PMNs in hemisected rats (Tai and Goshgarian, 1996; Tai et al., 1997; Golder and Mitchell, 2005).

While much of this work has focused on manipulation and strengthening of spared pathways to restore diaphragmatic activity after injury another, albeit elusive, therapeutic avenue exists which is the pursuit of a strategy to allow for frank regeneration of the interrupted bulbospinal axons to the phrenic motor nucleus. Indeed a significant number of laboratories in the field of SCI have focused on the regeneration of axons to denervated target nuclei, but as we will see there are the proverbial barriers to regeneration.

OBSTACLES TO THE REGENERATION OF RESPIRATORY PATHWAYS AND OVERCOMING THEM TO RESTORE FUNCTION

For the most part, without any kind of intervention, regeneration is not possible in the adult CNS. In Ramon y Cajal's seminal studies, the first descriptions of the dystrophic non-regenerating endbulbs of transected axons were made (Cajal, 1928). What was especially remarkable was that these endbulbs were formed in the face of an injury-induced milieu of reactive astrocytes and extracellular matrix molecules, which has been termed the "glial scar" (for an extensive review, please see Silver and Miller, 2004). Only when presented with a growth permissive environment can the stymied axon begin to regenerate. One especially useful substrate that robustly permits such re-growth is a segment of the peripheral nervous system (PNS) when placed into the CNS (David and Aguayo, 1981; Houle et al., 2006). This PNS "bridge" presents an alternative avenue for axonal regeneration around the lesion in SCI models.

There have been numerous attempts to promote regeneration of respiratory pathways into a PNS bridge, including bridging a cervical injury to reinnervate the PN (Gauthier and Rasminsky, 1988; Lammari-Barreault et al., 1991; Decherchi et al., 1996; Decherchi and Gauthier, 2002; Gauthier et al., 2002). It has been shown that respiratory bulbospinal axons can readily regenerate into a peripheral nerve graft into the medulla near the area of the RVRG or ventrolaterally at the C2 spinal cord. However, there was no significant functional recovery of the ipsilateral phrenic nerve owing to inadequate penetration of axons back into the CNS resulting in a paucity of reinnervated phrenic motor neurons (Gauthier et al., 2002). Later experiments by Houle et al. (2006) showed that modification of glial scar associated proteoglycans at the end of the bridge with chondroitinase ABC (ChABC) does allow for successful penetration and reinnervation back into the CNS. Following a C3 hemisection and insertion of an autologous peripheral nerve bridge, there was robust regeneration of bulbospinal axons into and out of the graft back into the C5 spinal cord as well as innervation of ventral horn motor neurons. This mediated meaningful behavioral recovery of the affected forelimb which was abolished after cutting the bridge (Houle et al., 2006).

ChABC digests glycosaminoglycans (GAGs) of the extracellular matrix class of molecules called chondroitin sulfate proteoglycans (CSPGs) (for extensive review please read Busch and Silver, 2007). A key component of the glial scar and the perineuronal net (PNN), first described by Golgi (1893), CSPGs have a strong inhibitory influence on plasticity during development as well as regeneration (Davies et al., 1997, 1999; Lander et al., 1997; Pizzorusso et al., 2002, 2006; Massey et al., 2006). Interestingly, CSPGs are increased at the site of injury as well as in the PNN enveloping denervated target nuclei where they exert their inhibitory influence. These inhibitory effects can be partially relieved through the administration of ChABC (Bradbury et al., 2002; Steinmetz et al., 2005; Barritt et al., 2006; Houle et al., 2006; Cafferty et al., 2008). In the C2 hemisected animal, ChABC administration alone also breaks down injury induced increases of CSPGs in the PNN and induces recovery through enhanced sprouting of spared pathways (Alilain et al., 2006, 2007). We are now currently attempting to combine ChABC treatment with peripheral nerve grafting to reinnervate the denervated PN and restore hemidicphragmatic function after C2 hemisection.

The rapid and intense growth of the glial scar and inhibitory proteoglycans of the PNN are just a few of the barriers to regeneration and sprouting of injured axons. Other potential factors which can contribute to CNS regeneration failure also include myelin inhibitors and infiltrating activated macrophages which actively retract axons (Brösamle et al., 2000; GrandPre et al., 2002; Horn et al., 2008). With all of these barriers to regeneration of supraspinal inputs after SCI, one might ask whether there might be another way to activate spinal motor neurons after injury without regeneration.

CHANNEL RHODOPSIN 2 MEDIATED RETURN OF FUNCTION AFTER C2 HEMISECTION

The algal protein Channelrhodopsin-2 (ChR2) is a light activated cation channel that has emerged as a powerful tool in neuroscience. Following expression in both mammalian and non-mammalian tissue, it can be induced by photostimulation to depolarize neurons, fire action potentials and control activity of neurons independent of any kind of pre-synaptic input (Boyden et al., 2005; Li et al., 2005; Zhang et al., 2006, 2007; Arenkiel et al., 2007; Herlitze and Landmesser, 2007). In SCI where one traumatic end result is diminished supraspinal input to motor neurons, we hypothesized that expression of ChR2 in spinal neurons and subsequent photostimulation might provide a powerful means to activate these otherwise quiescent cells and restore muscle activity. Therefore, in the C2 hemisection model, expression of ChR2 in the ipsilateral C3–C6 spinal cord and light stimulation could lead to a restoration of breathing activity in the fully adult animal (Alilain et al., 2008, for review please see Arenkiel and Peca, 2009).

Utilizing a sindbis virus at the same time as lesioning, approximately 600–700 neurons, including interneurons and motor neurons at the C3–C6 level of the spinal cord could be transfected to express ChR2 and GFP. Furthermore, retrograde tracing with dextran Texas red from the diaphragm revealed that phrenic motor neurons specifically expressed ChR2 as well. In the first series of experiments where we photostimulated the spinal cord, brief 1 Hz light stimulation (each flash of light being 0.5 s long) for about 30–60 s in duration was able to induce limited recovery. The restored activity was rhythmic and persisted even after photostimulation had ceased, but only up to 1 min after. Such brief hemidiaphragmatic activity could be restored multiple times in the same animal. In animals that received only the GFP control vector, there was no restoration in activity at all.

With these initial experiments as a starting point, we endeavored to induce a more long lasting type of recovery. In a stimulation protocol that we later termed "long light stimulation", we found that a pattern of 5 min baseline (no light) followed by 5 min of intermittent light (0.5 Hz, 1 s on, 1 s off) for at least three cycles led to a remarkable type of plasticity that eventually led to restored breathing by the initially paralyzed hemidiaphragm. After this stimulation protocol and sometimes during, a small amount of electromyographic (EMG) activity was detected in the hemidiaphragm ipsilateral to the lesion – including small but distinct bouts of activity coinciding with the pattern of breathing. This

activity would wax and wane in intensity in a highly regular fashion. Furthermore, evidence from the bilateral EMG recordings, suggested that there were changes in the contralateral (opposite the side of the lesion) hemidiaphragm as well. As the firing intensity declined on the lesioned side, the non-lesioned side would increase in intensity, and vice versa. This oscillating pattern continued to increase until there was a storm of EMG activity. Surprisingly, once past this peak activity, what remained was restored breathing that was rhythmic and synchronous, with significant increases in the duration and amplitude of each inspiratory burst. After restoring breathing activity with long light stimulation and then allowing the animal to recover for 1 day, we found that restored diaphragm function persisted for at least 24 h. The phenomenon of oscillating increases in activity bilaterally was independent of the injury since it could be evoked in an uninjured animal as well.

At the heart of this plasticity is the NMDA receptor, the long recognized mediator of plasticity and learning in the CNS (Seeburg et al., 1995; Tang et al., 1999). When the NMDA receptor antagonist MK-801 was applied prior to photostimulation there was no induction of the seizure-like activity and no restoration of breathing. From this set of data we hypothesized that photostimulation and subsequent depolarization is enough to relieve the NMDA receptor of the voltage sensitive Mg++ block and allow for the influx of Ca++ to occur, resulting in a signaling cascade that results in enhanced neurotransmission in the localized spinal region as well as super sensitivity to spared pathways, including the CPP. Taken together these experiments and observations demonstrate a capacity for spinal respiratory plasticity and adaptation that previously had been unknown or only speculated upon.

INSIGHTS INTO RESPIRATORY CIRCUITRY AND PLASTICITY

The transformation from the absence of activity in the paralyzed hemidiaphragm to the increasing waxing and waning of activity and oscillations between both sides, to rhythmic breathing activity demonstrates a remarkable form of plasticity in the adult spinal cord. It indicates a highly sophisticated level of connectivity between the two sides of the cord where one side can influence the other possibly through detection of the levels of activity. While the anatomical substrate that mediates this crossed communication is unknown, it is likely due to contralaterally projecting neurites from motor neurons and/or interneurons which were detected with GFP expression – and which are just now being described in the rodent (**Figure 1**) (Lane et al., 2008). What is also interesting as a possibility is that this newly described circuitry may play a role in generating a spinal respiratory central pattern generator.

While central pattern generation has been widely studied during locomotion, a localized spinal respiratory central pattern generator has only been speculated to occur (Aoki et al., 1980; Reinoso et al., 1996). It is conceivable that with our photostimulation pattern we are only igniting the first step in a sequence of events that begins to incorporate more and more spinal neurons and circuitry (that does not necessarily have the ChR2 protein), which is akin to the induction of seizure activity in models of epilepsy (Bertram, 2007). With this recruitment and activation we can begin to see this crude and vestigial manifestation of central pattern generated respiration. Furthermore, the process by which the circuitry corrects and rights itself and produces the refined, rhythmic, and synchronous activity of normal respiratory activity – be it adaptation or learning – is incredibly fascinating and will lead to more insight of spinal cord networks.

THE FUTURE

With the tools of molecular neuroscience and the emergence of light sensitive channels and proteins, its combined potential as a therapeutic application in CNS trauma and disease is only now being uncovered. Parkinson's disease and deep brain stimulation, epilepsy, and SCI are just a few of the injury models where there is a potential use for these technologies. With SCI, these powerful applications can be used to target interneurons and denervated motor neurons in the spinal cord so that they can express light sensitive proteins to activate them; or cell bodies in both spinal and supraspinal centers whose axons are damaged or spared to induce activity dependent plasticity and enhance connectivity. (Brus-Ramer et al., 2007). Furthermore, although, as we have shown in the C2 hemisection model of SCI, both pharmacotherapy and environmental changes, i.e. intermittent hypoxia, have induced recovery of the paralyzed hemidiaphragm, the nature of these therapies have the repercussions of system-wide alterations in the animal – not just in the spinal cord. Some of these effects can be deleterious (Gozal et al., 2001; Row et al., 2002). With the tools of molecular neuroscience, this concern can become irrelevant with the use of distinct promoters and the targeting of specific neuronal populations. Finally, utilization of these techniques can help us better understand the circuitry and network properties of the spinal cord and CNS, in both injured and uninjured paradigms. We are just now beginning to appreciate the sophistication of remodeled spinal pathways and other pathways that might be spared after SCI and fully exploring its nature can only help us to restore function and hopefully improve the quality of life of the SCI community (Courtine et al., 2008; Rosenzweig et al., 2009).

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Engineered G-protein coupled receptors are powerful tools to investigate biological processes and behaviors

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Understanding how discreet tissues and neuronal circuits function in relation to the whole organism to regulate physiological processes and behaviors is a fundamental goal of modern biological science. Powerful and important new tools in this discovery process are modified G-protein coupled receptors (GPCRs) known as 'Receptors Activated Solely by Synthetic Ligands (RASSLs),' and 'Designer Receptors Exclusively Activated by a Designer Drug (DREADDs).' Collectively, these are GPCRs modified either through rational design (RASSLs) or directed molecular evolution (DREADDs), that do not respond to native ligand, but functionally respond only to synthetic ligands. Importantly, the utility of these receptors is not limited to examination of the role of GPCR-coupled effector signal transduction pathways. Due to the near ubiquitous expression of GPCRs throughout an organism, this technology, combined with whole animal transgenics to selectively target expression, has the ability to regulate activity of discreet tissues and neuronal circuits through effector pathway modulation to study function and behavior throughout the organism. Advantages over other systems currently used to modify *in vivo* function include the ability to rapidly, selectively and reversibly manipulate defined signal transduction pathways both in short term and long term studies, and no need for specialized equipment due to convenient systemic treatment with activating ligand.

Keywords: G-protein coupled receptors, Receptors Activated Solely by Synthetic Ligands, Designer Receptors Exclusively Activated by a Designer Drug, signal transduction, muscarinic receptor, opioid receptor, serotonin receptor, transgenic

INTRODUCTION

The perturbation of normal biological processes has been one of the primary strategies employed to investigate the role of specific genes, proteins, tissues, and neural circuits in the intact organism or system. A primary methodology long used to modify neuronal activity and function has been with pharmacological tools. The discovery and utilization of agonist and antagonist ligands selective for specific neurotransmitter receptors and related signaling pathways and processes has allowed researchers to probe the role of select neuronal circuits in CNS function with tremendous power. Even so, there are certain limitations associated with pharmacological treatments, especially *in vivo*, that include off target affinities and undesired side-effects effects of drugs, and inability to effectively target a drug to act only in a restricted subset of tissues or neuronal circuits normally expressing the target. A more recent method to study neuronal function has involved the creation and use of transgenic animals. Initially it was possible to only knock-out a gene or knock-in a modified gene systemically. Modified genes usually represented hypo- or hyperactive versions, or particular splice isoforms. Subsequent developments both in insect and mammalian models allowed for the creation of expression systems where, through the use of a bipartite genetic system, transgene expression can be induced in defined tissues, and conditionally at defined time points by the administration of a drug, hormone, or temperature shift (Brand and Perrimon, 1993; Wells and Carter, 2001; McGuire et al., 2004; Aiba and Nakao, 2007; Gaveriaux-Ruff and Kieffer, 2007). Advantages of inducible genetic systems over constitutive ones include more precise control of expression and potential assessment of reversibility of expression. Targeted inactivation and activation of neurotransmitter related genes, as well as those for other neuronally expressed genes, has allowed for more precise elucidation of the function of neurotransmitters and circuits within the CNS of the intact organism. Other approaches that involve selective expression of toxins within defined neural circuits have also been informative (Drago et al., 1998; Martin et al., 2002; Yu et al., 2004; Nakashiba et al., 2008). These methods, however, are not without their limitations. Non-inducible systems are often associated with confounding developmental issues, inducible systems are slow to turn on and off, eliminating expression of an endogenous gene or introduction of a constitutively active isoform can have confounding effects on a particular cell's physiology with respect to its role in neuronal function, and toxins can damage or kill the neurons they are expressed in.

Recently, transgenic technologies have been used to create novel systems able to primarily influence neuronal activity states. These systems rely upon targeted expression of particular receptor proteins that, when activated, can inhibit or enhance neuronal activity. These approaches are more systems-based, allowing for functional analysis of distinct neuronal circuits as a whole, rather than examination of the role of a particular gene/protein within a neuron. One strategy has been to use potassium channels to inhibit neuronal activity. Both targeted expression of modified constitutively open potassium channels in Drosophila (Nitabach et al., 2002), and targeted expression of the inward rectifying Kir2.1

potassium channel (Yu et al., 2004) in mice, have been found to be effective in silencing neuronal activity. To modulate neuronal activity, a system has been developed to target expression of $GABA_A$ chloride channels that respond to the allosteric modulator zolpidem within a transgenic mouse model insensitive to zolpidem (Wulff et al., 2007). Difficulties with these approaches include potential developmental effects of constitutively expressed channels, and temporal induction methods of expression can take days or weeks to become effective.

A very interesting approach to perform targeted modification of neuronal activity has involved the use of light to activate ion channels and proteins. One method utilizes targeted expression of a light-activated non-selective cationic channel protein isolated from unicellular green algae, channelrhodopsin, to excite neuronal activity. (Nagel et al., 2003, 2005; Schroll et al., 2006; Lin et al., 2009). Whereas this is a very powerful method for rapidly activating neurons, channelrhodopsins are useful only for short-term neuronal modulation because they are hindered by rapid desensitization. Another light-based method that is used to promote neuronal inhibition uses lentiviral mediated expression of the light-activated halorhodopsin chloride pump from the microorganism *Natronomonas pharaonis* to hyperpolarize neurons in the CNS (Tonnesen et al., 2009). The most recent of these optical techniques are a collection of chimeras between rhodopsin and β_2 and α_1 adrenergic G-protein coupled receptors (GPCRs). Lentiviral mediated expression of these optoXR proteins in CNS tissues was found to modify firing rates of neurons in slice culture upon exposure to light consistent with the effects of neuronal Gαs or Gαq signal pathway activation to enhance or decrease firing respectively (Airan et al., 2009). Advantages of these optic techniques include very rapid activation, however, limitations include poor penetration of light into whole organisms and the need of specialized equipment including light sources and fiber optics. Furthermore, lentiviral methods involve surgical procedures and the effects are usually transient.

Other studies have employed transgenic expression of certain wild type GPCRs to induce neuronal silencing. For example, selective targeted restoration of $5\text{-}HT_{1A}$ serotonin receptor expression within a $5\text{-}HT_{1A}$ receptor knockout background followed by convenient administration of receptor selective agonist has been found to be effective (Tsetsenis et al., 2007) in mice. Similarly, selective targeting of different Drosophila 5-HT receptors to defined tissues has also had some success to modulate tissue function (Kerr et al., 2004). Whereas these methods utilizing native GPCRs are a promising and powerful avenue, care must be taken with respect to data interpretation due to potential confounding effects of endogenous neurotransmitters, and some of these models require specialized genetic backgrounds. Another approach involves heterologous targeted expression of the *Drosophila melanogaster* allostatin peptide GPCR within the mouse CNS followed by application of allostatin, an insect peptide hormone not normally found in mammals, to induce neuronal silencing in the CNS (Tan et al., 2006; Wehr et al., 2009). Peptides, however, have limited use because of their low solubility and must therefore be directly applied to tissues of interest.

A new approach to study biological function and behaviors that circumvents many of the disadvantages of other technologies has been developed that combines the tissue specificity of transgenics with the rapid and reversible effects of pharmacological agents. The methodology involves targeted expression of GPCRs that have been modified to respond only to non-endogenous chemicals to rapidly and reversibly modulate effector pathway activity in defined tissues and neural circuits. Importantly, these receptors can be used to produce both short-term and long-term modulation of activity, and only require simple and convenient systemic administration of ligand by feeding or peripheral injection. Invasive procedures including stereotactic injection, and specialized equipment like fiber optics and light sources are not required. These modified G-protein receptors receptors have been termed: 'Receptors Activated Solely by Synthetic Ligands (RASSLs)' and 'Designer Receptors Exclusively Activated by a Designer Drug (DREADDs)' (**Table 1**).

GPCRs

G-protein coupled receptors are 7-α-helical transmembrane proteins that transduce and amplify extracellular signals to multiple pathways inside the cell. GPCRs are the most widespread receptor class throughout the organism, and modulate not only cellular processes directly, but also the function of other receptor families, and are the primary mechanism of communication between cells (Kroeze et al., 2003; Armbruster and Roth, 2005). The physiological processes that GPCRs modulate are diverse and include neurotransmission, development, cardiovascular function, gut motility, and odor detection, among others. About 80% of hormones and

Table 1 | The major RASSL and DREADD receptors grouped according to the primary G-protein activated. The parent receptor of each is listed.

Name	Parent receptor	Effector	Reference
α_{2A}-AR	α_2-Adrenergic	Gαi	Pauwels (2003)
Ro1, Ro2	κ-Opioid	Gαi	Coward et al. (1998)
Rog, Rog-μ, Rog-μA	κ-Opioid	Gαi	Scearce-Levie et al. (2005)
hM_4D	hM_4 Muscarinic	Gαi	Armbruster et al. (2007)
β_2-AR-TREC	β_2-Adrenergic	Gαs	Small et al. (2001)
m $5\text{-}HT_{4A}$	m $5\text{-}HT_{4A}$	Gαs	Claeysen et al. (2003)
Rs1, Rs1.1–3	h $5\text{-}HT_{4B}$	Gαs	Chang et al. (2007)
Rm1, Rm2	MCR4	Gαs	Srinivasan et al. (2007)
GsD	rM_3	Gαs	Geuttier et al. (2009)
H_1	H_1	Gαq	Bruysters et al. (2005)
hM3D	hM_3	Gαq	Armbruster et al. (2007)

neurotransmitters involved in signal transduction are thought to act through GPCRs. Significantly, >50% of current drugs on the market target GPCR function as a major mechanism of therapeutics (Roth et al., 2004; Strachan et al., 2006). Thus, GPCRs remain the most popular family of targets used in drug discovery.

G-protein coupled receptors are functionally coupled to heterotrimeric G-proteins at the intracellular loops and C-terminus of the GPCR. Heterotrimeric G proteins are comprised of a Gα subunit and a dimeric Gβγ subunit, and are grouped into four classes: Gαs, Gαi/o, Gαq/11, and Gα12/13 (Gilman, 1987). Whereas Gαs stimulates the production of cAMP through activation of adenylate cyclase, Gαi/o produces the opposite effect, and reduces levels of intracellular cAMP through inhibition of adenylate cyclase. The Gαq subunit stimulates phospholipase-Cβ, which catalyzes the production of phosphoinositides and the release of intracellular calcium, among other processes. Gα12/13 interacts with a number of effectors including RhoGEFs to modulate cell growth and cytoskeleton structure (Kelly et al., 2007).

Because of the widespread expression of GPCRs (Regard et al., 2008), and the variety of effector pathways that they can couple to (Urban et al., 2007), targeted expression of modified GPCRs is an ideal tool to utilize to probe not only the role of how specific signal transduction pathways influence cellular function, but to also probe the role of specific tissues and neuronal circuits underlying physiological and behavioral processes. The primary RASSL/DREADD strategy is to engineer a collection of modified GPCRs, each coupling to different primary effector pathways, that respond to synthetic or non-endogenous compounds.

RASSLs

The first published report on the creation of a modified GPCR to respond to a non-endogenous ligand was by Strader et al. (1991). In the process of exploring the nature of ligand-receptor interactions for the β2-adrenergic receptor, a single point mutant (S113A) was found to eliminate binding of endogenous ligands while simultaneously conferring activity for a class of compounds with little affinity for the wild type receptor (Strader et al., 1991). Whereas the potencies of these synthetic compounds was relatively low at the modified β2AR, this work nevertheless demonstrated that GPCRs could be modified to lose native ligand recognition while maintaining affinity for synthetic ligands. The Strader et al. (1991) study was thus an important proof-of-concept study for this approach.

The next major development was the creation of modified human kappa-opioid receptors with dramatically reduced affinity for the natural peptide ligands (1000-fold reduction), but retained affinity for small molecule drugs like bremazocine and spiradoline (Coward et al., 1998). Two receptor variants were created: Ro1 and Ro2. Ro1 is a chimeric receptor of the κ-opioid receptor containing the second extracellular loop of the delta opioid receptor, and Ro2 is essentially the Ro1 receptor with an additional mutation at the top of the sixth transmembrane helix. The overall design of the modified receptors arose from previous studies of chimeras between κ and δ receptors indicating that the second extracellular loop contains a major determinant for binding of a κ selective ligand, dynorphin. What was achieved by this manipulation was a Gαi-coupled κ-opioid receptor with significantly reduced binding to κ-specific peptides, but retained affinity for κ-selective small molecule synthetic ligands whose binding regions were not determined by the second extracellular loop region (Coward et al., 1998). These receptors were termed RASSLs, and when expressed in rat fibroblast cells found to be functional for inducing proliferation, a process known to be stimulated by Gαi signaling, with the synthetic ligand spiradoline (Coward et al., 1998).

Many studies using the Ro1 RASSLs were subsequently published utilizing this receptor as a tool to explore the effects of activating the Gαi effector pathway in various tissues as defined by specific transgene targeting (Conklin et al., 2008; Pei et al., 2008). The first transgenic mouse study detailed the conditional expression and activation of the Ro1 receptor in mouse heart, liver, and brain (Redfern et al., 1999). Activation of Gαi signaling in the heart with the κ-opioid receptor agonist spiradoline produced significant and dose dependent bradycardia with a ~50% reduction in heart rate in less than a minute after drug administration (Redfern et al., 1999). In a subsequent study, it was found that the induction of expression of Ro1 in the heart induces cardiomyopathy in the absence of spiradoline (Redfern et al., 2000). Partially inhibiting expression of Ro1, treatment with the κOR antagonist nor-binaltomorphine, and treatment with pertussis toxin, restored normal function demonstrating that the heart defects were indeed due to excessive Gαi signaling from the Ro1 receptor in the absence of ligand stimulation (Redfern et al., 2000). Significantly, these experiments indicate that there is a level of basal constitutive activity associated with the Ro1 receptor that is able to influence physiological processes in the absence of pharmacological activation. Nevertheless, this system has proven valuable as a new model for the study of dilated cardiomyopathy (Baker et al., 2001; McCloskey et al., 2008). The inducible doxycycline-responding Ro1 mouse strain was also utilized by Sweger et al. (2007) to investigate the role of astrocytes in mouse brain. They created a transgenic κOR knockout mouse with conditional expression of Ro1 in astrocytes induced by discontinuation of feeding doxycycline and found that even in the absence of the κOR agonist spiradoline the mice developed severe hydrocephalus (Sweger et al., 2007) through mechanisms regulating CSF production, defining a new role for Gαi in astrocytes. Importantly, these results independently confirmed that the Ro1 receptor is associated with a certain physiologically relevant level of constitutive activity.

This constitutive activity of Ro1 was exploited to investigate the role of Gαi signaling in bone development by Peng et al. (2008). It had been previously known that inactivation of Gαs signaling, the main effector pathway coupled to the PTH/PTHrP (PPR) receptors in osteoblasts, resulted in a reduction of bone formation and turnover (Sakamoto et al., 2005). Furthermore, that various means of indirectly inhibiting Gαi pathways, increased bone turnover (Peng et al., 2008). To *directly* test the role of Gαi signaling, the Ro1 receptor was conditionally expressed in mouse osteoblasts at various developmental time points from embryogenesis to weaning. At each time point, expression of the Ro1 receptor resulted in decreased bone formation and turnover (Peng et al., 2008), mimicking the effects of Gαs inactivation. Together, these results indicate that proper bone formation results from a balance between Gαi and Gαs signaling activity in osteoblasts (Peng et al., 2008).

An interesting study using the doxycycline-responding Ro1 transgenic mice created by Redfen and colleagues was to examine the nature of the sweet and umami taste. Here, Ro1 was expressed in taste buds under the control of the T1R2 receptor promoter in T1R2 null mice. These mice responded to the κ-selective agonist spiradoline as control mice did to sweet taste, indicating that Gαi signaling through the T1R2 receptor is responsible for perception of sweet taste (Zhao et al., 2003).

Following the success of the initial Ro1 receptor, attempts were made to modify it. First, the Rog (RASSL opioid green) receptor was created by fusing GFP to the N-terminus of Ro1 to visualize receptor localization in the living cell (Scearce-Levie et al., 2005). After validating its ability to internalize upon agonist stimulation, signaling, and RASSL function, subsequent modifications included mutation of the four C-terminal phosphorylation sites to alanine (Rog-A), replacement of the entire C-terminus with the μ-opioid receptor (Rog-μ), and mutation of five C-terminal serine and glutamic acid residues of the Rog-μ receptor to alanine (Rog-μA) (Scearce-Levie et al., 2005). The loss of C-terminal phosphorylation sites in the Rog-A resulted in a significant reduction in agonist induced internalization and a resistance to desensitization, however maximal cAMP inhibition was unaffected (Scearce-Levie et al., 2005). The Rog-μ receptor demonstrated increased agonist induced receptor internalization, as was predicted based upon the ability of the μ receptor to more readily internalize than the δ receptor (Scearce-Levie et al., 2005). Whereas the Rog-μA receptor was predicted to be resistant to agonist induced internalization as the Rog-A receptor was, it surprisingly demonstrated constitutive internalization (Scearce-Levie et al., 2005). Addition of an antagonist, nor-BNI, was found to rescue cell surface expression (Scearce-Levie et al., 2005). Another physiological effect, adenylate cyclase superactivation, was examined in these receptors. Long-term activation of Gαi signaling pathways can lead to an increase in adenylate cyclase activity and an enhanced response to forskolin stimulation over baseline conditions (Watts and Neve, 2005). Neither the Rog-μ nor Rog-μA receptors showed this effect after overnight treatment with spiradoline followed by forskolin stimulation (Scearce-Levie et al., 2005). The authors speculated that it is not constitutive activity of Gαi from the Ro1 receptors that is responsible for cardiomyopathy observed in their previous studies, but rather an increase in Gαs signaling through adenylate cyclase superactivation that produces the phenotype (Scearce-Levie et al., 2005). It is possible that these newly modified Ro1-related RASSLs will address this issue as well as others.

Through a series of structure/function studies, another modified Gαi coupled receptor, the α_{2A}-adrenergic receptor, was demonstrated to have RASSL-like properties. Based upon previous work predicting the importance of two conserved serine residues within the putative transmembrane binding site the serine at position 200, and the serine at position 204 were each mutated to alanine and the resulting variants pharmacologically characterized. The resulting receptors had reduced (S200A), to negligent (S204A) affinity and activity for the native ligand, but high affinity and activity for certain classes of drug including synthetic imidazoline derivatives (Pauwels and Colpaert, 2000). Interestingly, several α_{2A}-adrenergic receptor antagonists, including atipamezole and SKF86466, retained antagonist properties at the S200A variant but demonstrated significant partial agonist activity at the S204A receptor (Pauwels and Colpaert, 2000). Significantly, these studies demonstrated that aspects of functional selectivity can be potentially engineered in to a RASSL where the response of a receptor to a particular ligand is not only lost or retained in mutant RASSL variants, but can be fundamentally altered to a different response.

There have been three independent RASSL families developed to probe Gαs signaling. The first was an attempt to modify the β2-adrenergic receptor as a tool for use in gene therapies and create a 'modified therapeutic receptor–effector complex'. In the human β2-adrenergic receptor, glutamine at position 27 was mutated to glutamate to reduce ligand-induced receptor internalization, aspartate at position 113 was mutated to serine to alter ligand binding properties, 15 potential phosphorylation sites were changed to alanine, and the entire open reading frame of the rat Gαs was fused to the C-terminus coding region (Small et al., 2001). This modified receptor complex was found to be unresponsive to catacholamines, but responsive to a single tested ligand L158870 at micromolar concentrations (Small et al., 2001). Due to the low potency of the modified receptor complex to the synthetic ligand its utility *in vivo* is likely limited.

The second approach at developing a Gαs RASSL involved modifying the serotonin 5-HT_{4A} receptor. Key amino acids in the native ligand binding pocket were deduced based upon the published crystal structure of the β-adrenergic receptor to identify Asp^{100} in TM3, which was predicted to interact with the amine in the indole ring of serotonin. Mutation of this Asp to Ala in the mouse 5-HT_{4A} receptor significantly reduced binding as well as activation of the receptor by serotonin and other tryptamines with respect to adenylate cyclase activation, while maintaining affinity and activity for other ligands without a protonated amine in the core structure (Claeysen et al., 2003). Interestingly, a number of antagonists at the native receptor were found to have agonist activity with respect to adenylate cyclase at the modified receptor (Rs1) (Claeysen et al., 2003). This may allow for selective activation of the modified receptor in animal models, while keeping native receptors inactive, something not possible with the Ro1 receptors. In a subsequent study, conditional expression of the Rs1 receptor in transgenic mouse osteoblasts resulted in increased bone mass in the absence of activating ligand, presumably by constitutive Gαs signaling from the Rs1 receptor (Hsiao et al., 2008). These bone-enhancing effects were observed to be different from a mouse expressing a constitutively activated form of the Gαs-coupled PPR receptor, indicating that additional factors are differentially recruited to the receptors to mediate physiological effects.

To refine the RASSL characteristics of a receptor like Rs1, a series of modifications of the human 5-HT_{4B} D100A receptor, which has high constitutive adenylate cyclase activity, were created (Chang et al., 2007). To reduce constitutive activity two separate mutations were made, based upon previous reported studies examining structure and activity, changing Asp at position 66 to Asn (Rs1.2), and Trp at position 272 to Ala. Although each resulting RASSL had significantly reduced constitutive adenylate cyclase activity, there was also a large reduction in the levels of activation by the ligand zacopride, limiting their utility (Chang et al., 2007). Nevertheless, the RASSLs were significantly stimulated by agonist. An interesting feature of the Rs1 receptor includes functional selectivity where

certain classes of ligand predominantly activate Gαs, while other classes, represented by zacopride, also induce coupling to Gαq. Surprisingly, the ability of zacopride to induce coupling to Gαq was lost in the two mutant 5-HT_{4B} RASSLs (Chang et al., 2007).

In an attempt to create a predominantly Gαq-coupled RASSL, the intracellular loops of the human Rs1 were replaced with those from the Gαq-coupled human 5-HT_{2C} receptor to create Rs1-5-HT_{2C} (Rs1.1). In this process, 12 different chimeras were created and tested that each had different splicing junctions and combinations of intracellular loops. It was found that replacement of the second or third intracellular loops eliminated both Gαs and Gαq activity, and only replacement of the C-terminus was able to increase coupling to Gαq (Chang et al., 2007). This new receptor, Rs1-C-5-HT_{2C}, however, still had significant constitutive and ligand-induced Gαs activity. Following similar techniques utilizing intracellular domains from the human 5-HT_{1A} receptor, attempts were made to engineer a Gαi-couple RASSL from Rs1. Replacement of the third intracellular loop was found to abolish Gαs and Gαq activity and to confer Gαi activity to the Rs1-i3-5-HT_{1A} (Rs1.3) receptor (Chang et al., 2007). The potency of this effect, however, was weak thus limiting the utility of this receptor. Importantly, these studies with the Rs1 series of receptors demonstrated that it was possible to continue to modify a RASSL to alter and refine physiological properties to something more desirable as a tool for *in vivo* use.

The third approach to develop a Gαs coupled RASSL involved modifying the melanocortin-4 receptor (MCR4). An extensive review of previous work examining the location and functional result of individual mutations in the MCR4 led to the identification of five candidate mutations for further study as potential RASSLs (Srinivasan et al., 2007). Of these, two were designated RASSLs: L106P-MCR4 (Rm1), and D122A-MCR4 (Rm2). The native RCM4 has significant constitutive basal activity associated with Gαs signaling, and although Rm1 has a 30% reduction in activity, Rm2 has a nearly twofold increase in basal activity over the native receptor (Srinivasan et al., 2007). Importantly, neither Rm1 nor-Rm2 respond to native melanocortin peptides, but do respond to nanomolar concentrations of the synthetic MCR4 selective ligand THIQ with respect to measured cAMP release (Srinivasan et al., 2007).

The third major class of G-protein effector is Gαq, and one attempt has been made to generate a RASSL coupled to this pathway. Based upon previous structure function studies examining ligand binding properties, the phenylalanine at position 435 of the human H_1 receptor was mutated to an alanine and the resulting receptor characterized (Bruysters et al., 2005). Rather than assess Gαq coupling by measuring PI turnover or calcium mobilization, a luciferase reporter assay for NFkB activity was used to measure activity. This was based upon previous work that demonstrated direct functional coupling of Gαq/11 to NFkB activation (Bakker et al., 2001). Therefore, RASSL activity from this receptor can only be reliably extended to activation of the NFkB signaling pathway. Interestingly, whereas the F453A H_1 receptor variant maintained affinity for histamine, affinity and potency at NFkB activation increased up to 1000-fold for certain synthetic ligands (Bruysters et al., 2005). In the case of ClPheHA, potency was increased from an EC_{50} of ~1 mM at the native receptor to ~1 nM at the F453A variant. Rather than engineering loss of affinity and potency for native ligand while retaining those properties for a synthetic ligand, greatly enhancing affinity and potency for a synthetic ligand through receptor modification was demonstrated here to be a new strategy to develop a RASSL. The F453A H1 receptor has negligent basal constitutive activity with respect to NFkB signaling, however, additional pathways coupled to Gαq have yet to be investigated, as well as the utility of this receptor to study *in vivo* processes.

Of significant concern with nearly all RASSLs created thus far is the presence of physiologically relevant basal constitutive activity with the primary Gα effector protein. Whereas this may effectively allow functional analysis of an effector pathway under certain conditions, constitutive activity present from embryogenesis has the potential to seriously confound results examining acute function. Another considerable shortcoming of the conventional RASSLs is that they by and large utilize synthetic ligands that have significant affinity and potency for the endogenous wild type receptor as well as potential off-target actions. Therefore, ligands used to stimulate RASSLs *in vivo* could induce behaviors and physiological processes from native receptors and confound results. Nonetheless, based on the large number of important papers, conventional RASSLs continue to be widely used with great success.

DREADDs

Ideally, a modified receptor would have no basal constitutive activity, and respond to a synthetic ligand that had no natural targets within the organism. To develop such a receptor system we followed a novel directed molecular evolution approach to generate a collection of modified GPCRs termed DREADDs (Armbruster et al., 2007). This strategy involved choosing a biologically inert metabolite of clozapine, clozapine-*N*-oxide (CNO) as the synthetic ligand, and the human M3 receptor (M3R) for initial mutagenesis. Whereas the parent compound clozapine has affinity at multiple GPCRs, including high affinity and weak partial agonist activity for the M3R, the metabolite CNO was shown to lack appreciable affinity for any receptor by screening of nearly the entire relevant recepterome (Armbruster et al., 2007). A yeast model system where activation of the heterologously expressed modified rat M3R was required for cell growth (Erlenbach et al., 2001) was used as a screening platform for random mutagenesis to generate a set of mutants that were each activated by clozapine. A subset of these clones were selected for a second round of mutagenesis and selection for activation by 1 μM CNO, followed by a third round of mutagenesis and selection for growth on 5 nM CNO to generate a final collection of two modified M3 receptors with high affinity for CNO (Armbruster et al., 2007) (**Figure 1**). Fortuitously, loss of affinity for the native ligand, acetylcholine, as well as carbachol was lost during this process. Whereas these modified rat M3 receptors demonstrated functional activation by CNO and insensitivity to acetylchole, they had high basal activity with respect to Gαq signaling and PI turnover in human HEK T cells. Based upon the results of the yeast screening experiments, a focused library of mutant full length human M3 receptors was created and screened in HEK T cells to identify a receptor demonstrating high affinity for CNO, extremely low affinity for acetylcholine, and minimal constitutive activity associated with Gαq signaling and PI turnover (Armbruster et al., 2007). The final receptor, hM_3D, contained two point mutations with tyrosine

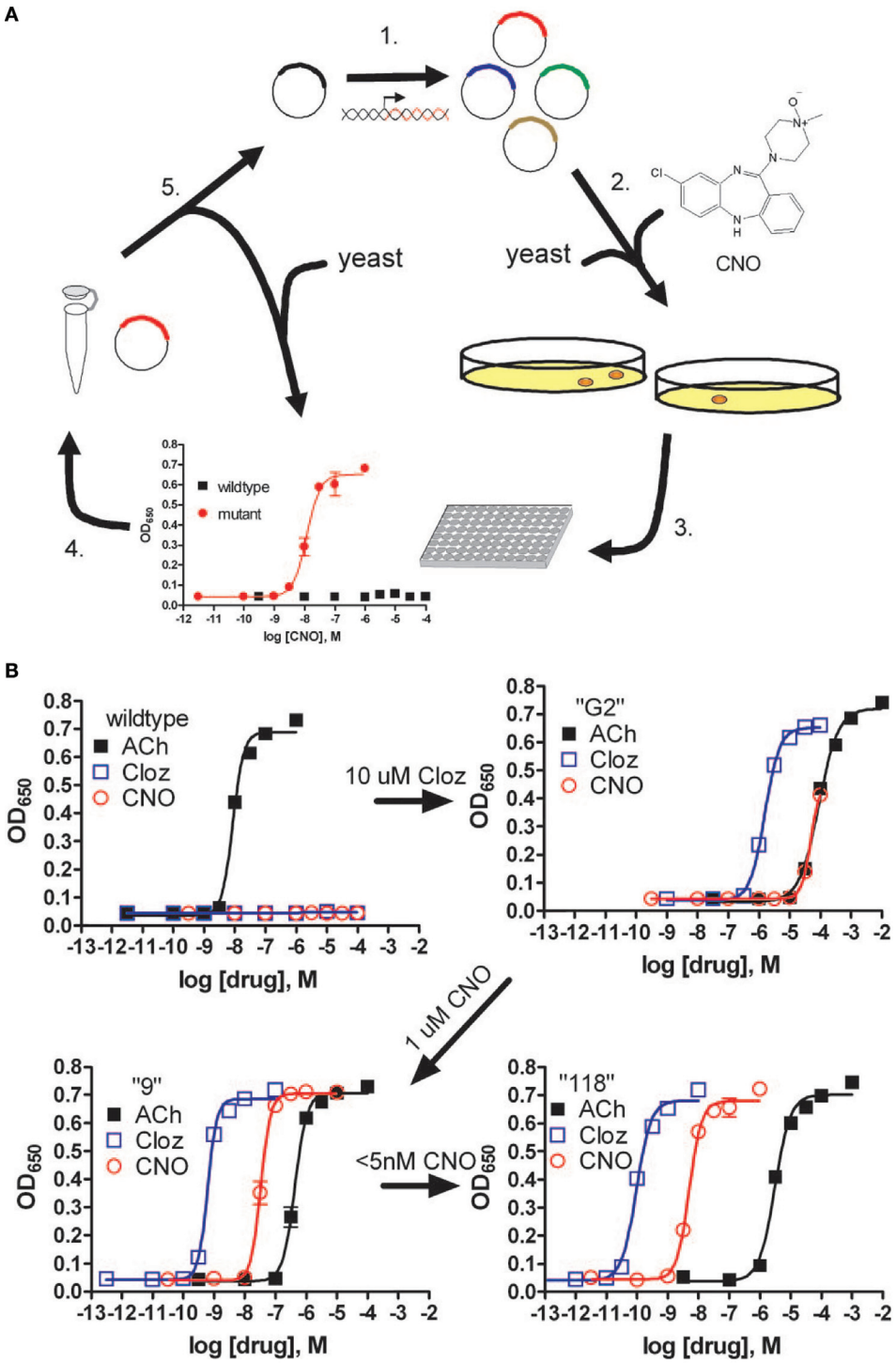

FIGURE 1 | Pharmacological profiles of an rM3Δi3 receptor mutant selected during directed molecular evolution for CNO responsiveness. (A) Experimental design for directed evolution of mammalian GPCRs in yeast to create DREADDs. (1) Libraries of randomly mutated rM3Δi3 receptors were produced by mutagenic PCR; (2) yeast-expressing mutant receptors activated by synthetic ligands (e.g., CNO) were selected for by growth on nutrient deficient medium; (3) mutants were verified by secondary liquid growth assays in 96-well plates; (4) plasmid DNA was isolated from yeast; (5) clones were retransformed into yeast to pharmacologically profile mutants by liquid growth assays, and those with desirable properties were sequenced and remutagenized for subsequent rounds of selection to yield receptors with higher potency. **(B)** Optical density at 650 nm of liquid cultures of yeast transformed with either wild type, clone "G2" (first library, 10 μM clozapine screen), clone "9" (second library, 1 μM CNO screen), or clone "118" (third library, 5-nM CNO screen) rM3Δi3 receptors incubated with ACh (■), clozapine (□), or CNO (E). Data shown are mean ± SEM values of a representative experiment performed with two independent yeast transformants grown for each clone. Figure used with permission: Copyright (2007) National Academy of Sciences, USA Armbruster et al. (2007).

at position 149 mutated to cystine, and alanine at position 239 mutated to glycine (Armbruster et al., 2007). Importantly, not only was CNO able to activate Gαq signaling and PI turnover, but CNO was also shown to activate MAPK signaling through interactions with β-arrestin, indicating that coupling to multiple effector pathways was preserved in the DREADD receptor. Mutation of the conserved tyrosine and glycine residues in the other human muscarinic receptors resulted in the creation of hM_2D and hM_4D DREADD receptors coupled to Gαi/o, and hM_1D coupled to Gαq. Additional functionality of the hM_4D was determined by demonstrating that stimulation of the receptor with CNO, but not with ACh, activated inwardly rectifying potassium channels (GIRKs) in both transfected HEK cells and transfected hippocampal neurons (Armbruster et al., 2007). These results indicate that the hM_4D receptor has potential as a tool for *in vivo* neuronal silencing.

To develop the final Gαs coupled DREADD (GsD), we modified the rat M_3D receptor by replacing the second and third intracellular loops with the corresponding loops from the Gαs-coupled turkey β_1-adrenergic receptor (Geuttier et al., 2009). Transgenic mouse lines expressing the hM_3D and GsD receptors in pancreatic beta-cells have been made that show stimulation of either receptor has significant effects on beta-cell function including aspects of glucose tolerance and insulin release (Geuttier et al., 2009). Additional transgenic mouse studies with all three DREADD receptors are currently being performed to analyze the role of G-protein signaling in a variety of tissues (**Figure 2**). We have demonstrated remote control of neuronal activity in mice expressing the hM_3D receptor in hippocampus (Alexander et al., 2009). Administration of CNO to these transgenic mice led to both increases in hippocampal neuronal activity as well as behavioral modifications including increased locomotor activity and seizures in a dose dependent fashion (Alexander et al., 2009). In slice cultures, the increases in neuronal activity from a single pulse of CNO returned to baseline levels in about 60 min. The locomotor effects of systemic CNO, however, did not return to baseline levels for about 9 h (Alexander et al., 2009). Importantly, these data indicate that this system is reversible with *in vitro* cellular effects of CNO extinguishing more rapidly than the *in vivo* behavioral effects.

We have recently created transgenic *Drosophila melanogaster* expressing the Gαs-coupled hM_4D, Gαi-coupled rM_3D-βar, and Gαq-coupled hM_1D receptors under the control of the bipartite GAL4/UAS system. The GAL4/UAS system is a genetic method to

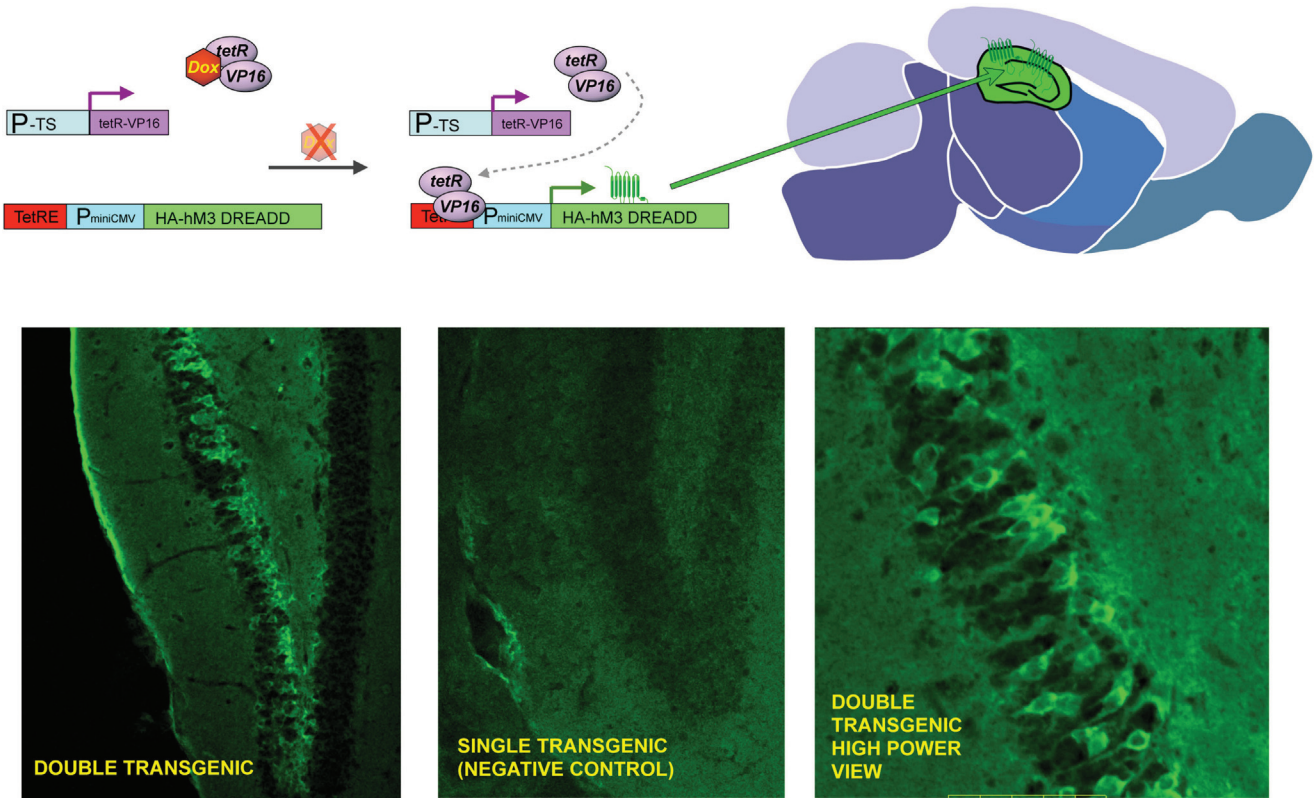

FIGURE 2 | General strategy to achieve inducible, tissue-specific expression of DREADDs. The upper panel shows the basic strategy to generate tissue specific expression of DREADDs in mouse brain. Double transgenic animals are made where one element contains a tissue specific promotor (P-TS) driving expression of the tetR-VP16 fusion protein, and the other contains the tetracycline response element (TetRE), followed by a minimal CMV promoter driving expression of HA-tagged DREADDs. Whereas both transgenic elements are present in every cell throughout the body, expression of tetR-VP19 is only in promoter defined areas (e.g. PET-1 for 5-HT neurons, GFAP for neuronal precursors and DARPP-32 for medium spiny neurons) and transgenic receptor expression is limited to the defined tissue (upper center and right panel). When doxycycline is present (DOX), it binds to the tetR factor and blocks it from interacting with the TetRE, preventing transcription of DREADDs (upper left panel). The lower panels show preliminary success with CAMKII-tet, which achieves forebrain-specific expression; in this set of founders, high levels of expression were achieved in hippocampus.

express transgenes selectively in defined tissues (Brand and Perrimon, 1993). In preliminary studies we have targeted DREADD expression to distinct neural circuits and have found that behaviors are dramatically altered when adult flies expressing DREADDS are fed CNO (unpublished results). Importantly, wild type and parental strains maintained on food containing CNO (at least to 10 mM) exhibit no overt abnormal developmental or behavioral effects, indicating that the effects of CNO on the transgenic flies expressing DREADDs are indeed due to DREADD activation. Given the conservation of biological processes between mammalian systems and the power of the fly as a genetic model (Nichols, 2006), studies performed in the fly are likely to be informative to both insect and mammalian models with respect to elucidation of signal transduction pathways and molecular processes underlying neuronal function as they relate to behaviors. Furthermore, this system is anticipated to be useful in the fly to probe the role of signal transduction pathways and discreet tissues in developmental processes.

Aside from using DREADDs to directly probe the role of individual signal transduction pathways in physiological processes and the role of specific tissues and circuits in behaviors, the hM_4D receptor has recently proven to be a valuable tool in understanding mechanisms of allosteric modulation in GPCRs. A known allosteric modulator of the M_4 receptor, LY2033298, was shown to act cooperativity with the orthosteric binding site to restore affinity and functionality of acetylcholine to the orthosteric site of the hM_4D receptor (Nawaratne et al., 2008). These muscarinic DREADD receptors, and potentially other similarly modified receptors, may represent novel tools to screen for allosteric modulators that act solely through interactions at distinct allosteric sites and have cooperativity with the orthosteric site. Compounds that would be able to restore affinity and activity to the native ligand would therefore potentially represent novel therapeutics that enhance the ability of native ligand to activate its receptor.

SUMMARY

An exciting and powerful method of probing the role of G-protein effector signaling, as well as the function of discreet tissues and neural circuits in mediating physiological processes and behaviors has recently been developed and refined. The first generation of engineered GPCRs, RASSLs, were primarily rationally designed based upon structure/function studies to eliminate native ligand binding, while maintaining affinity for synthetic ligands. These receptors have been used to generate conditionally expressing transgenic mice, where they have been used to study how G-protein effector pathways affect various processes including heart function, bone growth, and brain development. They have also been used to define the function of other GPCRs as well as particular tissues, as they were used to elucidate the receptor and effector pathway underlying the perception of sweet taste. Advantages that this system offers are the ability to very rapidly turn on and off signaling pathways by simply administering a synthetic ligand. Importantly, because GPCRs and their associated effector pathways are ubiquitous throughout an organism, studies are not limited to examining the native role of the particular receptor a RASSL is derived from, but can be used to define the function of entire tissues and neural circuits in a more systems-based approach. These first generation RASSLs, unfortunately, are frequently associated with physiologically relevant basal constitutive activity, as well as response to synthetic ligands that also target the endogenous wild type receptors. Whereas these properties may be advantageous for some studies, they likely present certain limitations and challenges for widespread use.

The next generation of engineered GPCRs, DREADDs, created through a process of directed molecular evolution overcome many of the limitations present in the first generation RASSLs. DREADDS have negligent basal constitutive activity associated with them, and are activated by synthetic ligands with no appreciable affinity for any of the known receptorome. Additionally, CNO has minimal activity at a variety of relevant kinases (**Figure 3**). Transgenic expression models in both mouse and fly have validated their functionality to probe both physiological processes and complex behaviors. Given the advantages of the DREADD receptors over the RASSLs, there are certain caveats that must be considered for them, as well as for the RASSLs, involving expression levels.

Under most normal transgenic conditions, expression levels are likely high, such that even under non-activated conditions the stoichiometric balance between receptor and precoupled effector may be perturbed such that the normal function of the tissue and circuit may be affected. Furthermore, the presence of high levels of activated receptor may induce activation of effector pathways not normally functionally coupled to the particular GPCR, confounding results. To achieve the most relevant data for a particular system, expression levels should be determined, and manipulated if possible, with those having expression closest to naturally occurring GPCRs used for experimentation. Nevertheless, both RASSLs and DREADDs present a very effective tool to elucidate biological function and behaviors. Advantages over other current systems include the specificity of transgenic targeting, the convenience of systemic administration of small molecule drugs with no other appreciable biological targets within the organism, rapidity and reversibility of effect, and no need for specialized equipment.

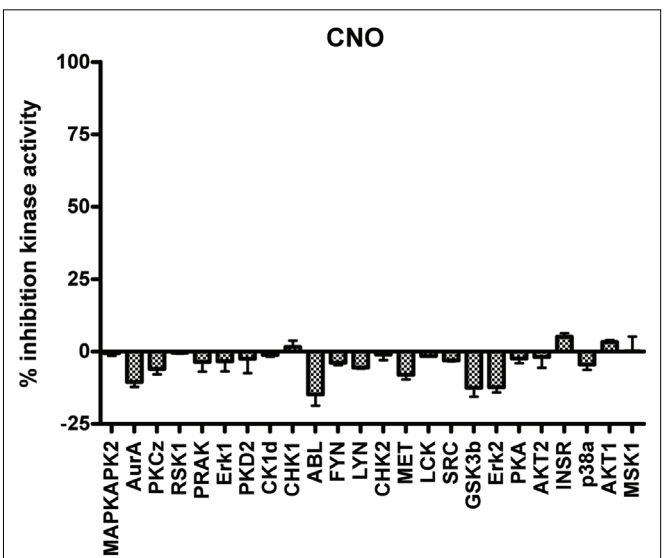

FIGURE 3 | Clozapine-*N*-oxide is devoid of activity at essential neuronal protein kinases. As is shown, CNO at a screening concentration of 10 μM is devoid of appreciable agonist or antagonist activity at a large number of the neuronal kinases implicated in signaling (V. Setola and B. L. Roth, unpublished observations).

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Studying cerebellar circuits by remote control of selected neuronal types with $GABA_A$ receptors

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Although $GABA_A$ receptor-mediated inhibition of cerebellar Purkinje cells by molecular layer interneurons (MLIs) has been studied intensely at the cellular level, it has remained unclear how this inhibition regulates cerebellum-dependent behaviour. We have implemented two complementary approaches to investigate the function of the MLI-Purkinje cell synapse on the behavioural level. In the first approach we permanently disrupted inhibitory fast synaptic transmission at the synapse by genetically removing the postsynaptic $GABA_A$ receptors from Purkinje cells (PC-Δγ2 mice). We found that chronic disruption of the MLI-Purkinje cell synapse strongly impaired cerebellar learning of the vestibular occular reflex (VOR), presumably by disrupting the temporal patterns of Purkinje cell activity. However, in PC-Δγ2 mice the baseline VOR reflex was only mildly affected; indeed PC-Δγ2 mice show no ataxia or gait abnormalities, suggesting that MLI control of Purkinje cell activity is either not involved in ongoing motor tasks or that the system compensates for its loss. To investigate the latter possibility we developed an alternative genetic technique; we made the MLI-Purkinje cell synapse selectively sensitive to rapid manipulation with the $GABA_A$ receptor modulator zolpidem (PC-γ2-swap mice). Minutes after intraperitoneal zolpidem injection, these PC-γ2-swap mice developed severe motor abnormalities, revealing a substantial contribution of the MLI-Purkinje cell synapses to real time motor control. The cell-type selective permanent knockout of synaptic GABAergic input and the fast reversible modulation of GABAergic input at the same synapse illustrate how pursuing both strategies gives a fuller view.

Keywords: γ-aminobutyric acid type A receptor, zolpidem, β-carboline, purkinje cell, memory consolidation

INTRODUCTION

"It seems likely that the cerebellum may be the first fragment of the higher levels of the nervous system to be understood in principle, all the way from peripheral input to peripheral output". So stated John Eccles in a Review Lecture at a Physiological Society meeting in the early 1970s (Eccles, 1973). At that time it was already known that the cerebellum was necessary for motor control and produces and stores motor memories. The question was how. Eccles expected the "cerebellar problem" to soon be solved; after all, it was considered the simplest part of the higher brain. During the 1960s, Eccles and collaborators had given Cajal's famous cerebellar circuit life and colour. They investigated the transmitters and polarity of the synapses, providing a view that has not changed fundamentally to this day and was captured in the slogan: "the cerebellum as a neuronal machine" (**Figure 1A**, Eccles et al., 1967a; Ito, 2006). Anticipating "systems biology", mathematicians such as Albus and Marr were captivated, and provided models that continue to guide investigations (Marr, 1969; Albus, 1971). Although Eccles' prophecy has not been fulfilled, reaching an understanding of how neuronal circuits work and what they compute remains a fundamental aim of neuroscience, perhaps even the most fundamental. For the cerebellum we remain in an excellent position to achieve this aim (Hansel et al., 2001; Linden, 2003; Nakanishi, 2009). The prosperous circumstances include: 1, the accurate, almost fully known circuit map (Grillner et al., 2005); 2, quantifiable behavioural tests for the structure's performance such as locomotion, vestibular-occular reflex (VOR), eye blink conditioning (Boyden et al., 2004; De Zeeuw and Yeo, 2005) and fear conditioning (Scelfo et al., 2008); 3, precise cell-type specific targeting, permitting interventional analysis of the circuit (e.g. Oberdick et al., 1990; Smeyne et al., 1995; Bahn et al., 1997; Barski et al., 2000; Aller et al., 2003; Wulff et al., 2007, 2009; Gajendran et al., 2009; Nakanishi, 2009); 4, techniques that enable specific cerebellar cell types to be reversibly modulated on a fast time scale *in vivo* (e.g. Tervo and Karpova, 2007; Wulff et al., 2007; Luo et al., 2008; Arenkiel and Ehlers, 2009). These techniques allow dynamic analysis of individual circuit components and how they relate to, or produce behaviour. Here we discuss our work on how two precise interventions within the circuit, one a permanent ablation of inhibitory synaptic input onto Purkinje cells, the other a selective fast reversible augmentation of the same inhibitory input have given complimentary insights into the function of molecular layer interneuron (MLI)-Purkinje cell synapses. Both strategies involved the same protein, the γ2 subunit of the $GABA_A$ receptor (Pritchett et al., 1989).

CIRCUITRY OF THE CEREBELLAR CORTEX

Conserved in its essential organization throughout the 550 million years of vertebrate evolution, from fish through to humans, the cerebellar cortex is a folded sheet of microcircuitry reiterated on a vast scale (Eccles et al., 1967a; Apps and Garwicz, 2005; Grillner

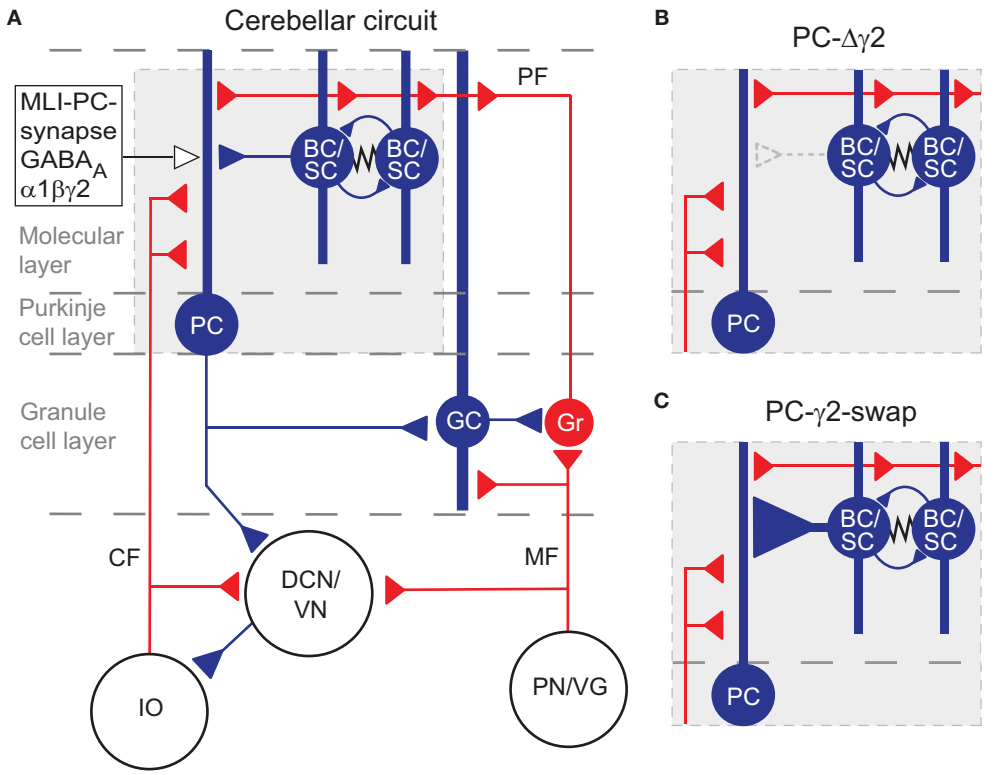

FIGURE 1 |The cerebellar circuitry and the interventions at the MLI-Purkinje cell synapses. Excitatory cells are shown in red, inhibitory cells are shown in blue. **(A)** All cells in the cortex are inhibitory apart from granule cells (Gr) which give rise to parallel fibres (PF), and unipolar brush cells (not shown). The cerebellar cortex receives excitatory input via mossy fibres (MF) and climbing fibres (CF). The only output of the cortex is via Purkinje cells (PC), which project to the deep cerebellar nuclei (DCN) and vestibular nuclei (VN). The activity of Purkinje cells is under inhibitory control from molecular layer basket and stellate cells (BC/SC) through α1βγ2 subunit containing GABA$_A$ receptors. Basket/stellate cells mutually inhibit each other and are coupled by gap junctions (zig-zag line). Diagram adapted from Grillner et al. (2005). **(B)** and **(C)** show the MLI-Purkinje cell synapse after chronic disruption in PC-Δγ2 mice **(B)** and during rapid enhancement with zolpidem in PC-γ2-swap mice **(C)**. GC, Golgi cell; IO, inferior olive; PN/VG, pontine nuclei and vestibular ganglion.

et al., 2005; Rokni et al., 2008). The same circuitry in different areas of the cerebellum probably computes similar operations linked to different parts of, for example, the neocortex (Ito, 2008). The conservation implies that there has been a selection pressure to maintain a core neural computation performed by this configuration of circuitry (Devor, 2000; Bell, 2002; Paulin, 2005). This core function is still not understood, but it probably measures or predicts time intervals and serves motor control, perception and cognition (Ohyama et al., 2003; Apps and Garwicz, 2005; De Zeeuw and Yeo, 2005; Paulin, 2005; Bastian, 2006; Ito, 2008; Rokni et al., 2008); in mammals, a notably similar and developmentally related cerebellar-like circuit, with analogous cell types, occurs in the dorsal cochlear nucleus of the hearing system (Devor, 2000). Specialized parts of the cerebellar cortex receive specific peripheral inputs and participate in defined behaviours e.g. the flocculus contributes to vestibular function and compensatory eye movements, and vermal lobules V-VI (an area of convergence of acoustic and nociceptive stimuli) to fear conditioning (Sacchetti et al., 2002; Gittis and du Lac, 2006). Purkinje cells form the only output from the cerebellar cortex, projecting to the deep cerebellar (DCN) or vestibular nuclei (VN) (**Figure 1A**). Thus all other cerebellar cortical synapses and cell types must exist with the sole aim of modifying, directly or indirectly, Purkinje cell output to the DCN and VN (**Figure 1A**; Pugh and Raman, 2009). Why are all these cortical components needed? Purkinje cells are spontaneously active at around 50 action potentials/second in the absence of synaptic input (Hausser and Clark, 1997; Raman and Bean, 1997; Pugh and Raman, 2009), so their inhibition or excitation by the surrounding cortical microcircuitry modifies either their firing rate or, for a given frequency, the temporal pattern of Purkinje cell action potentials (spikes).

INHIBITION ONTO PURKINJE CELLS

A clear feature from the static description of the circuit in **Figure 1A** is the dominance of inhibition: the majority of the cortex uses inhibitory synapses and GABA$_A$ receptors (Eccles, 1973; Brickley et al., 1996; Wisden et al., 1996; Vicini et al., 2001; Fritschy and Panzanelli, 2006); and the Purkinje cells themselves are inhibitory (GABAergic). In the adult circuit, molecular layer interneurons (MLIs) (stellate and basket cells) control Purkinje cells by feed-forward inhibition (Eccles et al., 1967b; Mittmann et al., 2005; Smith and Otis, 2005; Santamaria et al., 2007; Barmack and Yakhnitsa, 2008). Basket cells specialize in innervating the Purkinje cell soma and lower one third of the dendritic tree; stellate cells innervate the outer two thirds of the Purkinje cell dendritic tree. In the developing mouse cerebellum subsets of Purkinje cells receive another inhibitory input via Purkinje cell axon collaterals (Watt

et al., 2009). However, these Purkinje-Purkinje contacts are not common in adult mice (Watt et al., 2009). The cortical cerebellar circuit has a precise geometry, lattice like, with the Purkinje cell dendritic tree flattened out into a plane; and the innervations onto the Purkinje cell dendritic tree arriving orthogonal to this (Eccles et al., 1967a; Rokni et al., 2008). Each stellate/basket cell innervates (in the cat) about 20–50 Purkinje cells (Eccles et al.,1976a); the stellate/basket cells inhibit each other, and are coupled by gap junctions (Mann-Metzer and Yarom 1999; Mittmann et al., 2005). Adult Purkinje cells fire complex spikes in response to glutamatergic climbing fibre activity, and simple spikes (conventional action potentials) that reflect the integration of intrinsic pacemaker activity with glutamatergic and GABAergic synaptic inputs from parallel fibres and MLIs respectively (Pugh and Raman, 2009).

FEED-FORWARD INHIBITION AND PLASTICITY

Feed-forward inhibition emerges when inhibitory interneurons and their target cells receive common excitatory input (Smith and Otis, 2005): in the cerebellar cortex, parallel fibres excite Purkinje cells and stellate/basket cells (MLIs), and these in turn inhibit Purkinje cells (**Figure 1A**); this happens fast, about 1 ms after Purkinje cells are activated (Mittmann et al., 2005). The main role of feed-forward inhibition could be to sharpen up (reduce) the time window in which excitatory postsynaptic potentials onto the Purkinje cells summate to reach spike threshold, a way of coincidence detection of separate parallel fibre inputs (Mittmann et al., 2005). Previous studies on cerebellar learning have emphasized the role of glutamatergic excitation for plasticity and learning in the cerebellar cortex, e.g. long term potentiation (LTP) and long term depression (LTD) at the parallel fibre to Purkinje cell synapse. However, MLIs also display plasticity both at the input and output level (Kano et al., 1992; Jorntell and Ekerot, 2002; Duguid and Smart, 2004; Liu and Lachamp, 2006; Mittmann and Hausser, 2007; Scelfo et al., 2008; Lachamp et al., 2009). Some forms of plasticity at MLI-Purkinje cell synapses are expressed post-synaptically or have a postsynaptic component in their mechanism (Kano et al., 1992; Duguid and Smart, 2004; Mittmann and Hausser, 2007); others are expressed presynaptically (Liu and Lachamp, 2006; Scelfo et al., 2008; Lachamp et al., 2009). Indeed, the strength of MLI-Purkinje cell synapses increases after fear conditioning because of enhanced GABA release (Scelfo et al., 2008); this happens in parallel with LTP at the parallel fibre-Purkinje cell synapse (Scelfo et al., 2008). The potentiation of the MLI-Purkinje cell synapses may balance the LTP of the excitatory terminal in a form of scaling to preserve a narrow time window for coincidence detection of parallel fibre inputs. However, for lack of a suitably specific method, it has only recently become possible to study the function of the stellate/basket cell inhibitory network on the whole animal level.

REMOVAL OF SYNAPTIC $GABA_A$ RECEPTORS FROM PURKINJE CELLS REVEALS THAT FAST FEED-FORWARD INHIBITION OF PURKINJE CELLS CONTRIBUTES TO CEREBELLAR LEARNING

To understand how a system works, and in addition to simply observing it, one can break, inhibit or activate a component and then see how the system behaves, or in the words of a recent column in Nature: "most sciences are in the habit of poking that which they study to gauge its response" (Nature 458, p1077, 2009 – an editorial). Loss of function gene mutations, whether spontaneous or engineered, have given, and continue to give, essential information about the roles of a protein in the organism. Thus to investigate how MLIs contribute to cerebellum-regulated behaviour, our first approach was to disrupt selectively the GABA-gated chloride channel, the $GABA_A$ receptor, responsible for conferring fast inhibition onto Purkinje cells. Most $GABA_A$ receptors are complexes of α, β and $\gamma2$ subunits (Schofield et al., 1987; Pritchett et al., 1989; Seeburg et al., 1990; Luddens and Wisden, 1991; Rudolph and Mohler, 2004; Mohler, 2006; Goetz et al., 2007; Olsen and Sieghart, 2009); Purkinje cells express $\alpha1\beta2\beta3\gamma2$-type $GABA_A$ receptors (Laurie et al., 1992; Persohn et al., 1992; Pirker et al., 2000; Vicini et al., 2001; Fritschy et al., 2006). The subunits assemble as a pentamer, with the chloride channel at the centre. Although $GABA_A$ receptors will form as lower conductance (12–15 pS) $\alpha\beta$ complexes, the $\gamma2$ subunit enables a high single channel conductance (25–30 pS) and, importantly, is essential to target the receptor complex to the postsynaptic membrane (Lorez et al., 2000; Schweizer et al., 2003; Tretter and Moss, 2008). Mice with a $\gamma2$ gene knockout in every cell die in the first postnatal week, underscoring the importance of this subunit for neuronal function (Gunther et al., 1995). By crossing the L7Cre line, which has Cre recombinase restrictively expressed in Purkinje cells (Barski et al., 2000), with a line containing a floxed $\gamma2$ (*gabrg2*) allele (Wulff et al., 2007), we made mice (PC-$\Delta\gamma2$) where the $GABA_A$ receptor $\gamma2$ subunit was selectively removed from Purkinje cells (Wulff et al., 2007, 2009). This produced a Purkinje cell-selective loss of fast $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs), and thus the functional removal of stellate and basket cells from the cerebellar cortex (**Figure 1B**). When tested for motor performance, PC-$\Delta\gamma2$ mice showed no ataxia or gait abnormalities and no impairment in the rotarod test (Wulff et al., 2007). To test for cerebellar function at a higher resolution, we analysed the VOR. The VOR is a cerebellum-dependent reflex needed to stabilize the retinal image despite head motion by transforming vestibular input (from semicircular canals) into occulomotor output (eye muscle contraction), so that the eyes rotate to counteract the head movement (Boyden et al., 2004; Broussard and Kassardjian, 2004; De Zeeuw and Yeo, 2005).

The criteria which quantitatively assess the VOR are (i) gain, defined as eye velocity/head velocity (to avoid retinal slip, a gain of one is needed) and (ii), phase, defined as time shift in degrees between stimulus and eye velocity (to avoid retinal slip, 0° phase difference is needed) (Boyden et al., 2004). The VOR requires the flocculus, a specialized region of the cerebellar cortex, and the associated vestibular nuclei ("VN" in **Figure 1**; Ito, 2006). In agreement with the absence of clear motor impairments (see above), PC-$\Delta\gamma2$ mice had only mild abnormalities when tested for baseline VOR performance, suggesting that some compensation took place in the system (Wulff et al., 2007, 2009). However, the flocculus is also needed for VOR adaptation, a form of cerebellar learning (Boyden et al., 2004; Broussard and Kassardjian, 2004; De Zeeuw and Yeo, 2005; Gittis and du Lac, 2006; Ito, 2006). Unlike baseline VOR, any adaptations in the VOR need vision (via the accessory visual system, which signals errors – retinal slip – via climbing fibres to the Purkinje cells and stellate/basket cells). When a drum (visual stimulus), which surrounds the rotating platform with the head-restrained mouse, rotates in or against the direction of the platform,

the baseline VOR will lead to a retinal slip (Boyden et al., 2004). An error signal is transmitted by the climbing fibres (De Zeeuw and Yeo, 2005). After repeated trials, the VOR system learns to compensate by moving the eyes at a different speed. If for example, the surrounding drum (visual stimulus) moves repeatedly in the same direction as the head, the VOR system needs to slow down or even cancel the eye movements that would normally occur in the opposite direction (this is termed "gain down" adaptation) (Boyden et al., 2004). This adaptation can go to extremes: when the drum is rotated with higher velocity but in the same direction as the head, the direction of the eye movements becomes completely reversed (phase shift by 180°). When we assessed cerebellar learning (VOR adaptation) in PC-Δγ2 mice, we found that PC-Δγ2 mice showed a near normal capacity for gain-decrease adaptation when tested right after the training session, but a profound lack of memory consolidation when measurements were resumed the next day (Wulff et al., 2009). The degree of gain reduction (savings) carried forward from the previous day's learning was significantly smaller in PC-Δγ2 mice. The same prominent differences were observed in the consolidation of phase changes. Indeed phase adaptation was more broadly affected in PC-Δγ2 mice and also included strong deficits during acquisition. Thus, PC-Δγ2 mice were entirely unable to learn VOR phase reversal (i.e. to move their eyes in the same direction as the head instead of the opposite direction) (Wulff et al., 2009). Accordingly, some "resonance" or ongoing activity of feed forward-inhibition onto Purkinje cells is needed after or during learning for the memory to consolidate (see also Attwell et al., 2002; Cooke et al., 2004; Kassardjian et al., 2005). As the presynaptic form of plasticity at MLI-Purkinje cell synapses, due to enhanced GABA release (Scelfo et al., 2008; Lachamp et al., 2009), may be preserved in the PC-Δγ2 mice, we cannot rule out that this plasticity contributes to residual learning by dynamically regulating tonic inhibition. Certainly, PC-Δγ2 Purkinje cells receive inhibitory input from both metabotropic $GABA_B$ receptors (coupled to G proteins and second messengers) and extrasynaptic low conductance αβ $GABA_A$ receptors (Wulff et al., 2009). These forms of inhibition are probably too slow to precisely organize the simple spike intervals needed for motor learning.

Since Purkinje cells are the only output of the cerebellar cortex, any behavioural deficits in PC-Δγ2 mice must come from altered Purkinje cell activity. Extracellular recordings of Purkinje cell activity in the flocculus of PC-Δγ2 and control mice showed that the temporal patterns of Purkinje cell simple spike activity were changed in PC-Δγ2 mice. Simple spike activity was much more regular in PC-Δγ2 mice (Wulff et al., 2009). This is consistent with *in vitro* data: if Purkinje cells in acute slices are bathed in $GABA_A$ receptor blockers, the cells fire action potentials with more regularity (Hausser and Clark, 1997); without the inhibition, the regular "clockwork" pacemaker dominates. Other parameters of Purkinje cell activity such as simple spike firing frequency, phase modulation or modulation amplitude during optokinetic stimulation were not changed in PC-Δγ2 mice. In summary we found that chronic disruption of the MLI-Purkinje cell synapse leads to changes in the temporal pattern of Purkinje cell simple spike activity and deficits in the consolidation of motor memories. We hypothesize that the MLIs shape temporal activity patterns of Purkinje cells under conditions of increased excitatory plasticity (Wulff et al., 2009). Ultimately, VOR-based motor memories become stored in the VN (Kassardjian et al., 2005; Gittis and du Lac, 2006; Shutoh et al., 2006). The course of motor memory transfer from the cerebellar cortex to the VN is likely to correspond to the consolidation phase; during this time, the Purkinje cells must contribute to plastic changes in the VN by precise temporal interaction with the excitatory mossy fibre input onto the same cells (Nelson et al., 2003, 2005; Pugh and Raman, 2009). So from our studies on the PC-Δγ2 mice, we hypothesized that the precise spacing of the Purkinje cell action potentials would specify the plastic changes during the transfer from the cortex to the VN; in PC-Δγ2 mice the temporal control over Purkinje cell simple spike activity is impaired so that memory cannot be consolidated (transferred from cortex to the VN). Indeed, many believe that it is the fine scale patterning of Purkinje cell action potentials, combined with pauses in spike trains, that is crucial for information transfer to the deep cerebellar and VN (Walter et al., 2006; De Schutter and Steuber, 2009). The PC-Δγ2 mice, with their imposed regularity in Purkinje cell spike firing and failure to consolidate VOR adaptation, support this view.

REVERSIBLE APPROACHES

A potential problem in the subtractive analysis of brain function is compensation (Lomber, 1999; Wulff and Wisden, 2005; Marder and Goaillard, 2006). Quite often genetic knockouts produce clear effects (e.g. no consolidation of VOR adaptation in PC-Δγ2 mice, changes in regularity of Purkinje cell firing; Wulff et al., 2009). However, if no effect is seen (e.g. no change in rotarod or baseline VOR performance), it is possible that either the component is genuinely not important for that particular task or that its loss has been compensated for (e.g. Brickley et al., 2001; **Figure 2**). For example, a knockout of the α6δ-$GABA_A$ receptor that provides extrasynaptic tonic inhibition in cerebellar granule cells leads to transcriptional upregulation of three K2P potassium channel genes (TASK-1, TASK-3 and THIK-2) encoding leak conductances (Brickley et al., 2001; Aller and Wisden, 2008). The raised K^+ conductance prevents the cells undergoing any change in excitability (Brickley et al., 2001). Two alternative solutions to the same problem of maintaining granule cells in a certain range of excitability can be provided by either $GABA_A$ receptors or K^+ leak channels. Parallel

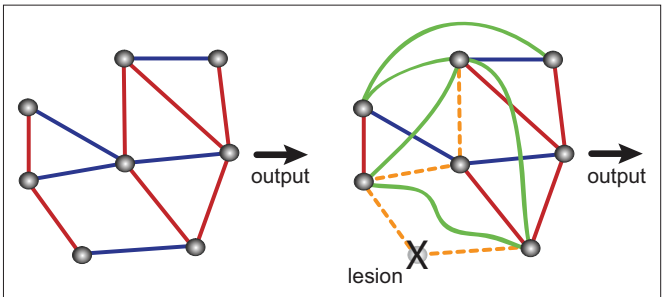

FIGURE 2 | Compensatory changes in an abstract biological network. Blue lines indicate inhibitory interactions, red lines indicate excitatory interactions. Shown are an intact network (left side) and the same network after ablation of one of its nodes. Lesioning one element causes the rest of the system to compensate for the loss. Some interactions are lost (orange dashed lines) and some new interactions emerge (green lines), so that the final output is unchanged. Adapted from Greenspan (2001).

solutions to neural network operations also appear in other circuits (Marder and Goaillard, 2006). How do we decide if there is really no deficit in a knockout or if there has been compensation? For example, in our PC-Δγ2 mice, is fast inhibition onto Purkinje cells genuinely not used to coordinate in real time e.g. motor performance, and is only used for memory formation? Or can the system develop without this inhibition and generate another solution? Indeed, we found that there is an apparent adaptive change in the PC-Δγ2 Purkinje cells: the ablation of GABAergic synaptic input onto Purkinje cells was accompanied by a reduction in parallel fibre-evoked excitatory responses (Wulff et al., 2009). This could arise either from an altered postsynaptic AMPA receptor function or from an alteration in presynaptic release.

The earlier during development we interfere with a system and the longer this interference persists, the larger the probability that the system compensates. Thus we wanted a method for fast (minutes in our case) and reversible modulation of the MLI-Purkinje cell synapse at any chosen time point. Such an approach would also allow us to investigate "when" and for "how long" the synapse is required in a particular process. We have temporal control. These thoughts are not new: reversibly inactivating whole brain parts by cooling (e.g. Eccles applied this for the cerebellum (Eccles et al., 1975)) or infusion of anesthetics or $GABA_A$ receptor agonists and glutamate receptor antagonists into the cerebellum has been used frequently (Lomber, 1999; Attwell et al., 2002; Nagao and Kitazawa, 2003; Cooke et al., 2004; Shutoh et al., 2006). Reversible approaches revealed a transfer of memory from cortex to deep cerebellar nuclei: inactivating the cerebellar cortex with muscimol at different times after eyeblink conditioning defined a consolidation time window of 1–2 h post training (Attwell et al., 2002; Cooke et al., 2004). Similar experiments with reversible inactivation have been carried out for VOR adaptation (Kassardjian et al., 2005).

USING GENETICS TO ENDOW NEURONAL SUBTYPES WITH UNIQUE SENSORS

These pharmacological approaches described above reveal what the regions in the cerebellar system do and when. Francis Crick encouraged molecular biologists to go one step beyond this and develop cell type-selective genetic techniques to turn neuronal subtypes on or off with fast (milliseconds) and relatively fast (seconds to minutes) timescales by "remote control" (Crick, 1999). The first methods for activating neurons by remote control used light, either to activate a retinal like transduction machinery heterologously expressed (Zemelman et al., 2002) or photo-uncageable capsaicin that activates a ligand-gated ion channel (TRPV1), again heterologoulsy experessed (Zemelman et al., 2003 – this paper, in its title, appears to have the first use of the phrase "remote control" in this context). Just a short while after the introduction of these clever methods came the introduction of the light-activated channel rhodopsins (see Boyden et al., 2005 and Han et al., 2009a,b; Sohal et al., 2009). The extremely easy applicability of the channel rhodopsin system in diverse animals and circuit settings has been a phenomenal breakthrough and captured the imagination of the neuroscience community (O'Connor et al., 2009; Scanziani and Hausser, 2009). Light activation has the advantage of high temporal precision (milliseconds) and the potential to deliver specific patterns of stimuli. But we were interested in remote control of the entire population of Purkinje cells. Light at 1 mW mm^{-2} can illuminate, through an optical fibre, a volume of 0.56 mm^3 in the mouse hypothalamus; this is the volume of a typical hypothalamic nucleus (Adamantidis et al., 2007). Thus, with current optical technology, we would not be able to easily influence many Purkinje cells *in vivo* with light. Thus for our particular system we decided to build in unique pharmacological sensors into Purkinje cells, such that we could add a ligand into the blood (or orally), that would then cross the blood brain barrier and influence the activity of only Purkinje cells and no other cell type. Such a method of remotely controlling Purkinje cells would have to work reasonably fast (effects within minutes of drug administration) and wear off within an hour or so.

Various ingenious ligand-receptor systems have been applied to selectively inhibit neuronal subtypes *in vivo*: these include the allatostatin system (Tan et al., 2006, 2008), a 5HT1A-receptor system (Tsetsenis et al., 2007), and the ivermectin-gated Cl^- channel system (Lerchner et al., 2007). Additionally, there is the promising metabotropic clozapine-N-oxide-hM(4)D receptor system, which has so far been published only using *in vitro* applications (Armbruster et al., 2007; Nichols and Roth, 2009). All of these systems rely on the ectopic expression of receptors or ligand-gated channels in the target neurons, and then giving a ligand which can only work on cells expressing the receptor (Wulff and Wisden, 2005; Arenkiel and Ehlers, 2009). The allatostatin method consists of a *Drosophila* peptide-7TM metabotropic receptor pair which promotes opening of GIRK K^+ channels. Given that the peptide does not cross the blood brain barrier or easily diffuse within neuronal tissue, it has to be pumped in to the relevant brain area at high concentrations. This method has been used successfully to study inhibitory V1 neurons in mouse spinal cord (Gosgnach et al., 2006; Tan et al., 2006) and to selectively silence somatostatin (Sst)-expressing neurons in the preBötzinger complex (Tan et al. 2008). By contrast, the 5HT1A system has a water soluble agonist (8-OH-DPAT) that can be given systemically and this receptor also promotes opening of GIRK K^+ cells, inhibiting neurons reversibly with very good kinetics; on the other hand, a 5HT1A receptor knockout mouse background has to be used, which could cause potential complications. The "5HT method" was applied successfully to inhibit selectively hippocampal dentate granule cells and the central nucleus of the amygdala *in vivo* and to probe how this affected anxiety-related behaviours (Tsetsenis et al., 2007). Thus, if expressed selectively in Purkinje cells, triggering either the allatostatin or 5HT1A ligand-receptor systems would probably mimic G-protein-coupled $GABA_B$ receptor activation. So these systems would be potentially useful to explore how metabotropic inhibition onto Purkinje cells influences motor memory consolidation. At the other end of the timescale, the *Caenorhabdtis elegans* ivermectin-gated Cl^- channel system, whose α and β subunits are encoded in the same gene superfamily as the $GABA_A$ receptor, effectively silences neurons for periods lasting around 8 h, but has slow onset kinetics (Lerchner et al., 2007). So although this method will aid certain types of experiment, it would not have been appropriate for studying the acute modulation of MLI-Purkinje cell synapses. Nevertheless, because the α and β subunits must co-express to assemble a functional ivermectin receptor, this does give the possibility of applying intersectional genetics to get more selective cell-type targeting (Luan and White, 2007).

ALLOSTERIC MODULATION OF GABA$_A$ RECEPTORS AS A TOOL TO MODULATE SELECTED INHIBITORY SYNAPSES

Given our focus on understaning how GABAergic synapses regulate the cerebellar circuit through precisely timed fast inhibition, the obvious and natural strategy for us was to take advantage of drugs which allosterically and selectively modulate GABA$_A$ receptors (Schoch et al., 1985; Luddens and Wisden, 1991; Korpi et al., 1997; Rudolph and Mohler, 2004; Goetz et al., 2007; Olsen and Sieghart, 2009). Many drugs bind at sites on the GABA$_A$ receptor distinct from the GABA-binding site; these drugs change the shape of the receptor oligomer so that the efficacy of GABA at opening the channel is either increased (positive allosteric agonists, e.g., diazepam or zolpidem) or decreased (negative allosteric agonists, e.g., the β-carboline, DMCM) (Korpi et al., 1997; Mohler, 2006; Hanson and Czajkowski, 2008; Olsen and Sieghart, 2009). Allosteric modulation of GABA$_A$ receptors is a very powerful way to modulate neuronal circuit function, as attested by the universal clinical use of such drugs as benzodiazpeines, steroids and propofol (Yentis et al., 2004). In the extreme case, by prolonging the duration of GABA-mediated IPSCs, propofol induces full anesthesisa (loss of consciousness); but changing the IPSC kinetics can profoundly alter most aspects of brain function. If we could make these drugs work at specific cell types (or more accurately at specific GABAergic synapses), without affecting other cell types, this would be an effective way to explore how an isolated neuronal pathway contributes to a behaviour. Thus we and our colleagues developed a method for fast reversible modulation of selected GABAergic synapses and applied it to the specific modulation with zolpidem of the stellate/basket cell inhibitory inputs to Purkinje cells (**Figures 1C and 3**; Wulff et al., 2007). Before we explain this method, we first describe the "benzodiazepine site".

Depending on the ligand, the "benzodiazepine site" can mediate different allosteric effects. These drugs require αβγ2-type receptors (Pritchett et al., 1989; Seeburg et al., 1990); the drug-binding site resides between the α and γ2 subunits (Sigel, 2002; Sancar et al., 2007). It is important to emphasise the modulator aspect of the benzodiazepine site. This is not the same site where GABA binds the receptor; and furthermore, the benzodiazepines and related drugs will not alter Cl^- gating unless GABA is also present as well. So the drugs modulate active GABAergic synapses, and also extrasynaptic GABA$_A$ receptors (if GABA is also present outside the synapse). Benzodiazepine antagonists like flumazenil (Ro 15-1788), which bind at the same site as the agonists, inhibit the effects of both agonists (positive allosteric modulators) and inverse agonists (negative allosteric modulators); this property, for example, makes flumazenil useful for diagnosing and treating benzodiazepine intoxication e.g. for treating prolonged sedation with benzodiazepines in the intensive care unit (Korpi et al., 1997; Yentis et al., 2004). The amino acids contributing to the binding sites of the benzodiazepines and similar drugs, found at the interface between the α and γ2 subunits, have been studied in detail (Wieland et al., 1992; Sigel, 2002; Sancar et al., 2007; Hanson and Czajkowski, 2008; Olsen and Sieghart, 2009). The best known is histidine at position 101 in the α1, α2, α3 and α5 subunits; mutating this histidine to arginine produces αβγ2 GABA$_A$ receptors insensitive to most benzodiazepine ligands without changing any other property of the receptor (Wieland et al., 1992). Placing

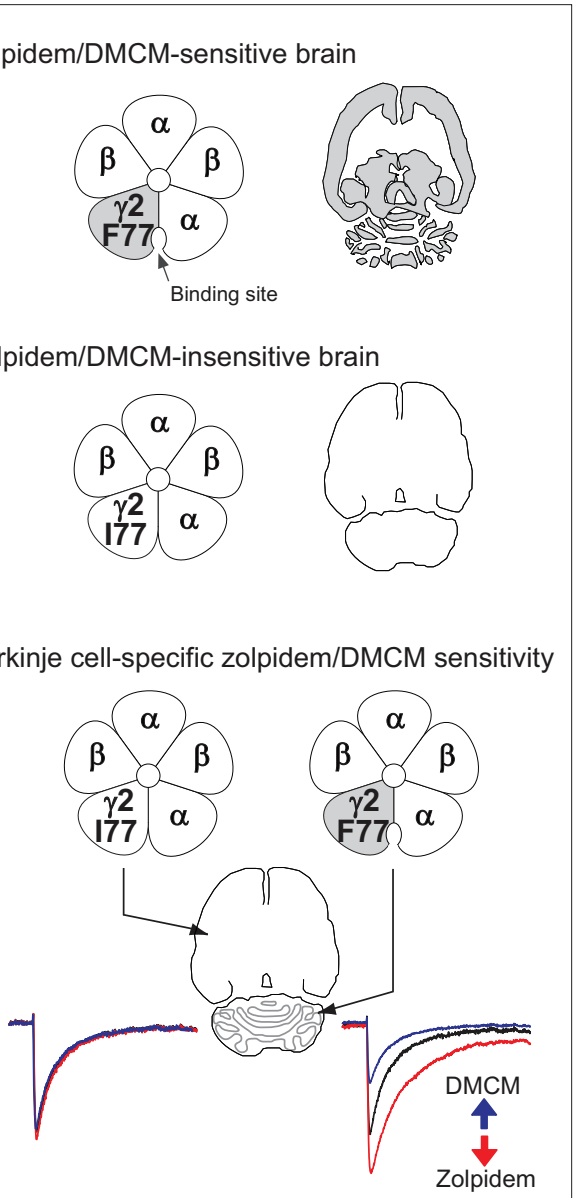

FIGURE 3 | The strategy for bi-directional modulation of selected GABAergic synapses. (A) shows the wild-type situation with a phenylalanine (F) at position 77 in the γ2 subunit of the GABA$_A$ receptor. Zolpidem/DMCM binding occurs at the interface of the α and the γ2 subunits and is widely distributed throughout the brain (indicated in grey in the cartoon of a horizontal brain section). In a first step **(B)** we have changed the phenylalanine at position 77 to isoleucine (I) by homologous recombination in mouse embryonic stem cells. In these mice zolpidem/DMCM binding is abolished. In the last step **(C)** we have reintroduced the drug-sensitive wild-type γ2 subunit under the control of the Purkinje cell-specific L7 promoter to selectively restore zolpidem/DMCM-sensitivity by a Cre-mediated Purkinje cell-specific swap of γ2 subunits (PC-γ2-swap mice). In these mice expression of zolpidem/DMCM-sensitive GABA$_A$ receptors is restricted to Purkinje cells of the cerebellum (grey). Note the difference to wild-type mice in **(A)**, which show drug binding throughout the cerebellar cortex. In Purkinje cells zolpidem (red trace) and DMCM (blue trace) now selectively enhance or reduce, respectively, GABA$_A$ receptor-mediated inhibitory post synaptic currents, illustrated by the artificial traces on the right (GABA alone: black trace). All other neurons in the brain are insensitive to these drugs (left traces).

this mutation into the α subunit genes in mice (knock-in mice) allowed the multiple actions of benzodiazepine ligands on the whole animal (sedation, hypnosis, anxyiolysis, muscle relaxing) to be explained in terms of particular $GABA_A$ receptor subtypes (Rudolph et al., 1999; Crestani et al., 2000; McKernan et al., 2000; Rudolph and Mohler, 2004).

As well as amino acid residues on the α subunits, key side chains that form or influence the benzodiazepine binding pocket are found in the γ2 subunit: replacing phenylalanine (F) by isoleucine (I) at position 77 of γ2 abolishes binding of zolpidem, DMCM and flumazenil in αβγ2 complexes (Buhr et al., 1997; Wingrove et al., 1997; Cope et al., 2004; Ogris et al., 2004). Zolpidem modulates GABA's action at three main types of $GABA_A$ receptor (α1βγ2 – comprising 43% of receptors in the brain, α2βγ2 – 18% of receptors, and α3βγ2 – 17% of receptors, where the β can be one of three variants); together, these α1–α3 receptors account for 78% of the total number of brain $GABA_A$ receptors, i.e. the majority of $GABA_A$ receptors will be sensitive to zolpidem modulation in the brain (McKernan and Whiting, 1996). Of these three receptor types, the most abundant and widely expressed in the brain, α1β2/3γ2, has the highest affinity for zolpidem, with an inhibition constant (K_i) of 20 nM (Niddam et al., 1987; Pritchett et al., 1989; Wisden et al., 1992; Duncan et al., 1995). At higher concentrations, zolpidem modulates receptors, and thus circuits, containing α2βγ2 and α3βγ2 receptors (K_i = 400 nM).

The γ2 subunit gene is widely expressed (Persohn et al., 1992; Wisden et al., 1992; Pirker et al., 2000), and contributes to most $GABA_A$ receptors (see above), and therefore most circuit functions in the brain. We reasoned that by engineering all neurons to express a γ2I77 gene (encoding zolpidem-insensitive receptors) and then reintroducing a zolpidem sensitive γ2F77 subunit back into selected cell types, we could explore the function of a variety of brain circuits by selectively manipulating the GABA input onto selected cell types. By homologous recombination at the γ2 subunit gene locus we made knock-in mice (γ2I77lox) such that all neuronal types had $GABA_A$ receptors insensitive to zolpidem (**Figure 3**), as assessed by behavioural, electrophysiological and pharmacological assays (Cope et al., 2004, 2005; Ogris et al., 2004). Normally, zolpidem is an effective hypnotic and induces sleep, presumably by enhancing $GABA_A$ receptor inhibition in sleep regulatory areas (Crestani et al., 2000). Thus on a rotarod task, normal mice when given zolpidem fall off the rod from a mixture of ataxia and sedation (Cope et al., 2004); however, γ2I77 mice showed no impairment even after high doses of zolpidem (Cope et al., 2004). As expected from recombinant studies (Buhr et al., 1997; Wingrove et al., 1997), the I77 mutation did not influence GABA's action at its receptor: in γ2I77 mice, mIPSCs from hippocampal and cerebellar neurons had normal rise times, amplitudes and decay kinetics (Cope et al., 2004, 2005; Wulff et al., 2007). We then arranged it by a cell type-specific genetic swap of the γ2 subunit gene, that only cerebellar Purkinje cells expressed the zolpidem-sensitive γ2F77 subunit, whereas all other neurons in the brain expressed the insensitive γ2I77 subunit. In other words, only $GABA_A$ receptors on Purkinje cells were sensitive to zolpidem – these mice were termed PC-γ2-swap (**Figures 1C and 3**). We could thus rapidly modulate a class of synapses (MLI-Purkinje cell synapses) in isolation from all other synapses in the brain to analyse their relevance for cerebellar tasks. Several minutes after intraperitoneal injection of zolpidem, PC-γ2-swap mice became ataxic and showed strong impairments in the rotarod and horizontal beam motor tasks. Thus rapid intervention at the MLI-Purkinje cell synapse revealed the involvement of MLIs in the control of real-time motor coordination. Accordingly, the chronic disruption of the MLI-Purkinje cell synapse in PC-Δγ2 mice must have induced compensatory mechanisms (Wulff et al., 2007).

BIDIRECTIONAL MODULATION OF GABAergic SYNAPSES

The opposite of inhibition, activation, is also important for understanding how specific neuronal cell types contribute to an animal's behaviour and/or perception or the function of a circuit (e.g. Boyden et al., 2005; Lima and Miesenböck, 2005; O'Connor et al., 2009; Zimmermann et al., 2009). In mice, cell type-specific activation *in vivo* can be done with light-activated channels (Adamantidis et al., 2007; O'Connor et al., 2009), capsaicin-activated TRPV1 channels (Arenkiel et al., 2008) and a synthetically evolved Gq-coupled metabotropic receptor (hM3Dq) activated by the otherwise inert ligand clozapine-N-oxide (Alexander et al., 2009; Nichols and Roth, 2009). Bidirectional modulation allows the exploration of a wider range of function (or potential function) of a particular cell type (or synaptic input). Ideally both approaches should be applied to the same cell. This can be done with light by co-expressing in neurons the genes coding for the blue light-activated cation channelrhodopsin-2 and the yellow light-driven chloride pump halorhodopsin (NpHR), and illuminating the cells with light pulses (Han and Boyden, 2007; Zhang et al., 2007; Han et al., 2009a; Scanziani and Hausser, 2009; Sohal et al., 2009). By delivering precisely timed blue and yellow light pulses, this enables the details of neural coding (e.g. spike timing) to be explored, a process termed "informational lesioning" by Han et al. (2009a) (see also Sohal et al., 2009).

Manipulating $GABA_A$ receptors with allosteric modulators also permits a bidirectional modulation (**Figure 3**). The mutation of the γ2 subunit that abolishes zolpidem binding also removes the β-carboline binding site (e.g. DMCM) (Buhr et al., 1997; Wingrove et al., 1997): we have shown that $GABA_A$ receptors in γ2I77 mice are about 1000-fold less sensitive to DMCM (Ogris et al., 2004). As DMCM is an inverse agonist at the benzodiazepine binding site, it decreases the effectiveness with which GABA opens the receptor and/or speeds the rate of channel deactivation. This means that DMCM is an excitatory agent; it decreases inhibition. In wild-type (γ2F77) mice DMCM (at low doses) causes severe seizures within minutes of an i.p. injection (Leppa et al., 2005). However, γ2I77 mice can receive high doses of DMCM without behavioural effects; at extremely high doses, there are still no seizures or neurological defects (e.g. ataxia), but some mild anxiolytic effects, most likely caused by another (undefined) $GABA_A$ receptor binding site or subtype (Leppa et al., 2005). An example of DMCM's utility or application with the γ2I77/F77 system could be as follows: after fear conditioning, the MLI-Purkinje cell synapses undergo long-term potentiation (Scelfo et al., 2008); we predict that selectively decreasing the strength at these synapses using DMCM in PC-γ2-swap mice would either undermine learning if given during acquisition, or disrupt consolidation if given directly after acquisition.

ADVANTAGES AND DISADVANTAGES OF THE $GABA_A$ RECEPTOR SYSTEM TO CONTROL NEURAL ACTIVITY CELL-TYPE SELECTIVELY

(1) As demonstrated by their continuing wide and successful clinical use, drugs that allosterically modulate $GABA_A$ receptors have a well-proven effectiveness in modulating all aspects of central nervous system function, from pain perception through to arousal and cognition (Korpi et al., 1997; Yentis et al., 2004; Mohler, 2006).

(2) Since zolpidem works on all α1, α2, α3, β, γ2 subunit containing $GABA_A$ receptors (it is selective for α1βγ2 receptors at low nM concentrations, but in the low μM range it modulates in addition α2βγ2 and α3βγ2 receptors), we can target many types of GABAergic synapses in the brain.

(3) Because the $GABA_A$ receptor is an ion channel, there are no complications with multiple second messenger pathways having different effects depending on cell type, or even in the same cell type.

(4) GABAergic circuit operations can be modulated bidirectionally with zolpidem (increase in amplitude and prolonged decay of inhibitory postsynaptic currents) and DMCM (decrease in amplitude and faster decay of inhibitory postsynaptic currents). The effect of both drugs can be, in principle, rapidly terminated with flumazenil, as this drug is an antagonist of the benzodiazepine binding site and blocks the actions of agonist benzodiazepine drugs if simultaneously present (Lauven et al., 1985). Indeed, the behavioural effects produced by zolpidem in PC-γ2–swap mice were blocked by pre-administration of flumazenil (Wulff et al., 2007).

(5) All drugs are water-soluble molecules and cross the blood brain barrier. Systemic drug applications (i.p. injections) work well for zolpidem, DMCM and flumazenil. For our cerebellar experiments targeting Purkinje cells, no surgery or catheters were needed – but for other situation or brain regions/nuclei the drugs could be given more locally using catheters. After i.p. injection, drug effects are produced within several minutes – the half life of zolpidem after i.p. injection in rodents is about 20 min (Benavides et al., 1988).

(6) Manipulations with these drugs can modulate the inhibitory synapses on a widely distributed population of neurons (e.g. Purkinje cells, subtypes of hippocampal and neocortical interneurons). With current technology, light delivered through optical cables could only reach few cells. Admittedly, activating even a few cells with light can produce defined outputs: for the hypothalamus a whole nucleus can be light activated to produce a change in arousal (Adamantidis et al., 2007), or dopaminergic cells in the ventral tegmental area can be selectively light-stimulated to cause changes in behavioural conditioning (Tsai et al., 2009). Indeed, inducing 14 action potentials (APs) in a single neuron using conventional microstimulation in rat barrel somatosensory cortex is detected by the animal (Houweling and Brecht, 2008). But some situations may require manipulating many more cells. Han et al. (2009a) suggest that tiling the brain with arrays of LED- or laser-coupled optical fibres will solve this problem.

(7) Altering fast GABAergic input with $GABA_A$ modulators uses the endogenous transmitter pathways, and differs in its effects from sudden activation (e.g. with light) or total silencing. We are thus modulating the natural "rhythm" of GABAergic communication at the synapse, which is a more subtle approach.

(8) The temporal resolution of our pharmacological manipulation is unsuited to probing events on the millisecond range, and it could not be used to deliver precise patterns of stimuli, but drug manipulations of selected cell types with zolpidem and DMCM are suited for reversibly manipulating neuronal activity during periods of a few minutes to hours to study processes like learning, which may require many repetitions over minutes to hours so that biochemical/structural changes can occur at the relevant synapses. Extended continuous modulation of this process by inhibition with zolpidem or excitation with DMCM may be useful.

(9) Because of the requirement for the γ2I77 background, selective manipulation with zolpidem can only be done in transgenic mice; it cannot, unlike the simple ligand-G-protein-coupled receptor systems such as the allatostatin or the hM3Dq receptors (Tan et al., 2006; Alexander et al., 2009) or light-activated channels and pumps (Boyden et al., 2005; Han et al., 2009a,b), be applied in diverse species. We are, however, currently developing a viral and a "γ2 switch mouse" system that will make the "zolpidem system" more accessible in the mouse.

CONCLUSIONS

One of the most enjoyable aspects of researching the brain is that so many methods and approaches can be used to study it (Wulff and Wisden, 2005; Arenkiel and Ehlers, 2009; O'Connor et al., 2009; Scanziani and Hausser, 2009). There is no "one size fits all" solution for the problem of which particular technique to use for investigating circuits. For the cerebellar system, we have used two complementary genetic approaches to investigate how MLIs regulate Purkinje cells and contribute to cerebellum-dependent behaviour at the whole animal level. Whereas chronic disruption of the stellate/basket cell to Purkinje cell synapse (PC-Δγ2 mice) did not produce any obvious deficits in motor coordination (Wulff et al., 2007, 2009), remote control of Purkinje cells by a reversible and selective enhancement of GABAergic transmission at the same synapse (PC-γ2-swap mice) following i.p. administration of a drug, zolpidem, revealed the involvement of interneurons in on-line motor control (Wulff et al., 2007). These results suggest that the circuit can compensate for the loss of MLIs despite their genuine involvement in motor coordination. Extended analysis of PC-Δγ2 mice, however, showed that MLI are indispensable for a different cerebellar task: motor learning (Wulff et al., 2009). By combining these two approaches we were thus able to delineate dispensable contributions and indispensable requirements of MLIs depending on the behavioural context. Reversible modulation of selected GABAergic synapses can now be applied to analyse the time window in which MLI activity is required for cerebellar learning. Modulating the inhibitory drive onto distributed populations of selected cell types with zolpidem could also assist the study of other neural circuits.

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Ion channels to inactivate neurons in *Drosophila*

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Ion channels are the determinants of excitability; therefore, manipulation of their levels and properties provides an opportunity for the investigator to modulate neuronal and circuit function. There are a number of ways to suppress electrical activity in *Drosophila* neurons, for instance, over-expression of potassium channels (i.e. Shaker Kv1, Shaw Kv3, Kir2.1 and DORK) that are open at resting membrane potential. This will result in increased potassium efflux and membrane hyperpolarisation setting resting membrane potential below the threshold required to fire action potentials. Alternatively over-expression of other channels, pumps or co-transporters that result in a hyperpolarised membrane potential will also prevent firing. Lastly, neurons can be inactivated by, disrupting or reducing the level of functional voltage-gated sodium (Nav1 paralytic) or calcium (Cav2 cacophony) channels that mediate the depolarisation phase of action potentials. Similarly, strategies involving the opposite channel manipulation should allow net depolarisation and hyperexcitation in a given neuron. These changes in ion channel expression can be brought about by the versatile transgenic (i.e. Gal4/UAS based) systems available in *Drosophila* allowing fine temporal and spatial control of (channel) transgene expression. These systems are making it possible to electrically inactivate (or hyperexcite) any neuron or neural circuit in the fly brain, and much like an exquisite lesion experiment, potentially elucidate whatever interesting behaviour or phenotype each network mediates. These techniques are now being used in *Drosophila* to reprogram electrical activity of well-defined circuits and bring about robust and easily quantifiable changes in behaviour, allowing different models and hypotheses to be rapidly tested.

Keywords: ion channels, receptors, membrane potential, electrical inactivation, intrinsic excitability, neural circuits, behaviour, *Drosophila*

INTRODUCTION

Drosophila with its 200,000 neuron brain displays a range of different behaviours such as learning, courtship, circadian rhythms, sleep, aggression and response to addictive drugs. Each of these behaviours has been mapped to well defined neural circuits with particular genes known to influence or control different aspects of each behaviour as determined by a powerful combination of genetic screens, promoter-based transgenic manipulations and studies of gene product expression (Baker et al., 2001; McGuire et al., 2004; Holmes et al., 2007). In *Drosophila*, like any other animal, information flows through the nervous system via patterned changes in membrane depolarisation along a neuron, interspersed with synaptic transmission between neurons. Ion channels are the electrical components of the circuit controlling membrane depolarisation and synaptic currents. Information is processed and stored in a network via changes in synaptic strength and connectivity (synaptic plasticity) as well as changes in excitability in the rest of the neuron connecting input and output synapses (intrinsic plasticity). *Drosophila* has a similar range of interacting intrinsic and synaptic plasticity mechanisms, some pre- and some post-synaptic. These have mostly been studied at glutamatergic or cholinergic synapses with conserved roles for CaMKII, PKA, CREB, potassium (K^+) channels and NMDA receptors in mechanisms of plasticity and learning between *Drosophila* and humans (Littleton and Ganetzky, 2000; Giese et al., 2001; Haghighi et al., 2003; Rohrbough et al., 2003; Gasque et al., 2006; Wu et al., 2007; Schmid et al., 2008; Turrigiano, 2008).

Approximately 350 genes encode ion channels in human (ca. 1–2% of the coding genome), with over 60 of these genes causing disease (channelopathies) when mutated (Ashcroft, 2006;

Abbreviations: 4-AP, 4-Aminopyridine, I_A Shaker blocker; CaMKII, Calcium/calmodulin sensitive protein kinase type 2; Cav2, Voltage-gated calcium channel subfamily 2, cacophony (CACNA); CCAP, Crustacean cardioactive peptide; CREB, cAMP responsive element binding protein; RMP, Resting membrane potential; DORK, *Drosophila* open-rectifier K^+ channel, TASK (KCNK); DORK-NC, *Drosophila* open-rectifier K^+ channel, non-conducting version; Eag-DN, Ether-à-go-go potassium channel (KCNH/Kv10) dominant negative; $GABA_A$ subunits, γ-aminobutyric acid ionotropic receptor type A; Gal4, Galactose 4 (yeast transcription factor); $Gal80^{ts}$, Temperature sensitive Galactose 80 (inhibits Gal4); GPCR, G-protein coupled receptor; GIRK, G-protein coupled inwardly-rectifying potassium channel, Kir3 (KCNJ); I_A, Voltage-sensitive transient outward potassium current; I_h, Hyperpolarisation and cyclic-nucleotide-activated potassium current; K_{ATP} channel, Inward-rectifier (ATP sensitive) potassium channel, (KCNJ11); Kir2.1, Inward-rectifier potassium channel, (KCNJ2); Kv1, Voltage-gated potassium channel subfamily 1, shaker (KCNA); Kv3, Voltage-gated potassium channel subfamily 3, shaw (KCNC); Kv4, Voltage-gated potassium channel subfamily 4, shal (KCND); LNv, large (l) or small (s) Lateral neuron ventral (clock neurons); LTD, Long term depression; LTP, Long term potentiation; MB, Mushroom body (insect learning centres); NaChBac, Voltage-gated bacterial Na^+ channel; Na^+/K^+ ATPase, Na^+/K^+ co-transporter/adenosine triphosphate-digesting enzyme; NMDA, N-methyl d-aspartate sensitive-ionotropic glutamate receptor; NMJ, Neuromuscular junction; P_2X_2, Purinergic receptor, ligand (ATP)-gated ion channel; PDF, Pigment Dispersing Factor, circadian neuropeptide; PDZ, Post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1); PKA, cAMP sensitive protein kinase; Rdl, Resistant to dieldrin (*Drosophila* $GABA_A$ subunit gene name); RMP, Resting membrane potential; SCN, Suprachiasmatic nucleus; SDN, Shaker dominant negative subunit; SUR1, Sulphonylurea sensitive β accessory subunit for K_{ATP} channel; TASK-1, TWIK-related acid-sensitive potassium channel subfamily 1, (KCNK); TRP, Transient Receptor Potential non-selection cation channel; UAS, Upstream activating sequence (Gal4 sensitive).

Cannon, 2006). *Drosophila* has around 150 ion channels (most of which have been mutated), again about 1–2% of its coding genome. *Drosophila* channels are clearly related to the human channels, with flies often having one prototypic member of an ion channel family containing many exons that are alternatively spliced (for instance the voltage-gated Shaker K^+ channel), while humans have many separate genes possibly through gene duplication of the prototypic channel gene (i.e. there are eight human Shaker genes called KCNA1-8 encoding channel proteins Kv1.1–Kv1.8). This makes it relatively trivial in flies to make an animal completely lacking a class of ion channels and then determining the functional consequence (Salkoff et al., 1992; Littleton and Ganetzky, 2000; Giese et al., 2001; Wicher et al., 2001).

WAYS TO MANIPULATE ION CHANNEL EXPRESSION TO SILENCE NEURONS

Since ion channels are the determinants of excitability, manipulation of their levels and properties allows one to modulate neuronal and circuit function. There are a number of ways to suppress electrical activity in neurons, for instance manipulating the expression of channels that regulate resting membrane potential (RMP) setting it below the threshold required to fire action potentials. This can be achieved in several ways (listed below, **Table 1** and sections 'Ion channel manipulations that have been used to electrically inactivate *Drosophila* neurons' and 'Gal4/UAS promoter system for broad spatial and temporal control of inactivation of neurons') mainly involving over-expression of K^+ channels that are open at RMP, causing increased K^+ efflux and therefore membrane hyperpolarisation. Alternatively one can over-express channels or pumps that conduct chloride (Cl^-) ions into the cell or over-express a sodium (Na^+)/K^+ ATPase co-transporter, again to hyperpolarise RMP and prevent action potential firing. Neurons can also be silenced by disrupting or reducing the level of functional voltage-gated Na^+ or calcium (Ca^{++}) channels that mediate the depolarisation phase of action potentials. Similarly, a strategy involving the opposite channel manipulation should allow net depolarisation and hyperexcitation in a given neuron (Wicher et al., 2001; Wulff and Wisden, 2005).

Combining these approaches with the versatile (Gal4/UAS) transgenic systems available in *Drosophila* allows one temporal and spatial control of (channel) transgene expression (McGuire, et al., 2004). This now makes it possible to electrically silence (or

Table 1 | Summary of ion channels to manipulate neuronal activity in *Drosophila*.

Name of channel (UAS) transgene	Description	Gal4 targeted neurons expressing this channel will be electrically:	Source e.g. reference (Bloomington stock number)
Kir2.1	Inward rectifier K^+ channel (GFP tagged)	Inactivated	Baines et al., 2001
DORK	Outward rectifier K^+ channel (GFP tagged)	Inactivated	Nitabach et al., 2002 (8928)
Shaw	Shaw K^+ channel (FLAG tagged)	Inactivated	Hodge et al., 2005
TrpA1-RNAi	TrpA1 cation channel, RNAi	Inactivated	Rosenweig et al., 2005
Cac-RNAi	Cacophony Ca_v2 channel, RNAi	Inactivated	Worrell and Levine, 2008
δ-ACTH-Hv1a	Tethered toxin inactivates *para* Na_v channel	Inactivated	Wu et al., 2008
EKO	Shaker K^+ channel (GFP tagged)	Inactivated	White et al., 2001a
GeneSwitch EKO	Shaker K^+ channel (GFP tagged), RU486 chemically inducible	Inactivated with (slow) temporal control	Osterwalder et al., 2001
DORK-NC	Inward rectifier K^+ channel (GFP tagged) non-conducting version	Wild-type	Nitabach et al., 2002 (6587)
SDN	Shaker K^+ channel (GFP tagged), dominant negative	Activated	Mosca et al., 2005
Eag-DN	Eag K^+ channel, dominant negative	Activated	Broughton et al., 2004 (8187)
Shaw-DN	Shaw K^+ channel (FLAG tagged), dominant negative	Activated	Hodge et al., 2005
Shaw-RNAi	Shaw K^+ channel, RNAi	Activated	Hodge and Stanewsky, 2008
Na^+/K^+ ATPase-DN	Na^+/K^+ ATPase, dominant negative	Activated	Parisky et al., 2008
GeneSwitch SDN	Shaker K^+ channel (GFP tagged), dominant negative, RU486 chemically inducible	Activated with (slow) temporal control	Mosca et al., 2005
TrpA1	Heat activated TrpA1 cation channel	Activated with (medium) temporal control	Pulver et al., 2009
TrpM8	Cold activated TrpM8 cation channel	Activated with (medium) temporal control	Peabody et al., 2009
P2X2	Purinergic receptor, activated by uncaging ATP	Activated with (fast) temporal control	Sjulson and Miesenböck, 2008
ChR2	Channelrhodopsin, blue light activated cation channel	Activated with (fast) temporal control	Schroll et al., 2006; Pulver et al., 2009 (24855)

hyperexcite) any neuron or neural circuit in the fly brain (Peabody et al., 2008, 2009; Gordon and Scott, 2009), and much like an exquisite lesion experiment, potentially elucidate whatever interesting behaviour or phenotype each mediate. Likewise, this approach can determine if the expression, mechanism of action or structure of a molecule, pathway or process is regulated by electrical activity. This special issue on new genetic techniques revolutionising the study of neural circuits, should facilitate the transfer of technology and channels between model systems and help researchers wishing to formulate a theoretical or computational understanding of this research.

HOMEOSTATIC PLASTICITY MECHANISMS

A key issue to consider when ion channels are expressed in order to silence neurons or reduce the activity of neurons, is will the resulting change in electrical activity induce homeostatic plasticity mechanisms that compensate and try to return the neuron back to its normal activity state. Put another way, neuronal networks have a tendency not to like to be silenced or, have extreme patterns of hyperexcitability imposed upon them. In fact, they may have evolved a diverse repertoire of homeostatic mechanisms to compensate for large net changes in synaptic and intrinsic excitability in order to preserve important network functions (Marder and Prinz, 2002; Davis, 2006; Turrigiano, 2008).

Ion channel mutants (i.e. nulls or 'knock outs'), although useful for determining the basic function of a channel, can be limited as tools for the spatial and temporal control of neural activity in a given circuit, as channels tend to be broadly expressed. Furthermore, removing a channel throughout development can induce compensatory mechanisms. An example from mouse is the $GABA_A$ subunits that mediate persistent inward Cl^- currents important for tonic inhibition of cerebellar granule cells when knocked-out trigger a homeostatic mechanism resulting in up-regulation of leak TASK-1 K^+ channels that preserve these particular neuron's characteristic electrophysiological properties (Brickley et al., 2001). Even single point mutations in *Drosophila* channels can result in a slew of transcriptional changes in activity dependent genes (Guan et al., 2005). These genes presumably form an inherently robust genetic network with loss of any branch (gene) of the network being compensated by a complementary rearrangement of the remaining interacting branches (genes) maintaining functional output from the network (van Swinderen and Greenspan, 2005).

Another example, this time from lobster, is when Shal (Kv4 or KCND) RNA is injected into neurons there is an increase in the fast transient I_A current. However this change is also accompanied by a compensatory up-regulation of a hyperpolarisation activated I_h cation current in order to maintain the neuron's firing behaviour. Interestingly expression of a non-conducting version of Shal also induced I_h up-regulation, suggesting that changes in channel protein level, as opposed to the accompanying changes in membrane activity, can be the trigger of this activity-independent homeostatic response. Not all changes in channel level are compensated, for instance the reciprocal increase in I_h channel was not compensated and did alter the neuron's behaviour (MacClean et al., 2003).

ION CHANNEL MANIPULATIONS THAT HAVE BEEN USED TO ELECTRICALLY INACTIVATE *DROSOPHILA* NEURONS

LOSS-OF-FUNCTION MUTATIONS IN NEURONAL Na^+ OR Ca^{2+} CHANNELS

Removing the main depolarising channels from neurons is one strategy to inactivate *Drosophila* neurons. However these ion channels tend to have a number of specific roles and serve a range of pleiotropic functions making their presence necessary for viability; this is reflected in these channels having widespread tissue or developmental expression. For instance, *Drosophila's* single member of the Cav2 family, the pan-neural expressed *cacophony*, when null mutated causes embryonic lethality, while partial loss of function alleles cause changes in synaptic transmission, increased convulsions and aberrant courtship and vision (Smith et al., 1998; Wicher et al., 2001). Likewise, null alleles of *paralytic*, the tetrodotoxin-sensitive *Drosophila* Na_v1 are lethal, while partial loss of function or temperature sensitive-alleles ($para^{ts}$ at restrictive temperature) have disrupted action potential propagation and paralysis (Suzuki and Wu, 1984; Wicher et al., 2001). Some point mutations may however, confer some spatial control of functional inactivation of neurons, for instance, *Smellblind* mutant alleles are thought to remove the olfactory specific splice form of *para* by specific exon-skipping events (Reenan et al., 1995). Similar splicing defects of human Cav and Nav channel genes can cause channelopathies resulting from brain region- or tissue specific-compromised channel function with accompanying pathophysiology such as cerebellar ataxia, familial hemiplegic migraine and cardiac arrhythmias (Ashcroft, 2006; Cannon, 2006).

GAIN-OF-FUNCTION MUTATIONS IN NEURONAL K^+ CHANNELS

K^+ channels are the most diverse ion channel family, with over 30 such channels in *Drosophila*, therefore mutations are seldom lethal with the possibility of having adult flies lacking two major classes of K^+ channel (Littleton and Ganetzky, 2000; Vähäsöyrinki et al., 2006). However K^+ channel mutants, like other channel chromosomal mutants, maybe of limited use to suppress neuronal electrical activity, especially as unlike human or worm channel mutants, few are gain-of-function alleles required for electrical inactivation (Ashcroft, 2006; Cardnell et al., 2006). Increased repolarising current can also be achieved by mutants that contain a chromosomal rearrangement that result in duplicated copies of a K^+ channel translocated onto an additional chromosome (Haugland and Wu, 1990). Similar changes in channel gene dosage likely occur in Downs (Trisomy 21) syndrome resulting in changes in neural development, long-term potentiation (LTP) and learning in mouse models of Downs (Morice et al., 2008). However all these approaches are limited in that they do not allow the experimenter both spatial and temporal control of electrical inactivation of just a chosen neural circuit.

Gal4/UAS PROMOTER SYSTEM FOR BROAD SPATIAL AND TEMPORAL CONTROL OF INACTIVATION OF NEURONS

The disadvantages of using mutants can be overcome by using selective over-expression strategies. The Gal4/UAS bipartite system has one part that consists of a fly containing a transgenic copy of the Gal4 transcription factor inserted into its genome. The expression of the integrated Gal4 transgene is dependent on whatever endogenous enhancers it is inserted next to (e.g. enhancer-trapping).

The second half of the system is a fly containing a transgene of interest downstream of a Gal4 upstream activation sequence (UAS), which by itself: is not expressed. By crossing a fly containing a Gal4 insert with a fly containing a UAS-transgene, the resulting progeny will have both, therefore expressing your gene of interest in a given pattern of cells. Over the last 15 years, UAS-transgenes to over-express most of the genes in the fly genome have been collected (http://flybase.org) some containing fluorescent tags, some point mutations and some homologues from other species such as human (Brand and Perrimon, 1993; White et al., 2001b). Recent inclusions to this arsenal include publicly available UAS-RNAi lines to all 14,000 genes in the fly genome (Dietzl et al., 2007).

Married to these are collections of 10,000s of Gal4 lines that have been screened for expression in different tissues and times of development using UAS-GFP. Many *Drosophila* promoter sequences are relatively compact so can be placed before Gal4, allowing the resulting Gal4 insertion to report the expression of the original gene promoter. Gene promoters for neuropeptide, neurotransmitter synthetic enzymes and different channels and receptors have revealed their neural expression pattern and also allowed mis-expression in dopaminergic, cholinergic and GABAergic neurons (Holmes et al., 2007). However the expression of Gal4 lines can be complex, for instance, there are many mushroom body (MB) Gal4 lines, however these seldom express in MB alone or just in the adult MB learning centre. This is because most genes are expressed in development and often in a number of tissues serving a number of functions (pleiotropic; Hall, 2005). For instance Rdl, a *Drosophila* $GABA_A$ receptor subunit is expressed in the MB and is important for learning (Liu et al., 2007), but is also expressed in clock neurons regulating sleep (Parisky et al., 2008). This is probably not a coincidence as sleep and memory influence one another and might be co-regulated by molecules expressed in over-lapping circuits controlling both behaviours. The combination of well-defined neural circuits, robust behavioural assays and powerful molecular genetics of *Drosophila* is now revealing the molecular nature of the plasticity mechanisms connecting sleep and memory (Donlea et al., 2009; Gilestro et al., 2009). Such circuit-breaking studies are more difficult in the more complex mammalian brain as there are many more cells of each type, a paucity of well-defined promoters, and neuronal groups (for instance GABAergic neurons) are often dispersed between brain structures (Wulff and Wisden, 2005).

A number of variations of the Gal4 promoter and similar systems are continually being developed in *Drosophila* (McGuire et al., 2004) to increase spatial and temporal control of transgene expression. For instance, Gal4 expression has been made to be dependent on the presence of an activator, a progesterone analogue called RU486. The resulting system called GeneSwitch makes flies only express Gal4 and hence transgenes such as Shaker when they are placed on RU486 containing food, thereby potentially bypassing any problems arising from lethality, developmental defects and compensation through long term (developmental) changes in excitability (Osterwalder et al., 2001). It is not exactly known why over-expressing K^+ channels sometimes causes apoptosis, however it maybe due to the steady depletion of K^+ from the cell (Nadeau et al., 2000). Likewise in order to circumvent lethality sometimes resulting from expression of K^+ channel transgenes, such as Kir2.1, the TARGET system can be employed (McGuire et al., 2004). At 18°C $Gal80^{ts}$ inhibits Gal4 activity blocking Kir2.1 expression and lethality. Whereas keeping the flies at 31°C inactivates $Gal80^{ts}$ releasing Gal4 driven Kir2.1. This technique allowed the critical window in development to be probed where electrical inactivation of a specific group of neurons resulted in lethality (Peabody et al., 2008).

In the following sections I will catalogue in detail these inactivation strategies that have been successfully used with the Gal4/UAS system.

HUMAN (KCNJ2/Kir2.1) CHANNELS

A pioneering study by Baines et al. (2001) wished to tease apart the relationship between intrinsic excitability and synaptic strength. In order to study the effect of electrical inactivation of fly neurons on synaptic transmission, the authors expressed an N-terminal GFP tagged human inwardly rectifying Kir2.1 channel. This channel had been used previously (Johns et al., 1999), to genetically suppress excitability of rat neurons in culture using an inducible promoter this time responsive for the *Drosophila* hormone, ecdysone. Gal4 promoters with different distributions of pre- and post-synaptic expression at defined synapses in the embryonic fly brain were used. Kir2.1 expression in the postsynaptic neuron (a motorneuron) removed evoked but not spontaneous release of neurotransmitter. No compensatory changes were seen. The same approach of developmental cell specific expression of Kir2.1 has also been used to determine the role of neural activity in generating the mouse olfactory sensory map (Yu et al., 2004). Also hyperpolarising Kir2.1 expression in hippocampal neurons was found to decrease firing rate, but this time in these mammalian neurons a homeostatic mechanism returned firing rate to normal even though Kir2.1 continued to be expressed (Burrone et al., 2002).

At the *Drosophila* larval neuromuscular junction (NMJ) synapse (Paradis et al., 2001), muscle expression of Kir2.1 resulted in an outward (~10nA) leak current accompanied by 10–15mV hyperpolarised shifts in muscle RMP. Despite this reduction in postsynaptic excitability, synaptic transmission was maintained by a compensatory increase in presynaptic release (quantal content), implicating an activity-dependent retrograde homeostatic mechanism.

Expression of the Kir2.1 transgene in insulin secreting cells of the fly was then used in order to help characterise a *Drosophila* model of diabetes (Kim and Rulifson, 2004). *Drosophila* has homologues of both the sulphonylurea receptor (SUR1 β-cell K_{ATP} channel β-subunit), and the K_{ATP} channel α-subunit (Kir6.2 or KCNJ11) it confers glucose sensitivity to. Sulphonylurea disrupted glucose stimulated insulin release in *Drosophila* by a mechanism involving SUR and Kir channels. Expression of the non-ATP sensitive human Kir2.1 in the *Drosophila* insulin secreting neurons prevented membrane depolarisation and release from these neurons resulting in flies with disrupted control of circulating glucose (Kim and Rulifson, 2004; Ashcroft, 2006).

In addition to learning and memory, another function prescribed to the MB and cAMP signalling within these cells is control of sleep. MB electrical inactivation using promoters that express Kir2.1 in these cells led to increased daily sleep while manipulations thought to increase excitability lead to a reduction in sleep (Joiner et al., 2006). It should be noted expression of Kir2.1 has the most extreme functional consequences of all the K^+ channel transgenes, for instance, Kir2.1 expression in as

little as 30–40 crustacean cardioactive peptide (CCAP) neurons is sufficient to cause lethality (Luan et al., 2006). So what are the longer-term consequences of electrical inactivation of different neurons using this approach, expression of Kir2.1 in a defined type of serotonin neuron, resulted in morphological changes in the neuron's neurites (Roy et al., 2007).

Lastly, in order to study the post-mating switch in behaviour of female flies (decrease in receptivity to courting males and increased egg laying), the Jan lab used Kir2.1 based techniques. The switch in behaviour is brought about by sex peptide that is transferred with the male's sperm into the female's reproductive organ where it acts on fruitless and pickpocket (ppk, a *Drosophila* Na^+ channel) neurons. Expression of Kir2.1 in ppk sensory neurons increased the post-mating change in behaviour, suggesting sex peptide normally inhibits neuronal transmission in these circuits. Clonal analysis using flies expressing GFP-Kir2.1 in ppk neurons downstream of FRT sites; showed that the virgins that displayed the most post-mating responses where the one's that had the most Kir2.1 expression in the ppk neurons on the uterus and which projected axons towards the flies brain (Yang et al., 2009).

DROSOPHILA SHAKER EKO (KCNA/Kv1) CHANNELS

Possibly the earliest attempt to electrically inactivate neurons using K^+ channel over-expression (Gisselmann et al., 1989), used a heat shock promoter to express either a Shaker cDNA or a dominant negative truncated version of Shaker (W404 to stop in the pore of the channel). Because a functional Shaker channel consists of four pore-forming α-subunits, the presence of one or more pore-truncated subunits would block the remaining pore intact endogenous Shaker subunits removing channel function. Heat-shock of the full-length or truncated Shaker over-expressing flies caused the predicted hypo- or hyper-excitability at the electrophysiological (NMJ recordings) and behavioural (ether-induced shaking) level.

In order to direct the inactivation of defined neurons, the Gal4/UAS was used to express Shaker channels that were genetically modified to have a more hyperpolarised voltage-dependence of activation, no N-terminal fast inactivation and an N-terminal GFP tag. This 'electrical knockout channel' or EKO allowed sustained activation in response to depolarisation suppressing electrical activity of the cells in which it was expressed. Pre or post-synaptic expression of EKO caused an increase in the sustained K^+ current, RMP hyperpolarisation and reduced firing. These changes were partially reversed by the Shaker channel blocker, 4-AP. No homeostatic changes were reported although the level of endogenous Shaker transient I_A current was reduced in muscles over-expressing EKO (White et al., 2001a,b).

As for Kir2.1 channels, EKO expression throughout the nervous system or musculature caused some paralysis and impaired locomotion. These effects increased with EKO gene dosage (it is possible to have multiple copies of UAS-EKO in a fly) resulting in increased embryonic lethality. Reduction in neuronal excitability with EKO over-expression caused some aberrant NMJ connectivity and in the eye a dose-dependent decrease in photoreceptor potential, again partially blocked by 4-AP. A307-Gal4 expression of EKO strongly in the ventral and abdominal ganglion of the fly resulted in adult flies with unexpanded wings and abnormally pigmented thorax. This developmental phenotype was suggested to be due to incomplete adult eclosion (insects divide their life into embryonic, larval, pupal and adult stages, completion of the later stage is called eclosion which involves wing expansion) requiring neuronal release of eclosion hormones (White et al., 2001a,b). Later studies have elegantly dissected the role of excitability in control of wing expansion by release of the hormone bursicon from CCAP neurons, three copies of EKO was found to be sufficient to block release of bursicon (Luan et al., 2006).

A similar approach was then used to increase excitability by expression of an N-terminal GFP-tagged Shaker dominant negative (SDN) subunit truncated after the first transmembrane domain (Mosca et al., 2005). As predicted SDN caused hyperexcitability associated with reduced I_A, enhanced synaptic transmission, enhanced synapse size and increased larval locomotion. In adults, hyperexcitable phenotypes included ether-induced shaking. Serendipitously the identical Shaker mutation was picked up in a forward genetic screen for flies with reduced sleep and called *minisleep*. This is a mutant that sleeps one-third as much as wild-type flies (Cirelli, et al., 2005). Electrical inactivation of MB neurons with EKO resulted in flies with a modest increase in sleep (Joiner et al., 2006).

Neuron specific expression of EKO can therefore be used to decrease intrinsic excitability, while expression of SDN can be used to increase excitability in neurons that endogenously express Shaker channels. Postembryonic expression of either transgene in motorneurons caused increased dendritic outgrowth (Duch et al., 2008). This study also showed that SDN changed the firing patterns of a motorneuron upon current injection, while EKO decreased firing but did not completely silence neurons. Interestingly, none of the transgenic manipulations effected RMP, input resistance or synaptic properties. Increases in flight behaviour were seen in the SDN flies and a decrease in flies expressing EKO in their motorneurons.

DROSOPHILA ORK (KCNK/TASK) CHANNELS

Drosophila open rectifier K^+ channels (DORK) exhibit no voltage or time dependence of opening, essentially forming K^+ selective holes in the membranes of the cells they are expressed. A C-terminally GFP tagged DORK transgene (DORK) was expressed in flies using the GAL4/UAS system, and an additional mutant non-conducting version of the channel (DORK-NC) was also expressed as a control (Nitabach et al., 2002). Unlike non-conducting versions of lobster Shal (MacClean et al., 2003), no homeostatic changes were triggered. However the trend of DORK-NC overexpression as the sole choice of wild-type control for over-expression of different transgenic channels should also be performed with caution, due to the increasing number of non-conducting functions of ion channels being discovered (Kaczmarek, 2006).

Not surprisingly, pan-neuronal expression of DORK like Kir2.1 caused embryonic lethality. In order to determine the effect of electrical inactivation on a well-defined behaviour: DORK or Kir2.1 were expressed in a subset of clock neurons (~20 neurons) called the lateral neurons ventral (LNv) using Pigment dispersing factor (*pdf*-Gal4) promoter (Nitabach et al., 2002). These form part of the ~150 neurons that form the fly clock. Circadian rhythms are generated by the molecular clock in each clock neuron which consists of rhythmically expressed clock genes (i.e. *timeless* and *period*)

that feedback and control their own expression with an ~24hour period (Hall, 2005).

It was claimed that DORK or Kir2.1 expression electrically silenced the clock neurons. Chronic silencing of neurons with such channels can be associated with apoptosis (Nadeau et al., 2000), however Pigment dispersing factor (PDF) staining of the 'silenced' LNv was qualitatively shown not to grossly disrupt their viability or morphology. In constant darkness, 'silencing' the LNv resulted in a higher proportion of flies showing arrhythmic or weakly rhythmic behaviour with respect to wild-type controls. LNv expression of DORK-NC resulted in some arrhythmic behaviour but otherwise appeared normal. The rhythmic expression of clock proteins using semi-quantification of antibody stain intensity on fixed brains was dampened in neurons expressing DORK or Kir2.1. The interruption of these results was that electrical silencing of the LNv had stopped the free-running circadian clock. Less dramatic effects were reported for the effect of these transgenic manipulations under normal lighting conditions (12 hours of light, 12 hours dark) (Nitabach et al., 2002). Expression throughout the clock circuit using *timeless*-Gal4 resulted in adult arrhythmia in constant darkness and disrupted the molecular clock as judged by timeless staining of the larval LNv (Nitabach et al., 2005). Later studies showed that electrical inactivation of LNv clock neurons had similar circadian molecular and behavioural effects as *pdf* mutants and that the molecular clock oscillations in remaining clock neurons were not abolished (Wu et al., 2008).

It is now thought that synchronisation of the rhythms between clock neurons and the circadian output from the clock that regulates rhythmic behaviour are mediated by, both chemical (predominantly PDF) and electrical signals (Hall, 2005; Holmes et al., 2007). Earlier mammalian suprachiamatic nucleus (SCN) recordings (Kuhlman and McMahon, 2004) and fly large (l)-LNv (Park and Griffith, 2005; Cao and Nitabach, 2008; Sheeba et al., 2008a,b; Wu et al., 2008a) recordings revealed clock neurons display circadian changes in their electrical properties including a (~10mV) depolarised shift in RMP during the day accompanied by an increase in frequency of spontaneous action potentials (Brown and Piggins, 2007; Holmes et al., 2007).

In order to extend the DORK behavioural genetic studies, whole-cell recordings were performed on l-LNv neurons expressing DORK this caused a 10mV hyperpolarising shift in LNv RMP and reduced but did not silence evoked action potential firing (Park and Griffith, 2005). The chromosomal location of a transgene can also effect expression and hence have functional effects, for instance the *pdf*-Gal4 expression of C1 insertion of DORK results in most flies being arrhythmic while the C2 insertion causes most flies to be weakly arrhythmic with Kir2.1 causing the greatest arrhythmicity (Nitabach et al., 2002). A later study confirmed *pdf*-Gal4 expression of DORK-C2 caused a significant hyperpolarisation of LNv RMP; that was greater for DORK-C1 and greatest for Kir2.1. This resulted in over 60% of flies expressing either Kir2.1 or DORK-C1 displaying arrhythmia in constant darkness while over-expression of the hyperpolarising DORK-C2 now gave the same level of arrhythmicity as control (~20%; Wu et al., 2008a).

Lastly, DORK expression has been used to explore plasticity mechanisms at fly synapses. It is widely thought that neurons can detect their level of activity via a range of calcium sensitive sensors prominent amongst which is CaMKII which autophosphorylates at T287 during LTP and T306 during long term depression (LTD), both events are thought to be important for learning in a range of animals including flies (Marder and Prinz, 2002; Davis, 2006; Nelson and Turrigiano, 2008). Electrical inactivation of postsynaptic cells using DORK caused an increase in synaptic phosphorylation of T306 and concomitant reduction in T287, these changes were regulated by the PDZ-scaffolding molecule, CASK (Lu et al., 2003; Hodge et al., 2006). Activated CaMKII is known to directly bind or modulate a number of K^+ channels and glutamate receptors regulating neuronal excitability in a range of systems (Griffith et al., 1994; Park et al., 2001; Yao and Wu, 2001; Haghighi et al., 2003; Sun et al., 2004; Nelson et al., 2005; Gasque et al., 2006), while CASK also interacts and changes the activity of a number of synaptic ion channels and receptors (Hsueh, 2006).

DROSOPHILA SHAW (KCNC/Kv3) CHANNELS

Drosophila Shaw is a member of the Kv channel family and encodes a slowly activating and non-inactivating K^+ current. Shaw is open at normal RMP and causes hyperpolarisation by K^+ efflux; the channel is widely expressed in the nervous system and helps regulate RMP in *Drosophila* central neurons (Salkoff et al., 1992; Tsunoda and Salkoff, 1995; Hodge et al., 2005; Parisky et al., 2008). Gal4 over-expression of full-length Shaw was detected via a C-terminal FLAG tag on the transgenic channel. A Flag tagged dominant negative form of Shaw was also made (Hodge et al., 2005) with the analogous pore truncation as the heat shock Shaker dominant negative transgene described previously (Gisselmann et al., 1989). This subunit behaved in a dominant negative fashion causing ~10mV depolarising shift in RMP and a doubling of spike frequency. This hyperexcitation did not lead to any homeostatic changes in the remaining K^+ currents. Like EKO expression, widespread neuronal expression of Shaw resulted in developmental lethality and wing expansion deficits consistent with the transgene causing electrical inactivation of the neurons it is expressed in. This wing expansion phenotype was mapped to the 30–40 CCAP positive neurons. Widespread expression of dominant negative Shaw caused hyperexcitable phenotypes such as ether-induced shaking and wing expansion phenotype. Endogenous Shaw was expressed widely in the nervous system including CCAP neurons (Hodge et al., 2005).

Because RMP-hyperpolarising DORK over-expression in clock neurons caused behavioural arrhythmicity (Nitabach et al., 2002, 2005), we wished to explore which channels might endogenously regulate clock neuron RMP. We started with Shaw as this channel is widely expressed in the *Drosophila* brain and regulates neuronal RMP (Hodge et al., 2005). Furthermore mammalian homologues of Shaw, Kv3, are widely expressed in SCN and the magnitude of their current varies between day and night and even in constant darkness. Blocking the currents prevented the daily rhythm in firing of SCN neurons (Itri et al., 2005). Using a C-terminal antibody, Shaw was found widely expressed in the adult brain including a subset of clock neurons. In normal light conditions expression of membrane hyperpolarising Shaw in all clock neurons (*timeless*-Gal4) increased locomotor activity at night. Under constant conditions, electrical inactivation of all clock neurons resulted in extreme arrhythmia as was also reported for expression of Kir2.1 or DORK.

This had little effect on the molecular clock as measured by per-luciferase oscillations in the dorsal clock neurons. The rhythmic accumulation of PDF in terminals of small LNv neurons was however disrupted by changing the level of Shaw throughout the clock. Hyperpolarising Shaw over-expression caused PDF accumulation in terminals while reduction in functional Shaw depleted the levels of the neuropeptide in terminals. Hyperexciting the clock by *tim*-Gal4 expression of dominant negative Shaw also caused weaker rhythms (Hodge and Stanewsky, 2008).

As mentioned over-expression of $GABA_A$ mediated inward Cl^- currents would inactivate neurons with GABAergic input and has been shown to inhibit *Drosophila* olfactory associative learning (Liu et al., 2007). In order to determine the role of GABA mediated regulation of sleep, Shaw was expressed in GABAergic neurons using glutamic acid decarboxylase (GAD)-Gal4, in order to decrease GABA release. This resulted in a reduction in the initiation and maintenance of sleep (Agosto et al., 2008). Likewise reduction of expression of $GABA_A$ receptors using RNAi in just the LNv neurons caused a similar reduction in sleep. Electrical inactivation of LNv using EKO also caused increased initiation and maintenance of sleep. In order to hyperactivate the LNvs in a physiologically relevant manner, Shaw-RNAi or dominant negative Na^+/K^+-ATPase was over-expressed. Both gave an ~10mV depolarised shift of RMP accompanied by increased firing of action potential, resulting in a reciprocal decreased initiation and maintenance of sleep. These results together suggest the LNvs are important for light-induced arousal (Parisky et al., 2008).

TARGETED REDUCTION IN DEPOLARISING CURRENTS IN ORDER TO INACTIVATE *DROSOPHILA* NEURONS

As Na^+ and Ca^{2+} channels gene products are large and do not tetramerise to form a function channel, dominant negative strategies targeting these channels are therefore more problematic. RNAi expression has proved affective at reducing *Drosophila* TrpA1 resulting in flies that do not show normal avoidance of elevated temperatures (Rosenweig et al., 2005). RNAi has also been used for targeted reduction of Cav2 currents, thereby decreasing evoked and spontaneous neuronal activity by a similar amount as hypomorphic *cacophony* mutants (Worrell and Levine, 2008). With genome-wide collections of Gal4 driven RNAi lines now available (Dietzl et al., 2007), one can expect this approach to be used increasingly to target *Drosophila* ion channels in inactivate neurons. Another new technology tested using the *Drosophila* circadian system, is cell autonomous expression of membrane-tethered toxins. *Pdf*-Gal4 expression of δ-ACTH-Hv1a inhibited the *para* Na_v channel inactivation and induced rhythmic action potential firing. This resulted in PDF accumulating in LNv terminals earlier and flies starting to become active earlier (anticipating) before lights on (Wu et al., 2008b).

Eag-DN, NARROW ABDOMEN, TRP-M, TRP-A AND NaChBac TRANSGENIC CHANNELS TO CHANGE *DROSOPHILA* NEURON ACTIVITY

An alternative Gal4 dominant negative K^+ channel strategy used an eag transgene truncated before the first transmembrane segment (eag-Dominant Negative). This was shown to increase excitability (Duch et al., 2008) and has been used to study courtship in flies (Broughton et al., 2004).

Another study exploring the relationship between the molecular clock and electrical activity used a range of mutations in Narrow Abdomen, a NALCN/ Na^+ non-selective leak channel endogenously expressed in the clock. Mutants are expected to hyperpolarise clock neuron RMP and caused arrhythmia in constant darkness, however as for Shaw expression, this activity manipulation did not disrupt the molecular clock but did change output from the clock via elevated PDF levels in LNv terminals (Lear et al., 2005).

In order to achieve finer temporal control of electrical activity, the heat-sensitive *Drosophila* TrpA1 channel has been used acutely increase LNv activity with temperature shifts sufficient to disrupt sleep (Parisky et al., 2008). Likewise, the cold and menthol sensitive rat TrpM8 channel has been expressed in CCAP neurons imposing a rapid cold-sensitive switch in network activity controlling wing expansion (Peabody et al., 2009).

Flies have also been made that express a voltage-gated bacterial Na^+ channel (NaChBac). Expression in CCAP neurons resulted in the wing expansion phenotype (Luan et al., 2006). *pdf*-Gal4 expression of NaChBac was found to cause hyper-excitation of LNvs in terms of causing massive increases in action potential amplitude and duration. However at the same time NaChBac expression was found to cause strong hypoexcitation of LNvs with RMP going from a wild-type value of −41.5 to −103mV with an accompanying drop in firing frequency. These changes in LNv excitability caused by NaChBac expression were not homeostatically compensated (Sheeba et al., 2008a,b). In addition, NaChBac LNv terminal PDF levels remain high at night. This resulted in subsets of clock neurons becoming desynchronised causing the generation of complex behavioural rhythms (splitting) (Nitabach et al., 2006), similar effects were seen with hyperpolarising expression of Shaw in some clock neurons (Hodge and Stanewsky, 2008). Likewise, l-LNv NacBac expression caused the normal day-night shift in excitability to be reversed, so that the flies were more active at night (Sheeba et al., 2008b). A mosaic technique allowed single cell manipulation of electrical activity. It was found the more l-LNvs neurons expressing NaChBac the more nocturnal the fly's behaviour became (Shang et al., 2008).

FUTURE DIRECTIONS

A current technology set to revolutionise the study of neural activity in circuits is the use of light-activated channels. Expression of the microbial channelorhodopsin using Gal4 has been used to depolarise and activate *Drosophila* neurons upon blue light stimulation. Likewise UV-uncaging of ATP has been used to cell autonomously stimulate rat P_2X_2 receptors expressed in *Drosophila* neural circuits. Conversely light stimulation of inhibitory channels, such as the *Natronomonas pharaonis* (NpHR) halorhodopsin chloride pump with yellow light has also been demonstrated (Zhang et al., 2007; Sjulson and Miesenböck, 2008). Generation of Gal4 inducible NpHR flies holds great promise for acute electrical inactivation of neural circuits in flies, however as it is a pump, it might be expected to have slower kinetics and different trafficking than ChR2. However, such Gal4 driven approaches would allow acute bi-directional changes in activation of *Drosophila* neurons that may bypass some of the confounding problems that can be caused by chronic channel activity mentioned earlier in this review.

Therefore these channels have the promise to provide millisecond control of membrane potential and neuronal spike firing (with

activated TRP channels allowing longer stimulation; Pulver et al., 2009) and allow robust remote control of different fly behaviours. For instance, blue-light photoexcitation of ChR2 expressed in acj6 (abnormal chemosensory jump 6 transcription factor) neurons was able to elicit the innate escape response of flies (Zimmermann et al., 2009). Furthermore, light induced activation of ChR2 expressed in larval dopaminergic neurons when paired with a specific odour could induce aversive memory formation, while activation of octopaminergic/tyraminergic neurons with another odour could induce positively reinforced memories (Schroll et al., 2006). Likewise, UV uncaging of ATP activated P_2X_2 receptors expressed in giant fibre system elicited the predicted escape behaviours of jumping, wing beating and flight. While expression in dopamine neurons caused increased locomotion (Lima and Miesenböck, 2005). Finally, photo-triggering P_2X_2 expressed in fruitless neurons that form part of the neural circuit that helps generate the male courtship song, caused flies of either sex to perform the courtship song (Clyne and Miesenböck, 2008).

Generally it should be noted that many of the ion channel manipulations described in this review did not cause any obvious compensatory changes, suggesting that *Drosophila* neurons and circuits may contain fewer compensatory mechanisms than mammalian ones. However in order to bypass any potential compensatory problems caused by chronic channel expression, pharmacological modulation of ectopically expressed transgenic channel could be added, allowing further temporal control of electrical activity to the investigator (Wulff and Wisden, 2005; Wulff et al., 2007). Likewise synthetic photoisomerisable small molecules have now been used to specifically target and inactivate endogenous Shaker channels causing their inactivation and acute changes in neural activity in rodent and leech preparations (Fortin et al., 2008). In order to get round any unwanted potential leaky expression of UAS channel transgenes independent of Gal4, it maybe possible to use new tools (retrovirus insulators and phiC31 integration) to minimise positional effects of host chromatin and target integration of transgenes to defined sites known not to be leaky. These technologies should help standardise reverse genetics in *Drosophila* by improving leakiness and allowing robust transgene expression at a defined level (Markstein et al., 2008).

So what else can we hope for in the future? Certainly, bi-directional translation of these new technologies between model organisms and also into new organisms that offer unique experimental features (Baker et al., 2001; Marder, 2002). Early examples of this trend include the fly's phototransduction pathway namely arrestin, rhodopsin G-protein coupled receptor (GPCR) and Gqα collectively termed chARGe being used to allow light triggered excitation of hippocampal neurons (Zemelman et al., 2002). Likewise, ectopic expression of the *Drosophila* allatostatin GPCR which couples to a $G_{i/o}$ to open inwardly rectifying K^+ channel (GIRK) can be used to cause a neuronal hyperpolarisation and resulting decrease in spiking on addition of allatostatin and has been used to investigate mouse spinal cord networks *in vivo* (Gosgnach et al., 2008; Zhang et al., 2008).

Also, as an increasing number of CNS disorders are found to involve abnormal neural activity of circuits (Saper et al., 2005; Ashcroft, 2006; Cannon, 2006; Beck and Yaari, 2008), modelling these diseases in a range of model organisms should in future elucidate the fundamental properties of these disorders at the molecular, electrophysiological and behavioural level (Mackay and Anholt, 2006; Zhang et al., 2007; Morice et al., 2008; Song and Tanouye, 2008). These model systems together have the potential to allow screening or validation of new candidate genes, drug targets, and therapies using small molecules, biologicals or even gene therapies using transgenic channels to repair or rescue functional output (Herlitze and Landmesser, 2007).

Researchers championing the different technologies described in this review can now think about reprogramming electrical activity in well-defined circuits and bring about predictable changes in behaviour. This approach can be used in a manner somewhat analogous to dynamic clamp (Prinz et al., 2004) except it is optically controlled, allowing the researcher to add or remove different currents to a circuit, testing theoretical models or replaying different activity patterns to try and instruct different changes in behaviour.

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Manipulating neuronal circuits with endogenous and recombinant cell-surface tethered modulators

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Neuronal circuits depend on the precise regulation of cell-surface receptors and ion channels. An ongoing challenge in neuroscience research is deciphering the functional contribution of specific receptors and ion channels using engineered modulators. A novel strategy, termed "tethered toxins", was recently developed to characterize neuronal circuits using the evolutionary derived selectivity of venom peptide toxins and endogenous peptide ligands, such as lynx1 prototoxins. Herein, the discovery and engineering of cell-surface tethered peptides is reviewed, with particular attention given to their cell-autonomy, modular composition, and genetic targeting in different model organisms. The relative ease with which tethered peptides can be engineered, coupled with the increasing number of neuroactive venom toxins and ligand peptides being discovered, imply a multitude of potentially innovative applications for manipulating neuronal circuits and tissue-specific cell networks, including treatment of disorders caused by malfunction of receptors and ion channels.

Keywords: tethered-toxins, cell-surface modulators, lynx1, receptors, ion channels

INTRODUCTION

Understanding complex processes such as neuronal activity or cell signaling malfunctions that result in human disorders or diseases relies on extensive knowledge about the function, structure and precision of ion channels, receptors and modulators. As a result of this, ion channels are at present the third biggest target class in drug discovery; yet still remain underexploited as drug targets. Recent reviews describe the increasing interest in peptide venom toxins for the development of drug therapies directed towards ion channels and receptors (Blumenthal and Seibert, 2003; Phui Yee et al., 2004; Lynch et al., 2006; Han et al., 2008; Twede et al., 2009). Specific areas in which peptide toxins have demonstrated their potential include Alzheimer's disease (candoxin) (Nirthanan et al., 2002), chronic pain (MVIIA) (Miljanich, 2004) and myasthenic autoimmune response (α-Bgtx) (Drachman, 1981; Mebs, 1989). For instance, snake neurotoxins bind to nicotinic acetylcholine receptors (nAChRs) with affinities within the pico and nanomolar range (Chiappinelli, 1991), which indicates that these would be among the best probes for investigating potential therapeutics that affect nAChR activity. The unique homologies of endogenous lynx1 prototoxins with venom toxins provided a biological scaffold for developing recombinant molecules to selectively modulate ion channels and receptors. Thus, based on the characteristics and mode of action of lynx1 cell-surface modulators, new classes of "tethered toxins" and "tethered ligands" were created as probes to characterize ion channels and receptors (Ibañez-Tallon et al., 2004; Fortin et al., 2009; Auer et al., 2009; Stürzebecher et al., 2009). Tethering peptide toxins or ligands close to their point of activity in the cell plasma membrane provides a new approach to the study of cell networks and neuronal circuits, as it allows selective targeting of specific cell populations, enhances the working concentration of the ligand or blocker peptide, and permits the engineering of a large variety of t-peptides (e.g., including use of fluorescent markers, viral vectors and point mutation variants).

This focused review describes the identification of lynx-1 and related endogenous cell surface modulators, the development of the t-peptide technology, and the application of the t-peptide strategy to basic research, cell-based therapies, and drug discovery.

ENDOGENOUS CELL-SURFACE MODULATORS OF LIGAND-GATED ION CHANNELS: THE LY6 SUPERFAMILY

Cell-surface receptors and ion channels are modulated by a rich variety of peptide neurotransmitters, hormones and ligands, but there are few examples of membrane-anchored modulators in nature. The Ly6 superfamily which includes lynx1-and slurp-1 cholinergic modulators, elapid snake venom toxins, and Ly6 molecules of the immune system, constitutes a unique class of short proteins that are either tethered to the cell surface via a glycosylphosphatidylinositol (GPI) anchor, like lynx1, or secreted as venom toxins (**Figure 1**). Members of this superfamily share the characteristic 8–10 cysteine motif that determines their compact three-finger structure. Examples of the GPI-anchor subgroup include molecules of the immune system such as CD59 (Davies et al., 1989), ly6A-E (Rock et al., 1989), ly6G (Mallya et al., 2006), and ly6K (de Nooij-van Dalen et al., 2003), the neuronal proteins lypd6 (Darvas et al., 2009), ly6H (Dessaud et al., 2006) and the urokinase plasminogen activator receptor (uPAR) (Blasi and Carmeliet, 2002). Members lacking the GPI-anchor comprise the cholinergic modulators SLURP-1 (Adermann et al., 1999; Chimienti et al., 2003) and SLURP-2 (Tsuji et al., 2003), cobra toxins, and other three finger venom toxins (Tsetlin, 1999) (**Figure 1**).

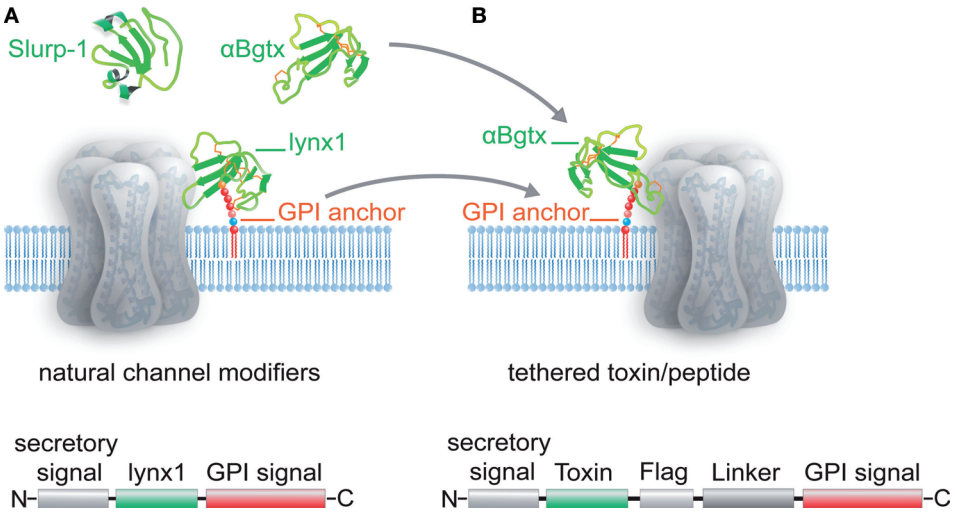

FIGURE 1 | Schematic representation of Ly-6/uPAR channel modifiers and engineered tethered toxins (t-toxins). (A) Examples of the Ly-6/uPAR superfamily include soluble Slurp-1, snake α-bungarotoxin (αBgtx) and the GPI-anchored cell-membrane bound lynx1. The schematic below the drawing of the channel indicates the coding sequences associated with lynx1, namely an N-terminal secretory signal region, followed by the amino acid residues that correspond to the lynx1 peptide, and a C-terminal GPI anchor. **(B)** The structural homology of lynx1 with αBgtx gave rise to the tethered-peptide strategy of using the biological scaffold of lynx1 (secretory signal and GPI signal) to generate recombinant membrane-bound toxins and peptide ligands such as the illustrated t-αBgtx. The schematic below the drawing of the channel indicates the coding regions that were conserved from lynx1 (shown in **A**), and those that were altered to accommodate the αBgtx (the toxins sequence, flag tag, and linker regions).

ALLOSTERIC MODULATION OF NACHRS BY Ly6 SUPERFAMILY MOLECULES

Lynx1 was the first identified member of the Ly6 superfamily capable of cell-surface modulatory action on a neurotransmitter receptor (Miwa et al., 1999). Lynx1 assembles and colocalizes with nAChR in the brain (Ibañez-Tallon et al., 2002) and in the lung (Sekhon et al., 2005). nAChRs stably associated with lynx1 are less sensitive to their ligand agonists acetylcholine and nicotine, display more rapid desensitization, and show a shift in the distribution of channel openings toward a faster inactivating species with more uniform, larger amplitude currents (Ibañez-Tallon et al., 2002). These findings, along with studies showing enhanced nicotine-mediated calcium influx and synaptic efficacy in lynx1 null mutant mice, are strong indicators that lynx1 is an allosteric modulator of nAChR function *in vivo* (Miwa et al., 2006). Other lynx-like molecules recently identified have similar properties. Lynx2 and ly6H, which are GPI-anchored to the cell membrane, have been localized to central and peripheral neurons in mice (Dessaud et al., 2006). Functional studies demonstrate that lynx2, but not ly6H, changes the agonist sensitivity and desensitization properties of nAChRs through direct association (Tekinay et al., 2009). Consistent with lynx2 enrichment in neurons that participate in circuits controlling fear and anxiety, lynx2 null mice display increased anxiety-like behaviors due to enhanced nAChR activity (Tekinay et al., 2009). Recently, a third allosteric modulator of nAChRs, named lypd6, has been identified in central neurons. Lypd6 is cell-membrane bound and selectively increases the calcium conductance of nAChRs (Darvas et al., 2009). The lynx1-related secreted ligands SLURP-1 and SLURP-2, also act as neuromodulators of nAChRs and have been linked to skin disorders (Chimienti et al., 2003; Arredondo et al., 2006).

Ly6 SPECIES DIVERSITY

Lynx1-like molecules are well conserved across species, both in structure and function, suggesting the importance of cell-surface modulators of nicotinic receptors in nature. Examples of Ly6 superfamily species diversity include molecules found in *C. elegans* (Odr-2; Chou et al., 2001), fireflies (Pr-lynx1; Choo et al., 2008), *Drosophila* (Hijazi et al., 2009) and chicken (recently identified prostate stem cell antigen PSCA; Hruska et al., 2009). Pr-lynx1 and PSCA are of particular importance as Pr-lynx1 is the first modulator of nAChRs in an insect species (Choo et al., 2008), and PSCA appears to prevent programmed cell death of neurons by antagonizing nAChRs (Hruska et al., 2009). The lynx1-like family of allosteric modulators of nAChRs constitutes a unique example of cell-surface channel modifiers that have evolved for fine-tuning of neurotransmitter receptor function *in vivo*.

VENOM PEPTIDE TOXINS: SPECIFIC MODULATORS OF ION CHANNELS AND RECEPTORS

Venom peptide toxins from predatory organisms such as scorpions, snakes and marine cone snails are suitable as agents for use in engineering cell-type specific modulators. Neurotoxins isolated from animal venom are disulfide-rich peptides that have been used extensively to characterize the structure–activity relationships of specific ion channels (ligand or voltage-gated) and cell-surface receptors (Catterall, 1986; Tsetlin, 1999; Norton and Olivera, 2006). α-bungarotoxin and other α-neurotoxins from Elapidae and Hydrophidae snakes were used to provide the first identification of nAChRs (Endo and Tamiya, 1991; Hucho et al., 1996; Léna and Changeux, 1998). Similarly, α- and β-neurotoxins from scorpions have been broadly used to modify voltage-gated sodium channels (VGSC or Na_vs) by delaying inactivation (α) or shifting the

membrane potential dependence (β) (Zuo and Ji, 2004; Bosmans and Tytgat, 2007). Neuropeptides from venomous marine cone snails (conotoxins) are prolific in their range and specificity for targeting various ion channels and receptors (Terlau and Olivera, 2004; Gowd et al., 2008; Han et al., 2009). As shown in **Table 1**, venom peptide toxins modulate a wide range of molecular targets. Peptide toxins vary in length from 12–30 residues in cone snails to 40–80 residues in toxins from scorpions and snakes. The relatively small size of these polypeptides, coupled with their structural integrity imposed by numerous disulfide bonds, have facilitated their use for investigating ion channels and receptors (Fontecilla-Camps et al., 1988; Pashkov et al., 1988; Tsetlin, 1999; Zhang et al., 2006).

PEPTIDE TOXINS SPECIFIC FOR nAChR AND VOLTAGE-GATED ION CHANNELS

α-Neuropeptides, which target nAChRs compete with cholinergic agonists and antagonists (Endo and Tamiya, 1991) and are characterized by four to five disulfide bridges in snake and scorpion venoms (Possani et al., 1999; Phui Yee et al., 2004) or two to three disulfide bridges in cone snail toxins (Sine et al., 1995; McIntosh et al., 1999; Ellison et al., 2006). α-neurotoxins that target nAChRs include α-Bgtx, MII and BuIA (**Table 1**), while other α-neurotoxins slow the sodium current inactivation in excitable membranes (Couraud et al., 1982). δ-Conotoxins, such as δ-SVIE, which bind to Na_vs also cause a delayed inactivation of sodium currents (Bulaj et al., 2001). They share a cysteine framework with the structure C-C-CC-C-C, that is identical to that of μO-conotoxins, like MrVIA and MrVIB which inhibit $Na_v1.2$, $Na_v1.4$ and $Na_v1.8$ (Terlau et al., 1996; Daly et al., 2004; Bulaj et al., 2006). μ-Conotoxins contain a framework of CC-C-C-CC and are by far the best characterized of all the conotoxins that target Na_vs. Several μ-conotoxins such as PIIIA, SmIIIA, and potentially BuIIIA, inhibit Na_vs in a similar manner to tetrodotoxin (TTX), by binding to site I on the channel (Catterall, 2000; Goldin, 2001). Despite the ever growing number of discovered natural toxins, only a few κ-conotoxins have been characterized so

Table 1 | Examples of venom peptide toxins used for generation of tethered modulators and corresponding targeted receptors/ion channels.

Tethered toxin	Origin	Length (aa)	Ion channel/receptor specificity	Original reference
AgaIIIA	*Agelenopsis aperta* (funnel web spider)	76	VGCC: $Ca_v2.2$ (N-type), Ca_v1 (L-type)	Mintz et al. (1991)
AgaIVA		48	VGCC: $Ca_v2.1$ (P/Q-type)	Mintz et al. (1992)
APETx2	*Anthopleura elegantissima* (aggregating anemone)	42	homomeric ASIC3 > heteromeric ASIC3-ASIC2b	Diochot et al. (2004)
α-AuIB	*Conus aulicus* (guilded cone snail)	15	nAChR: α3β4 >> α2β2	Luo et al. (1998)
α-Bgtx	*Bungarus multicinctus* (multi-banded krait)	74	nAChR: α7, α1β1δγ/ε, α3β2	Chang and Lee (1963), Nirthanan and Gwee (2004)
κ-Bgtx		66	nAChR: α3β2, α7, α4β2	Chiappinelli (1983)
μ-BuIIIA, B, C	*Conus bullatus* (bubble cone snail)	26	VGSC: $Na_v1.4$	Holford et al. (2009)
α-BuIA		13	nAChR: α6β2 > α3β2 > α2β2 > α4β2	Azam et al. (2005)
α-GID	*Conus geographus* (geography cone snail)	19	nAChR: α7 = α3β2 > α4β2, α3β4	Nicke et al. (2003)
HntxIII	*Haplopelma hainanum*	35	VGSC: DRG Na_v TTX-S	Xiao and Liang (2003)
HntxIV	(Chinese giant black earth tiger)	35	VGSC: DRG Na_v TTX-S	Xiao and Liang (2003)
Kurtoxin	*Parabuthus transvaalicus* (South African fattail scorpion)	63	VGCC: Ca_v3 (T-type), $Ca_v2.1$ (P/Q-type)	Chuang et al. (1998)
α-MI	*Conus magus* (Magician's cone snail)	14	nAChR: α1β1δε >> α1β1δγ	McIntosh et al. (1982)
α-MII		16	nAChR: α6/α3β2 > α3β2 > α3β4 = α4β2	Cartier et al. (1996)
ω-MVIIA		25	VGCC: $Ca_v2.2$ (N-type)	Bowersox and Luther (1998)
ω-MVIIC		26	VGCC: $Ca_v2.1$ (P/Q-type), $Ca_v2.2$ (N-type)	Hillyard et al. (1992)
μO-MrVIA	*Conus marmoreus* (marbled cone snail)	31	VGSC: Na_v 1.2, 1.4, 1.8	McIntosh et al. (1995)
μ-PIIIA	*Conus purpurascens* (purple cone snail)	22	VGSC: Na_v 1.2	Shon et al. (1998)
κ-PVIIA		27	VGKC: Kv1 shaker channel	Terlau et al. (1996)
α-PnIB	*Conus pennaceus* (penniform cone snail)	16	nAChR: α7 > α3β2	Fainzilber et al. (1994)
α-RgIA	*Conus regius* (crown cone snail)	12	nAChR: α9α10 >>> α7	Ellison et al. (2006)
κM-RIIIK	*Conus radiatus* (rayed cone snail)	24	VGKC: Kv1 shaker channel	Ferber et al. (2003)
μ-SmIIIA	*Conus stercusmuscarum* (fly-specked cone snail)	30	VGSC: Na_v 1.8	West et al. (2002)
SNX482 (1998)	*Hysterocrates gigas* (Cameroon red baboon spider)	41	VGSC: $Ca_v2.3$ (R-type)	Newcomb et al.
δ-SVIE	*Conus striatus* (striated cone snail)	31	VGSC: Na_v 1.4 > Na_v 1.2	Lu et al. (1999)

far. κ-conotoxins bind to voltage-dependent potassium channels (VGKC or K_v) altering either the repolarization phase of action potentials or the resting membrane potential. The most commonly identified κ-conotoxin is PVIIA, which blocks Shaker K channels cloned from *Drosophila* (Naranjo, 2002). Other gating modifiers of K_v channels are the phrixotoxins (PaurTX) I and II (Bosmans et al., 2006). Blockers of voltage-gated calcium channels (VGCC or Ca_v) include agatoxin IIIA and IVA. The most prominent VGCC toxin antagonist is conotoxin MVIIA, a highly specific blocker of N-type calcium channels which has been developed as an analgesic for the treatment of chronic pain (Miljanich, 2004; Staats et al., 2004).

The high degree of specificity with which venom peptide toxins bind to voltage and ligand gated sodium (Na_v), calcium (Ca_v), and potassium (K_v) ion channels, and receptors such as nAChRs, N-methyl-D-aspartate and G-protein coupled receptors (GPCRs), make them ideal for deciphering the connections between cell types, and for reversibly manipulating the activity of selective cell subtypes.

ENGINEERING TETHERED PEPTIDES FOR CELL-SURFACE MANIPULATIONS OF RECEPTORS AND ION CHANNELS

The unique functionality of endogenous lynx1-like cell-surface modulators provides a framework to use peptide toxins from predatory animals to manipulate ion channels and receptors in a novel manner. Peptide toxins that have been routinely employed for neuroscience research do not normally exist as cell-surface anchored molecules. Using the scaffold of the lynx1-like gene family, i.e., secretory signal and consensus sequences for GPI processing and recognition, it is possible to produce a series of tethered toxins (t-toxins) that are highly effective modifiers of neuronal activity (Ibañez-Tallon et al., 2004). This design directs any bioactive peptide to the secretory pathway, where the signal sequence is cleaved and the GPI targeting sequence is substituted by a covalent bond to GPI, anchoring the peptide to the extracellular side of the plasma membrane of the cell in which it is expressed (**Figure 1B**). Recombinant t-peptides have a modular framework consisting of a secretion signal, linkers, epitopes and/or fluorescent markers and membrane tethers (**Figures 1B and 2A**). The GPI-anchor targeting motif can be replaced with transmembrane (TM) domains fused with fluorescent marker genes (**Figures 2A,B**) (Auer et al., 2009). So far, approximately 40 different chimeric t-toxins derived from the venom of several predatory animals have been cloned and their activity has been characterized on voltage and ligand-gated ion channels (**Table 1**) (Ibañez-Tallon et al., 2004; Hruska et al., 2007; Wu et al., 2008; Stürzebecher et al., 2009; Auer et al., 2009). Furthermore, the t-peptide strategy has also been successfully extended to other bioactive peptides, such as ligand peptides for constitutive activation of GPCRs (Choi et al., 2009; Fortin et al., 2009), illustrating the general applicability of this approach for cell-surface modulation of receptors.

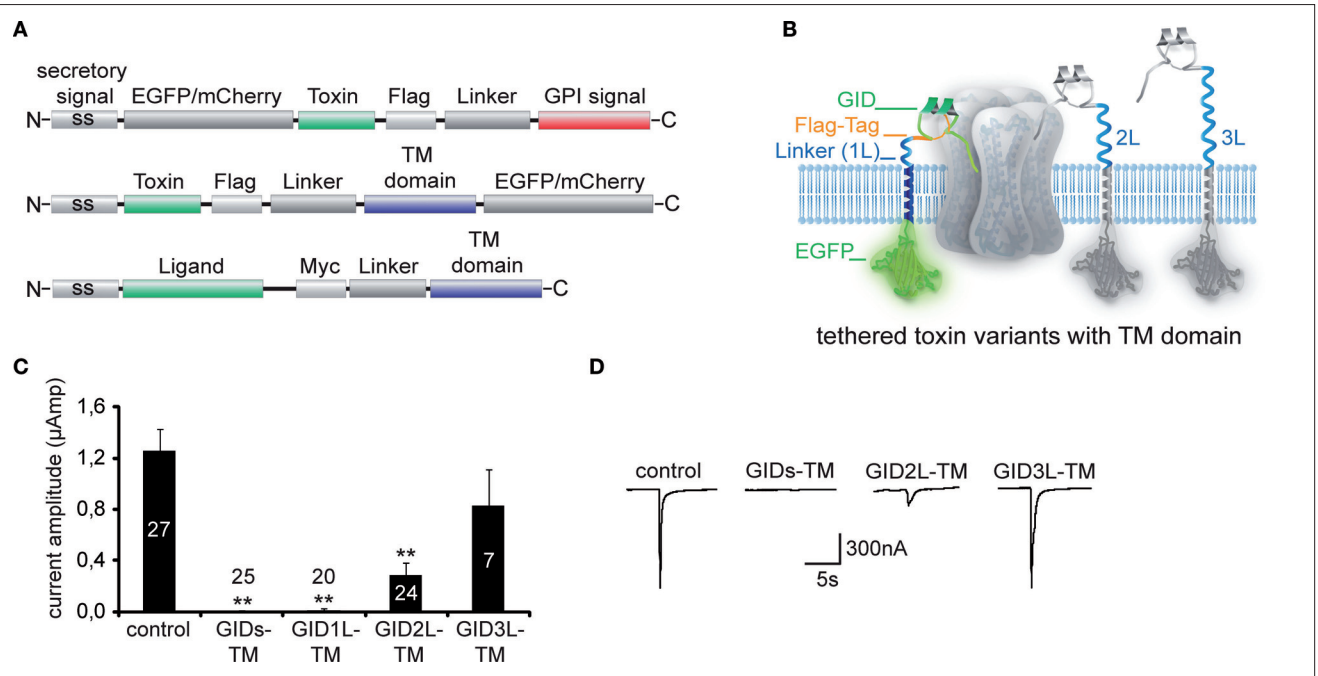

FIGURE 2 | Modular architecture of membrane-tethered toxin and ligand peptides. (A) Illustration of tethered-peptide variants consisting of secretory pathway signal sequence (ss), toxin/ligand cassettes, fluorescence markers (EGFP or mCherry), epitopes for immunostaining (Flag-tag, Myc-tag), flexible linker regions, and distinct functional modules for membrane attachment (GPI-signal, transmembrane-domain TM). **(B)** Illustration of t-toxin carrying the nAChR-specific snail-toxin GID with varying linker lengths [short (s), 1L, 2L and 3L]. **(C)** Electrophysiological recordings in *Xenopus laevis* oocytes expressing α7 nAChR alone (control) or together with tethered-GID (t-GID) with increasing linker lengths. t-GID expression results in complete block of nicotine-induced α7 nAChR current for short (6 aa, GIDs-TM) and long (20 aa, GID1L-TM) linker variants, while longer linkers (GID2L-TM and GID3L-TM, 40 aa and 60 aa) lead to decreased blocking capability. Number of recorded cells displayed in/above columns. **(D)** Representative traces of electrophysiological recordings in **(C)** suggest an optimal distance of the GID peptide from the plasma membrane of 9–22 aa to achieve complete inhibition of α7 nAChR.

COMPONENTS CRITICAL TO T-PEPTIDE SYNTHESIS

The biological activity of each tethered construct has to be individually evaluated and optimized per receptor/channel combination. While anyone with knowledge of cloning technology can construct a tethered-peptide, some forethought is required depending on how the t-peptide will be implemented. This is due to several factors including variability in the nature of the bioactive peptide and ion channel or receptor being targeted, and in the constraints imposed by the modular architecture of the tethers, linkers and epitope-tags. The vast amount of modular choices available for constructing tethered toxins point to its broad appeal and indicate the feasibility of tailor-made tethered manipulators for a wide range of different receptors/channels.

Amino acid composition of the bioactive peptide

Expression and functional assays have revealed that several elements are critical to achieve robust expression on the cell-surface and rotational flexibility for correct modulation of the t-toxin/peptide to the receptor or channel of interest. The affinity of the bioactive peptide for its cognate ion channel or receptor has to be taken into account. Toxins with a strong affinity are potentially more effective. It is also important to consider the composition and length of the peptides to be tethered, i.e., charges and hydrophobicity of the amino acid residues, number of cysteine bonds in the case of toxins, and existence of non-canonical residues, or terminal amidations. For example, substitution of hydroxylated or carboxylated amino acids with non-modified residues in highly post-translationally modified conotoxins, such as GVIA (Olivera et al., 1984), RIIIK (Ferber et al., 2003), PIIIA (Shon et al., 1998) and GID (Nicke et al., 2003), failed to yield satisfactory activity in their tethered constructs, except in the case of GID (see **Figures 2C,D**). Most likely these variations in efficacy are dependent on the location of the posttranslational modification in the toxin sequence. In general, the makeup of the peptide or toxin to be tethered has to be taken into account, but it is not predictive of the expression and activity of its tethered form. MrVIA and MrVIB, which despite their high hydrophobicity content and difficulties to be chemically synthesized (Terlau et al., 1996; Bulaj et al., 2006), are well expressed at the cell-membrane, and functionally active when tethered (Ibañez-Tallon et al., 2004; Wu et al., 2008; Stürzebecher et al., 2009). Other examples are the tethered forms of α- and κ-bungarotoxins which were well expressed and functionally competent (Ibañez-Tallon et al., 2004; Hruska et al., 2007) despite being long peptides (68–82 aa), while shorter conotoxins such as SmIIIA (30aa) or MVIIC (26aa) were not heterologously expressed, possibly due to folding disturbances or high proportion of charged amino acids.

Distance of the linker region

Another relevant feature when designing t-peptide constructs is the linker sequence bridging the toxin peptide to the GPI anchor or TM domain (**Figures 2A,B**). The distance of the t-toxin or t-peptide from the cell-surface has to be tailor-made for individual receptors and channels, and can be used for mapping active binding sites. Tethered constructs have been cloned using linkers consisting of glycine–asparagine repeats with lengths varying from 6 amino acids (short) to 20 amino acids (long), 40aa (2× long) or 60 aa (3× long) (**Figure 2B**) (Ibañez-Tallon et al., 2004). The longer flexible linker provides rotational freedom for the t-toxin to bind within the vestibule of voltage-gated channels (Ibañez-Tallon et al., 2004), or for ligand peptides to reach their binding site, such as onto class B1 GPCRs (Fortin et al., 2009). Experiments varying the length of the linker region of t-GID conotoxin indicate that a linker is necessary for inactivation of α7 nAChR currents. However, when the linker exceeds a certain length the inactivation is incomplete (**Figures 2B–D**). Similarly, the tethered form of the neuropeptide pigment dispersing factor (t-PDF) requires a short linker for effective binding to its receptor (Choi et al., 2009).

Choice of membrane tether: GPI vs. TM

The choice of membrane tether depends on the characteristics of the peptide as well as on the epitope-tags and markers to be used in combination (**Figures 1B and 2A,B**). GPI anchors, which are less bulky than TM domains, may facilitate the mobility of the t-peptide in close proximity to its receptor within the plasma membrane. If the toxin or peptide does not require a free N-terminus for interacting with its cognate receptor, GPI versions containing EGFP followed by the t-toxin may be used (**Figure 2**). However, GPI anchors are susceptible to cleavage by endogenous phospholipases, such as PI-PLC and phospholipase D (Paulick and Bertozzi, 2008). This has been suggested as a mechanism used by cells for selective and rapid release of certain GPI-anchored proteins at specific times. To avoid this potential problem, the GPI anchor can be replaced with a TM domain in t-toxins (**Figure 2**). TM domains can be used to retain t-peptides at the cell-surface and link fluorescent markers to the cytoplasmatic side of the plasma membrane avoiding hindrances between them.

CELL-SPECIFIC TARGETING AND CELL-AUTONOMOUS REGULATION IN MODEL ORGANISMS

Tethered toxins and peptides can be used for very diverse applications pertaining to experimental animal physiology. Because of their mode of action at the cell-surface, membrane-anchored peptide molecules act only on ion channels and receptors present in the membrane of the cell that is expressing the t-toxin or t-peptide, and not on identical receptors present on neighboring cells that do not express the tethered construct (Ibañez-Tallon et al., 2004). Several studies have shown that recombinant toxins as well as peptide ligands are not dispersed in solution and retain their high specificity for their cognate receptors, indicating that this approach can be used to restrict the site of neurotoxin or peptide ligand action to genetically targeted cells. For example, *in vivo* transgenic delivery of t-αBgtx in zebrafish using a muscle cell-specific promoter results in blockade of nAChR currents in muscle cells that express t-αBgtx but not in adjacent muscle fibers or in cells that express t-κBgtx, which has no activity on muscle-nAChRs (Ibañez-Tallon et al., 2004). Similarly, experiments in chicken employing a viral system to transduce ciliary neurons have revealed that expression of t-αBgtx blocks calcium currents via nAChRs and prevents programmed cell-death of these neurons during early development (Hruska et al., 2007). Further *in vivo* applications of the tethered toxin strategy have been carried out using transgenesis in *Drosophila melanogaster*. These studies have shown that cell-specific expression of the sodium channel toxin δ-ACTX-Hv1a in pacemaker clock neurons induces arrhythmicity (Wu et al., 2008). Transgenic targeting of the same

clock neurons with a tethered form of the PDF neuropeptide, which is normally rhythmically secreted by these neurons, constitutively activates its cognate GPCR, interfering with circadian control circuit (Choi et al., 2009). These examples illustrate the possibilities for cell-specific targeting and cell-autonomous regulation of channels and receptors with genetically encoded tethered toxins. As venom toxins are established tools for dissecting ionic currents in many animal species, the tethered-toxin strategy allows cell specific functional analysis of ion channels and receptors in model organisms (e.g., zebrafish, flies, rats, large mammals) for which transgenic methodologies are commonly used but gene targeting strategies are yet not available.

APPLICATIONS AND PERSPECTIVE USE OF CELL-SURFACE PEPTIDES TO BASIC RESEARCH, CELL-BASED THERAPIES AND DRUG DISCOVERY

Ion channels and receptors are involved in every physiological action from breathing to heart beating. Understanding the mechanics and functional activity of these macromolecular complexes is a grand challenge in science. The tethered-peptide method is one tool that has the potential to tackle certain aspects of this challenge, particularly in the area of cell-specific modulators (**Figure 3**). Genetically encoded cell-surface modulators can be adapted to a wide range of applications due to their small size, amenability to point mutagenesis, and relative ease to be combined with fluorescent markers, viral and transgenic vectors, Cre-dependent and transcriptional-control elements, and subcellular targeting motifs. Inhibition or constitutive activation of ion channels and receptors can be attained in a cell-type specific manner depending on the selectivity of the neuroactive peptide or hormone.

NEURONAL CIRCUITS: DISSECTING INDIVIDUAL IONIC CURRENTS

Manipulation of ion channels in specific neuronal populations within living animals can be achieved by transgenesis (Ibanez-Tallon et al., 2004; Wu et al., 2008). Similarly, viral methods can be employed to characterize the cellular functions mediated by specific ionic currents inactivated by delivery of t-toxins (Hruska et al., 2007). Gene knockouts led to a wide range of studies to characterize the function of receptors and ion channels (Capecchi, 2005). However, many receptors consist of multimeric assemblies of components, with common

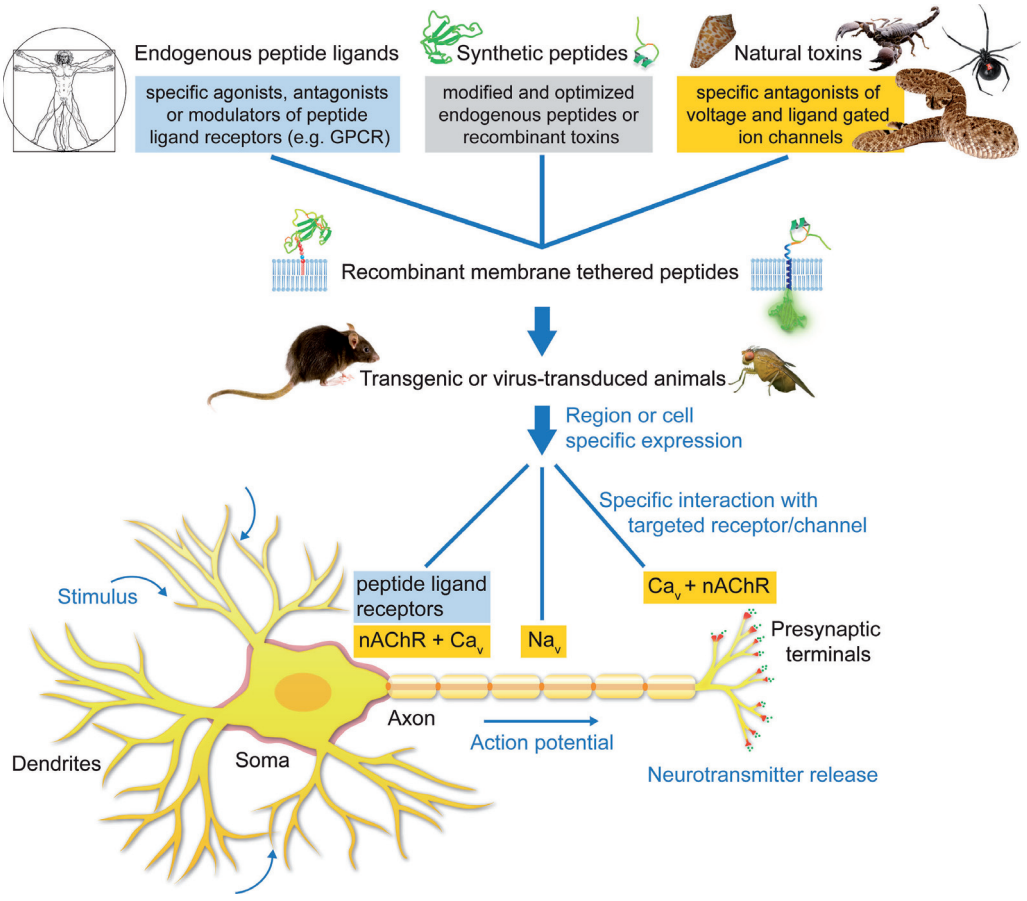

FIGURE 3 | Applications of the tethered-peptide strategy. Endogenous peptide ligands, natural toxins, and synthetic, modified versions of ligands or toxins can be integrated into recombinant membrane-attached fusion constructs and applied *in vitro* in transfected or transduced cells in cell-culture, or *in vivo* in transgenic or virus-transduced animals. The t-peptide retains the specificity of the toxin/peptide ligand allowing controlled manipulation of distinct subtypes of ion channels and receptors in a given neuronal circuit without affecting other channels/receptors in the cell.

components frequently shared by functionally diverse receptor types, modifying any one gene can potentially compromise the function of every complex with which it is associated. For example, it has proven difficult to separate the contribution of $Ca_v2.1$ and $Ca_v2.2$ VGCC channels by targeted deletion of one or the other alpha subunits because of functional compensation of ionic currents (Inchauspe et al., 2004; Takahashi et al., 2004). As venom toxins inactivate specific ionic currents produced by a receptor or channel complex, the t-toxin strategy could be an alternative to prevent compensatory ionic currents that may occur upon gene-deletion of receptor subunits.

The cell-autonomous modulatory action of tethered peptides and their selectivity for cognate cell-surface molecules can be further exploited by directing t-peptide molecules to subcellular compartments within the neuron (**Figure 3**). For instance, t-toxins could be directed to the axon initial segment where sodium channels are concentrated (Garrido et al., 2003), or to the dendritic compartment (Lewis et al., 2009). Further modifications of a number of optimized and novel tethered toxins for *in vivo* use will offer new possibilities for investigations regarding the physiology of neuronal circuits.

CHANNELOPATHIES AND OTHER DISEASES

The tethered-peptide strategy represents a potential new avenue for the development of genetic therapies for chronic diseases caused by malfunction of ion channels and peptide ligand receptors. Several human disorders that affect nervous system functions have been traced to mutations in genes encoding ion channels or regulatory proteins (George, 2005). These disorders, referred to as channelopathies, can be targeted by the tethered-peptide strategy when the disorder results in hyperactivity of the channel. Examples of hyperactive disorders include gain-of-function mutations in P/Q-type calcium channels, linked to familial hemiplegic migraine type 1 (Ophoff et al., 1996; Tottene et al., 2009), or mutations in neuronal nAChRs associated to autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al., 1995; Klaassen et al., 2006). One potential application would be to genetically introduce t-toxins into the corresponding mouse mutant models in a cell-specific manner to dissect the circuitry of the disease (**Figure 3**). Conversely, activation of receptors with t-peptide ligands could be beneficial to control GPCRs in a cell-selective manner (**Figure 3**). For instance, isoforms of glucagon-like and calcitonin-gene-related peptides are presently being used to regulate insulin release and bone remodeling in diabetes (Green and Flatt, 2007) and osteoporosis (Hoare, 2005). Similarly, feeding-regulation neuropeptides such as orexin or ghrelin (Shioda et al., 2008) could be targeted to circuits involved in appetite control, or tethered opioid peptides could be directed to nociceptive neurons. With an ever-growing interest in identifying the potential of naturally occurring venom peptide toxins (Blumenthal and Seibert, 2003; Han et al., 2008; Twede et al., 2009), as well as novel ligands for orphan GPCRs encrypted in the human proteome (Jiang and Zhou, 2006; Shemesh et al., 2008), an increasing number of peptide based therapies could be possible. Furthermore, parallel development on the safety of viral methods for genetic intervention will increase the number of diseases to which the t-peptide strategy is applicable.

IMPLICATIONS OF TETHERED PEPTIDES FOR DRUG DISCOVERY

Ion channels and GPCRs are some of the biggest molecular drug targets yet presently remain underexploited in drug discovery efforts. Peptide toxins, which are highly effective modulators of ion channels and GPCRs, offer an intriguing opportunity for increasing the drug development pipeline. Specific areas in which peptide toxins have demonstrated their potential include chronic pain (Miljanich, 2004) and myasthenic autoimmune response (Drachman, 1981). A major drawback to the universal usage of peptide toxins in the development of therapeutics has been the scarcity of obtaining the venom product. To circumvent this, most toxins are synthesized chemically, but this too has significant problems, one being obtaining the correct disulfide scaffold with *in vitro* folding. To combat these synthesis hurdles several structural strategies and characterization methods have been developed (Munson and Barany, 1993; Cuthbertson and Indrevoll, 2000; Han et al., 2009; Ueberheide et al., 2009; Walewska et al., 2009). However, even when the toxin is successfully synthesized, soluble toxins cannot be directed to single cell populations, are expensive, and have a limited time of application that makes their use *in vivo* problematic. The t-peptide strategy surmounts these limitations with the ability to recombinantly synthesize the toxins or peptide ligands in the cell itself, and co-express it with the molecular target (receptor or channel) to be screened. Such a cell-surface peptide tethering strategy can readily introduce point mutations to interconvert tethered agonists into antagonists. Several recent reports use the t-peptide technology to characterize point mutants of peptide hormones against class B1 GPCRs (Ibañez-Tallon et al., 2004; Fortin et al., 2009). In a similar manner, the t-peptide technology could be applied to screen gene libraries of t-peptides against specific membrane proteins by co-expression in the same cell. T-peptides with activating or blocking capabilities could be monitored with functional assays, i.e., calcium influx. This type of screen could be beneficial to block channels that are hyperactive in certain diseases, such as TRPP2, for which no natural toxins have yet been identified. These features make the t-peptide genetic approach a promising strategy for drug discovery and development of targeted therapeutics.

SUMMARY

The t-peptide strategy is an innovative technique for manipulating neuronal circuits in order to dissect the specific biological roles of ion channels and cell-surface receptors both *in vitro* and *in vivo*. The studies presented here illustrate how the t-peptide approach can be used to increase cellular specificity of neuropeptides by restricting their actions to targeted cell types through membrane tethering. The t-peptide method is relatively easy to implement and has the potential to significantly impact neuroscience research and cell-based drug screening of membrane proteins for targeted therapeutics.

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Neurotrapping: cellular screens to identify the neural substrates of behavior in *Drosophila*

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The availability of new tools for manipulating neuronal activity, coupled with the development of increasingly sophisticated techniques for targeting these tools to subsets of cells in living, behaving animals, is permitting neuroscientists to tease apart brain circuits by a method akin to classical mutagenesis. Just as mutagenesis can be used to introduce changes into an organism's DNA to identify the genes required for a given biological process, changes in activity can be introduced into the nervous system to identify the cells required for a given behavior. If the changes are introduced randomly, the cells can be identified without any prior knowledge of their properties. This strategy, which we refer to here as "neurotrapping," has been implemented most effectively in *Drosophila*, where transgenes capable of either suppressing or stimulating neuronal activity can be reproducibly targeted to arbitrary subsets of neurons using so-called "enhancer-trap" techniques. By screening large numbers of enhancer-trap lines, experimenters have been able to identify groups of neurons which, when suppressed (or, in some cases, activated), alter a specific behavior. Parsing these groups of neurons to identify the minimal subset required for generating a behavior has proved difficult, but emerging tools that permit refined transgene targeting are increasing the resolution of the screening techniques. Some of the most recent neurotrapping screens have identified physiological substrates of behavior at the single neuron level.

Keywords: circuits, neural networks, genetic, synaptic, excitability

INTRODUCTION

The fields of neuroscience and genetics face similar challenges: Both must explain how elementary components – neurons in the first case and genes in the second – interact to govern complex processes, such as behavior or development. Historically, the two fields have sought to meet their respective challenges in very different ways. Neuroscientists, having identified the basic unit of neural function early on, took a bottom-up approach, seeking to explain behavior and other integrative aspects of the brain by charting the connectivity of its component neurons and correlating their patterns of activity. Geneticists, lacking any knowledge of the basic unit of inheritance, were forced to take a different approach: They started at the top, working from the organismal phenotypes that resulted from unknown changes in the genetic material, and made their way down to the gene. The genetic approach was systematized by coupling methods for blindly inducing genetic changes with selective screens to identify mutations that affected particular biological processes of interest (**Figure 1A**). The nature of the gene and the identity of the particular genes involved in diverse biological phenomena were elucidated by this powerful, unbiased approach.

Historically, neuroscience research has lacked a counterpart to the unbiased screen of genetics: While electrophysiological, pharmacological, anatomical, and genetic manipulations have each provided productive avenues to perturbing brain function, there has been no general method for systematically and randomly altering neuronal activity in freely behaving animals to identify the neurons underlying a given behavior. Such a method would usefully complement classic circuit-mapping techniques by supplying causal links to circuit maps derived from tracing synaptic connections and correlating activities. Fortunately, the steady advance of genetic technologies is beginning to provide neuroscientists with the basic elements of such a method. This method, to which we apply the previously-introduced term "neurotrapping" (White et al., 2001a), is illustrated in **Figure 1B**. By analogy to mutagenic screens, neurotrapping involves the perturbation of neuronal, rather than gene, function, and uses not chemical mutagens, but instead, transgenes whose products inhibit (or in some cases stimulate) the activity of neurons in which they are expressed. The expression of these transgenes in random subsets of cells in the brains of living animals constitutes a process analogous to mutagenesis. By identifying animals that exhibit deficits in a behavior of interest as a consequence of inactivating different subsets of neurons, neurotrapping can be used to interrogate the nervous system to identify those cells that are essential for a given behavior, just as mutagenesis is used to interrogate the genome to identify genes required for a given biological process.

It is worth pointing out that while neurotrapping and mutagenesis are analogous techniques, there are differences between them. Perhaps the most important relates to the relative scale of the perturbations made: In contrast to mutagenesis, which is usually performed so as to affect the function of single genes, the transgene expression systems used for neurotrapping typically lead to the manipulation of multiple neurons – often hundreds to thousands. While this broad targeting is probably often necessary

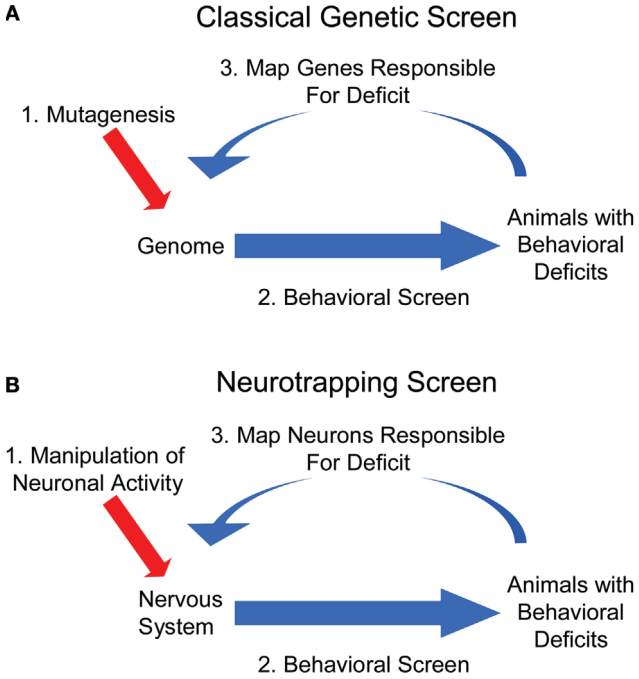


FIGURE 1 | Neurotrapping screens are analogous to genetic screens. **(A)** In a genetic screen, perturbations are introduced into the genome by mutagenesis, and animals bearing these mutations are analyzed for deficits in a behavior of interest. These heritable deficits are then traced back to the change(s) in the genetic material that are their source. **(B)** In a neurotrapping screen the activity of neurons, rather than genes, is perturbed. This type of screen requires genetic tools such as those listed in **Table 1** for manipulating neuronal activity, and a means of targeting these tools to particular neurons. Unlike traditional methods of circuit-mapping, neurotrapping identifies the functional components of a neural network without knowledge of synaptic connectivity (which often represents only part of a neuron's signaling capacity) or response properties (which may only coincidentally correlate with performance of a particular behavior).

to insure that enough neurons are functionally compromised to give rise to a behavioral deficit (either by inducing multiple changes that have small effects, or by compromising compensatory networks in addition to a primary circuit), it also means that identifying the essential neurons responsible for the deficit is often difficult. Indeed, neurotrapping has proved most feasible when the random targeting of gene expression can be performed reproducibly, allowing the same set of neurons to be repeatedly probed from animal-to-animal and generation-to-generation. This is possible when three conditions obtain: the targeting patterns are heritable, neuronal identities are relatively fixed, and behavioral circuits are more-or-less hard-wired. These conditions are largely met in *Drosophila*, where neurotrapping has been primarily developed. We review these developments here, first discussing the tools used for neuronal targeting and manipulation, and then, after reviewing early studies, focusing on recent innovations. These innovations have facilitated neurotrapping methodologies and have raised the prospects that neurotrapping will become as potent an engine of circuit discovery as random mutagenesis has been in elucidating transcriptional and signaling networks in genetics.

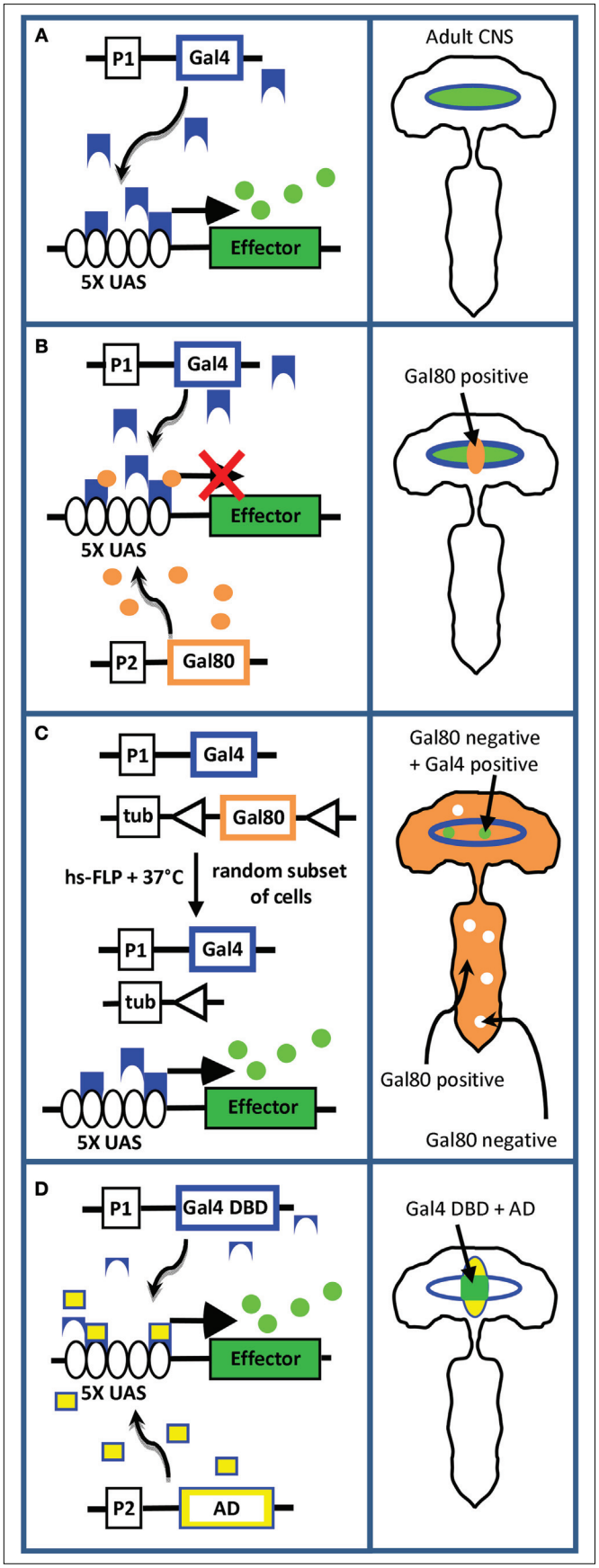
TOOLS FOR NEUROTRAPPING IN *DROSOPHILA*

A profound advantage of *Drosophila* in screen-based studies of neural function is the availability of powerful gene-targeting technologies based on the Gal4-UAS system (**Figure 2**). This expression system (**Figure 2A**), consists of two types of transgenic fly line, one that expresses the yeast transcription factor Gal4 in specific groups of cells under the control of a particular enhancer, and another that bears a transgene of interest downstream of the Gal4 binding site, which is called the "upstream activating sequence" (i.e. UAS). When flies from two such lines are mated, Gal4 drives expression of the transgene in the same cells in which Gal4 itself is expressed. Diverse manipulations of the same set of neurons can therefore be made at will by matching a single Gal4 driver with different UAS-transgenes. By the same token, multiple Gal4 lines with different expression patterns can be matched with a single UAS-transgene to perform identical manipulations on many different neuronal groups. The targeting of arbitrary groups of neurons is accomplished using so-called "enhancer-trap" lines, which are made by allowing the Gal4 gene to randomly integrate into the genome. In transgenic flies made by this method, genomic enhancers that normally regulate endogenous genes near the integration site determine the expression pattern of Gal4. Libraries of Gal4 enhancer-trap lines that have diverse expression patterns typically serve as the starting point for neurotrapping studies. Once an enhancer-trap line of interest has been identified (for example, one that alters a specific behavior when driving a suppressor of neuronal activity), its pattern of expression can be further refined to identify the subset of neurons essential for the behavior using strategies such as those illustrated in **Figures 2B–D**. These strategies are described in the context of specific studies in greater detail below.

The types of UAS-transgenes that have been used to manipulate neural function in *Drosophila* are listed in **Table 1**, which focuses on those whose products broadly affect neuronal function and are not cell-type specific. The products of most of these genes either suppress synaptic transmission or act to inhibit or enhance neuronal excitability. Some act constitutively, while others can be used to perform acute manipulations. The most relevant from the standpoint of the neurotrapping studies reviewed here are those that suppress either synaptic transmission or membrane excitability. These have received the greatest use for the simple reason that suppression of neuronal function allows one to determine whether a particular neuron or set of neurons is necessary for governing a particular behavior. It should be noted that many of the tools listed in **Table 1** are discussed in detail in the Frontiers in Molecular Neuroscience Special Topic titled "Genetic techniques and circuit analysis" (see Han et al., 2009; Hodge, 2009; Holford et al., 2009; Kasuya et al., 2009). Those used specifically in *Drosophila* in the context of circuit mapping are also comprehensively treated in recent reviews by Holmes et al. (2007) and Simpson (2009). Interested readers are referred to these reviews for details.

EARLY NEUROTRAPPING: SYNAPTIC SUPPRESSION WITH UAS-TNT

The first cellular screen of neuronal function in *Drosophila* by Sweeney et al. (1995) was facilitated by two developments: the coupling of the Gal4-UAS system to enhancer-trap technologies by Brand and Perrimon (1993) and the cloning of tetanus toxin light chain (TNT) by Mochida et al. (1990). TNT selectively



cleaves the neuronal isoform of synaptobrevin in *Drosophila* and thus suppresses synaptic transmission in the neurons that express it. Using UAS-TNT, Sweeney and colleagues screened a collection of enhancer trap-Gal4 lines and identified several that produced deficits in an olfactory escape response. The expression pattern of one line that produced particularly strong deficits was investigated and found to include neurons that projected into the leg nerves, but no further identification of the locus of TNT action was undertaken.

Although this early use of UAS-TNT as a screening tool was occasionally emulated, as in the investigation of larval locomotion by Suster et al. (2003), UAS-TNT was most extensively applied to investigating the functions of candidate neurons or brain regions already suspected of playing a role in particular behaviors (for a review of these studies see Martin et al., 2002). Gal4 enhancer-trap lines were typically screened anatomically to identify those that expressed in neurons of interest, and these lines were then used to suppress synaptic transmission so that the behavioral consequences could be observed. In this way, the neural components of various sensory and motor systems were investigated, as were neurons contributing to functionally interesting structures within the central nervous system, such as the mushroom bodies and the central complex.

Because of their focus on pre-selected groups of candidate neurons, these early UAS-TNT studies bear a closer relationship to reverse-genetic approaches than to the "forward" approaches that make use of random mutagenesis. In part, forward approaches to neuronal screens were handicapped by the broad expression patterns of most Gal4 enhancer-trap lines. Indeed, even in studies where the enhancer-trap lines were selected because their expression

FIGURE 2 | Methods of transgene targeting. (A) The Gal4-UAS system described in the text. Left panel: the schematic depicts the transgene construct for Gal4 (blue outline) on one fly chromosome, and the transgene effector construct (green rectangle) on another fly chromosome. The Gal4 gene lies downstream of the promoter/enhancer element, P1, which dictates its pattern of expression. The effector transgene lies downstream of five Gal4 binding sites (i.e. "UAS," black ovals). In flies bearing both constructs, neurons that express Gal4 protein (blue shapes) also express the effector protein (green circles). Right panel: schematic of the fly CNS depicting coincident expression of Gal4 (blue outline) and effector (green oval). **(B)** Subtractive restriction of effector gene expression using Gal80. Left panel: In cells that express the gene encoding Gal80 (brown outline) under the control of the promoter/enhancer P2, Gal80 protein (brown circles) will inhibit Gal4 by binding to its transcription activation domain and thus block effector gene expression (red X). Right panel: if P1 and P2 have overlapping expression patterns, Gal4 activity, and therefore effector expression, is eliminated in the region of overlap (i.e. in Gal80 positive cells, brown). **(C)** Random restriction using the "flp-out Gal80" system. Left panel: if the Gal80 transgene is placed downstream of the ubiquitously active tubulin promoter (tub) and is flanked by sites (triangles) that permit excision by heat-shock induced flp-recombinase activity, the Gal80 gene will be deleted in random subsets of cells in animals subjected to heat shock. Only cells that express Gal4, but not Gal80, will also express the effector gene. Right panel: Effector expression (green) is limited to cells within the Gal4 expression pattern (blue outline) that lack Gal80 expression. **(D)** Combinatorial restriction using Split Gal4. Left panel: If the DNA-binding (blue, DBD) and transcription activation (yellow, AD) domains of the Gal4 molecule are independently targeted to different neuronal groups using promoters P1 and P2, Gal4 activity will be reconstituted and the effector transgene expressed only in cells at the intersection of the P1 (blue) and P2 (yellow) expression patterns, as depicted in the right panel.

Table 1 | Tools for manipulating neuronal excitability in *Drosophila*.

Type	Tool	Action	Reference
Constitutive suppressors	Tetanus Toxin Light Chain (TNT)	Synaptic Block	Sweeney et al., 1995
	Inward Rectifier ($K_{ir}2.1$)	↓ Excitability	Baines et al., 2001
	Two-pored "leak" Channel (dORK)	↓ Excitability	Nitabach et al., 2002
	Voltage-gated K^+ Channels (EKO, Shaw)	↓ Excitability	Hodge et al., 2005; White et al., 2001b
Conditional suppressor	Dynamin Mutant (UAS-Shi^{ts1})	Synaptic Block	Kitamoto, 2001
Constitutive activator	Bacterial Na^+ Channel (NaChBac)	↑ Excitability	Luan et al., 2006a; Nitabach et al., 2006
	Tethered Toxin (δ-ACTX-Hv1a)	↑ Excitability	Wu et al., 2008
Conditional activators	Thermosensitive TRPs (TRPM8, dTrpA1)	Depolarize	Hamada et al., 2008; Peabody et al., 2009
	ChR2	Depolarize	Schroll et al., 2006
	Light Uncaged ATP (P_2X_2)	Depolarize	Lima and Miesenbock, 2005

included a particular set of neurons, concomitant expression outside the region of interest often meant that the effects of suppression could not be attributed with complete confidence to the silencing of the target neurons. This problem could sometimes be ameliorated by comparing results obtained with several lines that had overlapping expression patterns (see for example Suster et al., 2003; Broughton et al., 2004), but, in general, when expression cannot be restricted to a single group of neurons – a condition often difficult to meet – some ambiguity will remain.

ADDING TEMPORAL CONTROL: NEUROTRAPPING WITH THE SYNAPTIC SUPPRESSOR UAS-Shi^{ts1}

A second impediment to the implementation of unbiased screening strategies using UAS-TNT was the inability of investigators to temporally restrict UAS-TNT expression. The fact that synaptic transmission was constitutively compromised meant that synaptic suppression during development, and not acute suppression at the time of testing, might account for the observed behavioral deficits. There was also no guarantee that TNT would affect the same neurons, or neurons governing the same functions, during development and adulthood, since many enhancer-trap expression patterns are dynamic and change as the animal grows. The broad range of expression over development also meant that synaptic silencing was frequently lethal due to TNT expression in some subset of neurons essential for viability. An elegant solution to the problem of temporal control was developed by Kitamoto with his introduction of UAS-Shi^{ts1}, a UAS-construct encoding a temperature-sensitive mutant of Shibiri, the *Drosophila* ortholog of dynamin (Kitamoto, 2001). Shi^{ts1}, as is described more extensively in the Frontiers in Molecular Neuroscience Special Topic titled "Genetic techniques and circuit analysis" (see Kasuya et al., 2009), is a dominant inhibitor of synaptic vesicle recycling and thereby blocks synaptic transmission. Using flies bearing the UAS-Shi^{ts1} construct, Kitamoto demonstrated that synaptic suppression could, in many cases, be induced by a simple temperature shift within a matter of minutes.

Like UAS-TNT, UAS-Shi^{ts1} has been since used in many studies to inhibit synaptic transmission in candidate neurons for various processes, in some cases with only minimal pre-selection of enhancer-trap lines. In the latter cases, the resulting activity screens are essentially "forward" neurotrapping screens. For example, Pitman et al. (2006) screened 92 enhancer-trap lines with UAS-Shi^{ts1} that were pre-selected for their relatively restricted expression within different brain regions. These lines were chosen, however, because their expression patterns collectively covered most of the fly central nervous system. By using UAS-Shi^{ts1} to inhibit neurons in each of these patterns, the authors were able to demonstrate a specific role of the mushroom bodies in sleep.

Similarly, in a study of larval locomotion, Hughes and Thomas (2007) anatomically pre-screened approximately 1800 Gal4 enhancer-trap lines and selected only those that expressed in small subsets of CNS or PNS neurons. They then performed a secondary screen on the chosen lines using UAS-Shi^{ts1}, which allowed them to identify two classes of sensory neurons that provide proprioceptive feedback critical for larval crawling. A similar screen that was carried out without anatomical pre-selection was conducted by Song et al. (2007) and arrived at similar conclusions. Song et al. used UAS-Shi^{ts1} to functionally screen approximately 1000 Gal4 enhancer-trap lines and identified 10 that drastically reduced peristaltic rhythm and locomotion. All 10 were found upon subsequent investigation to express strongly in peripheral sensory neurons. One of these lines had no CNS expression, and, as in the study of Hughes and Thomas, led to the identification of multidendritic neurons as essential components of the circuitry required for normal peristalsis during larval locomotion.

It is perhaps not surprising that the reported neurotrapping screens often involve the identification of sensory neurons. The accessibility of these neurons to manipulation and physiological recording has long made them a favorite object of study and the availability of specific drivers for subsets of them has facilitated their identification in functional screens for the circuitry underlying specific behaviors. Another example is a pilot screen reported by Suh et al. (2004), which sought to identify neurons involved in a chemosensory-mediated avoidance response. This screen of approximately 250 Gal4 enhancer-trap lines uncovered one line that helped confirm the participation of a subclass of odorant receptor neurons in this behavior.

Fewer neurotrapping screens have reported identifying components of central circuits involved in more complex behaviors. One notable exception is a study by Katsov and Clandinin (2008) which used UAS-Shi^{ts1} to screen approximately 400 enhancer-trap lines for specific visual processing defects using a high-throughput behavioral assay. These authors identified one line in which expression was limited to two brain regions, one of which (the mushroom body), could be ignored because its ablation did not cause the visual processing defect. Remarkably, this left only a small set of

three to four bilaterally represented neurons in each optic lobe as the presumptive circuit components responsible for the processing defect.

FURTHER NEUROTRAPPING METHODOLOGIES FOR RESTRICTION IN TIME…

Although UAS-Shi^{ts1} affords excellent temporal control of synaptic transmission under many circumstances, it can also perturb basic cellular functions. These effects can lead to ambiguities in the interpretation of manipulations made with this tool (see Kitamoto, 2002b). An alternate method for temporally regulating synaptic block is the flp-out TNT method introduced by Keller et al. (2002), which employs a construct with a transcription stop cassette interposed between the UAS and the TNT transgene. This stop cassette can be excised by the flp recombinase, which can be ubiquitously activated by heat shock in flies bearing the flp transgene expressed behind the heat-shock promoter.

More general temporal control can be achieved by techniques that permit conditional transgene expression such as the GeneSwitch system (Osterwalder et al., 2001; Roman et al., 2001) or the TARGET system (McGuire et al., 2004). The latter uses a temperature-sensitive mutant of the Gal4 inhibitor Gal80 (i.e. Gal80ts), which is capable of repressing Gal4-mediated transcription at lower, restrictive temperatures, but not at higher, permissive temperatures. This technique has been particularly useful in suppressing the expression of TNT during development to prevent lethality, and then releasing it in the adult where it can suppress circuit function. This approach was used in two recent studies (Gordon and Scott, 2009; Zhu et al., 2009). The general applicability of the TARGET system in temporally regulating Gal4-mediated transgene transcription also means that it can be used with constitutive suppressors of neuronal activity besides TNT (see **Table 1**).

…AND RESTRICTION OF EXPRESSION PATTERN

Gal80 (in its native, non-temperature-sensitive form) has also been useful for narrowing down sets of neurons to identify those that specifically function in a behavioral circuit. The general utility of Gal80 in restricting the range of Gal4 activity was first demonstrated by Lee and Luo with their introduction of the MARCM system (Lee and Luo, 2001), which is routinely used to limit Gal4 activity to individual neurons. Due to its inherently mosaic expression, the MARCM system does not permit reproducible expression of effectors in the same cells from animal to animal, and has not typically been used in neurotrapping screens. However, Gal80 itself has proved useful in evaluating the lines generated by such screens (**Figure 2B**) as was first demonstrated by Kitamoto, who used Gal80 to suppress the cholinergic subset of neurons within the expression pattern of a particular Gal4 enhancer-trap line (Kitamoto, 2002a,b). This line was isolated from a screen designed to analyze the neural basis of courtship behavior. When subjected to transient suppression, males resulting from crosses of this line to UAS-Shi^{ts1} engaged in aberrant male–male courtship. By selectively relieving suppression in the cholinergic neurons of this pattern using a Gal80 construct expressed behind the choline acetyltransferase promoter, Kitamoto showed that male courtship patterns returned to normal. By this means he was able to demonstrate that cholinergic neurons within the expression pattern of the enhancer-trap line were responsible for the behavioral deficit seen in response to synaptic block.

While this example illustrates the utility of Gal80 in restricting an expression pattern to isolate a cell group of interest, it also illustrates one of the challenges of this approach. Because Gal80-mediated restriction represents a negative selection strategy that subtracts neurons from a pattern of interest (see **Figure 2B**), these neurons are then no longer available for manipulation. Further narrowing of the pattern to identify the minimal set of behaviorally relevant neurons therefore becomes difficult. A positive selection strategy that does not share this drawback couples the use of Gal80 to the flp recombinase in such a way that the behavioral phenotype of interest is seen only in conjunction with restricted expression patterns. This "flp-out Gal80" system, illustrated in **Figure 2C** relies on the stochastic activation of flp by heat shock and can lead to the restoration of Gal4 activity in small sets of cells within an expression pattern, in some cases as few as one. This method was recently used in conjunction with a neurotrapping screen by Gordon and Scott (2009) to identify individual neurons involved in a sensorimotor taste circuit.

The latter study is perhaps the most elegant implementation to date of the neurotrapping strategy and provides a good example of what can be done using some of the latest tools. Starting with a suppressor screen of 534 Gal4 enhancer-trap lines, Gordon and Scott set out to identify neurons involved in taste-related behaviors. They used Gal80ts to prevent developmental mortality and identified 47 lines that gave rise to adult deficits in a sensorimotor taste reflex. These deficits fell into four different categories, including motor deficits. By analyzing one of the six motor defective lines using the flp-out Gal80 technique, they were able to identify and characterize a single pair of motor neurons that were necessary for the execution of a subprogram of the taste reflex. In addition to the synaptic blocker, TNT, the authors used a potent suppressor of excitability, Kir2.1, to conduct their screen and also made use of the light-sensitive channel, ChannelRhodopsin2 (i.e. ChR2). Indeed, by using ChR2 to selectively stimulate the pair of motor neurons identified using TNT and Kir2.1, they were able to demonstrate that activation of these neurons was sufficient to induce the same behavioral subprogram that was lacking in the flies in which these neurons were suppressed.

EMERGING TECHNOLOGIES FOR NEUROTRAPPING

While the study of Gordon and Scott convincingly demonstrates the efficacy of existing neurotrapping techniques in *Drosophila*, emerging technologies promise to facilitate both the consistent targeting of small groups of neurons and the rational dissection of a large group of neurons into smaller subsets. In addition, an ever broadening palette of tools for manipulating excitability promises to increase the range of manipulations of neuronal activity that can be made for neurotrapping. Each of these is discussed in turn in the sections below.

RESTRICTION OF TRANSGENE EXPRESSION

Unbiased transgene targeting is at the heart of neurotrapping. Given the large number of neurons in even the modestly-sized brain of the fruitfly, it is clear that generating Gal4 lines to target each individual neuron would be overwhelming and quite possibly also

unproductive. Redundancy of function both within and between circuits will undoubtedly obscure the behavioral contributions of individual neurons in many cases. On the other hand, if one wants to target multiple neurons, it is unclear what number should be targeted: Targeting too many neurons risks hitting multiple circuits and confounding the behavioral effects of any given manipulation; on the other hand, targeting too few neurons risks missing circuits that are broadly distributed and rely on the participation of many weakly contributing components.

The data from neurotrapping screens conducted with Gal4 enhancer-trap lines indicates that 1–10% of lines can be expected to produce a phenotype of interest when used to drive suppressors, but only one to two per 1000 will have an expression pattern small enough to analyze easily using existing methods. This would suggest that having more Gal4 lines with small expression patterns would be beneficial. Pfeiffer et al. (2008) recently described a method for generating such lines using random 3 kb fragments of putative regulatory DNA from the flanking and intronic regions of genes known to be expressed in the nervous system. They report that Gal4 lines made with 44 such enhancer fragments labeled only about a quarter as many central brain neurons on average (approximately 100) as conventional enhancer-trap lines. Happily, these authors are in the process of making several thousand such lines using putative enhancer regions from many different genes. They estimate that these lines will collectively provide coverage of most neurons in the *Drosophila* brain.

Even enhancer-trap lines with small expression patterns, however, will at best identify a set of candidate neurons in neurotrapping screens. This set will then have to be winnowed down to the minimal subset that gives rise to the behavioral deficit of interest when suppressed. In addition to the flp-out Gal80 method, there are several other combinatorial techniques that use the flp recombinase to restrict transgene expression. These include the UAS > STOP > TNT system described above, and UAS > STOP > Shi^{ts1}, which was introduced by Stockinger et al. (2005). One drawback of these techniques as they are traditionally used (i.e. in combination with hs-flp) is that excision of the FRT-flanked cassette is stochastic, and the restricted expression patterns are therefore not reproducible from animal to animal.

An alternative methodology that provides reproducible expression patterns is the Split Gal4 system introduced by Luan et al. (2006b). In this system (**Figure 2D**), the Gal4 molecule is split into its two component domains, the DNA-binding domain (DBD) and transcription activation domain (AD), which can then be independently targeted to different neuronal groups. Gal4 transcriptional activity, and therefore the expression of UAS transgenes, is thus restricted to the intersection of the expression patterns of the two domains. By making a fly line in which the expression of one domain (e.g. the DBD) is restricted to a cell group of interest, and crossing this line to enhancer-trap lines made with the other domain (i.e. the AD), one can produce many different patterns of expression in subsets of the original group (Luan et al., 2006b; Luan and White, 2007). These patterns are reproducible from animal-to-animal and can be generated at will.

The Split Gal4 system can be used in neurotrapping screens in two principal ways. In the first, a primary screen can be conducted using enhancer-trap (ET) lines made with one component (e.g. ET-DBD) while the other component is expressed throughout the nervous system (e.g. the AD driven by a pan-neuronal promoter). Each ET-DBD line that yields a behavioral deficit of interest in the primary screen can then be used in a secondary screen with AD enhancer-trap lines. In this way, the patterns of the individual ET-DBD lines from the primary screen can be subdivided in the secondary screen. Alternatively, if a Gal4 enhancer-trap line has been isolated in a neurotrapping screen, it can be converted into a Split Gal4 line by P-element swap (Sepp and Auld, 1999) to subdivide its expression pattern. Gao et al. (2008) have recently demonstrated the efficacy of converting an existing enhancer-trap Gal4 line into a DBD line which shares the expression pattern of the original. The process of swapping a Split Gal4 component for Gal4 could also be streamlined by using Gal4 constructs that permit recombinase-mediated cassette exchange (RMCE, Horn and Handler, 2005). In any case, application of the Split Gal4 technique provides a strategy for systematically parsing the pattern of an identified enhancer-trap line into smaller groups so that their function can be analyzed.

BEYOND SYNAPTIC SILENCING: EXCITABILITY SUPPRESSION AND NEURONAL STIMULATION

Although most neurotrapping screens conducted thus far have used UAS-TNT or UAS-Shi^{ts1}, these tools will not block communication through electrical synapses or necessarily impede signaling by hormones and neuromodulatory factors (Thum et al., 2006). In principle, neuronal function can be more universally suppressed by inhibition of membrane excitability, which should block all signaling mechanisms used by neurons. As described in the Frontiers in Molecular Neuroscience Special Topic titled "Genetic techniques and circuit analysis" (see Hodge, 2009) numerous tools have been developed to block neuronal excitability, from the potent inward rectifying Kir2.1 K^+-channel to the relatively weak EKO channel. As noted above, UAS-Kir2.1 was used in conjunction with UAS-TNT to perform the neurotrapping study of Gordon and Scott (2009), though no differences in the efficacy of the two tools was reported.

In contrast, TNT and EKO display clear differences in efficacy in suppressing wing expansion behaviors (Luan et al., 2006a). These behaviors are regulated by the hormone bursicon, which is expressed in a subset of neurons that express the neuropeptide CCAP. In contrast to EKO, TNT poorly blocks wing expansion when expressed in CCAP-expressing neurons and Kir2.1, because of its greater potency, causes developmental lethality at the pupal stage. Luan et al. (2006a) therefore chose to use the EKO channel to conduct a screen of enhancer-trap lines to identify neurons involved in wing expansion. This screen identified 24 lines (from a total of 114) that produced wing expansion deficits when crossed to EKO. Using a CCAP-Gal80 construct, 23 of these lines were shown to act by suppressing CCAP-expressing neurons. Because of its selective expression in a subset of bursicon-secreting neurons, one of these lines permitted a functional dissection of the bursicon-expressing group.

Although there is, as yet, no conditional suppressor of excitability available for use in *Drosophila*, an increasing number of options are available for acutely stimulating neurons using either light or temperature (see **Table 1**). As indicated by the use of ChR2 to stimulate motor neurons in the study of Gordon and Scott (2009), or the recent use of the temperature-sensitive TRP channels to selectively

stimulate neurons promoting wakefulness (Parisky et al., 2008) or wing expansion (Peabody et al., 2009), these tools can be used to identify sets of central neurons that are capable of inducing behavioral programs. More generally, they have the potential of being used to identify neurons that act as "command systems" in generating behavioral outputs. Neurotrapping screens using activators rather than suppressors of neuronal activity thus offer an additional important tool for exploring behavioral control in *Drosophila*.

In addition to screens that involve suppressing and activating neurons, the general strategy described here can be applied to study any neuronal function of interest. Indeed, screens have been carried out using numerous other effectors, including UAS-transformer to feminize neurons in studies of courtship behavior (Ferveur et al., 1995), UAS-PKA^{inh}, a PKA inhibitor, to study brain regions sensitive to alcohol (Rodan et al., 2002), and UAS-mC*, a constitutively active form of PKA, to study neural substrates of sleep (Joiner et al., 2006). With the recent availability of UAS-RNAi constructs to virtually every gene in the *Drosophila* genome (Dietzl et al., 2007), diverse neurotrapping strategies can now be envisaged that ask not only which neurons are necessary for executing different behaviors, but which genes in those neurons are necessary.

FUTURE PROSPECTS

With recent advances, neurotrapping screens are beginning to realize their full potential. Although technical challenges to implementing them remain, the most recent advances provide proof that success is possible and suggest that whole circuits will soon yield to the neurotrapping approach. Whether all circuits can be "cracked" by this approach remains to be determined, but the answer is soon likely to depend less on the availability of techniques than it will on biology. Like all nervous systems, the *Drosophila* nervous system is densely interconnected and we have much to learn about how it is organized: How many behaviors are governed by "command" systems as opposed to "multifunctional" networks that broadly coordinate the animal's responses to environmental inputs? How are conflicts between motor outputs resolved and what mechanisms govern behavioral choice? What compensatory mechanisms and circuits exist to allow for behavioral flexibility in the face of environmental challenge or developmental damage? How are complex behavioral programs assembled? All these questions remain, but given recent developments, neurotrapping in *Drosophila* seems poised to provide at least some of the answers.

The answers derived from *Drosophila* will undoubtedly inform our thinking about the functional architecture of other nervous systems, including our own. Will it be possible to also gain direct insights into the function of vertebrate nervous systems from neurotrapping studies? Despite the technical challenges of longer generation times, larger chromosome numbers, and increased costs encountered in working with vertebrate preparations and the fact that neuronal identities in such organisms are often epigenetically sculpted and less stereotyped between individuals than they are in *Drosophila*, the answer is almost certainly yes. Indeed, the feasibility of neurotrapping in fish has already been demonstrated by Wyart et al. (2009). Using multiple zebrafish Gal4 enhancer-trap lines that drive expression in spinal cord neurons, these authors expressed a light-activated cation channel to identify the stimulatory inputs to the swim central pattern generator in the spinal cord.

Demonstrations of neurotrapping's feasibility in mammalian preparations may well follow. Sophisticated approaches for gene targeting and neuronal manipulation are available in the mouse (Deisseroth et al., 2006; Luo et al., 2008), and have already been extensively used in what might be called "reverse" neurotrapping studies in which targeted neuronal manipulations have been used to demonstrate the functional roles of defined neuronal populations (see for example Yu et al., 2004; Adamantidis et al., 2007; Tan et al., 2008; Sohal et al., 2009; Tsai et al., 2009). A particularly elegant example is the study of Kim et al. (2009), who used an intersectional expression system to synaptically silence a subclass of serotonergic neurons and show that silencing correlated with a reduction in anxiety-related behaviors. Because the technical challenges mentioned above are a particular impediment to neurotrapping in the mouse, progress in this animal may have to exploit different strategies than those used in *Drosophila*. It would be particularly helpful if random neuronal manipulations could be correlated with behavioral changes in single animals. This would be facilitated if advances in gene expression profiling of mouse neurons (Lein et al., 2007; Ng et al., 2009) could be leveraged to identify the neurons randomly targeted by viral transduction. Whether such strategies will prove tractable will be decided by future developments. However, as the work reviewed here indicates, technical development is unlikely to limit progress for long, and neurotrapping, in *Drosophila* and in other organisms, is likely to play an increasing role in elucidating the circuitry that governs behavior.

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Neuronal mechanisms of learning and memory revealed by spatial and temporal suppression of neurotransmission using *shibire*^{ts1}, a temperature-sensitive dynamin mutant gene in *Drosophila melanogaster*

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The fruit fly *Drosophila melanogaster* is an excellent model organism to identify genes and genetic pathways important for learning and memory. However, its small size makes surgical treatment and electrophysiological manipulation technically difficult, hampering the functional analysis of neuronal circuits that play critical roles in memory processing. To circumvent this problem, we developed a unique experimental strategy that uses the temperature-sensitive allele of the *Drosophila* dynamin gene, *shibire*^{ts1} (*shi*^{ts1}), in combination with the GAL4/UAS expression system. This strategy allows for rapid and reversible perturbation of synaptic neurotransmission in identifiable neurons, and analysis of the behavioral consequences of such manipulation in free-moving animals. Since its introduction in 2001, this GAL4/UAS-*shi*^{ts1} strategy has been widely used to study the neuronal basis of learning and memory. This review focuses on how this strategy has revitalized *Drosophila* memory research, and contributed to our understanding of dynamic neuronal processes that control various aspects of memory.

Keywords: learning and memory, *Drosophila*, dynamin, temperature-sensitive mutant

INTRODUCTION

An exciting and challenging task in modern neuroscience is to gain a comprehensive understanding of how we learn and remember – at the molecular, cellular and systems levels. As a versatile model organism for behavioral genetics, the fruit fly *Drosophila melanogaster* has been successfully used to study individual genes and genetic pathways that are important for different aspects of memory (McGuire et al., 2005). Notably, forward genetic screens for "memory mutants" in *Drosophila* have resulted in the identification of molecular cascades that are critical to learning and memory, and also remarkably well-conserved among evolutionarily diverse animal species. In order to fully elucidate how these genes and genetic pathways control memory processes, we must understand their roles in the context of the neurons and neuronal circuits responsible for the acquisition, consolidation and retrieval of memories. To link neuronal circuits to memory processes, it is necessary to manipulate the activity of identified neurons within intact animals, and to analyze the direct effects of this manipulation on memory-based behavioral modifications. Unfortunately, it is difficult to perform such experiments in *Drosophila melanogaster* through surgical or electrophysiological means, mainly because of the small size of this animal. To circumvent this problem, we have developed a strategy that couples the *Drosophila* dynamin mutant gene, *shibire temperature-sensitive1* (*shi*^{ts1}), with the GAL4/UAS binary expression system, to rapidly and reversibly suppress synaptic neurotransmission from targeted neurons in intact free-moving animals, through a mild temperature shift (Kitamoto, 2001, 2002). Here we explain the principles of this strategy and discuss how it has been utilized to gain a better understanding of neuronal mechanisms of *Drosophila* learning and memory. We focus in particular on the aversive associative olfactory learning paradigm.

RAPID AND REVERSIBLE SUPPRESSION OF SYNAPTIC NEUROTRANSMISSION BY GAL4-DRIVEN EXPRESSION OF *shibire*^{ts1}

The *Drosophila* GAL4/UAS system (Brand and Perrimon, 1993) has been widely used to drive the expression of UAS-linked transgenes in specific sets of identified neurons in order to study their roles in behavior. For example, particular set of neurons can be eliminated by introducing a cell death-inducing gene. Alternatively, functional properties of targeted neurons can be altered by expressing genetically engineered ion channels (to change neuronal excitability) or neurotoxins (to disrupt synaptic transmission). The relationships between particular neurons/neuronal functions and behavior can then be assessed using established assays. These non-conditional gene expression systems, however, have serious limitations with respect to studying the roles of neurons in behavior – due to the inherent plasticity of the nervous system. When a group of neurons is eliminated or their functions are continuously altered, the rest of the nervous system is likely to adjust (either structurally or functionally) to the perturbation. Such homeostatic responses from the nervous system may obscure the direct consequence of the targeted expression of UAS-linked transgenes. A second problem is that most, if not all, GAL4 driver lines exhibit substantial GAL4 activity during development. Thus, even if GAL4-driven expression of a transgene is restricted to a small number of neurons in the adult brain, the

behavioral changes observed in adult animals could nevertheless arise as a consequence of undefined expression of the transgene during development. These considerations underscore the importance of the inducibility of transgene expression for analyzing the neuronal basis of behavior using the GAL4/UAS system. Only if a transgene exerts its expected function in the targeted neurons while the behavior is being examined, the observed behavioral changes can be directly link to the neurons expressing these genes.

UAS-linked transgenes can be conditionally expressed using the drug-inducible GeneSwitch GAL4 system (Osterwalder et al., 2001) or the temperature-dependent TARGET system (McGuire et al., 2004), and these conditional GAL4 systems are useful for eliminating any developmental effects of transgene expression. However, they are not suitable for analysis of the dynamic neuronal processes responsible for the temporal regulation of memory because of the lag time (several hours) between drug administration/temperature shift, respectively, and transgene expression. In addition, these conditional expression systems need several hours to terminate transgene activity once they are "turned off". This kind of time resolution limits the usefulness of these systems for the functional study of neurons involved in temporally regulated memory processes.

The *Drosophila* gene *shibire* encodes dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991), a protein that plays a critical role in synaptic vesicle recycling in nerve terminals (Kosaka and Ikeda, 1983). The dynamin molecule encoded by the mutant gene *shibire temperature-sensitive1* (*shi^{ts1}*) has a single amino acid substitution in the GTPase domain, making it reversibly temperature-sensitive. *shi^{ts1}* mutants are paralyzed in a temperature-dependent manner, because the functions of *shi^{ts1}*-encoded dynamin are impaired at restrictive temperature. This results in depletion of synaptic vesicles at the nerve terminals, and thus leads to suppression of neurotransmission in the neurons responsible for motor control (**Figure 1**). The *shi^{ts1}* allele is semidominant regarding the paralytic phenotype (Kim and Wu, 1990). Therefore, it was expected that would be possible to perturb neurotransmission in a temperature-dependent manner by overexpressing *shi^{ts1}* in the presence of its endogenous wild-type counterpart. We have demonstrated that this is indeed the case, using *Cha*-GAL4 to drive UAS-*shi^{ts1}* expression in the major excitatory (cholinergic) neurons in the *Drosophila* nervous system (Kitamoto, 2001). Adult flies expressing *shi^{ts1}* in cholinergic neurons were apparently normal at permissive temperature, but became paralyzed within 2 min of being moved to the restrictive temperature; activity was immediately regained when the flies were returned to permissive temperature. A more specific behavioral defect was observed when *shi^{ts1}* expression was directed to the photoreceptor cells; both larvae and adults displayed a temperature-dependent defect in light-induced behavior, yet other behaviors were intact (Kitamoto, 2001). These results demonstrated that UAS-*shi^{ts1}* can be used as a molecular switch for synaptic transmission in targeted neurons (**Figure 1**).

NEURONS INVOLVED IN AVERSIVE OLFACTORY ASSOCIATIVE MEMORY

Drosophila has a remarkable ability to acquire, store and recall memory in various learning and memory paradigms. The most extensively studied of these paradigms is aversive olfactory conditioning (Davis, 2005). Previous studies indicated that single odor-shock training (single training) induces protein synthesis-independent memory that lasts for hours. The memory induced by single training is composed of at least three temporally distinct memory phases: short-term memory (STM), middle-term memory (MTM) and anesthesia-resistant memory (ARM). STM and MTM are labile memories and disrupted by anesthetic treatment such as a cold shock, whereas ARM, which develops during the first 30 min after training, is a form of consolidated memory and resistant to anesthetic treatment. Two protocols that are more intensive than single training, known as mass training and spaced training, are used to study the consolidation of aversive olfactory memory. In mass training, paired odor-shock stimuli are repeatedly presented without intervening rest periods. As in single training, mass training generates STM, MTM and ARM. Spaced training, in which repeated odor-shock stimuli are presented at intervals, is used to generate long-term memory (LTM), a protein synthesis-dependent form of consolidated memory that lasts for at least several days.

ROLES OF NEURONS INTRINSIC TO THE MUSHROOM BODIES

Which neurons are involved in the regulation of distinct memory phases? How does neuronal activity contribute to information processing relevant to the acquisition, consolidation and retrieval of memories? The UAS-*shi^{ts1}* transgene has been effectively used to gain important insights into these key questions. The primary brain region that has been studied in the context of olfactory memory is the mushroom body (MB) (Heisenberg, 2003). Previous studies showed that aversive olfactory memory is disrupted in flies with genetically or chemically ablated MBs (Heisenberg et al., 1985; de Belle and Heisenberg, 1994), yet the MB-deficient flies are able to discriminate odors and respond to an electric shock. Although these results indicated that MBs play an essential role in olfactory memory, little was known about how MB-intrinsic neurons contribute to memory processing. The MB-intrinsic neurons known as Kenyon cells are broadly classified into three subtypes, the α/β, α′/β′ and γ neurons, according to the projection patterns of their axons (Lee et al., 1999) (**Figure 2**). The use of UAS-*shi^{ts1}* in combination with various GAL4 drivers that are specific to different subsets of Kenyon cells has revealed that these structurally distinct neurons are also functionally diverse in the temporal processing of olfactory memory.

The α/β neurons, which constitute approximately 50% of the Kenyon cells, are labeled in the majority of the "MB-specific GAL4" lines (Aso et al., 2009). When UAS-*shi^{ts1}* was preferentially expressed in the α/β neurons and neurotransmission from these neurons was temporally blocked by maintenance at the restrictive temperature only during single training, the flies avoided the shock-associated odor during the test period, showing that memory is formed, stored and retrieved under these conditions. Similarly, inhibiting α/β neuron output between the training and test periods did not significantly affect the memory-based avoidance behavior during testing. These results indicate that neurotransmission from the α/β neurons is dispensable for the acquisition and storage of olfactory memory. In marked contrast, blocking neurotransmission only during the test period (30 min to 3 h after training) eliminated the avoidance behavior, demonstrating that neurotransmission from the α/β neurons is required for

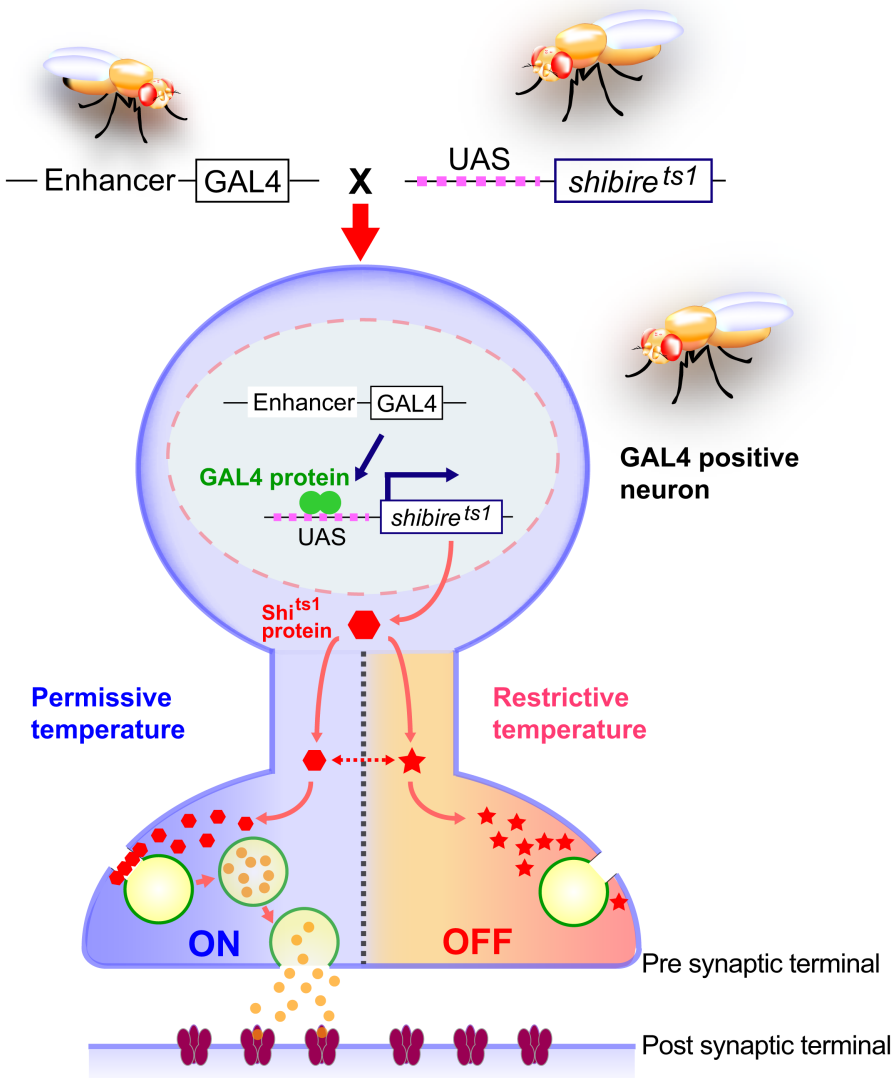

FIGURE 1 | Spatially and temporally restricted suppression of neurotransmission using the UAS-*shi*^{ts1} transgene. A GAL4 driver specific to neuronal subsets is crossed to the UAS-*shi*^{ts1} line. Progeny ectopically expressing *shi*^{ts1} in GAL4-positive neurons are raised at permissive temperature. When the temperature is shifted from permissive to restrictive, the *shi*^{ts1} product (temperature-sensitive dynamin) is rapidly inactivated and synaptic vesicle recycling is interrupted. As a result, the GAL4-positive neurons are depleted of synaptic vesicles and synaptic transmission is blocked. Behavioral consequences of spatial and temporal suppression of neurotransmission can be observed in free-moving animals. The *shi*^{ts1} product regains its activity and synaptic vesicles are restored immediately after the animals are returned to permissive temperature.

the retrieval of aversive olfactory memory (Dubnau et al., 2001; McGuire et al., 2001). An important implication of this finding is that any aversive olfactory memory formation (presumably as changes in structures and/or functions of cellular components) occurs at or upstream of the α/β output synapses of the neuronal circuits involved in the processing memory. Memory is manifested as behavioral alterations in response to learned unconditioned stimuli (US) through neurotransmission from the α/β neurons in the MBs.

Either single or mass training can generate the consolidated form of memory known as ARM. Are the α/β neurons also required for ARM? ARM can be measured separately from STM and MTM by exposing animals to a cold shock 1 h after training, because labile STM and MTM are eliminated by this treatment. Blocking the output of the α/β neurons using UAS-*shi*^{ts1} has also been shown to impair ARM, indicating that neurotransmission from the α/β neurons is necessary for the retrieval of three types of short-lasting, protein synthesis-independent memory (Isabel et al., 2004). In contrast to ARM, LTM is a protein synthesis-dependent, consolidated form of memory that is generated only after spaced training. Recent optical imaging studies demonstrated that spaced training, but not single or mass training, results in a robust increase in calcium influx into the α lobes, in a protein synthesis-dependent manner (Yu et al., 2006). In addition, analysis

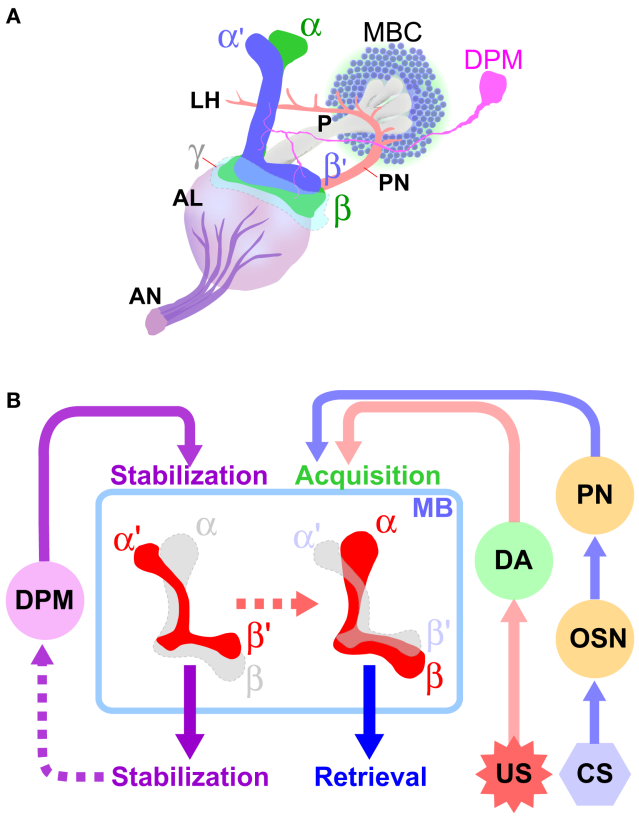

FIGURE 2 | Diverse roles of intrinsic and extrinsic mushroom body-associated neurons in the processing of aversive olfactory memory. **(A)** The mushroom body and associated neurons in one brain hemisphere are schematically represented (adapted from Armstrong et al., 1998). The cell bodies of Kenyon cells (MBCs), the mushroom body-intrinsic neurons, are located in the dorsal and posterior cortices of the brain. They extend axons anteriorly through a structure called the peduncle (P). The axons of α/β and α′/β′ neurons bifurcate to form vertical (α and α′) and horizontal (β and β′) lobes. The axons of γ neurons do not bifurcate, and form only a horizontal lobe (γ). The primary olfactory information received by the olfactory neurons is transmitted through the antennal nerve (AN) to the first olfactory center antennal lobe (AL), where the information is processed and further transmitted to the mushroom bodies by the projection neurons (PN). The dorsal paired medial (DPM) neuron extends an axon that branches and terminates in all lobes of the mushroom body. **(B)** Conditioned stimuli (CS; e. g. odors) and unconditioned aversive stimuli (US; e.g. electric shock) are simultaneously presented to flies. The olfactory information received by the olfactory neurons (OSN) is conveyed to the mushroom bodies (MB) through the first-order interneuron, the projection neurons (PN). The neuronal circuits that transmit the aversive sensory information include dopaminergic neurons (DA). The information generated by CS and US converges at the MB, where aversive olfactory memory is formed. Neurotransmission from DPM neurons to the α′/β′ neurons, as well as that from the α′/β′ neurons, contributes to the stabilization of memory. Retrieval of both protein synthesis-independent, short-lasting memory and protein synthesis-dependent, long-term memory (LTM) require neurotransmission from the α/β neurons.

of the *alpha-lobes-absence* (*ala*) mutants, which lack either the two vertical lobes (α and α′) or two of the three horizontal lobes (β and β′), suggested that LTM depends on the vertical lobes (Pascual and Preat, 2001). These results suggested that neurons forming vertical lobes, in particular those of the α lobe, are important for LTM. Experiments with UAS-*shi*^{ts1} provided data in support of this notion. Indeed, the retrieval of memory 24 h after spaced training is significantly impaired when neurotransmission from the α/β neurons is blocked only during testing, indicating that the output of the α/β neurons is required for the retrieval of LTM. As in the case of STM, a memory trace for LTM is likely formed, at least partly, at or upstream of the α/β neuron output synapses (Isabel et al., 2004).

The original model for aversive olfactory memory proposes that pairing conditioned stimuli (CS) with US leads to the sequential formation of STM and MTM, and that the latter is then processed into either ARM and/or LTM, depending on the training protocol (DeZazzo and Tully, 1995). In this model, both ARM and LTM derive from MTM, and ARM and LTM coexist for 24 h after spaced training. Interestingly, however, it was shown that spaced training that produces LTM leads to disappearance of ARM in the *ala* mutants lacking vertical lobes (Isabel et al., 2004). In addition, the experiments using UAS-*shi*^{ts1} indicate that ARM and LTM involve the same α/β subset of the MB neurons. These results suggest that ARM and LTM are mutually exclusive. Furthermore, normal ARM can be detected in mutants defective for STM (*rut*) or MTM (*amn*). Based on these collective results, it has been proposed that ARM is processed largely independently of the sequential STM-MTM-LTM pathway, but stored in the common group of neurons in the MBs (Isabel et al., 2004).

With respect to aversive olfactory memory, the functional significance of the γ neurons, which account for approximately 30% of Kenyon cells (Aso et al., 2009), has thus far not been shown. The α′/β′ neurons are relatively minor components of the MBs, comprising approximately 20% of the Kenyon cells (Aso et al., 2009). Although they extend axons parallel to those of the α/β neurons, experiments using UAS-*shi*^{ts1} demonstrated that the roles of the α′/β′ neurons in olfactory memory processing are distinct from those of the α/β neurons. When neurotransmission from the α′/β′ neurons was blocked only during the test period by introducing UAS-*shi*^{ts1} in combination with GAL4 drivers that preferentially label the α′/β′ neurons, the memory-based avoidance behavior during the test period was not affected. This result shows that, unlike neurotransmission from the α/β neurons, that from α′/β′ neurons is dispensable for the retrieval of olfactory memory. Interestingly, however, when the α′/β′ neurons were blocked either during or after training, olfactory memory was severely impaired. Thus, output from the α′/β′ neurons appears to be required for the acquisition and stabilization of olfactory memory (Krashes et al., 2007). An exciting line of future investigation will focus on how neurotransmission from the α′/β′ neurons contributes to the processing the memory that eventually depends on output of the α/β neurons for its retrieval.

ROLES OF NEURONS EXTRINSIC TO THE MUSHROOM BODIES

The Dorsal Paired Medial (DPM) neurons are two large neurons that project extensively to all the lobes and the base of the peduncle of MB neurons. They were first identified as primary cells in the adult brain that express the putative neuropeptide encoded by the *amnesiac* gene (*amn*) (Waddell et al., 2000), whose product plays an important role in stabilizing memory (Quinn et al., 1979). *amn* mutants can learn and form STM, but their memory decays abnormally rapidly, within 30–60 min after training, and

this results in severe defects in MTM. Because the *amn* memory defect is rescued by expressing the wild-type *amn* gene product preferentially in DPM neurons, these neurons are considered to be the site at which *amn* acts to stabilize olfactory memory. Results obtained from UAS-*shi*^{ts1} experiments revealed the significance of DPM neuron output in memory stabilization. When neurotransmission from DPM neurons was constantly blocked (during the training, storage and test periods), the flies displayed defects in memory that could be measured 1 h after training, as was the case in *amn* mutants. However, temporally blocking neurotransmission from DPM neurons only during the training or test period did not affect olfactory memory. Therefore, DPM neuron output is dispensable for memory acquisition and retrieval. In contrast, blocking DPM output for 30 min during the storage period (between training and testing) resulted in a significant impairment of 1-h memory, demonstrating that the DPM neuron is critical for stabilizing memory (Waddell et al., 2000).

Considering that neurotransmission from the α′/β′ neurons is similarly required to stabilize memory during storage, and that DPM neurons heavily innervate the MB lobes – including the α′ and β′ lobes – it is possible that direct information flow from DPM neurons to the α′/β′ neurons is essential to stabilizing the newly formed memory. This possibility was tested by using flies that express an isoform of *Drosophila* Down syndrome cell adhesion molecule (Dscam17-2) in DPM neurons. The expression of Dscam17-2 in DPM neurons affected their development such that projections of DMP neurons to the α, β and γ lobes are significantly reduced, but those to the α′ and β′ lobes are relatively intact. In these genetically engineered flies with limited DPM projections to the MB α′/β′ neurons, aversive olfactory memory was not significantly affected. Importantly, a temporal block in DPM neuron output to the α′/β′ neurons, but not to most α/β and γ neurons, was enough to induce the *amn*-like memory phenotype (Krashes et al., 2007). These results strongly suggest that stabilizing memory during the storage period mainly depends on neurotransmission from DPM neurons to a subset of MB neurons – the α′/β′ neurons.

It has been proposed that aversive olfactory memory is formed in the MBs, where sensory information concerning conditioned odor stimuli and unconditioned aversive stimuli converges, and that this establishes an association between CS and US (Davis, 2005). Although significant progress has been made in understanding the molecular and neuronal mechanisms responsible for olfactory information processing (Hallem and Carlson, 2004), little is known about how unconditioned electric shocks are sensed as aversive stimuli, and which neurons are involved in conveying US information to the MBs. In other organisms, monoaminergic interneurons are thought to transmit such information. In *Drosophila*, a study investigating mutants for *dopa decarboxylase,* which encodes an enzyme involved in the synthesis of both dopamine and serotonin, implicated these neurotransmitters in aversive olfactory learning (Tempel et al., 1984). However, this result could not be reproduced by another study (Tully, 1987). Given the controversial nature of these findings, UAS-*shi*^{ts1} was used to revisit this issue (Schwaerzel et al., 2003). Tyrosine hydroxylase (TH) is the rate limiting enzyme for dopamine and expressed specifically in dopaminergic neurons. *TH*-GAL4, which was generated using regulatory DNA of the TH gene (Friggi-Grelin et al., 2003), was used to direct UAS-*shi*^{ts1} to dopaminergic neurons. Although blocking neurotransmission from dopaminergic neurons before and during testing did not affect memory, blocking them only during the training period severely impaired aversive olfactory memory (Schwaerzel et al., 2003). Interestingly, dopamine transmission was not required for appetitive olfactory learning using sugar as the US. These results provide strong support for the notion that dopaminergic neurons are part of the neuronal circuit for aversive US in *Drosophila*. There are approximately 120 dopaminergic neurons in the adult fly brain, and some of which extensively innervate the MBs (Riemensperger et al., 2005). Future studies using GAL4 lines specific to a subset of brain dopaminergic neurons should reveal functional differences between dopaminergic neurons within the brain, and identify the particular neuronal circuit involved in aversive olfactory memory.

SUMMARY AND FUTURE PERSPECTIVES

It is almost 30 years since the genetic analysis of learning and memory was initiated using *Drosophila melanogaster* as a model system. Since then, considerable progress has been made in identifying and characterizing genes and genetic interactions that are important for memory processes. Currently the *Drosophila* memory research field is aiming to attain a comprehensive understanding of the mechanisms that underlie learning and memory, by an approach that integrates molecular, cellular and systems analyses. Since its introduction to *Drosophila* behavioral research, the UAS-*shi*^{ts1} transgene has contributed significantly to the studies of temporal and spatial regulation of the neuronal activities responsible for learning and memory. **Figure 2** summarizes the findings of the UAS-*shi*^{ts1} studies regarding the roles of intrinsic and extrinsic neurons of the MBs in different aspects of aversive olfactory memory. This knowledge provides a strong foundation for detailed functional and structural analyses in the future. Recently Pfeiffer et al. (2008) have generated thousands of transgenic *Drosophila* lines in which GAL4 expression is directed to distinct (most of them small) subsets of cells in the adult brain (Pfeiffer et al., 2008). In the case of the MBs in particular, an increasing number of GAL4 enhancer-trap strains have been identified that label specific subsets of its intrinsic and extrinsic neurons (Tanaka et al., 2008). These new GAL4 lines will be used in combination with UAS-*shi*^{ts1}, as well as other equally useful UAS-linked effector transgenes, to enable the conditional activation of identifiable neurons (Lima and Miesenbock, 2005; Schroll et al., 2006) and the optical imaging changes in the levels of important messengers including Ca^{2+}, H^{+} and cAMP (Yu et al., 2004). The same approach should be applied to other learning paradigms in *Drosophila* that involve sensory stimuli and brain neurons distinct from those involved in aversive olfactory learning. With a handful of valuable molecular and genetic tools, *Drosophila* research on memory and learning is poised to continue providing essential knowledge of the basic principles of learning and memory in the coming years.

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Activity-dependent modulation of neural circuit synaptic connectivity

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In many nervous systems, the establishment of neural circuits is known to proceed via a two-stage process; (1) early, activity-independent wiring to produce a rough map characterized by excessive synaptic connections, and (2) subsequent, use-dependent pruning to eliminate inappropriate connections and reinforce maintained synapses. In invertebrates, however, evidence of the activity-dependent phase of synaptic refinement has been elusive, and the dogma has long been that invertebrate circuits are "hard-wired" in a purely activity-independent manner. This conclusion has been challenged recently through the use of new transgenic tools employed in the powerful *Drosophila* system, which have allowed unprecedented temporal control and single neuron imaging resolution. These recent studies reveal that activity-dependent mechanisms are indeed required to refine circuit maps in *Drosophila* during precise, restricted windows of late-phase development. Such mechanisms of circuit refinement may be key to understanding a number of human neurological diseases, including developmental disorders such as Fragile X syndrome (FXS) and autism, which are hypothesized to result from defects in synaptic connectivity and activity-dependent circuit function. This review focuses on our current understanding of activity-dependent synaptic connectivity in *Drosophila*, primarily through analyzing the role of the fragile X mental retardation protein (FMRP) in the *Drosophila* FXS disease model. The particular emphasis of this review is on the expanding array of new genetically-encoded tools that are allowing cellular events and molecular players to be dissected with ever greater precision and detail.

Keywords: neurotransmission, synapse, pruning, fragile X syndrome, FMRP, *Drosophila*, development

INTRODUCTION

The development of neural circuits is initiated with the "soft-wiring" of speculative synaptic connections that form the foundation of specific penultimate circuits, and culminates with a process of refinement in which the correct synaptic connections are solidified, while improper connections are systematically weakened and removed from the circuit. The initial phase of synaptic overgrowth is presumably required to ensure that the nervous system is completely wired in the proper manner without missing essential synaptic targets. It is hypothesized that a less inclusive, more precise growth, extension and connection program would run the risk of missing important synaptic connections and thus leave the brain with impaired processing capacity and unable to efficiently respond to the myriad of sensory demands from the environment. Moreover, the removal of inappropriate synapses, coupled to the addition of newly acquired synapses, provides a vital segregation mechanism to distinguish connections with common functionality. Thus, before so-called "hard-wiring" can be complete, circuits must go through a period of neuronal process pruning.

The refinement of neural circuitry depends on a period of neuronal activity, which is known to be necessary for the final specification of the synaptic map. This activity-dependent process has been classically investigated in the vertebrate neuromusculature and visual sensory system, with less exploration in higher order central brain integration circuits (Cang et al., 2005b; Chandrasekaran et al., 2005; Liu et al., 1994; Wiesel, 1982). In these systems, an early soft-wiring program of activity-independent mechanisms involving guidance cues, diffusible signaling ligands and transmembrane receptors appears sufficient to direct pre- and postsynaptic processes to proper locations and culminate in synaptogenesis (Cang et al., 2005a; Cutforth et al., 2003; Feinstein and Mombaerts, 2004; Feinstein et al., 2004; Imai et al., 2006; Yates et al., 2001). The final refinement of both dendritic and axonal projections to specify the mature synaptic map then requires intrinsic synaptic firing (for review see Wong and Ghosh, 2002). In some circuits, such as the mammalian olfactory system, the relative role of activity-dependent refinement is more controversial, as different groups have found evidence for and against this process (Lin et al., 2000; Marks et al., 2006; Yu et al., 2004; Zheng et al., 2000).

In invertebrates, the involvement of synaptic activity in modulating circuits is much less clear. The dogma has long been that invertebrate circuits are hard-wired from the early stages of development, and do not employ activity-dependent refinement in a manner comparable to vertebrates. Indeed, a strong body of experimental evidence supports this position, in both primary sensory circuits and higher order systems (Berdnik et al., 2006; Hiesinger et al., 2006; Jefferis et al., 2004; Oland et al., 1996; Scott et al., 2003; Srahna et al., 2006). However, both classical and recent evidence has shown that environmental experience in invertebrates is capable of modulating the connectivity of sensory and higher order circuits

(Chiba et al., 1988; Devaud et al., 2001, 2003; Fahrbach et al., 1995; Sachse et al., 2007; Withers et al., 1993). Very recently, detailed structural analysis of single neuron architecture *in vivo* has shown activity-dependent refinement of circuits as a late-occurring phase of development (Tessier and Broadie, 2008; Tripodi et al., 2008). These new insights have resulted from advances in the powerful *Drosophila* genetic system, and novel transgenic tools position a field now poised to dissect the cellular and molecular mechanisms of circuit map refinement.

Multiple neurological disorders of mental retardation and autism likely arise from defects in neural circuit refinement. The most common of these is Fragile X syndrome (FXS), an X-linked inherited genetic disorder of mental retardation (IQ < 40), autism and hyperexcitability (Cohen et al., 2005; Hagerman et al., 2005; Rogers et al., 2001; Sullivan et al., 2006). FXS patients commonly exhibit hypersensitivity to sensory stimuli, hyperactivity and attention deficit disorder, with approximately 20% of patients manifesting epileptic seizures during childhood (Berry-Kravis, 2002; Incorpora et al., 2002; Musumeci et al., 1999). A great deal of recent interest has focused on FXS at the intersection of developmental circuit refinement and neuronal activation. FXS is caused by loss of function of the *fragile X mental retardation* (FMR1) gene, a member of a tripartite gene family (Pieretti et al., 1991; Siomi et al., 1995; Zhang et al., 1995). In rodents, the expression and function of the FMR1 product (FMRP) is regulated by neuronal activity levels, with the FMRP expression peak during the early postnatal period of synaptic refinement (Ferrari et al., 2007; Khandjian et al., 1995; Ostroff et al., 2002; Singh et al., 2007; Tessier and Broadie, 2008; Wang et al., 2004, 2008). A hallmark of FMRP loss is the failure to remove immature synaptic connections (Comery et al., 1997; Galvez and Greenough, 2005; Galvez et al., 2003, 2005; Irwin et al., 2001, 2002; McKinney et al., 2005; Nimchinsky et al., 2001; Rudelli et al., 1985). Thus, FMRP is a leading candidate for a molecule mediating activity-dependent synaptic refinement.

A powerful FXS model has been long established in *Drosophila* (Wan et al., 2000; Zhang et al., 2001). There is only a single homologous *Drosophila* FMR1 gene (*dfmr1*), so its deletion is presumably more comparable to loss of the tripartite gene family in mammals. Consistently, null *dfmr1* mutants exhibit strikingly similar molecular, cellular and behavioral phenotypes compared to mouse FMR1 knockouts, but with the great advantage of increased robustness of phenotype manifestation (Zhang and Broadie, 2005). A particular advantage of the *Drosophila* model is the UAS-GAL4 promoter/transcriptional activator transgenic tools which permit spatially and temporally targeted genetic manipulation of this system (Fischer et al., 1988). For example, transgenic expression of modified ion channels can be used to potentiate or depress neuronal function to probe roles of neuronal activity in identified circuits, and transgenic reporters can be similarly introduced to monitor this activity (Mosca et al., 2005; Reiff et al., 2005; White et al., 2001). The Mosaic Analysis with a Repressible Cell Marker (MARCM) clonal approach permits such manipulation down to the level of single neurons within defined circuits (Lee and Luo, 2001). The inducible GeneSwitch system can control these tools at specific temporal or developmental time windows (Osterwalder et al., 2001). The vast array of targeted activator lines available allows interrogation of relevant circuitry throughout the fly brain.

This review focuses on recent work in the *Drosophila* FXS model showing that activity-dependent refinement of synaptic architecture in defined brain circuits proceeds into the adult stage of the life cycle. The many genetic tools being used to dissect the roles of the critical FMRP synaptic regulator will be discussed. In this review, we distinguish between early activity-dependent refinement of synaptic connections and maintained activity-dependent synaptic plasticity. Developmental refinement includes architectural and functional remodeling of a circuit in response to initial-use neuronal activity, which is necessary to sculpt the final synaptic map. This mechanism is restricted to precise developmental windows corresponding to activation by external experience. In contrast, synaptic plasticity involves activity-dependent alterations in synaptic structure and function required for the generation of higher order brain activities, such as learning and memory. This is a maintained property of neuronal circuits, which is temporally separable. However, it may be that the developmental refinement sets the stage to permit the plastic modulations later in life, or that these two activity-dependent processes may involve an overlapping cast of molecular players.

DEVELOPMENTAL WINDOWS OF ACTIVITY-DEPENDENT CIRCUIT MODULATION

Sensory systems are particularly attractive for the study of activity-dependent development, owing to the stereotypic structuring of sensory neurons and the ease of manipulating appropriate activity input. In the *Drosophila* visual system (**Figure 1A**), the dogma has been that development proceeds solely via intrinsic genetic elements such as receptor/ligand interactions, and specifically that neuronal activity is not required for the final hard-wired map to form. For example, blocking visual activity by dark-rearing animals reportedly caused no clear changes in dendritic structure in the lamina (Scott et al., 2003). A more rigorous experiment using the UAS/GAL4 system to express the cell death *head involution defective* (*hid*) gene to destroy photoreceptors in the imaginal discs as soon as they are born, similarly failed to detect significant alterations in

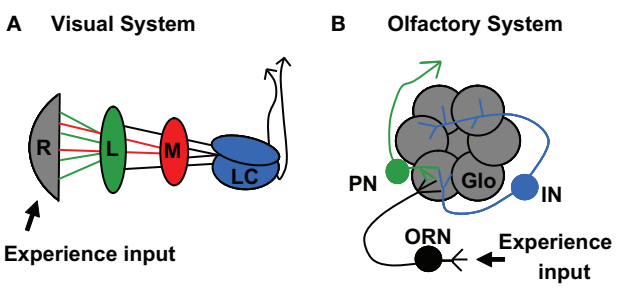

FIGURE 1 | Organization of *Drosophila* olfactory and visual systems. **(A)** The visual lobe receives input from retinal photoreceptor cells (grey). Photoreceptors 1–6 project to the lamina neuropil (green) while photoreceptors 7 and 8 project to the medulla neuropil (red). Signals are further processed in the lobula complex (blue) before proceeding to higher order brain regions. **(B)** The olfactory system receives input from olfactory sensory neurons (black), which each project axons to unique olfactory lobe glomeruli (grey). Local interneurons (blue) process information from multiple glomeruli. Projection neurons (green) transmit signals to higher order brain regions, including the Mushroom Body.

laminal dendritic structures (Scott et al., 2003). Likewise, a large scale high-resolution electron microscopy study revealed no differences in synapse number, or the location of synaptic boutons, in photoreceptor contacts from 43 different genetic mutants altering the levels of synaptic activity (Hiesinger et al., 2006). These studies therefore concluded that neuronal activity is not required for circuit map refinement. Similarly, the *Drosophila* olfactory system is reportedly largely stable throughout pupal morphogenesis (**Figure 1B**), and olfactory lobe innervations by either the pre- or postsynaptic cell are still patterned in the absence of the opposing synaptic partner (Berdnik et al., 2006). In contrast, serotonergic interneurons in the *Drosophila* antennal lobe display dramatic reorganization throughout development, which is dependent on evoked and spontaneous neural activity (Roy et al., 2007). It is important to note that the *Drosophila* studies have all focused on developmental time points during pupal metamorphosis, as circuit activity has been shown to be functioning during this developmental transition (Hardie et al., 1993). However, it is not clear when the development of these circuits ends and a "mature state" is achieved. The moment when the insect emerges from the pupal case (eclosion), is commonly considered the end of development and the start of adulthood, without any defined transitory period. This delineation appears as naïve as considering birth to be the end of development in mammals: the postnatal period is the active stage of activity-dependent refinement and synapse elimination. Similarly, changes in the volume of *Drosophila* olfactory glomeruli and visual lamina occur within days after eclosion (Barth et al., 1997; Devaud et al., 2001, 2003; Sachse et al., 2007). Recent work in both flies and ants has shown pruning of axonal processes, dendrites and synaptic connections occurs during the post-eclosion period (Seid and Wehner, 2009; Tessier and Broadie, 2008). Thus, during the initial early-use period following eclosion, *Drosophila* shows developmental refinement of synaptic processes, which we believe will become increasingly apparent with advances in genetic tools and high-resolution imaging.

The fragile X mental retardation protein (FMRP) appears to be one player modulating the refinement of synaptic processes. FMRP is an mRNA-binding protein implicated in transcript stability, transport and translational repression (for review, see Bassell and Warren, 2008). A popular hypothesis is that FMRP represses mRNA translation during transport to synapses and locally modulates translation in response to synaptic activity. In this mechanism, FMRP provides the means of ensuring that proteins required for synaptic structure and function are efficiently translated in response to use-dependent need. Importantly, FMRP expression peaks sharply during the developmental period of synaptic refinement, both in vertebrates and invertebrates, which suggests a predominant role in this transitory mechanism. In *Drosophila*, dFMRP is strongly expressed in the brain during late stages of pupal metamorphosis and shortly after eclosion (Tessier and Broadie, 2008). Following the early-use period, dFMRP protein levels drops precipitously to a low level, which is thereafter maintained throughout adulthood. Interestingly, the *dfmr1* mRNA profile mimics the dFMRP protein levels throughout development, but then diverges in the adult animal when *dfmr1* transcript levels rise to high levels but the dFMRP protein remains scarce (Tessier and Broadie, 2008). This suggests two distinct mechanisms of dFMRP regulation during (1) the post-eclosion refinement period and (2) presumed synaptic plasticity mechanisms in the mature animal. Further support for a transient role of dFMRP is apparent in the earlier larval period of development (Gatto and Broadie, 2008). The conditional GeneSwitch system was used to control the temporal expression of dFMRP in the nervous system (Osterwalder et al., 2001). GeneSwitch is a pharmacologically controlled version of the UAS-GAL4 system in which the GAL4 activator protein requires the cofactor RU486 (an analog of mifepristone) to promote transcription at UAS promoter sites. The drug may be mixed with standard fly food, or applied topically to induce targeted gene transcription. After removal of the drug, expression of the UAS transgene once again stops. Thus, the GeneSwitch system adds temporal control of gene expression to the inherent spatial control of the classical UAS-GAL4 method. Conditional dFMRP expression in *dfmr1* null animals is effective in reducing synaptic defects only when the induction window is during the early-use period immediately after larval hatching (Gatto and Broadie, 2008). Late dFMRP induction at maturity only very weakly alleviates a subset of synaptic defects. Thus, dFMRP peak expression and functional requirement both correspond to the restricted developmental windows of early use refinement.

Several lines of evidence suggest that FMRP functions directly downstream of neuronal activity (reviewed in Bassell and Warren, 2008). FMRP associates with polyribosomes in an activity-dependent manner and phosphorylated FMRP functions to locally repress the translation of critical synaptic proteins. After activation, FMRP is rapidly dephosphorylated to relieve this repression, but is subsequently re-phosphorylated to prevent excessive translation. In rodents, FMRP expression itself is regulated by sensory input activity (Irwin et al., 2005; Todd and Mack, 2000). Similarly in *Drosophila*, rearing in sensory deprived conditions results in decreased levels of both *dfmr1* mRNA and dFMRP protein in the brain (Tessier and Broadie, 2008). Likewise, dFMRP expression is significantly reduced in mutant animals with genetic blocks in olfactory and visual sensory pathways. Importantly, this activity-dependent regulation occurs during the period of early circuit use-dependent refinement, when dFMRP levels are transiently elevated compared with the mature animal (Tessier and Broadie, 2008). Thus, it is probable that dFMRP is acting as a monitor of circuit activity during this time window, with its expression controlled by sensory input and activity-dependent processes regulated by the translation of subsets of synaptic mRNAs.

The translation of many FMRP mRNA targets (e.g. MAP1B, Arc/Arg3.1 and PSD95) is rapidly upregulated by synaptic activation of metabotropic glutamate receptors (mGluRs), (Davidkova and Carroll, 2007; Park et al., 2008; Todd et al., 2003; Waung et al., 2008). The overextension of synaptic complexity in FMR1 knockout mice can be rescued either by antagonizing mGluR signaling or by more broadly enriching environmental stimulation (de Vrij et al., 2008; Restivo et al., 2005). The latter is also effective at eliminating hyperactive behaviors in FMR1 mutant mice (Restivo et al., 2005). Together, these studies suggest that FMR1 mutant animals exist in a state of heightened activity, which therefore precludes cellular responses to additional input. In the *Drosophila* FXS model, the translation of the dFMRP mRNA targets (e.g. chickadee/profilin) is elevated in genetic mutants blocking sensory activity inputs

(Tessier and Broadie, 2008). To examine structural consequences of functional cell-autonomous requirements, Mosaic Analysis with a Repressible Cell Marker (MARCM) can be used to visualize single mutant neurons in an otherwise wildtype brain (**Figure 2**). This genetic technique might be compared to Golgi staining, with the important addition that neurons labeled via MARCM are mutant for the gene of interest while all unlabeled neurons remain unaffected. The MARCM method uses a GAL4 activator expressed in a subset of neurons to turn on expression of a UAS-promoter controlled GFP transgene (Lee and Luo, 2001). However, the animals also ubiquitously express a GAL80 repressor, which inhibits GAL4 activation. To alleviate this repression, a heat shock controlled flipase is used to induce mitotic recombination between targeted FRT sites on the chromosome carrying the GAL80 repressor and the chromosome carrying the mutant gene of interest (in this case, *dfmr1*). By timing the recombination to coincide with the development of a known neuronal population, the result is a mutant clone lacking the GAL80 repressor. Thus, GFP is expressed, effectively labeling the mutant clonal population and permitting mutant cell structural analyses. The *Drosophila* Mushroom Body (MB) learning/memory center is critical for the integration of sensory experience (**Figure 2A**). MARCM analysis of single MB neurons (**Figure 2B**), comparing control to *dfmr1* mutant cells, revealed structural over-elaboration of synaptic connections, both in axonal processes and dendritic arbors (Pan et al., 2004). By definition, this requirement is cell-autonomous and also bidirectional, since dFMRP over-expression greatly reduced the number of synaptic connections. The fluorescent GFP signal from MARCM clones was then photoconverted to an electron-dense signal for ultrastructural analyses (**Figure 2C**). This powerful method showed that synaptic differentiation was altered in the absence of dFMRP, with a loss of regulation in synaptic bouton size and establishing synaptic vesicle pools (Pan et al., 2004). These MARCM defects were reversed by pharmacologically antagonizing mGluRs with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), showing that dFMRP functions to control synaptic connectivity via a pathway mediated by synaptic mGluR activity (Pan et al., 2008). It was subsequently established that an axonal pruning and synapse elimination program in these MB neurons normally occurs shortly after eclosion, during the initial use period, and that this refinement mechanism is lost in *dfmr1* null neurons during this specific developmental window (Tessier and Broadie, 2008).

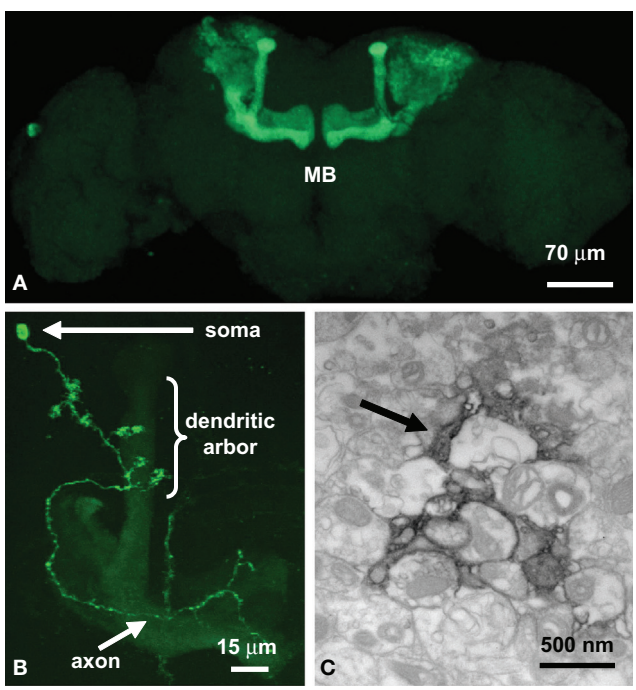

FIGURE 2 | Imaging the mushroom body learning and memory center. **(A)** OK107-GAL4 line driving GFP in the Mushroom Body (MB). **(B)** A single MB neuron clone generated by MARCM. **(C)** The MARCM fluorescent signal photoconverted to an electron-dense signal in the presence of diaminobenzene (DAB) with high intensity 490 nm light. The arrow points to a labeled MB process.

A recently developed means of directly manipulating neuronal activity *in vivo* employs the UAS-GAL4 system to target expression of a *Chlamydemonas* light-gated ion channel, channelopsin (Boyden et al., 2005; Nagel et al., 2003; Schroll et al., 2006). In the presence of the cofactor, all-trans retinal, this exogenous channel, referred to as channelrhodopsin (CHR2), conducts depolarizing current when stimulated with 480 nM blue light (reviewed in this issue). In *Drosophila*, this technique has recently been used broadly to control the activity of selected subsets of neurons by targeting UAS-CHR2 expression with a range of GAL4 driver lines (Borue et al., 2009; Hornstein et al., 2009; Pulver et al., 2009; Zhang et al., 2007). In addition to the targeting advantages, the electrophysiological functioning of the channel is now being manipulated, which will permit even more precise control of synaptic events (Nikolic et al., 2009; Radu et al., 2009; Wang et al., 2009a). When targeted to MB neurons, activation of the CHR2 channel effectively augmented activity-dependent synaptic pruning in control animals, but completely failed to do so in *dfmr1* null animals (Tessier and Broadie, 2008). This pruning function was restricted to the early-use developmental window in post-eclosion animals; reintroduction of environmental stimulation in mature animals failed to induce pruning. Thus, precise timing of events must be required for the activity-dependent pruning mechanism of dFMRP function, further suggesting that dFMRP itself may be the specific molecule "reading" the activity input required for refinement of neuronal circuits.

More generally, developing *Drosophila* neurons have also been recently imaged in genetic mutants that either lack the ability to produce neurotransmitter, or were targeted with the UAS-GAL4 system to express tetanus light chain toxin (TNT-LC) to eliminate neurotransmitter release (Tripodi et al., 2008). The TNT-LC protease cleaves the integral synaptic vesicle protein n-synaptobrevin in the presynaptic terminal and thereby eliminates evoked, but not spontaneous, neurotransmitter release (Broadie et al., 1995; Sweeney et al., 1995). This transgenic tool provides an excellent means of ascertaining cellular responses and developmental requirements of evoked neurotransmission. Earlier studies have clearly shown that the synaptic outputs of motor neurons at the neuromuscular junction (NMJ), are sculpted by activity at multiple levels of structural and functional refinement (Broadie and Bate, 1993; Budnik et al., 1990; Jarecki and Keshishian, 1995; Mosca

et al., 2005). In a recent study on embryonic motor neurons, it was determined that the elongation of dendritic branches onto which silenced synapses input was mitigated simply by the act of contact between the pre- and postsynaptic cell, and was independent of synaptic activity (Tripodi et al., 2008). However, presynaptic activity was required for synaptic refinement, as neurotransmission was involved in regulating local dendritic architecture. Blocking evoked transmission with TNT-LC resulted in an increase in the length of dendritic branches which did not themselves make synaptic contacts, but were derived from the same ordered branch as a branch which did make a synaptic contact (Tripodi et al., 2008). Thus, the growth of these so-called "non-synaptic sister branches" was locally controlled by nearby synaptic activity. This study further suggested that global changes in dendritic structure may be used to compensate for alterations in synaptic input. In other words, if the postsynaptic cell does not receive the correct amount of presynaptic input, it can elaborate its dendritic arbor to ensure proper synaptic coverage. These intriguing findings show that the arrangement of the final synaptic systems in the *Drosophila* neuromusculature involve activity-dependent mechanisms.

LEVELS OF ACTIVITY CONTROL

Neurotransmitter-activated G-protein coupled receptors participate in neurotransmission via G-protein dependent downstream signaling cascades (Conn and Pin, 1997). The role of metabotropic glutamate receptors (mGluRs) in particular has been well documented in regulating both synaptic architecture and function. In mammals, these receptors are divided into 3 classes of 8 subtypes (Pin and Duvoisin, 1995), with marked distribution and functional differences. Group I mGluR5 functions upstream of FMRP, and FMRP functions in mGluR-induced forms of synaptic plasticity (Hou et al., 2006; Nosyreva and Huber, 2006; Wilson and Cox, 2007; Zhang et al., 2009). The mGluR (or Gq) theory of Fragile X proposes that synaptic Gq signaling through such receptors regulates FMRP to control the translation of specific mRNAs modulating synapse structure and function (Bear et al., 2004; Volk et al., 2007). However, the application of this theory to neural circuit development is not clear. In support of such a developmental model, mGluR5 is important for synaptic development, although this role may be region-specific (Hannan et al., 2001). For example, recent studies show that formation of the mouse somatosensory barrel cortex is dependent on mGluR5, with knockout mice exhibiting a nearly complete loss of barrel segregation in developing layer 4 neurons (Wijetunge et al., 2008). Mutant neurons exhibit a reduced density of dendritic spines. In contrast, no difference was seen in dendritic spines from mGlur5+/– heterozygote layer 3 pyramidal neurons (Dolen et al., 2007). Consistently, expression pattern analysis and pharmacological disruption of mGluR signaling demonstrates these receptors function differentially during development and at maturity to regulate differentiation of dendritic spines as well as mediate synaptic plasticity (Chen and Roper, 2004; Doherty et al., 2004; Mares, 2009; Mateo and Porter, 2007; Reid and Romano, 2001; Reid et al., 1997; Vanderklish and Edelman, 2002; Wang et al., 2007). Since FMRP functions downstream of mGluR signaling, and is itself developmentally regulated during a transient window, FMRP may be the molecule providing developmental specificity to this pathway.

The *Drosophila* genome encodes only a single functional mGluR, DmGluRA, allowing a single gene knockout of all mGluR signaling that is unencumbered by potential multi-gene interactions (Bogdanik et al., 2004). Since DmGluRA is the only *Drosophila* mGluR, it must mediate all conserved functions of the mammalian sub-types. Genetic ablation of DmGluRA clearly demonstrates the importance of mGluR signaling in regulating synaptic structure and function in *Drosophila* (Bogdanik et al., 2004). Null mutants exhibit reduced synaptic bouton number and a concomitant increase in bouton size at the larval NMJ. In addition, activity-dependent synaptic facilitation is dramatically augmented in animals lacking DmGluRA providing further evidence that this receptor functions at the interface of synaptic structure and function. Based on mammalian studies, it is reasonable to hypothesize that DmGluRA may interact with dFMRP. Indeed, the expression of DmGluRA and dFMRP is inversely correlated in the *Drosophila* nervous system (Pan et al., 2008), as observed in null mutants of each gene respectively. Moreover, co-removal of dFMRP and DmGluRA is able to restore normal coordinated behavior, which is impaired in *DmGluRA* single null mutants. Similarly, co-removal of DmGluRA and dFMRP rescues the increased synaptic arborization and branch number characterizing the *dfmr1* single null NMJ. This genetic rescue is paralleled using a pharmacological mGluR antagonist (MPEP), which restores the *dfmr1* null synaptic architecture towards normal, both at the larval NMJ and the adult brain MB learning/memory center (Pan et al., 2008). The challenge now is to extend these central brain studies to determine the relationship between mGluR signaling and the role of dFMRP in establishing and modulating synaptic function.

Fortunately, new techniques are emerging that make functional assays in defined *Drosophila* central brain neurons more accessible. For example, in recent years a primary neuronal culture system has been created by deriving *in vitro* isolated neurons from pupal brains (**Figure 3**) (Gu and O'Dowd, 2007; Phillips et al., 2008; Su and O'Dowd, 2003). These neurons send out processes that form synaptic connections over the course of a couple days, and are capable of both spontaneous and evoked neurotransmitter release, which can be monitored *in vitro* (Gu et al., 2009). Using the UAS-GAL4 system, primary cultures are derived from animals expressing visual markers (e.g. UAS-GFP) in a defined subset of neurons in order to interrogate functional properties. For example, the lypophilic FM dyes are commonly applied to visually monitor the synaptic vesicle cycle (**Figure 3B**) (Betz and Bewick, 1992). Depolarizing stimulation causes the dye to be loaded into vesicles and then unloaded upon subsequent depolarization, allowing readouts of both endocytosis and exocytosis. This technique can also be coupled to the process of photoconversion, such that the fluorescent dye signal is converted into an electron dense product visible via high-resolution electron microscopy (**Figure 3C**). Thus, by photoconverting dye-loaded synapses, quantitative measurements can be made of the capacity of defined synapses to respond to stimulation. Similarly, the genetically encoded synaptopHluorin reporter can be expressed in a neuron type specific manner to also monitor the synaptic vesicle cycle (Poskanzer and Davis, 2004; Poskanzer et al., 2003). SynaptopHluorin is a fusion protein of a pH-sensitive GFP (pHluorin) and the integral synaptic vesicle protein synaptobrevin, such that the phluorin moiety is situated in

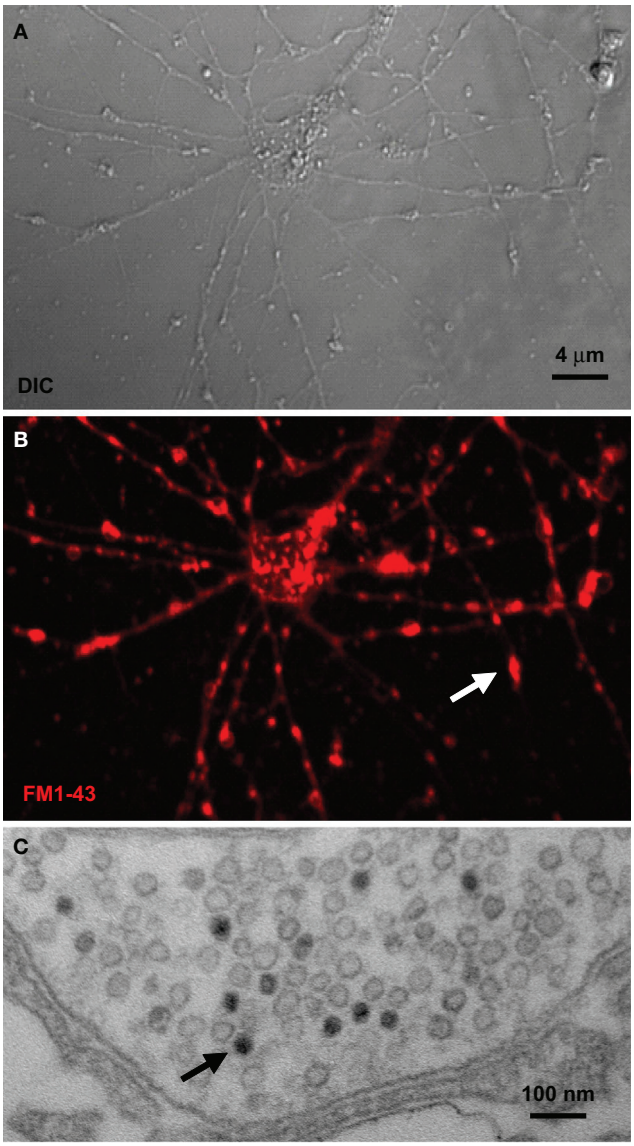

FIGURE 3 | Synaptic vesicle cycle imaging. (A) Nomarski (DIC) image of *Drosophila* primary brain neuron culture at 8 days *in vitro*. **(B)** The lipophilic dye FM1-43 loaded into vesicles by depolarization. Arrow points to a single synaptic bouton. **(C)** The fluorescent FM1-43 signal can be photoconverted to an electron-dense signal in the presence of diaminobenzene (DAB) with high intensity 490 nm light. Arrow points to a single labeled synaptic vesicle.

the acidic lumen of the vesicle (Burrone et al., 2006; Miesenbock et al., 1998). Upon stimulation, vesicles fuse with the presynaptic membrane and synaptopHluorin is exposed to the relatively basic extracellular space, causing an increase in fluorescence intensity. Likewise, when the reporter is endocytosed back into a vesicle, the acidic vesicular lumen quenches the synaptopHluorin fluorescence. Monitoring these fluorescence changes in *dfmr1* and *DmGluRA* single and double mutants will extend the functional analysis of this critical pathway.

Of course, it is best to characterize synaptic function in the context of native circuits within the brain. To that end, a *Drosophila* non-dissociated whole brain explant system has been developed that provides access to relatively undisturbed central circuits for extended periods (~1 week) (Ayaz et al., 2008; Wang et al., 2003). In principle, the explant brain can be subjected to repeated imaging/recording from the same neuron or circuit over time. As with primary neuronal cultures, these whole brain explants reportedly maintain synaptic properties and are amenable to both imaging and electrophysiological studies. Thus, it should be possible to monitor activity-dependent processes of development in this form of *ex vivo* system. As with cultured neurons, synaptic function can be assayed with FM dye labeling using lipophilic dye incorporation into cycling synaptic vesicles and this marker photoconverted to allow ultrastructural analysis at a given time point (**Figure 2C**). Again the UAS-GAL4 system, or the more powerful MARCM technique described above, can be employed to study single neurons or described circuits. Importantly, both explants and minimally dissected intact brains can be imaged with GFP reporters to visualize circuit neurons, in order to direct cellular electrophysiological recordings (Gu and O'Dowd, 2007; Wilson and Laurent, 2005; Wilson et al., 2004). Exploiting these tools to study temporal regulation of adult circuit development in both *dfmr1* null and *DmGluRA* null animals will be critical components to determine the contribution of these genes to the functional wiring of the brain.

In mammals, one component of mGluR-dependent FMRP function is to regulate the cycling of AMPA glutamate receptors to/from postsynaptic membranes (Moga et al., 2006; Muddashetty et al., 2007; Nakamoto et al., 2007; Waung et al., 2008). There have been many proposed mechanisms by which FMRP may regulate receptor endocytosis and trafficking (Volk et al., 2007). A recent model proposes translational regulation of the FMRP target mRNA encoding Activity Regulated Gene of 3.1 kb (Arg3.1; also called Arc) (Park et al., 2008). Arc expression is increased in synapses in response to synaptic activity and specifically in response to mGluR activation (Chowdhury et al., 2006; Moga et al., 2004; Park et al., 2008; Wang et al., 2009b). In *Drosophila*, two AMPA-R subtypes, GluRIIA and GluRIIB, are differentially regulated by dFMRP (**Figure 4**) (Pan and Broadie, 2007). The GluRIIB class of receptors is downregulated in the absence of dFMRP, whereas the GluRIIA class of receptors is upregulated. Conversely, *DmGluRA* single mutants exhibit a moderate elevation of both receptor classes, and the *DmGluRA; dfmr1* double null mutant shows an additive effect: higher synaptic abundance of GluRIIA receptors and lower levels of GluRIIB receptors than in the *dfmr1* null alone (Pan and Broadie, 2007). These two classes of AMPA-R differ markedly in their functional properties; for example, GluRIIA conducts larger current compared to GluRIIB (**Figure 4**) (DiAntonio et al., 1999; Sigrist et al., 2002). Thus, this mechanism of glutamate receptor subclass regulation may illustrate a means to directly modulate postsynaptic transmission or, alternatively, to functionally compensate for changes in presynaptic efficacy. Interestingly, FMRP in mammals has been shown to stabilize the mRNA of Postsynaptic Density Protein of 95kDa (PSD-95), which modulates postsynaptic density formation and glutamate receptor localization/function (Zalfa et al., 2007). Similarly in *Drosophila*, the PSD-95 homolog Discs Large (DLG) strongly regulates synaptic development and modulates the expression of GluRIIB class of receptors, but not the GluRIIA class (Chen and Featherstone, 2005). Thus, an attractive model is forming whereby downstream of DmGluRA synaptic signaling,

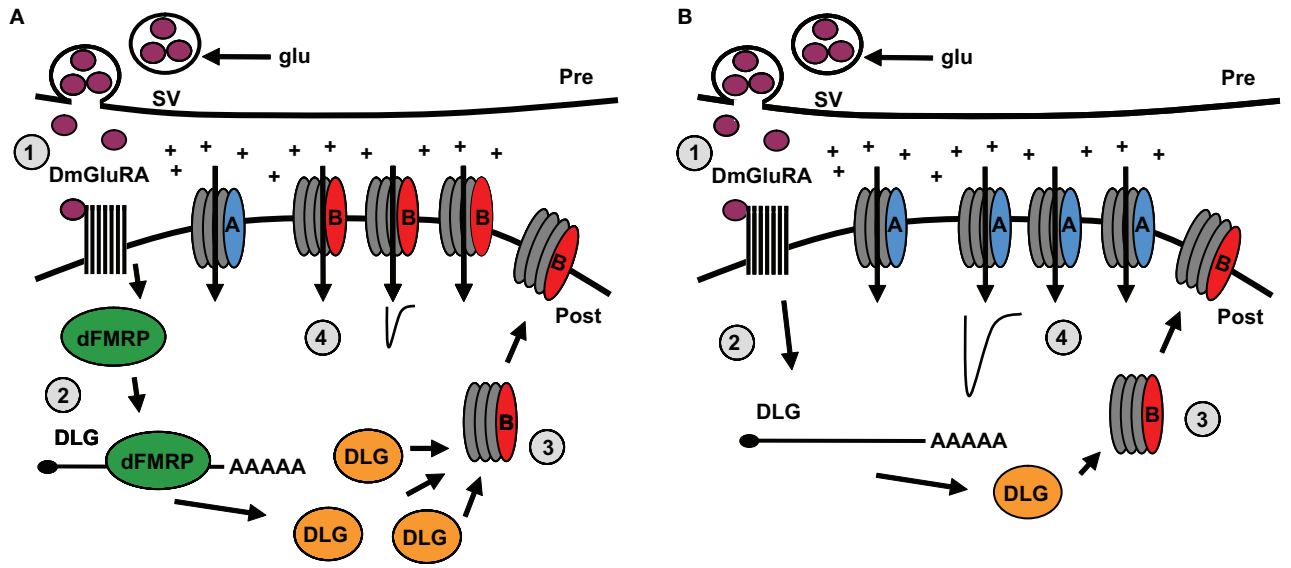

FIGURE 4 | Model of DmGluRA signaling and dFMRP function regulating postsynaptic changes. (A) In step 1, presynaptic glutamate (glu) release from synaptic vesicles (SV) stimulates the DmGluRA receptor to regulate dFMRP. In step 2, dFMRP regulates the stability of DLG/PSD-95 mRNA resulting in an increase in protein levels. In step 3, DLG facilitates the insertion of GluRIIB class of *Drosophila* AMPA-like receptors in postsynaptic membrane. In step 4, GluRIIB receptors support reduced excitatory junctional currents. **(B)** In the absence of dFMRP, DLG mRNA exhibits reduced stability and DLG protein levels are reduced. As a consequence, GluRIIA receptors dominate the synaptic membrane, causing increased excitatory junctional currents.

dFMRP regulates DLG mRNA stability and thereby promotes the specific insertion of GluRIIB receptors into postsynaptic membranes, at the expense of GluRIIA receptors, and thereby modulates synaptic transmission on the basis of GluRIIB functional properties (**Figure 4**).

This regulation of glutamate receptor subtypes may relate to recently reported changes in the functional synaptic transmission properties of *dfmr1* and *DmGluRA* null mutants (Repicky and Broadie, 2009). Neurotransmission defects in both mutants are most apparent following prolonged periods of moderate to high-frequency stimulation (e.g. 1 min, 10 Hz). During such a stimulus train, *DmGluRA* null animals show dramatic augmentation of the synaptic response, which is delayed in double mutant combination with the *dfmr1* null. Similarly, the strikingly aberrant premature long term facilitation (LTF) present in *DmGluRA* null animals is again delayed in the double null mutants (Repicky and Broadie, 2009). Following the high-frequency train, *DmGluRA* nulls manifest grossly elevated post-tetanic potentiation (PTP), a defect prevented by co-removal of dFMRP. These data suggest that DmGluRA functions in a negative feedback loop in which excess glutamate released during high-frequency transmission binds the DmGluRA receptor to dampen synaptic excitability, and dFMRP functions to suppress the translation of proteins regulating this synaptic excitability. Removal of the translational regulator partially compensates for loss of the receptor and, similarly, loss of the receptor weakly compensates for loss of the translational regulator (Repicky and Broadie, 2009). The precise mechanism of this compensation may involve either pre- or postsynaptic functions, or potentially both.

The above studies clearly indicate a mechanistic requirement for both DmGluRA signaling and dFMRP function in modulating synaptic excitability and neurotransmission strength, but it is still unknown how these properties relate to circuit development. To begin to identify the intersecting pathways with activity, numerous characterized genetic ion channel variants can be expressed in *Drosophila* neurons in a UAS-GAL4 targeted fashion to alter neuronal firing rates. For example, mutants in the *Drosophila* Shaker potassium channel have been made to perpetually activate this channel and effectively shunt electrical activity in the neurons expressing it (White et al., 2001). Conversely, a dominant negative (DN) form of Shaker can be expressed to specifically inactivate these channels and thus produce hyperexcitable neurons (Mosca et al., 2005). More specific blocks to neurotransmission may also be used, for example, by expressing the tetanus toxin light chain described above to block synaptic vesicle fusion, or by using DN temperature-sensitive mutants of the *shibire* gene encoding dynamin, which inactivates the synaptic vesicle cycle by inhibiting endocytosis (Chen et al., 1991; Koenig and Ikeda, 1989; van der Bliek and Meyerowitz, 1991). Combining these techniques in *dfmr1* and *DmGluRA* single and double mutants will be a powerful means to dissect the requirements of neuronal and synaptic excitability in order to test functional interactions in neural circuit developmental refinement.

In addition to electrophysiological approaches, recent advances in *Drosophila* calcium imaging permit monitoring cellular responses to neuronal activation. Several GFP reporters have been generated which alter their fluorescence properties in the presence of calcium, thus allowing the visual monitoring of calcium influx and buffering dynamics (Reiff et al., 2005). Reporters including the camgaroos and gCAMPs are GFP fusion proteins coupled to calmodulin Ca^{2+} binding domains (Pologruto et al., 2004; Yu et al., 2003). Upon binding with calcium, the GFP reporter undergoes a conformational change to enhance fluorescence intensity (**Figure 5**). In

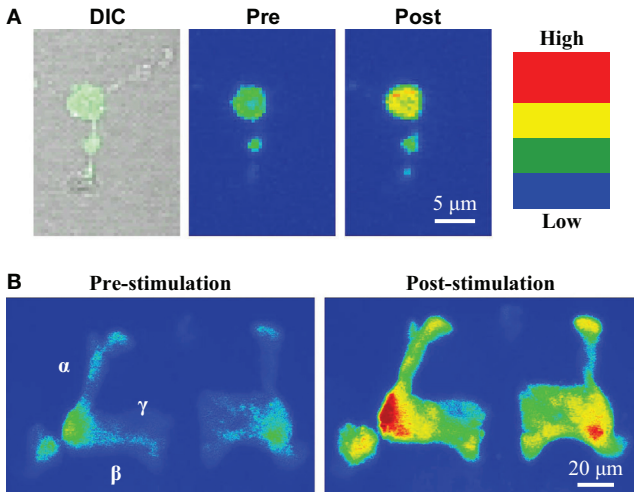

FIGURE 5 | Use of transgenic calcium reporter gCAMP to visualize synaptic activity. (A) OK107-GAL4 driving UAS-gCAMP in primary neuron culture before and after depolarization. **(B)** OK107-GAL4 driving UAS-gCAMP in whole brain Mushroom Body axonal lobes (α, β, γ). Depolarization causes fluorescence increases throughout the Mushroom Body.

Drosophila, these reporters can be used either in primary neuronal cultures of identified neuronal subtypes (**Figure 5A**) or in the intact brain (**Figure 5B**). In addition, the commonly utilized cameleon reporters are a fusion of the calmodulin binding domain situated between CFP and YFP (Miyawaki et al., 1997). The cameleon reporter relies on a FRET based mechanism to generate fluorescence changes in response to calcium binding. With this reporter, Ca^{2+} binding is monitored by a concomitant decrease in CFP emission and an increase in YFP emission (Fiala and Spall, 2003). Similarly, reporters based on the calcium-binding troponin C protein have been optimized by mutageneisis (TN-XXL) to produce high affinity calcium binding and reliable responses to stimulation (Mank et al., 2008). TN-XXL is sensitive over a range of stimulations and functions in both flies and mice as a stable monitor of calcium dynamics. Each transgenic reporter has distinct advantages and disadvantages, most notably in the respective signal to noise ratios, but they are continually being modified to provide the best physiological responses to neuronal activation.

BEHAVIORAL OUTPUTS OF ACTIVITY REGULATED CIRCUITS

The activity-dependent refinement of neural circuits results in a final wiring optimized for behavioral outputs. Of course, circuits remain plastic in mature animals, permitting constant modulation to adapt to changing conditions, to learn from environmental stimuli and to remember lessons learned. It may be that activity-dependent refinement during development is required to lay a foundation for maintained synaptic plasticity. *Drosophila* is well suited for the analysis of circuits driving quantifiable output behaviors. For example, MB-dependent olfactory associative learning and progressive memory consolidation has been a particularly important focus in this system (Akalal et al., 2006; Connolly et al., 1996; de Belle and Heisenberg, 1994; Dubnau et al., 2001; Heisenberg et al., 1985; Tully and Quinn, 1985). *Drosophila* possess at least four distinct phases of memory (Isabel et al., 2004); short term memory (STM) and middle term memory, which persist each on the order of minutes, and anesthesia–resistant memory (ARM) and long term memory (LTM), which persist on the order of days to, theoretically, the end of the animal's lifespan. Critically, only the latter two memory phases are dependent on *de novo* translation, with LTM alone being absolutely dependent on protein synthesis, both for its formation and maintenance (Isabel et al., 2004; Tully et al., 1994; Yu et al., 2006). Experimentally, memory has been best characterized using an olfactory associative model whereby an animal is exposed to an odor/electrical shock pairing, learning to avoid this association in favor of an odor which has not been coupled to an electric shock (Tully and Quinn, 1985). While short and middle term memories are generated by multiple training sessions performed back to back with no breaks in between, this "mass training" protocol does not induce LTM (Yin et al., 1994). LTM is only induced by spacing training sessions (e.g. ~15 min apart) and is blocked in the presence of protein synthesis inhibitors. Thus, there is an obvious potential link between protein synthesis-dependent LTM and the translational repressor FMRP whose loss is hallmarked by cognitive and memory impairments in FXS patients. FMRP may be required in an activity-dependent manner to establish the circuit map necessary for both learning and memory formation.

In *Drosophila*, null *dfmr1* mutants have a relatively mild defect in learning formation and more profound defect in memory consolidation in the above associative olfactory paradigm (Bolduc et al., 2008). Importantly, the strong LTM defect only manifested after spaced training. dFMRP expression was broadly upregulated in the brain specifically in response to spaced training, but was unaffected by mass training protocols (Bolduc et al., 2008). Surprisingly, this upregulation appeared widespread and not restricted to any specific brain region or defined circuitry (e.g. Mushroom Body). Acute over-expression of dFMRP prior to training also blocked LTM, suggesting that dFMRP acts in acute memory consolidation; elevating dFMRP after training had no effect. Blocking protein synthesis in *dfmr1* null animals rescued these memory deficits, suggesting that enhanced protein synthesis is sufficient to explain the failure to consolidate memories (Bolduc et al., 2008). In parallel with the dFMRP requirement, mutants of the *staufen* gene, encoding a protein involved in mRNA translocation, share a similar defect in LTM consolidation. The two proteins appear to interact in protein synthesis-dependent memory formation. Heterozygous mutation of each gene alone causes no memory defects, but double heterozygotes (*staufen*/+; *dfmr1*/+) show dramatic loss of LTM consolidation after spaced training, with no effect on mass training memory (Bolduc et al., 2008). As predicted by the Gq hypothesis, DmGluRA antagonists significantly rescued memory consolidation defects.

dFMRP is also involved in *Drosophila* courtship, a learning and memory behavior that requires the integration of multiple sensory modalities (Joiner and Griffith, 2000; McBride et al., 2005). Null *dfmr1* males fail to effectively court females and have defective memory associated with this social interaction. These defects were rescued by feeding animals mGluR antagonists, which provides further support for this synaptic signaling mechanism in regulating dFMRP function (McBride et al., 2005). Interestingly, mGluR antagonists provide the most effective rescue of *dfmr1* defects when supplied throughout development, as opposed to acutely in adult animals, which caused an adverse affect on memory formation

in wildtype animals. Mechanistically, it is of note that in oocytes dFMRP binds to and negatively regulates the *Drosophila* Orb protein, a homolog of mammalian cytoplasmic polyadenylation element-binding protein (CPEB) (Costa et al., 2005). In the brain, the CPEB RNA-binding protein is proposed to "mark" synapses locally in response to signaling via mGluRs and the *Drosophila* neural specific Orb2 is essential for *Drosophila* courtship conditioning induced LTM (Keleman et al., 2007; Si et al., 2003). Thus, it will be critical to ascertain whether dFMRP interacts with Orb2 to regulate general, or paradigm specific LTM. Unfortunately, the identity of the neural circuits involved in this complex social behavioral repertoire are unknown, precluding close developmental analysis of circuit structure and function at this time.

In contrast, the circuitry of another dFMRP-dependent complex behavior, circadian rhythm cycling, is particularly well characterized, including the large and small lateral clock neurons in the central brain (Helfrich-Forster, 2003). Large lateral neurons exhibit overextension and apparent defasiculation in *dfmr1* null animals, phenotypes that are proportional to the dosage of gene removal (Morales et al., 2002). The mechanism of these structural changes is dependent on the dFMRP mRNA target, actin-binding chickadee/profilin (Reeve et al., 2005). Reducing chickadee/profilin protein levels in *dfmr1* null animals can rescue over-elaboration phenotypes. Consistent with clock circuit dysfunction, null *dfmr1* animals exhibit profoundly disrupted circadian activity cycles and prolonged bouts of sleep (Bushey et al., 2009; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002). So far, these defects have not been attributed to either aberrant mGluR activation or direct defects in clock control though the abundance of the clock protein period (Per) is elevated throughout the circadian cycle in *dfmr1* null animals. Thus, it will be necessary to further investigate the developmental defects within this circuitry to understand the role of dFMRP in these behavioral manifestations. Lastly, there are numerous other well-characterized *Drosophila* behaviors, such as visual discrimination, aggression and pain avoidance which all require the integration of multiple sensory and higher order neural circuits (Chen et al., 2002; Duistermars and Frye, 2008; Guo and Gotz, 1997; Manev and Dimitrijevic, 2004; Schuster et al., 2002; Tracey et al., 2003; Xu et al., 2006; Yurkovic et al., 2006). Using the tools discussed in this review, and elsewhere in this issue, it will soon be possible to dissect these circuits to understand the role of activity and dFMRP in laying the foundation for these diverse behavioral repertoires.

CONCLUDING THOUGHTS

It still remains unclear the extent to which dFMRP functions in mediating activity-dependent developmental refinement versus maintained plasticity to drive the proper manifestation of complex behavioral outputs. Currently, the most disease-relevant behavioral data in *Drosophila* is the defective MB circuit associative learning and memory consolidation, and this will certainly remain an important brain region for understanding the circuit requirements of dFMRP. But what about other circuits? Does dFMRP regulate neuronal activity the same way in all circuits? Is metabotropic receptor signaling upstream of dFMRP function in every circuit? Does dFMRP play a similar late-stage role in activity-dependent refinement in all circuits? If there are differences, then can those differences be attributed to specific molecular functions of dFMRP? Special attention will need to be paid to the developmental control mediated by dFMRP, and particularly the synaptic pruning function. The key molecular components of this mechanism still need to be determined. Is pruning broadly used to establish maps in all circuits, or is it a specialized program for select brain regions or neuronal subtypes? As advances in *Drosophila* transgenic tools accelerate our ability to investigate these processes, these vital tools will continue to aid in our understanding of the molecular mechanisms of disorders such as FXS, hastening our ability to engineer effective intervention strategies.

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Genetic control of active neural circuits

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The use of molecular tools to study the neurobiology of complex behaviors has been hampered by an inability to target the desired changes to relevant groups of neurons. Specific memories and specific sensory representations are sparsely encoded by a small fraction of neurons embedded in a sea of morphologically and functionally similar cells. In this review we discuss genetics techniques that are being developed to address this difficulty. In several studies the use of promoter elements that are responsive to neural activity have been used to drive long-lasting genetic alterations into neural ensembles that are activated by natural environmental stimuli. This approach has been used to examine neural activity patterns during learning and retrieval of a memory, to examine the regulation of receptor trafficking following learning and to functionally manipulate a specific memory trace. We suggest that these techniques will provide a general approach to experimentally investigate the link between patterns of environmentally activated neural firing and cognitive processes such as perception and memory.

Keywords: neural circuits, genetics, memory, fear conditioning, amygdala, tetracycline, fos, creb

INTRODUCTION

How does the brain represent the surrounding world in discrete percepts? How does the pattern of retinal activity produced by looking at the image of Halle Berry ultimately lead to the perception of the actress rather than a violin? How do the myriad different associations with Halle Berry (actress, specific roles) or violin (inanimate object, music) form through learning and memory? These are basic questions at the heart of many areas of neuroscience. They have been approached by a variety of techniques that fall into two broad categories, watching brain activity and disrupting brain function. In this review we will discuss the development of genetic techniques that bridge the divide between these two approaches and allow the targeting of molecular changes specifically to anatomically dispersed neural representations that are activated by discrete environmental stimuli. These new tools allow the establishment of causal relationships between the activation of sparsely distributed neural ensembles and changes at the behavioral level.

The use of single unit recordings in awake behaving animals provides an exquisitely precise measure of the temporal activity of neurons. This has been used to extract information about how the brain encodes information by studying the correlation between neuronal activity and the presentation of specific sensory stimuli. The best-studied example is probably in the primate visual system where a hierarchical pathway has been defined (Van Essen et al., 1992). Neurons in the primary visual cortex (V1) fire in response to very general visual features such as orientation whereas following processing through the ventral visual pathway, neurons in inferior temporal cortex respond to complex object features. This visual information is then relayed to the medial temporal lobes, which integrate multimodal sensory information and play a critical role in memory.

Single unit recording studies in the medial temporal lobe in humans have detected neurons with responses to highly defined categories. In the limit, units were found that responded to the presentation of a single individual in a variety of contexts (Quiroga et al., 2005). One neuron in the right anterior hippocampus responded to the actress Halle Berry, presented in a photograph, as a masked character (catwoman), as a drawing, or as the letter string "Halle Berry". This level of response specificity shows that the cells are not tuned to general visual features common to images of Halle Berry but to the concept of the specific actress. The striking degree of responsive specificity of these units strongly suggests that they participate in the neural representation of specific individuals. However, these studies are still correlative in the sense that they allow us to watch neurons that fire in a manner suggesting a role in encoding specific information, but they do not allow us to disrupt these neurons specifically to test this hypothesis.

Historically much of what we know about the functional parceling of the brain has been obtained from lesion studies in experimental animals and in patients with damage to specific brain regions (Squire, 2004). In learning and memory, studies of patient HM, who underwent a bilateral resection of the medial temporal lobe, have helped define this area, and in particular the hippocampal formation, as critical in the formation of long-lasting declarative memories. At a more molecular level both pharmacological and genetic manipulations have been used to test ideas about the cellular signaling mechanisms that underlie behavioral plasticity. However, each of these approaches is limited in that they act as sledgehammers, altering every neuron in a given brain region, when the electrophysiological studies suggest that it is really a very sparse group of neurons that is truly of interest in any given experimental context.

CIRCUITS AND REPRESENTATIONS

One approach to circuit analysis is to describe the precise pattern of wiring within specific processing units like the hippocampus or a cortical column. For example, in the hippocampus there is the classic tri-synaptic circuit where information from the entorhinal

cortex enters through the dentate gyrus, is relayed to CA3 neurons via the mossy fiber pathway and then to CA1 neurons via the Schaffer collateral pathway and finally back out to the entorhinal cortex (Squire, 2004). This connectivity diagram can be obtained to finer and finer levels of resolution and in principle an entire wiring diagram of a single brain at a single time point could be produced to the level of individual synaptic connections, similar to that obtained in C. elegans (White et al., 1986). However, even if this were precisely defined down to the level of single synapses it is not likely that the mechanisms that give rise to a neural representation or memory trace would become apparent. As the example of the Halle Berry neuron indicates, these representations are likely to be quite sparse and embedded within a matrix of apparently identical neurons. The particular response patterns of an individual neuron are likely determined by the strength of specific synaptic connections that have been altered through experience. Moreover, even if one could explain how these specific firing patterns arise through circuit plasticity, it would be difficult to experimentally establish the contribution of a particular ensemble of neural activity to an actual representation of the environment.

An alternate view is that what defines a circuit is the environmental contingencies that lead to its activation (**Figure 1**). In the mammalian brain this is generally referred to as an ensemble code or neural representation of the particular environmental stimulus. In the case of simple systems or reflex pathways, the wiring diagram often predicts the location of the neural ensembles that encode specific environmental information. For example, in the Aplysia gill withdrawal reflex a group of sensory neurons are activated by tactile stimulation and synapse directly onto motor neurons to control withdrawal behavior (Kandel, 2001). Here the primary sensory neurons are defined by their enervation of the gill and their activation by tactile stimulation of the gill. There is no further higher order processing prior to motor output. The behavioral plasticity of the withdrawal reflex is controlled by synaptic plasticity within these sensory neurons. Because of the uniformity and anatomical isolation of this group of cells, it has been possible to apply techniques for both watching and manipulating neurons within the context of a defined circuit (representation) in a behaving animal. The application of these convergent approaches has proven quite powerful in defining the cellular and molecular mechanisms that underlie behavioral plasticity in this system. The goal of this review is to discuss recent attempts to develop approaches that allow similar convergent molecular and physiological access to the more dispersed neural representations of the mammalian brain.

LINKING NEURAL ACTIVITY TO MOLECULAR CHANGE

One technique that has been used for many years to watch brain activity has taken advantage of a class of immediate early genes or IEGs that are expressed in response to high-level neural firing (Sagar et al., 1988). The three most commonly used IEGs for this purpose are cfos, arc, and zif268. The expression of these genes is induced by action potential firing, and the ½-life of the gene products are relatively short. Thus the expression pattern of IEGs in brain sections from an animal provides a record of the neural activity from several hours prior to sacrifice and has been used extensively to map brain activation from a wide variety of environmental stimulation and in learning and memory relevant paradigms (Guzowski et al., 2005).

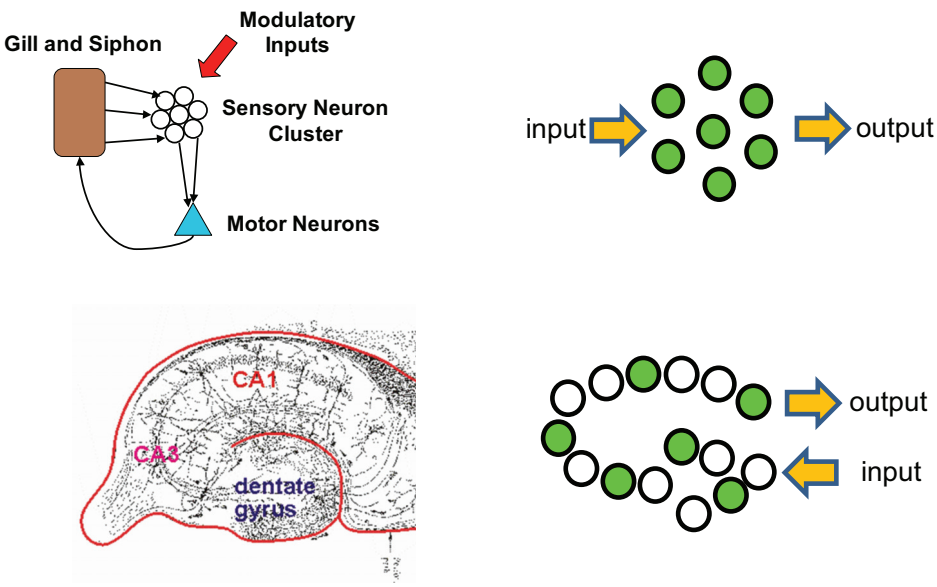

FIGURE 1 | Clustered and distributed neural ensembles. Two examples of neural circuits are shown. The top panel shows a simplified version of the gill and siphon withdrawal circuit in Aplysia. The sensory neuron cell bodies are located adjacent to each other in a cluster and possess similar biochemistry and response properties. The bottom panel represents the hippocampal circuit of the mammalian brain. The green circles represent neurons that are activated by a specific pattern of sensory stimulation and that when activated contribute to a specific behavioral response. The hippocampal circuit, like many other circuits in the brain, responds to sensory stimulation with activation patterns that can not be predicted from their wiring diagram. Each of these neural ensembles involves a sparse subset of neurons that have an unpredictable spatial distribution. The Aplysia neurons are primary sensory neurons and their response properties can be predicted by their physical location in the ganglion.

One of the limitations of this approach is that it provides only a single time point record of activity patterns, making it difficult to determine how plasticity modulates activity or even how stable this pattern of gene expression is in relation to an identical stimulus. This problem was addressed using the expression of the IEG arc (Guzowski et al., 1999). By using fluorescent *in situ* hybridization to examine expression of arc mRNA they were able to detect the pattern of arc expression at two separate time points in the same animal. They took advantage of the fact that they could detect the expression of the arc precursor RNA while it was still in the nucleus as well as the mature mRNA which was present in the cytoplasm and dendrites. The nuclear arc signal represented very recent and ongoing expression reflecting neural activity several minutes prior to sacrifice of the animal, while the cytoplasmic signal reflected activity that had occurred 30 min or more prior. They used this approach to examine the consistency of activation of the hippocampus when an animal was repeatedly exposed to the same environment. They found that when animals were allowed to explore the same environment, they re-expressed arc in many of the same neurons that had also expressed it on the first exposure. This is a critical result in that it demonstrated for the first time that IEG expression could be used to consistently reflect patterns of activity associated with a discrete representation and provided results that were qualitatively and quantitatively similar to results obtained with electrophysiological recordings of the hippocampus.

The temporal information regarding neural activity that can be obtained using IEG expression is clearly limited relative to electrophysiological recordings. For example, while it is clear that high-level firing induces expression, it is not clear what the precise threshold is and how this might vary among different neuronal cell types. One advantage is that large brain regions can be surveyed and precise anatomical information can be obtained. A second advantage is that the promoter regulatory elements that confer neural activity dependence can, in theory, be used to drive expression of any linked heterologous transgene. This was first demonstrated in transgenic mice by (Smeyne et al., 1992) using the *cfos* promoter to drive activity dependent expression of E. coli β-galactosidase. More recently, an axonally targeted β-galactosidase was expressed from the *cfos* promoter, providing the potential to trace the projections of specific active neuronal populations (Wilson et al., 2002). The use of these promoter elements is general and provides the potential to introduce functional effector molecules directly into activated neural ensembles to allow their molecular manipulation.

The use of IEG promoters as tools for both watching and potentially manipulating functional neural circuits is limited in a number of ways. For example, the direct introduction of toxins or other molecular regulators via the cfos promoter could be complicated by developmental effects of their expression. In addition, it would be useful for many studies to allow the molecular change introduced into the activated neurons to be maintained for more prolonged periods than the short (minutes to hours) times afforded by the promoters themselves. We therefore set out in a recent study to develop a genetic system with the following features. (1) The expression of any transgene of interest should be linked to neural activity only during a specific experimenter controlled time window. (2) The transgene expressed in those active neurons should be maintained for a prolonged period, but no further labeling of active cells should occur following closure of the permissive time window.

We achieved activity dependent regulation of transgene expression with these two features by combining elements of the tetracycline system for gene regulation with the cfos promoter as shown in **Figure 2** (Reijmers et al., 2007). The approach uses two separate transgenes in the same animal. The first uses the cfos promoter to drive expression of the tetracycline transactivator (tTA or TET-off). In mice carrying only this transgene high-level neural activity will result in the induction tTA, which is a transcription factor that can be blocked by the antibiotic Doxycycline (Dox). In the absence of Dox tTA drives expression of genes linked to a tetO-promoter sequence. The second transgene incorporates both a tetO-linked reporter (in this case the somato-axonal marker taulacZ) as well as a transcriptional feedback loop to maintain tetO-linked gene expression indefinitely once it is activated. The tTA (tTA*) in this construct was made Dox insensitive by introduction of a point mutation in the Tet binding domain. In the presence of Dox the tetO-linked reporter is not activated even in those neurons in which the cfos-linked tTA is expressed. However, if Dox is withdrawn then both taulacZ and tTA* are expressed, but only in those neurons that were active to a high enough level to induce the cfos-linked tTA. Once activated, the tTA* sets up a transcriptional feedback loop that can be maintained even in the presence of added Dox. In this manner discrete time windows for genetic tagging of active neurons can be opened and closed through the use of Dox. In the absence of Dox, any neuron that has sufficient induction of cfos-linked tTA to activate the feedback loop will persistently activate the taulacZ reporter, as well as any other tetO-linked transgene that is introduced into the mouse (Aiba and Nakao, 2007). This expression will be maintained even when the time window for sampling active neurons is closed by the readministration of Dox. In this way a persistent record of neurons that were active during the off-Dox period can be maintained. We called this the TetTag mouse, which stands for TETracycline transactivator controlled genetic TAGging of active neural circuits.

We used the TetTag mouse to examine the neural circuit that mediates fear memory (Reijmers et al., 2007). We asked whether neurons that are stimulated during learning in a Pavlovian fear-conditioning paradigm were reactivated during retrieval of the memory as shown in **Figure 2**. We subjected TetTag mice to a learning trial consisting of paired presentations of a tone (CS) and a foot-shock (US). This results in a long-lasting fear memory for both the tone (cued conditioning) and the conditioning box in which the animals were shocked (context conditioning). The learning trials took place during a time window in which the animals were free from Dox, allowing activated neurons to be tagged with long-lasting expression of taulacZ. The animals were then returned to Dox to prevent further tagging of activate neurons, tested for retention of the memory in a retrieval trial, and sacrificed after 1 h for analysis using the endogenous IEG zif268 as a measure of recent neural activity. By comparing the expression of lacZ (activity during learning) and zif268 (activity during memory retrieval) we could determine the degree of circuit reactivation. We found that the

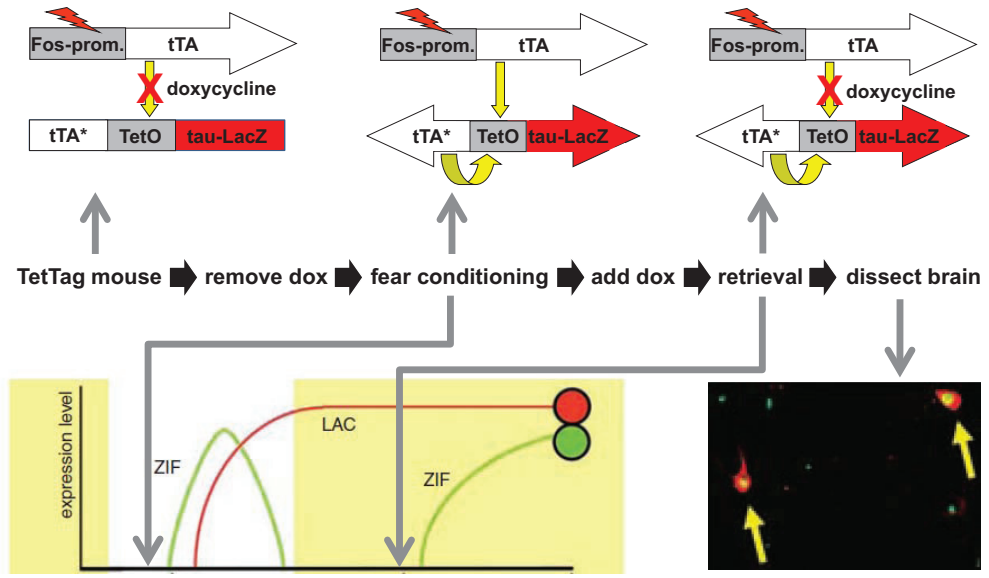

FIGURE 2 | The TetTag mouse. The figure summarizes an experiment from Reijmers et al. (2007). Mice carrying two transgenes were used. The first transgene uses the cfos promoter to drive expression of the tetracycline transactivator (tTA). tTA activates the tetO promoter in the absence but not presence of doxycycline (Dox). The second transgene uses the tetO promoter to drive expression of a Dox insensitive tTA (tTA*), which, once expressed, sets up a positive feedback loop that continuously drives expression of a β-galactosidase reporter coupled to the tau protein (taulacZ). Neurons activated during fear conditioning (while off Dox) were tagged with long-lasting expression of taulacZ (LAC; red circle). Mice were put back on food with doxycycline and a retrieval test was done 3 days later, followed by analysis of the brains 1 h after retrieval for expression of lacZ and zif268. Neurons activated during learning expressed lacZ and those active during retrieval expressed zif268 (ZIF; green circle). The number of neurons in the amygdala that expressed both LAC and ZIF, indicating that they were activate during both learning and retrieval, was positively correlated with the strength of the fear memory that the animal displayed.

number of reactivated neurons in the amygdala, a region critical in fear conditioning (LeDoux, 2007), correlated with the retrieval of the fear memory.

We hypothesized that these reactivated neurons represent a component of the memory trace for conditioned fear. To test this idea we weakened the strength of the memory by extinction training; giving repeated CS presentations without the shock US. Animals were first fear conditioned while free from Dox to tag the learning activated neurons. The extinction training then took place following re-exposure to Dox (to prevent further labeling) and a memory retrieval trial was conducted 1 h prior to analysis. Extinction is never complete and varied significantly from animal to animal. We found that there was a significant correlation between the strength of the remaining fear response and the degree of circuit reactivation; animals with a high fear response during retrieval showed strong reactivation of the learning circuit while those with low fear responses showed a low degree of reactivation. In addition, we found some specificity in the anatomy of the responses so that reactivation in the basal amygdala was correlated with context fear while reactivation in the lateral amygdala was associated with the strength of the cued (tone) fear memory. These results are consistent with the known role of these subdivisions of the amygdala with the two different forms of fear memory (Quirk et al., 1995; Herry et al., 2008).

These results demonstrate that memory retrieval results in a reactivation of some of the same neurons that were active during the initial learning. We suggested that neurons activated by the US (shock) but also receiving weak CS inputs were altered during learning such that the CS alone could now activate them. In this way presentation of the CS alone after learning would recapitulate a portion of the aversive US leading to downstream fear responses. This approach represents a somewhat elaborate way of simply watching neural activity and the results are still purely correlative. However, by using a regulatable and binary genetic system, it should be relatively easy to introduce additional effector transgenes into the mice to control the activity or biochemistry of these neurons and directly test their role in memory. One recent study by Josselyn and coworkers has achieved this direct manipulation of neural ensembles associated with a specific memory trace using a somewhat different approach.

DRIVING MEMORIES INTO SPECIFIC NEURAL ENSEMBLES

The use of neural activity to introduce genetic alterations into neurons offers the possibility of obtaining direct molecular control over the neurons that participate in a *specific* neural representation or memory trace. An alternate approach that realized this goal took advantage of the finding that certain molecular manipulations could recruit neurons to participate in control of a specific memory. In one recent study, (Han et al., 2007), it was found that over expression of the transcription factor CREB in neurons resulted in their preferential recruitment into a fear memory trace. In this study, neurons in the amygdala were randomly infected with a viral vector that over expressed CREB and the animals were then trained in fear conditioning. They then performed a retrieval trial and examined the expression of the IEG arc in the amygdala. They found that the CREB over expressing neurons were more likely to be activated

during the memory retrieval. This result suggests that these CREB over expressing neurons were predisposed to participate in the memory trace. The mechanism by which these neurons are preferentially recruited is unclear but it does demonstrate that, at least in the amygdala, there is a good deal of flexibility in which neurons can be used to encode a specific memory. The system is not hard wired at the level of individual neurons but there is a sort of competition, with CREB over expression favoring a neuron's recruitment into the memory trace.

Josselyn and coworkers went on to take advantage of this CREB priming trick to directly manipulate a specific fear memory trace (Han et al., 2009). In this study the viral vector that delivered CREB to amygdala neurons also carried a gene that allowed for the expression of the diphtheria toxin receptor (DTR) (**Figure 3**). The CREB over expression recruited the neurons to the memory trace and expression of the DTR allowed for the selective ablation of these specific neurons with diphtheria toxin (DT). Ablation of the CREB over expressing neurons disrupted the fear memory while ablation of a similar number of random neurons in the amygdala did not. The memory effect was long lasting and specific (the same animals could learn a second fear association) demonstrating that this limited group of neurons played a critical role in the specific memory encoded during the CREB expression time window. This is the first example of the disruption of a specific memory within a distributed network.

ALTERING ACTIVATED NEURAL ENSEMBLES

While the ability of CREB over expression to recruit neurons to participate in a specific memory is interesting in its own right, it would be useful to have an approach to manipulate neurons that were naturally activated by any general environmental stimulus. This has recently been accomplished using a technique in which neurons expressing β-galactosidase can be specifically disrupted with a pharmacological agent (Koya et al., 2009). The study used rats that carry a cfos-promoter driven β-galactosidase to label activated neurons. To manipulate the neurons they use a drug that is inactive in the absence of β-galactosidase (daun02) but can be hydrolyzed to a compound that can reduce Ca^{2+} dependent action potentials (Santone et al., 1986). They examined context specific sensitization to cocaine, which is an associative paradigm where the response to a drug of abuse is potentiated when it is administered in the same environment in which it has been repeatedly taken. Animals were given repeated injections of cocaine over 1 week in context A to produce the context specific sensitization (measured as increased locomotor response to the drug). Following the training, a final sensitization trial to induce β-galactosidase was given and 90 min later daun02 was injected into the nucleus accumbens to disrupt the β-galactosidase expressing neurons. Previous studies had suggested that the nucleus accumbens was a critical site of plasticity mediating this behavior (Mattson et al., 2008). The injected animals showed a reduction in the context specific component of the sensitization but retained normal responses to cocaine in a novel context B when tested several days later.

Like the results with the CREB over expression, this study suggests that a specific associative representation (context A + cocaine) is being interfered with selectively. While the behavioral results in this study are intriguing and the linkage to cfos based expression provides a potentially general approach for manipulating discrete neural representations, there are a number of important

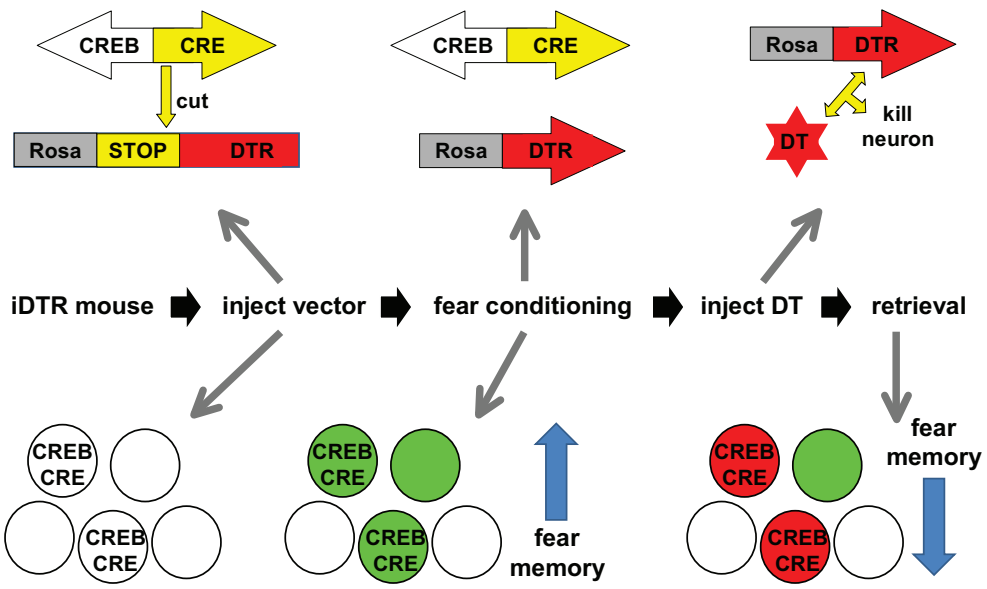

FIGURE 3 | Disrupting a specific memory in the mouse. The figure summarizes the experiments of Han et al. (2009). Transgenic mice were used that express an inducible diphtheria toxin receptor (iDTR). A viral vector expressing both CREB and CRE recombinase was injected into the amygdala of iDTR mice leading to the expression of both CRE and CREB in a random subset of neurons (circles with CREB/CRE). The CRE recombinase removed a transcriptional STOP sequence and allowed for expression of DTR in these CREB expressing neurons. The mice were then subjected to fear conditioning. An earlier study from the same authors (Han et al., 2007) demonstrated that the CREB expressing neurons participate in the storage of the fear memory (green circles symbolize neurons that participate in the storage of the memory). After fear conditioning, mice were injected with diphtheria toxin (DT), which killed the CREB expressing neurons which participated in the encoding of that memory (red circles). This caused a significant reduction in the strength of the fear memory measured during retrieval.

questions that remain to be addressed with this technique. First, the electrophysiological effect of the daun02 treatment was not examined directly in the neurons but inferred from studies in cell lines. Whether the effects are mediated by suppression Ca^{2+} dependent action potentials or some other effect of the treatment remains to be determined. The time course of any neural excitability or Ca^{2+} channel changes is also a critical parameter. The effect of the daun02 treatment was examined 3 days after the initial injection of the compound and it is unclear whether the observed effect was due to ongoing suppression of activity or to a persistent effect manifest during the initial treatment. Nevertheless, it demonstrates the general principles of this approach, which could be combined with the host of recently developed genetic regulators of neural activity.

SYNAPTIC TAGGING

While neural representations are encoded in the specific ensemble of neurons that are activated in response to a stimulus, the plasticity that molds these patterns of activation is thought to occur at the synapse. It has been known for some time that long-term memory lasting 24 h requires new gene expression initiated at the time of learning, while short-term memory lasting a few hours lacks this requirement (Davis and Squire, 1984). Since the short and long-term memories for the same event presumably involve the same pattern of synaptic changes, it raises the question of how the required gene products exert their effects selectively on the appropriate synapses. A potential answer to this question was suggested by Frey and Morris in studies of long-term potentiation (LTP), a form of synaptic plasticity thought by many to underlie memory (Frey and Morris, 1997, 1998). They found that synaptic activity could produce a sort of molecular "tag" at a synapse that would allow it to utilize newly expressed gene products to maintain LTP for long periods. We recently used the cfos based genetic approach to demonstrate a similar mechanism in behavioral learning and memory.

A number of studies have implicated the regulated trafficking of the glutamate receptor GluR1 to synapses as an important mechanism in both LTP and fear learning (Kessels and Malinow, 2009). In addition to allowing the genetic tagging of activated neurons, the IEG promoters like cfos show a very rapid onset and offset of expression, making them useful for cellular trafficking and turnover studies. We took advantage of this property to examine the trafficking of GluR1 following learning in the fear-conditioning paradigm (Matsuo et al., 2008). Mice carrying both a cfos-tTA and tetO-GFPGluR1 fusion transgene were used in this study (**Figure 4**). In the absence of Dox neural activity will induce a pulse of expression of the GFP tagged GluR1 and the distribution to synapses of this newly synthesized receptor can be followed histologically. Animals were fear conditioned in the absence of Dox to both induce a contextual fear memory and to induce synthesis of the GFP tagged receptor in activated neurons. The distribution of the receptor to dendritic spines (the site of most excitatory synapses) in the hippocampus was examined 24 h after the conditioning. We found that the receptor was not evenly distributed but present in only about 50% of spines, even in controls. In the fear conditioned animals we found a similar distribution except that there was an increase in trafficking to one morphological type of spine, the mushroom spines.

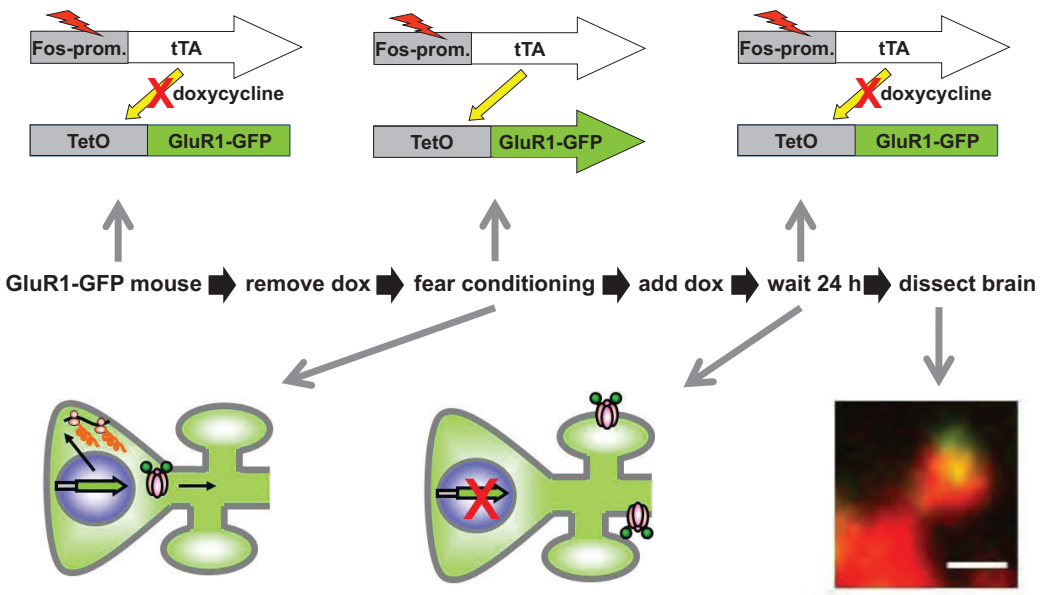

FIGURE 4 | Learning regulated targeting of glutamate receptors. The figure summarizes an experiment from Matsuo et al. (2008). Mice carrying two transgenes were used. The first transgene is identical to the one described in **Figure 2** and uses the cfos promoter to drive expression of a tetracycline transactivator (tTA). The second transgene was a tetO-promoter GFP tagged glutamate receptor subunit (GluR1-GFP). Animals were fear conditioned in the absence of Dox to produce both a fear memory and a pulse of GFP-GluR1 expression in active neural ensembles in the hippocampus. The distribution of GFP-GluR1 in dendritic spines was analyzed 24 h following the conditioning using DiI to label all spines on a given neuron. Fear conditioning led to an increase in trafficking of the receptor specifically to mushroom type spines. This experiment demonstrates how genetic tools can be used to image a specific molecular event selectively within an activated neural circuit.

This preferential trafficking only happened when the conditioned stimulus (CS: novel box) and the unconditioned stimulus (US: foot shock) were paired, but not when CS or US were presented separately. The newly synthesized GluR1 requires 2 h to begin to reach the dendritic spines, yet is somehow preferentially recruited to a specialized class of spine based on the associative conditioning that occurred 2 h prior to its arrival. This is indicative of a synaptic tagging event acting in behavioral memory similar to that described for LTP (Frey and Morris, 1997).

NEW DIRECTIONS AND NEW QUESTIONS

The ability to genetically manipulate activated neuronal ensembles or neurons participating in a sparsely encoded memory trace offers a number of advantages that are only beginning to be realized. A parallel line of technological development has focused on generating genetic tools for manipulating neuronal activity. The light regulated channelrhodopsin ChR, developed by Deisseroth and colleagues (Zhang et al., 2006), allows for the very precise light regulated control of action potential firing in neurons expressing the channel. There is an expanding tool box of genetic effectors like ChR that are light or ligand controlled and can be used to either stimulate or suppress neural activity (Luo et al., 2008). In addition, there are a number of similar effectors that can be used to regulate second messenger signaling pathways when expressed in heterologous cells (Isiegas et al., 2008; Pei et al., 2008; Airan et al., 2009; Alexander et al., 2009).

The combination of these new tools with activity based genetic delivery and multi time point brain activity mapping at cellular resolution will open up a variety of new questions to experimental analysis. To return to the initial discussion of neural representations, the fusion of these approaches should allow one to address the question of what neural firing patterns "mean" to the animal. To take the example of the Halle Berry neurons (actually the equivalent in genetically accessible animal models) one could ask what would be the consequences of silencing this specific group of neurons. Would the recognition of Halle Berry be inaccessible? What fraction of neurons in the representation need to be silenced in order to impair recognition? How does the silencing of a specific group of neurons in one brain region affect the activation patterns in downstream areas?

An alternate approach is to ask whether a representation can be built by experimenter driven stimulation of the appropriate neurons. In one recent study, Svoboda and colleagues delivered the ChR2 molecule to random populations of neurons in the somatosensory cortex. They found that stimulating as few as 300 neurons could be detected by the animals and used to alter behavior in a conditioning task (Huber et al., 2008). However, the coordinated activation of those neurons presumably does not form any natural representation. Suppose that instead of a random group of neurons one could activate a subset of neurons that responded to a natural stimulus, say the tone CS in a fear-conditioning task. The artificial stimulation of those neurons paired with a foot shock would presumably lead to a conditioned fear of the extrinsic stimulation CS. But the more interesting question now is would the animal also fear a tone? If so would all the features of this sensory representation be maintained, for example frequency selectivity? With what fidelity does artificial stimulation of the tone representation in one brain region recapitulate the brain activity patterns produced by the natural tone itself? This type of approach should now be achievable and allow direct functional investigation of the structure of neural representations.

Finally, the ability to genetically alter activated neural ensembles provides an entre into a more specific biochemistry of the brain. In the GluR1 trafficking studies discussed above, the GFP tagged receptor provides not only a signal to watch molecular movements, but also a tag for specific biochemical analysis. For example, one could ask how the synapses from activated neurons that received new receptor differ biochemically from those that did not by using the antibodies to the GFP tag to affinity purify positive from negative material. A similar approach could be used to tag specific cellular compartments or molecular complexes so that biochemical studies can be limited to just the activated neurons, sparsely embedded in a matrix of inactive neurons and glia.

It should also be possible to improve the specificity of the genetic modifications using a variety of genetic tricks such that only neurons active in one brain region or active at time point 1 but not time point 2 would be tagged. The increased specificity along with the new genetic tools and biochemical tagging should provide a new level of circuit analysis in the brain and break down the barrier between watching neural firing and manipulating neural function.

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Genetically encoded optical sensors for monitoring of intracellular chloride and chloride-selective channel activity

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This review briefly discusses the main approaches for monitoring chloride (Cl^-), the most abundant physiological anion. Noninvasive monitoring of intracellular Cl^- ($[Cl^-]_i$) is a challenging task owing to two main difficulties: (i) the low transmembrane ratio for Cl^-, approximately 10:1; and (ii) the small driving force for Cl^-, as the Cl^- reversal potential (E_{Cl}) is usually close to the resting potential of the cells. Thus, for reliable monitoring of intracellular Cl^-, one has to use highly sensitive probes. From several methods for intracellular Cl^- analysis, genetically encoded chloride indicators represent the most promising tools. Recent achievements in the development of genetically encoded chloride probes are based on the fact that yellow fluorescent protein (YFP) exhibits Cl^--sensitivity. YFP-based probes have been successfully used for quantitative analysis of Cl^- transport in different cells and for high-throughput screening of modulators of Cl^--selective channels. Development of a ratiometric genetically encoded probe, Clomeleon, has provided a tool for noninvasive estimation of intracellular Cl^- concentrations. While the sensitivity of this protein to Cl^- is low (EC_{50} about 160 mM), it has been successfully used for monitoring intracellular Cl^- in different cell types. Recently a CFP–YFP-based probe with a relatively high sensitivity to Cl^- (EC_{50} about 30 mM) has been developed. This construct, termed Cl-Sensor, allows ratiometric monitoring using the fluorescence excitation ratio. Of particular interest are genetically encoded probes for monitoring of ion channel distribution and activity. A new molecular probe has been constructed by introducing into the cytoplasmic domain of the Cl^--selective glycine receptor (GlyR) channel the CFP–YFP-based Cl-Sensor. This construct, termed BioSensor-GlyR, has been successfully expressed in cell lines. The new genetically encoded chloride probes offer means of screening pharmacological agents, analysis of Cl^- homeostasis and functions of Cl^--selective channels under different physiological and pathological conditions.

Keywords: fluorescent proteins, noninvasive monitoring, ion-sensitive microelectrodes, fluorescent dyes, quinolinium indicators, glycine receptor channel, FRET

INTRODUCTION

Fluorescent indicators designed for quantitative monitoring of intracellular ions and analysis of the distribution of various proteins have brought about a revolution in obtaining important information about the functioning, development and pathology of cells and cellular components of biological organisms.

In this review we will briefly discuss the main approaches for monitoring chloride (Cl^-), the most abundant physiological anion. Cl^- is present in every cell of biological organisms and participates in a variety of important cellular processes, such as neurotransmission, regulation of cell volume, pH and water–salt balance. The concentration of intracellular Cl^- and its permeance is highly regulated by a variety of Cl^--selective channels and Cl^- transporters (revs. Chen and Hwang, 2008; Jentsch, 2008). Dysfunction of these proteins results in a various diseases. For instance, the most prevalent lethal genetic disease, cystic fibrosis (Kerem et al., 1989), arises from mutations in the specific regulator of Cl^- permeability, cystic fibrosis transmembrane conductance regulator (CFTR) protein. This voltage-independent Cl^- channel is found in the epithelial cells of many tissues (intestine, lung, reproductive tract, pancreatic ducts). Mutations in the gene encoding CFTR affect 1 in 2000–2500 people (Ashcroft, 2000). Several other human diseases have been linked to dysfunction of Cl^- channels or transporters: myotonia congenita (Koch et al., 1992), congenital chloride diarrhoea (Kere et al., 1999), inherited hypercalciuric nephrolithiasis (Lloyd et al., 1996), Bartter's and Gitelman's syndromes (Simon and Lifton, 1996), hyperekplexia/startle disease (Shiang et al., 1993) and epilepsy (Macdonald et al., 2004; Lerche et al., 2005; Heron et al., 2007; Dibbens et al., 2009).

Direct measurement of intracellular Cl^- concentration ($[Cl^-]_i$) in neurons and in other cell types is a challenging task owing to two main difficulties: (i) low transmembrane ratio for Cl^-, approximately 10:1 (for Ca, for instance, 10000:1); and (ii) a small driving force for Cl^-, as the Cl^- reversal potential (E_{Cl}) is usually close to the

Abbreviations: CFP, cyan fluorescent protein; CFTR, cystic fibrosis transmembrane conductance regulator (protein); CHO, chinese hamster ovary (cells); CMV, cytomegalovirus (promoter); FRET, fluorescence resonance energy transfer; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GlyR, glycine receptor; HEK, human embryonic kidney (cells); HTP, high-throughput (screening); YFP, yellow fluorescent protein; MEQ, 6-methoxy-N-ethylquinolium; MQAE, N-(6-methoxyquinolyl)-acetoethyl ester; SPQ, 6-methoxy-N-(3-sulfopropyl) quinolinium; TFP, topaz fluorescent protein; VCF, voltage-clamp fluorometry; WT, wild type; YFP, yellow fluorescent protein.

resting potential of the cells. Consequently, sensitive probes with high dynamic range at physiological $[Cl^-]_i$ are necessary for reliable analysis of $[Cl^-]_i$ distribution and its functional variations.

For $[Cl^-]_i$ monitoring several methods have been proposed. The most used are Cl^--selective microelectrodes; chloride-sensitive fluorescent dyes and genetically encoded chloride-sensitive probes. We will briefly describe these approaches with the main focus on genetically encoded chloride-sensitive probes, which are the most promising tools for effective analysis of Cl^- homeostasis in various cell types.

Cl^- SELECTIVE MICROELECTRODES

In the 1960s, 70s and 80s the use of ion-selective electrodes was the main available technique for intracellular Cl^- detection. It allowed valuable information on Cl^- distribution and dynamics in a number of cell types of biological organisms to be obtained. In very early studies, AgCl electrodes were used as tools for $[Cl^-]_i$ estimation (Mauro, 1954; Keynes, 1963; Strickholm and Wallin, 1965; Sato et al., 1968). With an electrode consisting of a fine AgCl wire protruding from the end of a glass capillary, $[Cl^-]_i$ was measured in giant axons of squid (Mauro, 1954; Keynes, 1963) and crayfish (Strickholm and Wallin, 1965). However, later observations demonstrated that all microelectrodes that use AgCl as the sensitive element develop "the same type of error in the intracellular environment and thus all give erroneously high values of $[Cl^-]_i$" (Neild and Thomas, 1974).

The improved method was based on the use of siliconized borosilicate glass micropipettes, the tips of which were filled with liquid chloride ion exchanger. This technique was introduced by Walker (1971) and used with some modifications in a number of studies (Walker and Brown, 1970; Neild and Thomas, 1974; Ascher et al., 1976; Vaughan-Jones, 1979). These electrodes (**Figure 1A**) had a small tip (1–2 μm) and gave complete responses to changes in Cl^- within 1–2 min. Preparing these electrodes is a time-consuming procedure and penetration of cells without damage is difficult.

Despite these complications, Cl^--sensitive microelectrodes were successfully used to measure the intracellular Cl^- activity in giant neurons of molluscs *Aplysia* (Brown et al., 1970; Ascher et al., 1976) and *Helix aspersa* (Neild and Thomas, 1974; Kennedy and Thomas, 1995; Schwiening and Thomas, 1996), in frog heart cells (Ladle and Walker, 1975; Vaughan-Jones, 1979) and in other preparations (Walker and Brown, 1977; Thomas, 1978).

An intracellular Cl^--sensitive microelectrode records the algebraic sum of the membrane potential (E_m) and a voltage proportional to changes in Cl^- activity. It means that E_m must be separately determined using an independent electrode (**Figure 1B**). To diminish the damage from insertion of two microelectrodes into a single cell, double-barrelled pipettes were proposed (Aickin, 1981) and used for monitoring the intracellular Cl^- activity in smooth muscle cells (Aickin and Brading, 1982; Davis et al., 2000), retinal pigment epithelium (Bialek et al., 1995; La Cour et al., 1997) and in other cell types (Kitano et al., 1995; Debellis et al., 2001; Ianowski et al., 2002).

Early studies with Cl^--sensitive microelectrodes already demonstrated that $[Cl^-]_i$ in cells differs substantially from a predicted passive distribution, suggesting that Cl^- ions must be actively transported through cellular membranes. These observations were confirmed by the more recent discovery of several mechanisms of transmembrane Cl^- transport (rev. Russell, 2000; Lauf and Adragna, 2004).

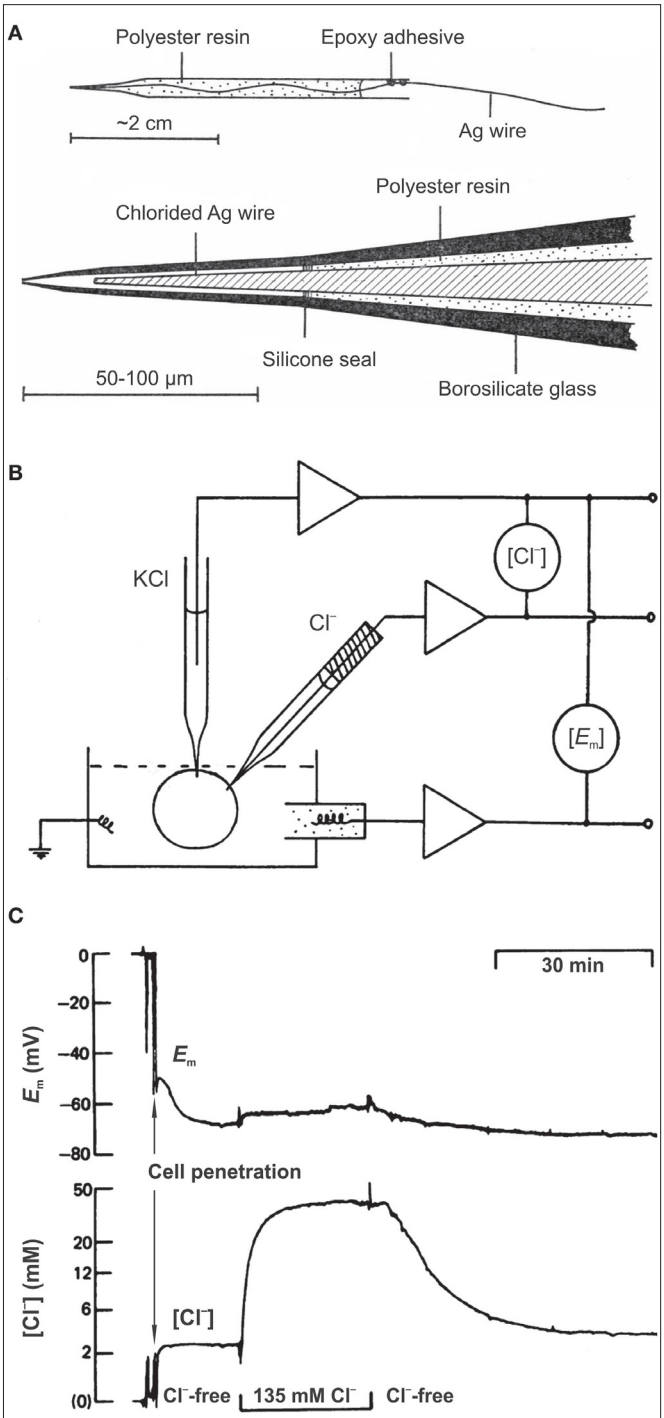


FIGURE 1 | Cl^--sensitive microelectrodes and intracellular chloride concentration measurements. (A) Construction of recessed-tip Cl^--sensitive microelectrode. The complete electrode (*top diagram*) and enlarged view of the sensitive tip (*bottom diagram*) are shown. (From Neild and Thomas, 1973). **(B)** Diagram showing the basic experimental arrangement for chloride concentration measurements using microelectrodes. (Modified from Thomas, 1977). **(C)** Example of simultaneous recording of membrane potential (E_m) and $[Cl^-]_i$ in smooth muscle cell of the guinea pig vas deferens using double-barrelled microelectrode. Cl^--free and normal solutions were applied. Note the very slow (min) recorded Cl^- transients. (Modified from Aickin and Brading, 1982).

Three main obstacles limited the spread of the Cl^--sensitive microelectrode technique: (i) time-consuming procedure for microelectrode preparation; (ii) slow kinetics (**Figure 1C**); and (iii) the need to use relatively large cells for reliable recording without cell damage. In addition, penetration of cells could change the native intracellular Cl^- distribution. Methods based on imaging techniques are more promising as they provide an opportunity to monitor Cl^- activity noninvasively and in populations of cells.

FLUORESCENT Cl^- SENSITIVE DYES

Because of the possibility of monitoring noninvasively the distribution and dynamics of ion concentration changes, fluorescent Cl^--sensitive dyes are the most popular approach for analysis of Cl^- and Cl^--dependent physiological processes in different cells types.

The fluorescence of many fluorophores is decreased on application of heavy-atom anions, such as bromine and iodine. Cl^- ions are less effective in this respect and relatively few fluorophores are quenched by Cl^- (Geddes et al., 2001). The background for Cl^- monitoring was established by George Stokes, who described in 1852 the phenomenon of fluorescence. In 1869 Stokes observed that the "fluorescence of quinine in dilute sulfuric acid was reduced after the addition of hydrochloric acid, i.e., chloride ions" (Geddes et al., 2001). Perhaps the fact that quinine, which is sensitive to chloride, contains a quinoline ring has stimulated the production of many quinoline analogues in the search for efficient Cl^- probes.

Quinolinium Cl^- indicators are based on the capability of halides to quench the fluorescence of heterocyclic organic compounds with quaternary nitrogen (Chen et al., 1988; Verkman, 1990; **Figure 2A**).

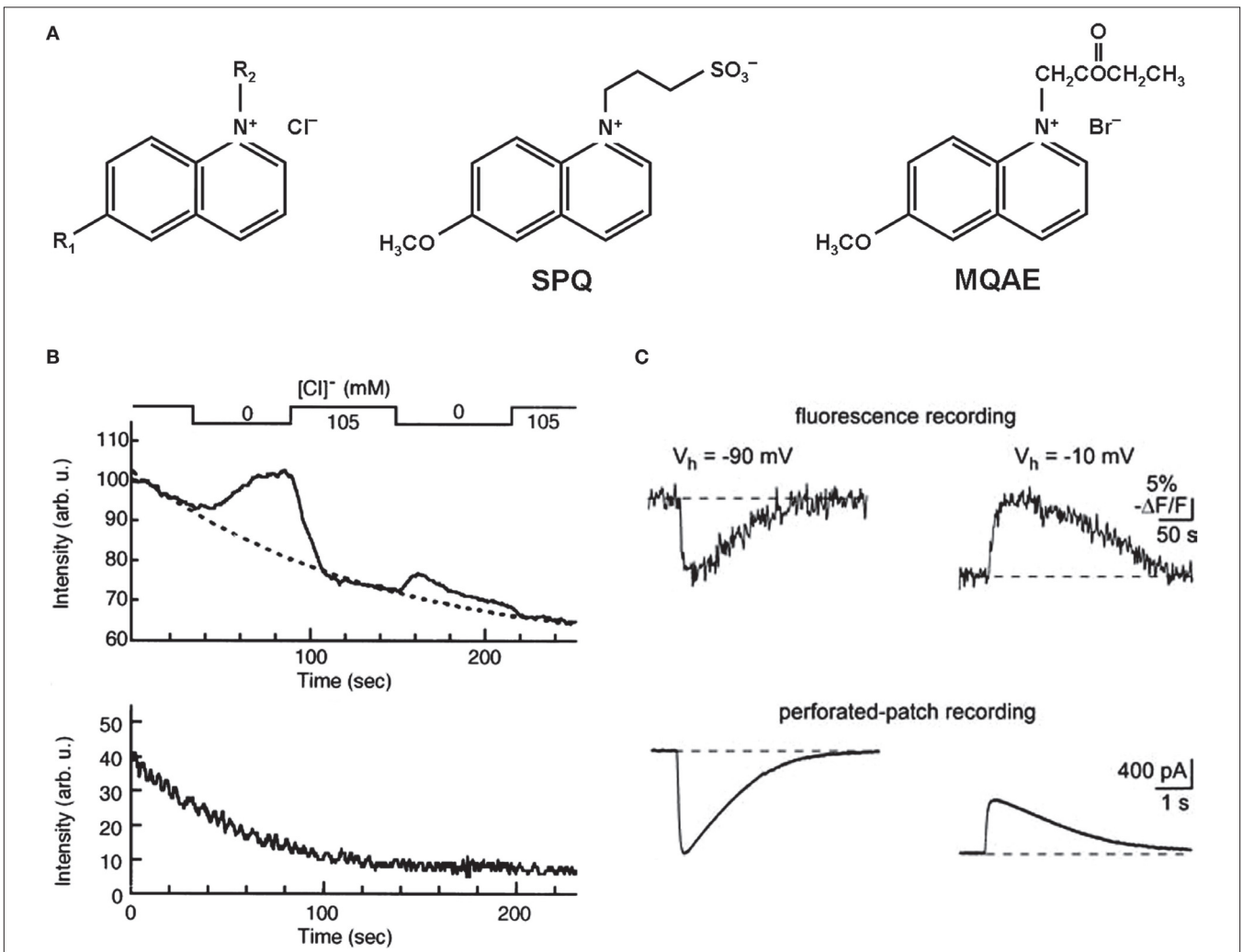


FIGURE 2 | Chemical structure and fluorescent properties of widely used Cl^--sensitive dyes. (A) Chemical structure of quinolinium Cl^--sensitive dyes. *Left:* Common structure, R_1 and R_2, radicals. *Middle and right*: Structural formula for SPQ and MQAE dyes respectively. **(B)** Examples of continuous fluorescence measurements of the MQAE-loaded cells. Note the rapid degradation in baseline fluorescence value (*top and bottom traces*) and dramatic decrease in the fluorescent response for the second transition from 105 to 0 mM of Cl^- (*top trace*). (Modified from Nakamura et al., 1997). **(C)** Two-photon imaging in MQAE-loaded cerebellar neurons. Changes in fluorescence (*top traces*) and corresponding transmembrane currents (*bottom traces*) caused by 150-ms-long pressure applications of GABA. Note about 50-times slower fluorescent transients in comparison with currents. (Modified from Marandi et al., 2002).

A relation between the Cl^- concentration and the fluorescence intensity is described by the Stern–Volmer-equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Cl^-]$$

where F_0 is the fluorescence in the absence of halide, F is the fluorescence in the presence of halide and K_{SV} is the Stern-Volmer quenching constant (in M^{-1}). From this equation the EC_{50}, the concentration of Cl^- causing a 50% decrease in fluorescence, is $1/K_{SV}$.

Table 1 shows values of the Stern-Volmer constant and corresponding EC_{50} values for some quinolinium indicators. The most-used quinolinium indicators are 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ), 6-methoxy-N-ethylquinolium (MEQ) and N-(6-methoxyquinolyl)-acetoethyl ester (MQAE).

The first designed quinolinium-based Cl^- indicators was SPQ (Wolfbeis and Urbano, 1983; Illsley and Verkman, 1987; **Figure 2A**, **Table 1**). SPQ is excited at ultraviolet wavelengths with absorbance maxima at 318 nm and 350 nm. This fluorescent dye has a single broad emission peak centred at 450 nm with a quantum yield of 0.69 in the absence of halides. In aqueous buffers the Stern-Volmer constant for quenching of SPQ by Cl^- is 118 M^{-1}, giving EC_{50} ~ 8.5 mM. The fluorescence of SPQ is not altered by cations, phosphate, nitrate or sulfate, but it is quenched weakly by other monovalent anions including citrate, acetate, gluconate and bicarbonate (Illsley and Verkman, 1987; Krapf et al., 1988b; Jayaraman et al., 1999).

MQAE, the other quinolinium-based dye, which has been used in a number of studies during recent years, has high Cl^- sensitivity (**Figure 2A**, **Table 1**) and, unlike SPQ, easily permeates through the plasma membrane (Mansoura et al., 1999). As a result the time of incubation with this dye can be rather short. For instance, 10-min incubation of brain slices is sufficient for bright staining of neurons (Marandi et al., 2002). However, this substance has to be used with prudence, as incubation of slices for 30–45 min caused a deterioration in the properties and even death of many neurons in neocortex and hippocampal slices (Holmgren, Zilberter, Mukhtarov, personal observations). For these reasons, results obtained after as long as 1–2 h of treatment with MQAE (e.g. Servetnyk and Roomans, 2007) have to be interpreted with caution. The other disadvantages of this dye lie in the significant leakage rate and bleaching. The leakage rate seems to be preparation-specific, ranging from 3% per hour in liposomes (Verkman et al., 1989) to 30% per hour in brain slices (Marandi et al., 2002).

Quinolinium-based Cl^- indicators have been used for measurements of Cl^- in a variety of preparations, including isolated growth cones (Fukura et al., 1996), neurons (Schwartz and Yu, 1995; Inglefield and Schwartz-Bloom, 1997; Dallwig et al., 1999; Frech et al., 1999; Marandi et al., 2002), glia (Bevensee et al., 1997), different types of epithelial cells (Krapf et al., 1988b; Lau et al., 1994), fibroblasts (Chao et al., 1989; Woll et al., 1996; Munkonge et al., 2004), human gastric cancer cells (Miyazaki et al., 2008) and pancreatic beta-cells (Eberhardson et al., 2000). In general, quinolinium compounds have relatively good sensitivity and selectivity to Cl^- and rapid responses to changes in Cl^-. They are also insensitive to physiological changes in pH.

The major disadvantage of these probes comes from their spectral properties, i.e. excitation at ultraviolet wavelengths. As a result, they are prone to strong bleaching (Inglefield and Schwartz-Bloom, 1997; Nakamura et al., 1997, **Figure 2B**). This restricts the duration of the measurements and allows only a very low data acquisition rate (0.2–2 frames per minute) (Inglefield and Schwartz-Bloom, 1997; Fukuda et al., 1998; Sah and Schwartz-Bloom, 1999). However, the use of these dyes in combination with two-photon microscopy strongly reduces bleaching and, consequently, photochemical damage (Marandi et al., 2002; Funk et al., 2008).

GENETICALLY ENCODED Cl^- INDICATORS

YFP AS A TOOL FOR INTRACELLULAR Cl^- MONITORING

An alternative to exogenously added indicators is the use of an endogenously expressed chromophore such as green fluorescent protein (GFP). Using appropriate targeting sequences, GFPs have been directed selectively to numerous intracellular sites. Various applications of GFP in physiological studies of living cells have been described (Tsien, 1998; Zaccolo and Pozzan, 2000; Bizzarri et al., 2009). GFP derivatives with different colors have been used in FRET models to monitor Ca^{2+} (Miyawaki et al., 1997, 1999), pH (Kneen et al., 1998; Llopis et al., 1998; Miesenbock et al., 1998) and protein–protein interactions (Heim, 1999).

During the last decade, a new method of noninvasive $[Cl^-]_i$ monitoring using genetically encoded optical probes has been developed. This approach is based on the halide-binding properties of yellow

Table 1 | Structures and chloride sensitivity of some quinoline-based Cl^- fluorescent indicators.

IUPAC name	R_1	R_2	K_{sv} (M^{-1})	EC_{50} (mM)	References
6-Methoxy-*N*-ethylquinolinium (MEQ)	OCH_3	C_2H_5	145	7	Biwersi and Verkman, 1991
N-(Ethoxycarbonyl methyl)-6-methoxy	OCH_3	$CH_2COOC_2H_5$	200	5	Verkman et al., 1989
quinolinium (MQAE)			77*	13	Marandi et al., 2002
			24.7#	40	
6-methoxy-N-(-3-sulfopropyl)quinolinium (SPQ)	OCH_3	$(CH_2)_3SO_3^-$	118	8.5	Krapf et al., 1988b
6-methyl-N-(-3-sulfopropyl)quinolinium	CH_3	$(CH_2)_3SO_3^-$	83	12	Krapf et al., 1988b
6-methoxy-N-(-4-sulfobutyl)quinolinium	OCH_3	$(CH_2)_4SO_3^-$	78	13	Krapf et al., 1988b
6-methoxy-N-(-8-octanoic acid) quinolinium Br^-	OCH_3	$(CH_2)_7COOH$	52	19	Geddes et al., 1999
6-methoxy-N-(-11-undecanoic acid)quinolinium Br^-	OCH_3	$(CH_2)_{10}COOH$	34	29	Geddes et al., 1999
6-methoxy-N-(-15-pentadecanoic acid)quinolinium Br^-	OCH_3	$(CH_2)_{14}COOH$	34	29	Geddes et al., 1999

**Data from experiments in vitro. #Hippocampal pyramidal rat neurones P9. (Modified from Geddes et al., 2001).*

fluorescent protein (YFP) and it derivatives (Wachter and Remington, 1999; Jayaraman et al., 2000). YFP is a derivative of GFP, which contains four point mutations (T203Y/S65G/V68L/S72A). It has improved brightness and red-shifted excitation/emission spectra compared with GFP (Ormo et al., 1996; Elsliger et al., 1999). The halide sensitivity of YFP was conferred on this protein using a rational mutagenesis strategy based upon crystallographic data (Wachter et al., 1998) and confirmed experimentally (Wachter and Remington, 1999).

It was found that YFP fluorescence is sensitive to various small anions with relative potencies $F^- > I^- > NO_3^- > Cl^- > Br^- >$ formate$^-$ > acetate$^-$ (Jayaraman et al., 2000). YFP sensitivity to these small anions results from ground-state binding near the chromophore (Jayaraman and Verkman, 2000), which apparently alters the chromophore ionization constant and hence the fluorescence emission. As with other GFP derivatives, the fluorescence of YFP is pH-dependent. The EC_{50} values for Cl^- varied from 32.5 mM (pH = 6) to 777 mM (pH = 7.5) (**Table 2**).

This analysis demonstrates that at the physiological range of intracellular pH (7.2–7.4) the sensitivity of YFP to Cl^- is low, which creates difficulties and limitations in using "wild type" (WT) YFP as a Cl^- indicator. Indeed, different methods of $[Cl^-]_i$ estimation in various cell types gives its range of variations from 3 to 60 mM (**Table 3**). Consequently, the resolution of the indicator with EC_{50} more then 100 mM is low and it can lead to errors in noninvasive estimation of $[Cl^-]_i$.

Table 2 | Sensitivity of YFP and YFP-H148Q to chloride (Modified from Wachter et al., 2000).

pH	EC_{50} YFP (mM)	EC_{50} YFP-H148Q (mM)
6.0	32.5	22.1
6.5	82.1	34.0
7.0	245	66.2
7.5	777	154.4
8.0	2160	431

To further improve spectral characteristics, YFP was subjected to additional mutagenesis and the most successful variants were selected. It has been demonstrated that at pH 7.5 the EC_{50} for Cl^- of the mutant YFP H148Q is 154 mM (**Table 2**; Wachter et al., 2000), which is still high; however, it is closer to the physiological range of $[Cl^-]_i$ than WT YFP (777 mM).

To enhance sensitivity of YFP-H148Q to Cl^-, libraries of mutants were generated in which pairs of residues in the vicinity of the halide binding site were randomly mutated (Galietta et al., 2001a). Analysis of over a thousand clones revealed improved anion sensitivity with EC_{50}s down to 40 mM for Cl^- (V163S), 10 mM for NO_3^- (I152L) and 2 mM for I^- (I152L). To check physiological applicability, the I152L mutant, which exhibited the best I^- and NO_3^- sensitivities, was expressed in Swiss 3T3 fibroblasts carrying CFTR. Transfected cells were brightly fluorescent with a uniform cytoplasmic and nuclear staining pattern. Replacement of 20 mM Cl^- with I^- produced a fluorescence decrease of 53 ± 2% with YFP-I152. It was much greater than that of <10% for the same experiment with YFP-H148Q, indicating that this mutant is a good tool for monitoring I^- and NO_3^-. The same results were obtained when the activity of CFTR was studied using a Cl^-/NO_3^- exchange protocol (Galietta et al., 2001b).

Some other mutants of YFP also showed greatly improved Cl^- sensitivities (**Table 4**), which stimulated further development of Cl^- indicators. It provided the basis for using YFP mutants as genetically encoded Cl^- sensors that can be targeted to specific organelles in living cells or expressed in specific cell types for monitoring $[Cl^-]_i$ distribution, to study the functioning of Cl^- channels and pumps.

Table 3 | Intracellular chloride in different preparations registered by different methods.

Preparation	Method	$[Cl^-]_i$ (mM)	References
Snail neurons	Cl-sensitive microelectrodes	3–8	Neild and Thomas, 1974
Rabbit epithelial cells	Cl-sensitive microelectrodes	16	Abdulnour-Nakhoul et al., 2002
Rat sympathetic neurons	Cl-sensitive microelectrodes	30	Ballanyi and Grafe, 1985
Retina bipolar cells	Perforated patch	21–25	Billups and Attwell, 2002
Hippocampal slices (P16-P20)	Perforated patch	6–12	Khirug et al., 2008
Salamandra cones	Perforated patch	20–50	Thoreson and Bryson, 2004
Spinal neurons	Perforated patch	11–27	Rohrbough and Spitzer, 1996
Retina bipolar cells	Perforated patch	8–23	Satoh et al., 2001
Retina amacrine and ganglion cells	Perforated patch	14–29	Zhang et al., 2006
Hippocampal slices	Cell attach	4–18	Tyzio et al., 2006
Hippocampal cultures	MQAE	11–35	Hara et al., 1992, 1993
Epithelial cells	SPQ	27	Krapf et al., 1988a
Renal cells	MQAE and flow cytometry	43	Miyazaki et al., 2007
Cockroach salivary gland cells	MQAE and two-photon fluorescence lifetime imaging	42–80	Hille et al., 2009
Human neurophils	Zimosan-conjugated Cl probe	73	Painter and Wang, 2006
Hippocampal slices	Clomeleon	5–30	Berglund et al., 2008
Hippocampal culture	Clomeleon	20–60	Kuner and Augustine, 2000
Retina bipolar cells	Clomeleon	10–60	Duebel et al., 2006
Hippocampal neurons	Cl-Sensor	11	Markova et al., 2008
CHO cells	Cl-Sensor	23	Markova et al., 2008
Retina photoreceptor cells	Cl-Sensor	30–50	Markova et al., 2008; Mukhtarov et al., 2008

Table 4 | Sensitivity of YFP mutants to chloride at pH 7.5.

Mutant	EC_{50} (mM)
H148Q	106[a]–197
H148Q/I152L	85[a]–88
H148Q/V150A/I152L	61
H148Q/V163S	39[a]–62
H148Q/V163L	77
H148Q/V163T/F165Y	55
H148Q/V150S/V163T	92

[a]*Purified protein. EC_{50} was measured in bacterial lysate with YFP-H148Q containing additional single or double mutations. (Modified from Galietta et al., 2001a).*

APPLICATION OF YFP IN HIGH-THROUGHPUT SCREENING

In a number of experimental models YFP derivatives have been used as suitable probes for high-throughput (HTP) screening. These allow the testing of tens of thousands of different compounds (Ma et al., 2002a,b). We present here several examples.

YFP-H148Q was transfected in Fisher rat thyroid cells (FRT) and in Swiss 3T3 fibroblasts for quantitative HTP screening of potential modulators of CFTR halide permeances (Galietta et al., 2001b). In these assays small increases in CFTR activity by the agonists forskolin and genistein were detected. Because YFP fluorescence is sensitive to H^+ a simultaneous monitoring of pH by specific pH-probe, BCECF, was also conducted (Galietta et al., 2001b). Using HTP screening on cells expressing the YFP-H148Q mutant, new classes of CFTR modulators were identified: inhibitors (Ma et al., 2002a), activators (Galietta et al., 2001c; Ma et al., 2002b; Muanprasat et al., 2004, 2007), potentiators (Yang et al., 2003) and correctors (Pedemonte et al., 2005a,b) of wild-type CFTR, as well as of ΔF508-CFTR, the major CFTR mutation causing cystic fibrosis (see rev. Verkman and Galietta, 2009).

To screen potential activators of CFTR-mediated Cl^- flux, a YFP mutant with higher halide sensitivity (YFP-H148Q/I152L) has been co-expressed with defective ΔF508-CFTR in FRT epithelial cells (Yang et al., 2003; Xu et al., 2008). It has been shown that natural cumarine compounds rescue defective ΔF508-CFTR chloride channel gating (Xu et al., 2008).

A mutant with enhanced sensitivity to halides (YFP-V163S) has been used in M1 cortical collecting-duct cells to monitor changes in Cl^- mediated by CFTR or by stimulation with cAMP- and Ca^{2+}-increasing agonists (Adam et al., 2005).

Important observations have been obtained with YFP-H148Q/I152L, which shows a 30-fold selectivity to I^- over Cl^-. It was proved to be a sensitive biosensor of Na^+/I^- symporter-mediated I^- uptake in thyroid cells and nonthyroidal cells following gene transfer (Rhoden et al., 2007, 2008). As defective iodide transport occurs in several inherited and acquired thyroid disorders, using this YFP mutant for detection of I^- represents a useful tool for studying the pathophysiology and pharmacology of this Na^+/I^- symporter (Rhoden et al., 2007).

YFP derivatives were also used for testing ligands of glycine receptors (GlyRs) and ionotropic GABA receptors. Using HEK 293 cells expressing YFP-I152L or YFP-V163S mutants with these Cl^--selective receptor-operated channels has established the optimal conditions for pharmacological screening of Cl^- (Kruger et al., 2005) and detection of functional and non-functional mutations in the GlyRs (Gilbert et al., 2009).

These observations have demonstrated advantages of using genetically encoded YFP derivatives in HTP in comparison with other techniques.

ADVANTAGES AND DISADVANTAGES OF GENETICALLY ENCODED Cl^- INDICATORS

Of the many advantages of YFP-based Cl^- indicators in comparison with fluorescent dyes, we will mention only the four most important.

First, the peak of absorbance is at a wavelength of more then 480 nm, i.e., in contrast to quinolinium-based halide indicators, YFP can be excited in the visible range, permitting more stable fluorescence signal and less cell damage. Consequently, it allows long-lasting Cl^- monitoring with repetitive stable responses and bright fluorescence signals using conventional imaging equipment.

Second, genetically encoded probes can be targeted to specific cell types by cell-specific promoter, or to defined cellular compartments by fusion to short sequence tags or to specific proteins. This would allow Cl^- monitoring in specific cell types or cellular compartments. For instance, transgenic mice expressing enhanced YFP (EYFP) under control of the Kv3.1 K^+ channel promoter (pKv3.1) have been generated (Metzger et al., 2002), making possible neuron-specific expression of EYFP in the hippocamus, thalamus and granule cell layer of cerebellum. This model has been used for analysis of glutamate-induced changes in intracellular Cl^- and pH (Metzger et al., 2002; Slemmer et al., 2004). The thy1 promoter has been successfully used to drive specific neuronal expression of Clomeleon in the hippocampus and in neocortical areas as well as in the dentate gyrus, cerebellar mossy fibers and piriform cortex (Berglund et al., 2008).

Third, the intracellular concentration of the YFP-based Cl^- indicators is only a few micromolar, which is several orders of magnitude less than the $[Cl^-]_i$. This avoids buffering effects, which are a substantial problem in, for instance, fluorescence measurements of intracellular Ca^{2+} at using conventional fluorescent dyes.

Finally, the molecular weight of the YFP-based Cl^- indicators is about 27 kDa, which prevents diffusion of the indicator from cells. In cells that are imaged without simultaneous electrophysiological recordings, indicator levels remains constant over hours.

YFP-based sensors also have several disadvantages. One of them is pH sensitivity (**Table 2**). Changes in intracellular pH or in specific compartments can lead to errors in observations and interpretation of results. To overcome this problem, in some cases independent monitoring of pH is necessary.

YFP-based Cl^- sensors have rather low kinetics of Cl^- association/dissociation. The double mutant YFP-H148Q/V163S, which exhibits relatively high Cl^- sensitivity (EC_{50} = 39 mM), has an association time constant τ = 1900 ms (Galietta et al., 2001a), which would cause limitations in the time resolution when using this mutant for analysis of rapid Cl^- dynamics. The other double mutant, YFP-H148Q/I152L, has much faster association/dissociation kinetics (association time constant τ = 52 ms; Galietta et al., 2001a); however, its sensitivity to Cl^- is relatively low (EC_{50} = 85 mM). Thus, the YFP-based probes can be used to reliably detect changes in Cl^- concentrations with time course resolution in the range of hundreds of milliseconds or even seconds.

A significant limitation in the use of Cl^- indicators was a lack of a Cl^--dependent change in spectral shape, which precludes ratiometric measurements. For quinolinium-based Cl^--sensitive dyes, synthesis of a series of dual-wavelength fluorescent indicators has been achieved using conjugation of Cl^--sensitive and Cl^--insensitive dye molecules with different spacers (Jayaraman et al., 1999). Only one "chimera" (MQa4AQ) was cell-permeating and it turned out to be four times less Cl^--sensitive than MQAE. YFP-derivatives also do not have a clear isosbestic point in spectral shape at different Cl^- concentrations. This precludes ratiometric measurements and, consequently, gives rise to limitations in the estimation of $[Cl^-]_i$ values.

The important development of genetically encoded Cl^- probes was achieved by Kuner and Augustine (2000), who constructed a ratiometric YFP-based Cl^- indicator, termed Clomeleon.

RATIOMETRIC MONITORING OF Cl^- USING GENETICALLY ENCODED PROBES

Clomeleon

Clomeleon consists of two fluorescent proteins, Cyan Fluorescence Protein (CFP) and a variant of YFP, Topaz Fluorescence Protein (TFP, GFP/S65G/S72A/K79R/T203Y/H231L), connected with a polypeptide linker of 24 aminoacids (**Figures 3A,B**).

The work of this probe is based on the phenomenon of Fluorescence Resonance Energy Transfer (FRET) between two fluorescent proteins (see **Box 1**). Binding of a Cl anion to TFP reduces its emission, leading to a decrease in FRET efficiency. This process can be visualized as a reduction in the ratio of fluorescence emission between the TFP acceptor and CFP donor fluorophores. Analysis of emission spectra of this construct revealed that the intensity of fluorescence depends on Cl^- concentration. Moreover, presence of the isosbestic point in normalized spectra (**Figure 3C**) allows the use of this indicator as a ratiometric probe for estimation of Cl^- concentration (**Figure 3D**) The construct was named Clomeleon as an allusion to the FRET-based genetically encoded Ca^{2+} indicator, Cameleon (Miyawaki et al., 1997).

Unlike organic probes, Clomeleon possesses several valuable features: excitation at visible wavelengths, good signal-to-noise ratio, safer loading procedures for cells, absence of leakage from cells and the possibility of targeting the probe to different cell types using specific promoters. Moreover, the construct exhibits high fluorescence stability: absence of Clomeleon bleaching during 2 h of recording has been reported (Pond et al., 2006). Proteolitical stability of Clomeleon in transgenic mice has been observed during 9 months. It is also a low toxicity probe, which did not cause any behavioural aberration in Clomeleon-expressing mice in the course of 2 years (Berglund et al., 2006).

The main advantage of Clomeleon is the possibility of performing ratiometric measurements of $[Cl^-]_i$. The ratiometric capabilities of Clomeleon allow optical measurements that are minimally influenced by the thickness of the specimen, intensity of the excitation light and concentration of the indicator. This, in turn, makes it possible to accurately determine Cl^- values even in cells with complicated geometry, such as neurons.

Clomeleon has been used for measurements of $[Cl^-]_i$ in cultured hippocampal neurons (Kuner and Augustine, 2000), in plant cells (Lorenzen et al., 2004) and in cells of retina and brain slices (Duebel et al., 2006; Pond et al., 2006). The widest field

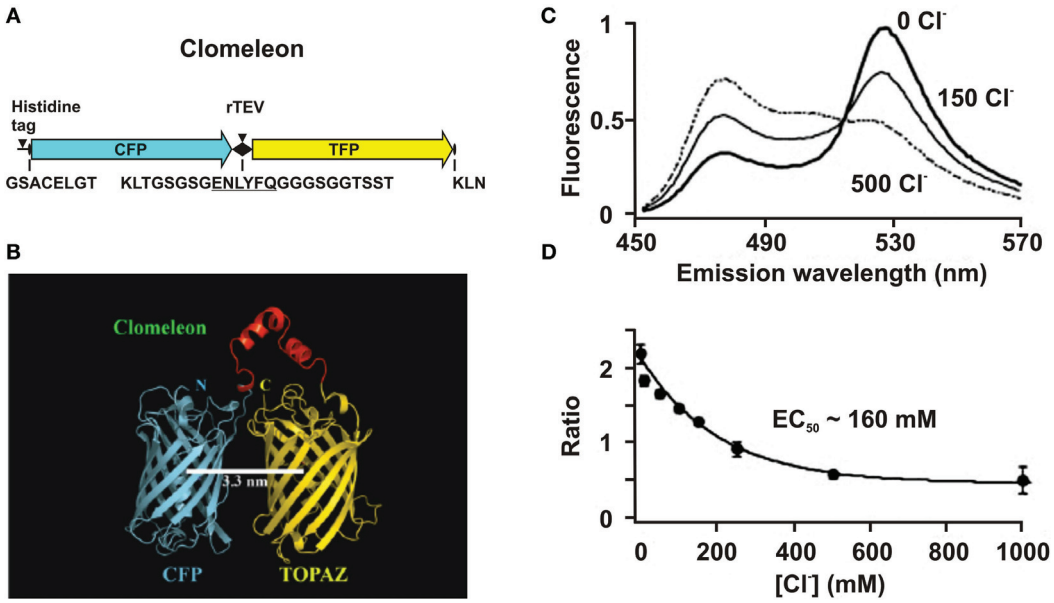


FIGURE 3 | Structure and fluorescence properties of Clomeleon. **(A)** Scheme of Clomeleon construct and **(B)** schematic reconstruction of Clomeleon structure. The fluorescent domains, CFP (cyan) and Topaz (yellow) were set in parallel orientation as N- and C-terminal ends of either domain were on the same site. The distance between CFP and Topaz chromophores was calculated to be 3.3 nm. (From Kuner and Augustine, 2000 (*top*) and Jose et al., 2007 (*bottom*). **(C)** Emission spectra of Clomeleon in the presence of different Cl^- concentrations. In all cases, the recombinant protein was excited at 434 nm and the emission spectra were normalized to their peaks at 527 nm. **(D)** The relationship between fluorescence emission ratio (527 nm/485 nm) and Cl^- concentrations. (Data from Kuner and Augustine, 2000).

BOX 1 | FRET.

Phenomenon of fluorescence resonance energy transfer (FRET) represents interaction between two fluorophores, when excitation energy from a donor (D) molecule is directly transferred to a molecule of acceptor (A).

Four main conditions have to be fulfilled for this phenomenon development:

(i) overlapping of emission spectrum of donor and excitation spectrum of acceptor;
(ii) small distance between molecules (less then 10 nm);
(iii) good orientation the dipole moments of donor emission and acceptor absorption;
(iv) high quantum yield of fluorophores.

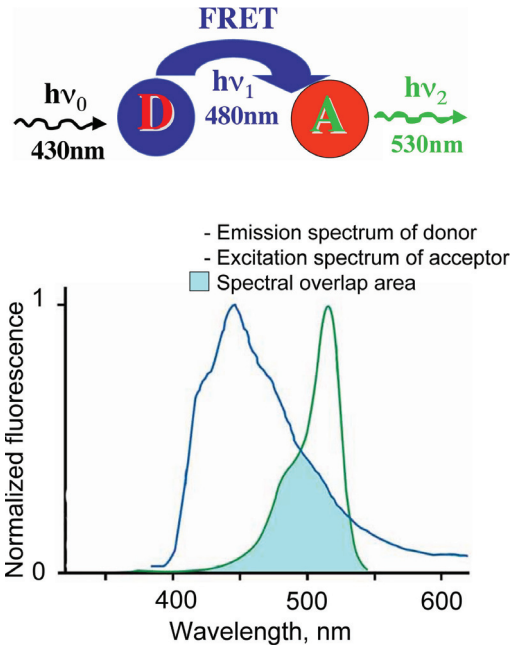
The FRET efficiency (E) is dependent on the inverse sixth power of the distance between fluorophores (r):

$$E = 1/[1 + (r/R_0)^6]$$

where R_0 is the distance at which the energy transfer efficiency is 50%.

This makes FRET technique a sensitive tool for analysis of protein–protein interaction and changes in intermolecular distances.

For more details see: Tsien et al., 1993; Pollok and Heim, 1999; Jares-Erijman and Jovin, 2003; Sekar and Periasamy, 2003; Wallrabe and Periasamy, 2005.



of application for genetically encoded probes comes from the possibility of targeting them to specific cell types using unique promoters, or to cellular compartments and membrane domains by fusion to respective tags or to proteins with known location. Several transgenic mouse lines have been created by insertion of Clomeleon, under control of the thy1 promoter, into their genome (Berglund et al., 2008). Details and functional implications of these models are described in recent papers (Duebel et al., 2006; Berglund et al., 2008).

Potential limitations of this probe are that it, as other YFP-based Cl^- indicators, is sensitive to pH and that the time course of reaction to Cl^- is relatively slow. The other potential problem is that the fluorophores, CFP and TFP, may bleach at different rates, which would distort the calibration of the indicator signal.

The important disadvantage of Clomeleon is that at physiological pH it has a rather low sensitivity to Cl^-. The EC_{50} of Clomeleon is more than 160 mM (Kuner and Augustine, 2000; **Figure 3D**, **Table 5**) which is far from physiological $[Cl^-]_i$ (3–60 mM). For this reason, the development of ratiometric probes with high sensitivity to Cl^- is required.

Cl-Sensor

Recently a new genetically encoded indicator, termed Cl-Sensor, has been proposed (Markova et al., 2008; **Figure 4A**). Analysis of the spectral properties of this construct during simultaneous monitoring of fluorescence signals and whole-cell recordings with different Cl^- concentrations in the pipette solution revealed two important features.

First, the normalized excitation spectra obtained at different $[Cl^-]_i$ have a common point near 465 nm (**Figure 4B**), meaning that Cl-Sensor allows ratiometric monitoring using the fluorescence excitation ratio. This feature allows recordings using conventional setups with devices for a rapid change of excitation wavelength.

Table 5 | Halide sensitivity and ionic selectivity of Clomeleon (Modified from Kuner and Augustine, 2000).

Halide	EC_{50} (mM)	Hill coefficient
F^-	5.9 ± 2.4	1.25 ± 0.09
I^-	46 ± 14	0.90 ± 0.07
Br^-	111 ± 21	0.82 ± 0.09
Cl^-	167 ± 13	0.87 ± 0.07

Second, due to triple YFP mutation (YFP-H148Q/I152L/V163S) this construct exhibits a relatively high sensitivity to Cl^- with an estimated EC_{50} of about 30 mM (28 ± 5 mM). With about 5-fold higher sensitivity than Clomeleon, this indicator has a good dynamic range at physiological intracellular concentrations (**Figure 4C**), providing a good basis for reliable monitoring of $[Cl^-]_i$ in different cell types.

Cl-Sensor demonstrates the same advantageous features as Clomeleon, i.e. excitation at visible wavelengths, good signal-to-noise ratio, safer loading procedures, absence of leakage from cells and ability to be targeted to different cell types using specific promoters.

Similarly to other fluorescent proteins from the GFP family, Cl-Sensor exhibits a relatively high pH sensitivity with pKa ranging from 7.1 to 8.0 pH units at different Cl^- concentrations.

One widespread problem with GFPs is their low transfection efficiency in neurons. To overcome this difficulty, Cl-Sensor was subcloned at two different vectors driven by mutated CMV and ubiquitine promoter. These plasmids carrying Cl-Sensor reveal higher transfection efficiency and brightness of probe in the CHO cell line, retinal cells and spinal or hippocampal neurons (Waseem et al., paper in preparation).

The Cl-Sensor was used for noninvasive estimation of $[Cl^-]_i$ in CHO cells, hippocampal neurons and photoreceptor cells from retinal slices (**Table 3**; Markova et al., 2008; Mukhtarov et al.,

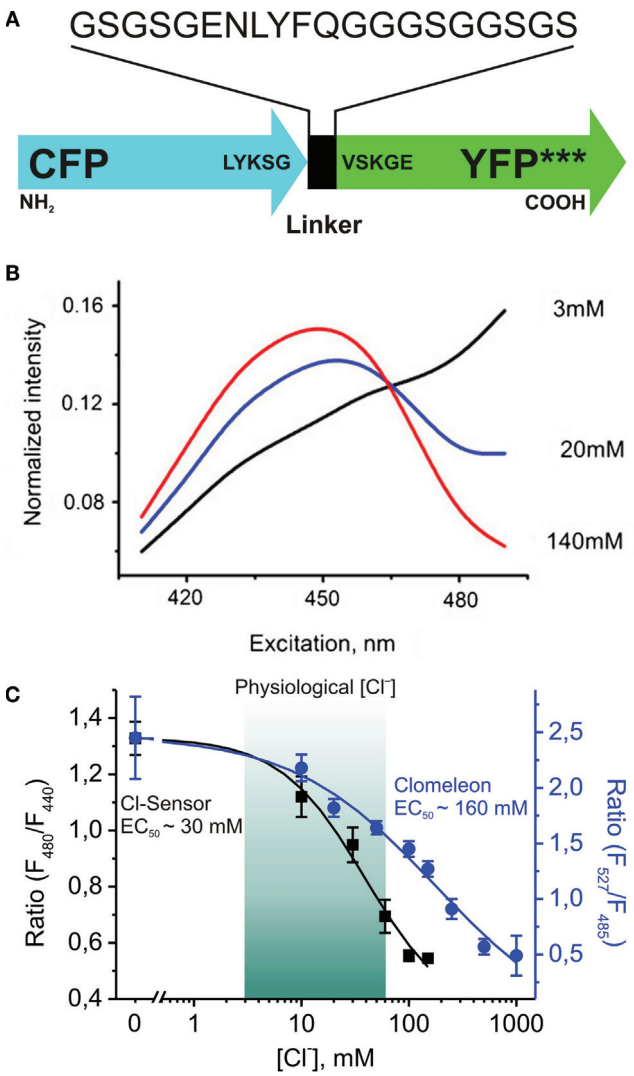


FIGURE 4 | Design and fluorescence properties of Cl-Sensor. **(A)** Schematic representation of Cl-Sensor construct; *** indicates three mutations: YFP-H148Q, -I152L and -V163S in the YFP sequence. **(B)** Normalized spectra of Cl-Sensor. Whole-cell recordings from CHO cells with pipettes containing different Cl^- concentrations (shown in the graph). Note that spectra have a common point at 465 nm. **(C)** Comparison of Cl^--sensitivities of Cl-Sensor and Clomeleon. *Cl-Sensor* (*black squares and line*): the relationship between fluorescence excitation ratio (F_{480}/F_{440}) and $[Cl^-]_i$ obtained from whole-cell recordings with pipettes containing solutions with different Cl^- concentrations (from 0 to 150 mM) (from Markova et al., 2008). *Clomeleon* (*blue circles and line*): the relationship between fluorescence emission ratio (F_{527}/F_{485}) and $[Cl^-]_i$ plotted from Kuner and Augustine, 2000 (see **Figure 4D**). Note that main part of calibration curve for Clomeleon is out of physiological range of $[Cl^-]_i$.

2008). For Cl-Sensor gene delivery to retinal cells an *in vivo* electroporation technique was used (Matsuda and Cepko, 2004; Mukhtarov et al., 2008). The efficiency of electroporation into the developing postnatal retina (at P0) was high, and transgene expression persisted for more then 1 month (Mukhtarov et al., 2008), indicating that *in vivo* electroporation of Cl-Sensor cDNA is a powerful tool for monitoring $[Cl^-]_i$ under different experimental conditions and through age-dependent changes in Cl^- in neurons.

BioSensor-GlyR – tool for monitoring Cl^--selective channel activation

Investigation of brain functioning requires methods allowing dynamic analysis of network activity combined with determination of single-cell properties. This strategy has been developed for monitoring calcium transients using rapid two-photon microscopy (Cossart et al., 2005). Chemically engineered proteins that are directly sensitive to light are also powerful optical methods of protein function control for modulation of signalling circuits inside cells and in cell circuits (Gorostiza and Isacoff, 2008). However, analysis of networks formed by neuronal circuits for specific synapses (glutamatergic, GABAergic or glycinergic) is hampered by lack of adequate techniques. This problem could be solved by genetic incorporation of molecules capable of changing their fluorescence on activation of specific synapses. The best candidates for these molecules would be fluorescently modified postsynaptic receptor-operated channels. Genetic incorporation of a molecular domain which could change fluorescence upon channel activation would provide the possibility of noninvasive monitoring of ion channel activity. Development of these molecules is a highly challenging task.

One of the approaches uses the voltage-clamp fluorometry (VCF) technique, based on covalently attaching a small environmentally sensitive sulfhydryl-labeled fluorophore to a cysteine introduced into a domain of interest on the protein. This approach has been successfully used to analyze the conformational rearrangements underlying gating of voltage-gated potassium channels (Mannuzzu et al., 1996) and ligand-gated glycine receptor (GlyR) channels (Pless and Lynch, 2008, 2009).

The other way consists of inserting a genetically encoded fluorescent sensor in the protein's sequence without changing its functional properties. Recently, a new genetic probe, termed BioSensor-GlyR, has been developed (Mukhtarov et al., 2008). This construct is a Cl^--selective GlyR channel with Cl-Sensor incorporated into the long cytoplasmic domain (**Figure 5A**).

The functioning of this modified GlyR is not perturbed by the inserted Cl-Sensor. This fact was proved in whole-cell recordings of cells expressing BioSensor-GlyR, where rapid application of glycine elicited ionic currents with kinetics similar to those of wild-type GlyR (**Figure 5B**). The main functional properties of BioSensor-GlyR, i.e. kinetics, agonist sensitivity and Cl^- selectivity, are also similar to those of the wild-type GlyR.

Application of glycine to cells expressing BioSensor-GlyR induced changes in fluorescence (**Figure 5C**). The amplitude and direction of fluorescence signals correlated with the amplitude and direction of glycine-induced currents (**Figure 5D**), demonstrating that BioSensor-GlyR is a good probe for spectroscopic monitoring of GlyR activation in live cells. The sensitivity of BioSensor-GlyR was high enough to resolve changes in $[Cl^-]_i$ induced by activation of postsynaptic receptors in glycinergic synapses. The decay kinetics of fluorescence responses were slow compared with those of ionic currents. This might be partially due to the kinetics of Cl^- binding by YFP. However, this may also reflect slow intracellular Cl^- transients, as monitoring with MQAE, the rapid quinolinium indicator, showed a similarly slow rise and decay of Cl^--dependent fluorescence in cerebellar neurons (see **Figure 2C**).

In spite of these limitations, BioSensor-GlyR is a promising tool for spectroscopic monitoring of $[Cl^-]_i$ changes in the local surroundings of glycine receptor ion channels. Development of

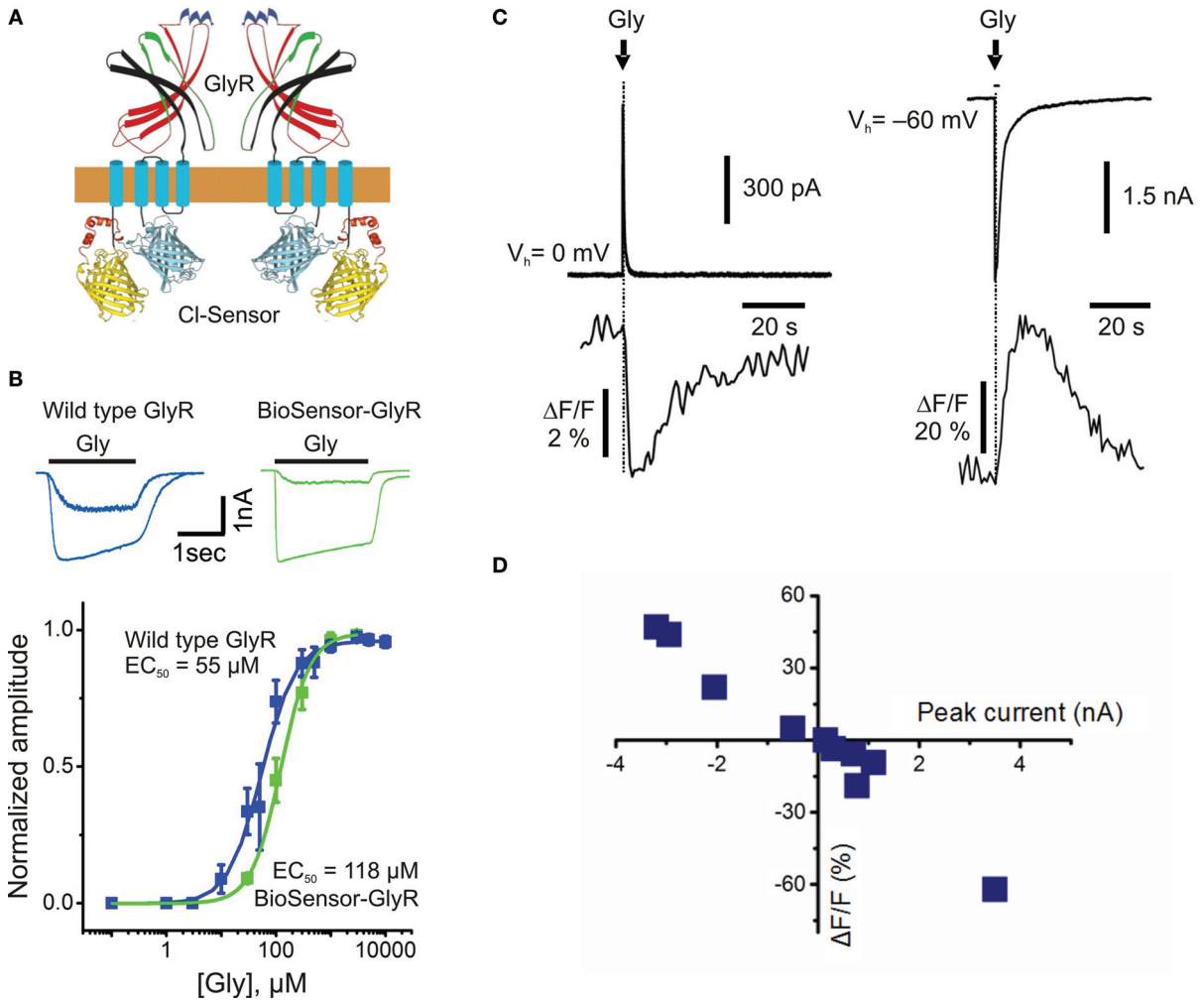


FIGURE 5 | Structure and fluorescence properties of BioSensor-GlyR. **(A)** Scheme of BioSensor-GlyR construct. Two subunits are shown. **(B)** Whole-cell currents induced by rapid application of glycine (30 or 300 μM) and dose-response curves obtained from CHO cells transfected with either wild-type human GlyR (*blue*) or BioSensor-GlyR (*green*). Note similar kinetics and agonist sensitivity for wild-type GlyR and BioSensor-GlyR. **(C)** Examples of simultaneous whole-cell and fluorescence recordings from BioSensor-GlyR transfected BHK cells with pipette containing either nominally 0 (*left traces*) or 150 mM (*right traces*) Cl^-. Glycine (1 mM) was pressure applied for 10 ms duration. **(D)** Relationship between the amplitude of glycine-induced currents and changes in fluorescence of BioSensor-GlyR at 480 nm. The amplitude of currents was regulated by the changing of V_h. (Modified from Mukhtarov et al., 2008).

transgenic animals expressing Cl-Sensor and BioSensor-GlyR will be particularly useful for studies of inhibitory neuronal networks in brain slice preparations using two-photon microscopy. For the Biosensor-GlyR it might, however, be a difficult task as expression of the GlyR, containing an additional CFP-YFP module in the long cytoplasmic loop, may modify the function of glycinergic synapses.

CONCLUSIONS

The development of imaging techniques and specific genetically encoded chloride-sensitive probes has opened new avenues for non-invasive monitoring of this ion in different cell types and cellular compartments in normal and pathological conditions. The main steps stimulating the development of these probes were as follows. First, the discovery of the halide sensitivity of YFP. Second, the production of YFP mutants exhibiting usefully high sensitivity to Cl^- at concentrations close to the physiological range of $[Cl^-]_i$. Third, the construction of molecules (Clomeleon and Cl-Sensor) consisting of two fluorescent proteins, allowing ratiometric noninvasive estimation of $[Cl^-]_i$. Finally, the incorporation of Cl-Sensor into the sequence of the Cl^--selective glycine receptor channel (BioSensor-GlyR), which opens up the ability to monitor synaptic activation of these proteins using imaging techniques. This provides an intriguing background for the development of biosensors which make it possible to monitor the activity of ionotropic GABA receptors and other Cl^--selective channels.

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Second and third generation voltage-sensitive fluorescent proteins for monitoring membrane potential

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Over the last decade, optical neuroimaging methods have been enriched by engineered biosensors derived from fluorescent protein (FP) reporters fused to protein detectors that convert physiological signals into changes of intrinsic FP fluorescence. These FP-based indicators are genetically encoded, and hence targetable to specific cell populations within networks of heterologous cell types. Among this class of biosensors, the development of optical probes for membrane potential is both highly desirable and challenging. A suitable FP voltage sensor would indeed be a valuable tool for monitoring the activity of thousands of individual neurons simultaneously in a non-invasive manner. Previous prototypic genetically-encoded FP voltage indicators achieved a proof of principle but also highlighted several difficulties such as poor cell surface targeting and slow kinetics. Recently, we developed a new series of FRET-based Voltage-Sensitive Fluorescent Proteins (VSFPs), referred to as VSFP2s, with efficient targeting to the plasma membrane and high responsiveness to membrane potential signaling in excitable cells. In addition to these FRET-based voltage sensors, we also generated a third series of probes consisting of single FPs with response kinetics suitable for the optical imaging of fast neuronal signals. These newly available genetically-encoded reporters for membrane potential will be instrumental for future experimental approaches directed toward the understanding of neuronal network dynamics and information processing in the brain. Here, we review the development and current status of these novel fluorescent probes.

Keywords: genetically-encoded voltage sensors, fluorescent proteins, fluorescence, optical imaging, neurons, patch clamp, neuronal circuit dynamics

INTRODUCTION

Significant progress in our understanding of neuronal network dynamics underlying brain function requires the ability to monitor the activity of multiple neurons simultaneously. Optical imaging based on voltage-sensitive dyes offers the spatio-temporal resolution necessary to fulfill this requirement (reviewed in Baker et al., 2005; Grinvald and Hildesheim, 2004; Grinvald et al., 1988; Knöpfel et al., 2006). These organic dyes have been successfully used during the past 20 years to report changes in membrane potential from single or large numbers of neurons in a variety of preparations, including mammalian brain tissue (Ferezou et al., 2007; Grinvald and Hildesheim, 2004). However, conventional voltage-sensitive dyes are generally not suitable for the labeling of specific cell populations. In the absence of targeting to a defined cell population, the optical signal is often drowned out either by background fluorescence from inactive cells or by signals from cells that are not the focus of interest. Moreover, unspecific staining of brain tissue prevents the unequivocal attribution of the fluorescence output signal to a defined cell population. To overcome these limitations of classical voltage-sensitive dyes, it is greatly desirable to find a way to target specific cell populations.

With the molecular cloning of green fluorescent protein (GFP) from *Aequorea victoria* (Chalfie et al., 1994) and subsequent generation of new and improved spectral variants derived from various sea organisms (reviewed in Shaner et al., 2007; Verkhusha and Lukyanov, 2004), the construction of genetically-encoded sensors for visualization of cellular dynamics became conceivable. A now broadly used approach to fluorescent biosensor engineering involves the molecular fusion of a GFP-based reporter protein to a second protein that undergoes conformational transitions in response to a physiological signal such as fluctuations in calcium or membrane potential (recently reviewed in Knöpfel et al., 2006; Qiu et al., 2008; Van Engelenburg and Palmer, 2008). Since protein-based sensors are encoded in DNA, they can be expressed under the control of cell specific promoters and introduced *in vivo* using gene transfer techniques. In a transgenic animal, a genetically-encoded voltage sensor could be expressed in practically any cell type and would have the advantage of staining only the cell population determined by the promoter used to drive the expression.

During recent years, several designs of genetically-encoded optical probes for membrane potential have been explored. FlaSh, the first prototype, was obtained by inserting GFP within the C-terminal tail of the voltage-gated Shaker potassium channel (Siegel and Isacoff, 1997). Concomitantly, our laboratory explored a FRET (Fluorescence Resonance Energy Transfer) design principle based on the voltage-dependent conformational change associated with the voltage-sensing domain of the Kv2.1 potassium channel, resulting in a voltage sensor we named VSFP1 (Sakai et al., 2001). Finally, the third prototype, SPARC, was generated by introducing GFP into a reversibly nonconducting form of the rat

μI skeletal muscle sodium channel (Ataka and Pieribone, 2002). Although these first generation fluorescent protein voltage sensors were shown to optically report changes in membrane potential, their application in mammalian systems is severely hindered by their poor targeting to the plasma membrane in transfected cells (Baker et al., 2007). Indeed, confocal microscopy analysis revealed a prominent intracellular expression for Flare (a Kv1.4 FlaSh variant), VSFP1 and SPARC with little, if any, fluorescence associated with the cell surface in both HEK293 cells and hippocampal neurons. Unfortunately, neither the mutagenesis of potential ER retention sites nor the introduction of ER export motifs has resulted in a significant improvement of the low plasma membrane expression displayed by the first generation FP voltage-sensitive probes (Baker et al., 2008). Despite this setback, the functional concept underlying VSFP1 (Sakai et al., 2001) has proven to be the most successful for the following generation of VSFPs.

SECOND GENERATION VOLTAGE-SENSITIVE FLUORESCENT PROTEINS

Recently, a self-contained voltage sensing domain (VSD) was isolated from the non-ion channel protein *Ciona intestinalis* voltage sensor-containing phosphatase (Ci-VSP) (Murata et al., 2005). Interestingly, a single VSD was shown to be functional in Ci-VSP (Kohout et al., 2008) while four VSD-containing subunits are required for the gating of the Kv potassium channel pore region (Bezanilla, 2000). Furthermore, the VSD of Ci-VSP operates as a sensor by itself since robust sensing currents were shown in the absence of the enzyme region (Murata et al., 2005). In contrast, sensing or gating currents of voltage-gated ion channels have so far been elusive if the voltage sensor is separated from the pore region (Okamura et al., 2009).

We thus reasoned that the limited cell surface targeting of first generation voltage-sensitive proteins could be resolved by using the structurally much simpler Ci-VSP scaffold and tested this hypothesis by exchanging the VSD of VSFP1 with that of Ci-VSP (Dimitrov et al., 2007; He et al., 2007). The resulting series of voltage sensors were termed VSFP2s (Dimitrov et al., 2007; Lundby et al., 2008; Mutoh et al., 2009). The initial series included the variant VSFP2.1 in which a positive arginine was mutated to a neutral glutamine at position 217 (R217Q) within the charged S4 membrane segment of the VSD. As a result of this charge neutralization, the voltage-activity curve of the native Ci-VSP showed a leftward shift to the physiological range of membrane potential fluctuations in electrically active mammalian cells (Dimitrov et al., 2007). As expected from the self-contained properties of Ci-VSP, VSFP2s displayed a predominant targeting to the plasma membrane in transfected PC12 cells. **Figure 1A** illustrates the expression and clear membrane localization of VSFP2.1, which represents a major advance when compared to the poor cell surface trafficking exhibited by the first generation FP voltage sensors (Baker et al., 2007).

VSFP2.1 responded to depolarizing voltage pulses by a decrease in cyan fluorescence and a concomitant raise in yellow florescence, resulting from increased energy transfer from the cyan to the yellow-emitting FP chromophore following the translocation of the S4 transmembrane segment (Dimitrov et al., 2007). Moreover, VSFP2.1 showed relatively fast kinetics with an apparent on-time constant of ~15 ms upon a depolarization from a holding potential of −70 to

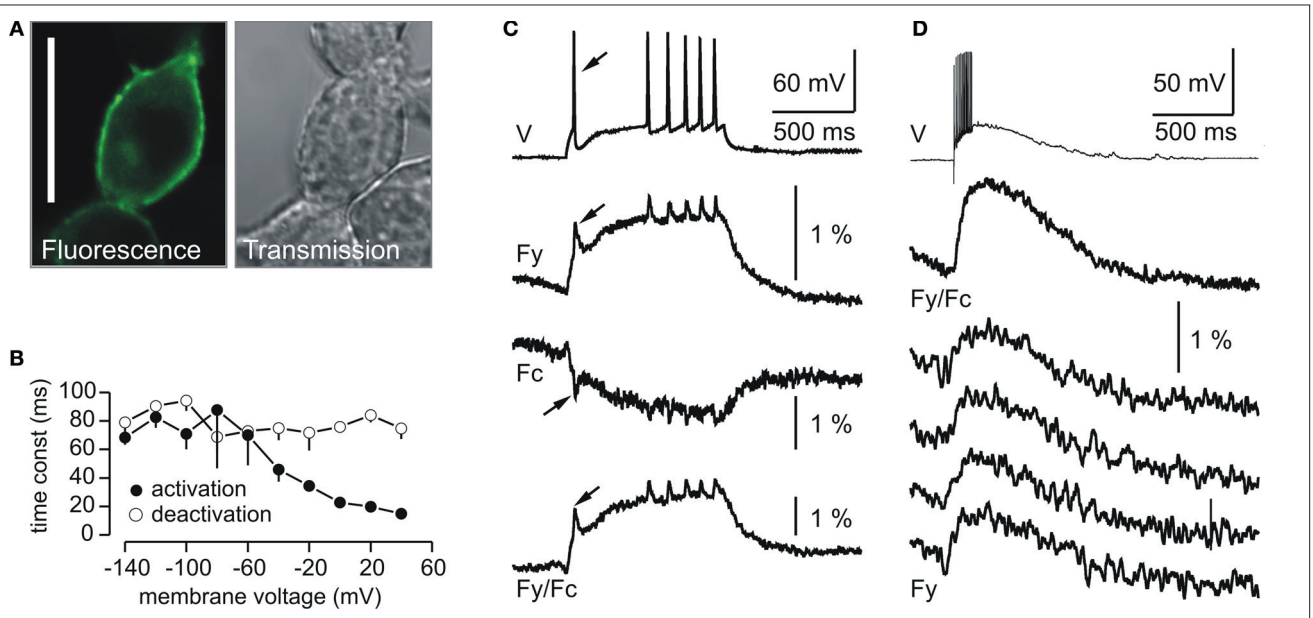

FIGURE 1 | Plasma membrane expression and fluorescence response properties of VSFP2.1. (A) Confocal fluorescence and transmission images of PC12 cells transfected with VSFP2.1. Note the targeting of VSFP2.1 to the plasma membrane. Scale bar is 25 μm. **(B)** Voltage dependence of apparent activation and deactivation time constants of VSFP2.1 fluorescence signals in PC12 cells at 35°C. **(C,D)** VSFP2.1 fluorescence responses to physiological neuronal membrane signals. VSFP2.1-expressing PC12 cells were voltage-clamped with membrane voltage traces recorded from olfactory mitral cells that were stimulated to generate a series of action potentials by direct current injection **(C)** or by electrical stimulation of the olfactory nerve **(D)**. Traces in **(C)** and upper trace in **(D)** are the average of 50 and 90 sweeps, respectively. The lower four traces in **(D)** are single sweeps. Traces show membrane potential (V), yellow fluorescence (Fy), cyan fluorescence (Fc) and the ratio of yellow and cyan fluorescence (Fy/Fc). Fluorescence signals were digitally low pass filtered (0.2 kHz) and were not corrected for photobleaching. Recordings were done at 35°C (from Dimitrov et al., 2007).

+40 mV as shown in **Figure 1B**. To investigate whether VSFP2.1 could be a candidate for optical measurements of neuronal activity, PC12 cells were voltage-clamped with membrane voltage transients recorded from olfactory mitral cells that were stimulated either by direct current injection to generate a series of action potentials (**Figure 1C**) or synaptically to induce a burst of fast action potentials (**Figure 1D**). Fluorescence traces revealed that VSFP2.1 could clearly resolve individual action potentials as well as the underlying membrane depolarization (**Figure 1C**). However, VSFP2.1 mainly reported the slow components of these voltage transients, as expected from its response kinetics (Dimitrov et al., 2007). Indeed, the optical readout of the fast action potentials was significantly reduced relative to the slower component of the membrane potential change. This phenomenon was also clearly noticeable when using the membrane voltage transients from a mitral cell succeeding a single shock electrical stimulation of the olfactory nerve (**Figure 1D**). Most importantly, it should be noted that the responses shown in **Figure 1D** could be resolved in single sweeps. Optimizing the length of the amino acid linkers connecting either the donor chromophore to the VSD or the FRET donor/acceptor pair in VSFP2.1 resulted in VSFP2.3 with both improved fluorescence response kinetics and FRET efficiency (D. Dimitrov et al., unpublished; Akemann et al., 2009; Lundby et al., 2008; Mutoh et al., 2009; Villalba-Galea et al., 2008, 2009).

Spectral variants of VSFP2.1

Cyan- and yellow-emitting variants of GFP from *A. Victoria* are most often used as a fluorescent reporter component for FRET-based sensors. However, the photophysical properties of this FP pair are less than ideal for FRET imaging since both chromophores have broad emission/absorption spectra with relatively small Stokes shift (Chapman et al., 2005) and considerable FRET donor emission within the acceptor emission band. Furthermore, red-shifted emitting variants would ultimately yield a higher signal-to-noise ratio if tissue autofluorescence is an issue. Indeed, red fluorescence should provide better spectral separation from the intrinsic green autofluorescence of brain tissue given that the fluorescence of flavins, vitamins and NADPH is considerably lower in the red region of the spectrum than in the blue-green region. Additionally, long-wavelength light is usually associated with reduced phototoxicity and deeper penetration into biological tissue. To this aim, we generated a red-shifted VSFP2.1 variant comprising a pair of yellow and far-red emitting FPs that we termed VSFP2.4 (Mutoh et al., 2009). The absorption spectrum of the acceptor, mKate2, shows considerable overlap with the emission spectrum of the donor, Citrine, with a calculated Förster distance of 5.82 nm (Mutoh et al., 2009). Furthermore, both spectra are well enough separated to allow independent excitation of the chromophores, limiting spectral bleed through from the donor emission into the acceptor channel. In parallel, Tsutsui et al. (2008) reported another VSFP2.1 spectral variant based on FPs isolated from corals (mUKG and mKOκ) referred to as Mermaid. The conceptual design of the original series of VSFP2s and its color variants is illustrated in **Figure 2A**.

Quantitative comparison of these three most advanced FRET-based voltage probes (VSFP2.3, VSFP2.4 and Mermaid) revealed relatively similar steady state spectrally-resolved maximal change in fluorescence (ΔR/R) upon a depolarization from −100 to +40 mV (13.3 ± 3.4, 12.4 ± 1.0 and 12.9 ± 4.8% for VSFP2.3, VSFP2.4 and Mermaid, respectively) (Mutoh et al., 2009). Acceptor and donor fluorescence signals in response to voltage steps from a holding potential of −70 mV to test potentials of −140 to +60 mV are shown in **Figure 2B**. Likewise, VSFP2.1 spectral variants displayed comparable voltage dependencies ($V_{1/2}$ = −54.2 mV, $V_{1/2}$ = −54.2 mV and $V_{1/2}$ = −43.6 mV for VSFP2.3, VSFP2.4 and Mermaid, respectively). Upon depolarization from a holding potential of −70 mV, all three sensors exhibited fluorescence signals that could be fitted with two main time constants (**Table 1**; Akemann et al., 2009; Lundby et al., 2008; Mutoh et al., 2009; Tsutsui et al., 2008; Villalba-Galea et al., 2009) that likely correspond to the conformational transition states of Ci-VSP (Villalba-Galea et al., 2008). The values for these on-time response components were very similar except that the fast on-time constant contributed to a larger fraction of the total signal in VSFP2.4 when compared to Mermaid (40 ± 4 and 23.5 ± 5% at +60 mV, respectively) (Mutoh et al., 2009). The off-time kinetics did not differ among the VSFP2.1 variants under this assay protocol. The fluorescence response properties of the VSFP2.1 variants detailed above are summarized in **Table 1**.

To validate the expression pattern of the latest FRET-based voltage-sensitive probes in neurons, we transfected primary hippocampal neurons after 6 days of culture with VSFP2.3, VSFP2.4 and Mermaid and evaluated them by confocal fluorescence imaging 1 week later. As shown in **Figure 3**, VSFP2.3, VSFP2.4 and Mermaid fluorescence was distributed over the cell body, dendrites and axons of a variety of neurons including pyramidal cells.

In particular, VSFP2.3 showed efficient targeting to the plasma membrane as indicated by arrows in magnified views. Likewise, VSFP2.4 fluorescence was mainly found at the cell surface while some fluorescence was also observed intracellularly within a juxtanuclear trans-Golgi network-like structure (arrowheads) which is likely involved in endosome trafficking. Mermaid was also in part targeted to the plasma membrane but the extent of membrane-associated fluorescence was largely overwhelmed by much stronger fluorescence derived from structures reminiscent of intracellular vesicles as indicated by arrowheads in **Figure 3**. These punctuate structures have previously been reported for fluorescent proteins isolated from reef coral anthozoan species which are known for their high tendency to form aggregates (Hirrlinger et al., 2005; Katayama et al., 2008). Indeed, bright fluorescent clusters were observed in cell somata and processes of a series of transgenic mouse lines expressing various reef coral FPs within early postnatal weeks, which were shown to increase substantially with the age of the animal (Hirrlinger et al., 2005). In contrast, fluorescent aggregates were not detected in mice expressing *A. victoria* GFP variants even at older ages (Hirrlinger et al., 2005; Nolte et al., 2001). Since the establishment of transgenic mice expressing VSFP2s requires long-term expression of the reporter proteins, VSFP2.3 and VSFP2.4 would likely be better candidates due to their reduced intracellular accumulation and aggregation (**Figure 3**). Accordingly, with the relatively small signal amplitude of these second generation FP voltage sensors, optimal responses are largely dependent on proper trafficking to the plasma membrane since intracellular expression principally contributes to background fluorescence, decreasing the signal-to-noise ratio ($R_{S/N}$) significantly.

In order to investigate the relationship between VSFP activation kinetics and VSFP-mediated optical report of neuronal activity, we

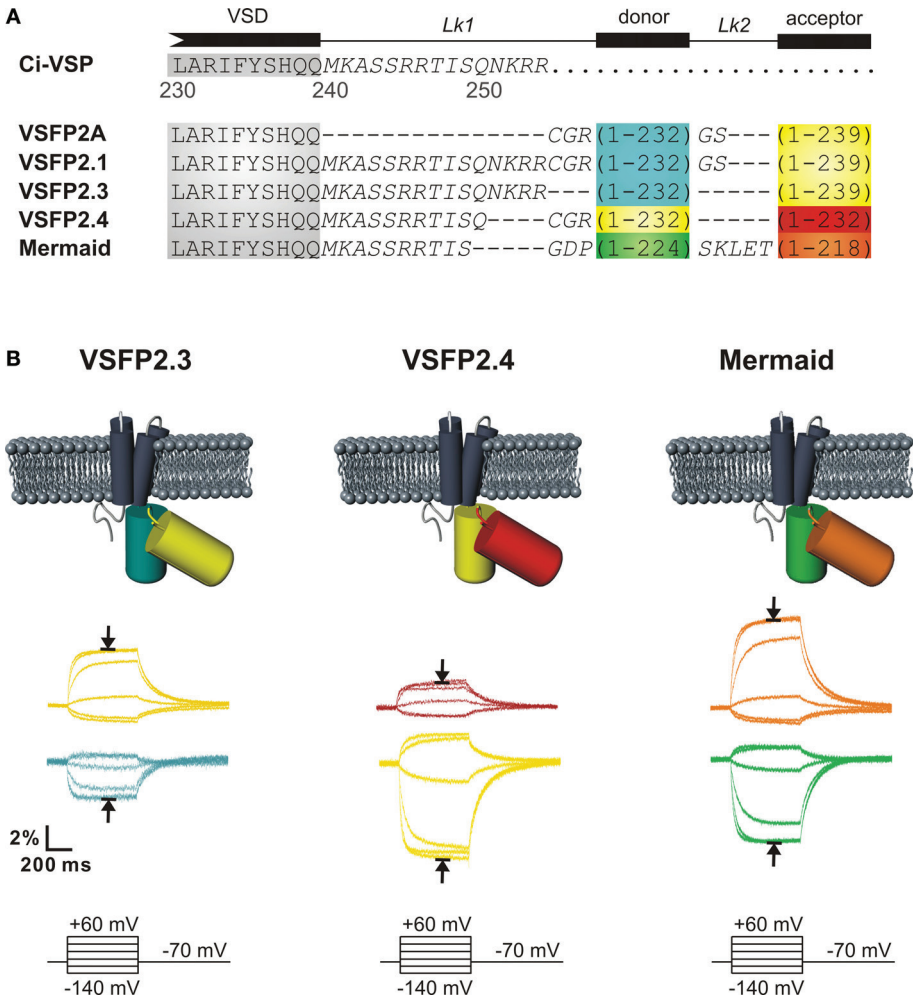

FIGURE 2 | Second generation voltage-sensitive fluorescent proteins. **(A)** Alignment of the amino acid sequences of VSFP2.1 variants. The C-terminal residues and downstream segment (240–254) of the VSD of Ci-VSP are shown in gray and italic text, respectively. FPs are mCerulean (blue), Citrine/mCitrine (yellow), mKate2 (red), mUKG (green) and mKOκ (orange). **(B)** Acceptor (upper color traces) and donor (lower color traces) signals of PC12 cells in response to a family of 500 ms voltage steps from a holding potential of –70 mV to test potentials of –140 to +60 mV recorded at 35°C. Traces corresponding to a depolarization to +60 mV are indicated by arrows (from Mutoh et al., 2009).

Table 1 | Fluorescence response properties of VSFP2.1 variants. Steady state spectrally-resolved maximal changes in fluorescence acquired from voltage-clamped PC12 cells. The $\Delta R/R$ values represent the average of 14–37 measurements from hyperpolarized (–100 mV) to depolarized (+40 mV) membrane potential from five representative cells (data are from Mutoh et al., 2009).

	$\Delta R/R$ (%)	τ_{on} (to +60 mV, ms)	τ_{off} (from +60 mV for 500 ms) (ms)	Steady state $V_{1/2}$ (mV)
VSFP2.3	13.3 ± 3.4	1.9 ± 0.9 and 24.5 ± 0.3	78.4 ± 0.5	–49.5 ± 1.1
VSFP2.4	12.4 ± 1.0	2.3 ± 0.2 and 24.2 ± 0.2	72.2 ± 0.4	–54.2 ± 2.0
Mermaid	12.9 ± 4.8	3.2 ± 0.2 and 24.8 ± 0.3	76.4 ± 0.4	–43.6 ± 1.0

represented VSFP2.3 and VSFP2.4 by an eight state Markov process kinetic model reflecting their experimental response properties (**Figures 4A1,B1**; see Akemann et al., 2009).

Inclusion of these kinetic models into a realistic conductance-based computational version of a rat somato-sensory layer 5 pyramidal neuron given by Mainen and Sejnowski (1996) enabled us to predict *in silico* the possible VSFP fluorescence readouts that would be obtained from neuronal activity (Akemann et al., 2009). The simulations indicated that the second generation VSFPs can provide an activation mechanism sufficiently fast to track burst firing

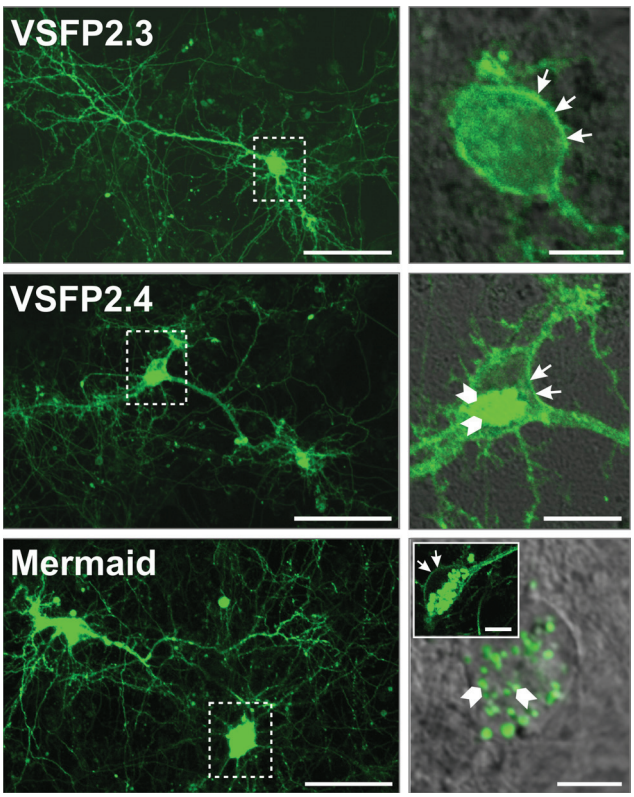

FIGURE 3 | Expression pattern of VSFP2.1 variants in transfected hippocampal neurons. Primary hippocampal cultures derived from mouse E18 embryos were transfected with either VSFP2.3, VSFP2.4 or Mermaid 6 days after plating and imaged by confocal fluorescence microscopy 1 week later. Fluorescence was allowed to saturate locally to optimize the visualization of neuronal processes. Boxed areas in the left panel are shown magnified on the right panel. Arrows indicate cell surface expression while arrowheads show intracellular expression. Note the targeting of VSFP2.3 and VSFP2.4 to the plasma membrane and Mermaid-associated intracellular aggregates in magnified views. The insert in the lower right image shows an example of a cell with clear expression of Mermaid at the cell surface. Scale bars are 50 and 10 μm for left and right panels, respectively.

of action potentials albeit with significant attenuation as shown in **Figure 4**, wherein individual action potentials were resolved as narrow peaks superimposed on a slowly rising (VSFP2.3; **Figure 4A2**) or declining (VSFP2.4; **Figure 4B2**) curve representing the activation state of both voltage reporters. Additionally, simulation of VSFP2.3 and VSFP2.4 optical readouts in response to a subthreshold or suprathreshold activation of a distal synaptic conductance (causing an excitatory postsynaptic potential, EPSP) showed that both FP voltage sensors are able to readily resolve synaptic potentials in individual L5 pyramidal neurons (**Figures 4A3,B3**). However, as the fluorescence response properties of VSFP2s to membrane potential changes consist of a slow and fast kinetic response with time constants differing by more than an order of amplitude (Lundby et al., 2008; Mutoh et al., 2009; Villalba-Galea et al., 2009), the optical resolution of any membrane voltage transient rising faster than the slow kinetic component will critically depend on the relative contribution of the slow versus fast component of the VSFP activation response.

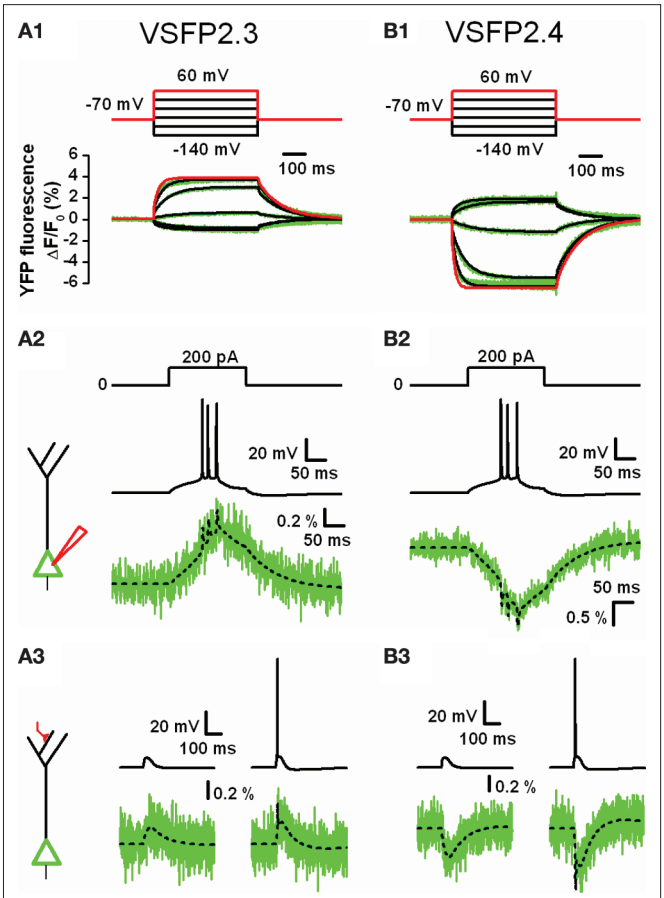

FIGURE 4 | Computer simulations of VSFP2.3 and VSFP2.4 optical response signals in cortical layer 5 (L5) pyramidal neurons. (A1) Family of YFP fluorescence ($\Delta F/F_0$) responses (lower panel; green traces) to 500 ms voltage steps (upper panel) from a holding membrane voltage of –70 mV to test potentials of –140 to +60 mV recorded from voltage-clamped PC12 cells expressing VSFP2.3. Overlaid to the experimental traces are simulated traces obtained from a VSFP2.3 model wherein the kinetics are represented as an eight state Markov process as given in (Akemann et al., 2009). The simulated response to +60 mV is highlighted in red. **(A2)** Predicted VSFP2.3 fluorescence signal ($\Delta F/F_0$; lower panel) in response to an action potential burst (middle panel) in the cell body of a simulated L5 pyramidal neuron evoked by constant current injection (upper panel). The schematic to the left depicts the neuron with a point current source attached to the cell body (red electrode). The electrical response was simulated using a conductance-based model of a reconstructed rat L5 neuron given by Mainen and Sejnowski (1996). VSFP2.3 was homogenously inserted within the membrane (all 177 compartments) at a density of 800 units/μm^2. The fluorescence signal (lower panel) represents the response of VSFP2.3 in the somatic membrane with (green) or without (dashed black) photon quantum noise calculated assuming a sampling rate of 2 kHz and excitation light level that bleaches GFP within 10 s of illumination. **(A3)** VSFP2.3 fluorescence signal ($\Delta F/F_0$; lower panel) as predicted by the simulation in response to a subthreshold (left column) or suprathreshold (right column) activation of a distal synaptic conductance (schematically depicted to the left with the activated synapse in red). The voltage signal in the cell body (upper row left: EPSP; upper row right: EPSP plus evoked action potential) together with the associated fluorescence signals (lower row), with (green) or without (dashed black) photon quantum noise are shown. **(B1–B3)** Same as in **(A1–A3)**, but using a model of VSFP2.4 instead of VSFP2.3. VSFP2.4 was simulated as an eight state Markov chain model analogously to VSFP2.3. For methodological details see Akemann et al. (2009).

THIRD GENERATION VOLTAGE-SENSITIVE FLUORESCENT PROTEINS

To address whether the relatively slow fluorescence response kinetics of the second generation FP voltage probes is due to intrinsically slow operations of Ci-VSP, we measured fluorescence signals along with sensing currents (i.e. currents resulting from the displacement of charges within the VSD) in VSFP2.3-expressing PC12 cells and found that the voltage dependency of the fluorescence read-outs closely resembles the activation curve of the sensing currents, indicating that the fluorescence signal effectively reports the voltage-dependent conformational change of the VSD (Lundby et al., 2008). However, the sensing charge movement was found to be two orders of magnitude faster than the dominant slow component of the fluorescence response (~1 versus ~100 ms), suggesting a relatively weak coupling between the VSD and the VSFP2 class reporter proteins (Lundby et al., 2008).

Already during the analysis of our initial series of Ci-VSP-based VSFP2s, we noted that the ratio between the CFP and YFP signal components increases when the length of the linker connecting the VSD to the donor chromophore is shortened (Dimitrov et al., 2007). Furthermore, a short linker version of VSFP2.1 (VSFP2A R217Q) clearly exhibited a fast on-time component in the CFP response which was not observed in the YFP channel (CFP: 7 ± 2 and 180 ± 15 ms, YFP: 171 ± 15 ms; **Figure 5A**), suggesting the presence of a FRET-independent response component (Lundby et al., 2008). To test this hypothesis, we measured the response characteristics of VSFP2A(R217Q) before and after photobleaching of the acceptor chromophore (YFP) as illustrated in **Figures 5A,B**, respectively. Single cell spectrofluorometry confirmed the disappearance of the 530-nm peak in the emission spectrum and revealed an increase in CFP emission at 470 nm due to donor dequenching (**Figure 5B**) (Lundby et al., 2008). Most importantly, a significant signal remained in the cyan channel after acceptor photobleaching (**Figure 5B**, lower panel), indicating that the fast CFP response component does not require the presence of a FRET acceptor. To investigate whether the removal of YFP would favor the fast intrinsic CFP response further, we generated a novel Ci-VSP-based voltage sensor comprising a single cyan fluorescent reporter protein that we termed VSFP3.1. Interestingly, VSFP3.1 responded to voltage steps with a very fast initial transient (activation time constant of 1.3 ± 0.1 ms at +70 mV; **Figure 5C**), indicating that truncation of the acceptor chromophore significantly improved the coupling of the VSD to the fluorescent reporter protein. Indeed, the fluorescence output of VSFP3.1 showed a dominant fast response component matching closely the sensing currents of Ci-VSP (Lundby et al., 2008; Villalba-Galea et al., 2009).

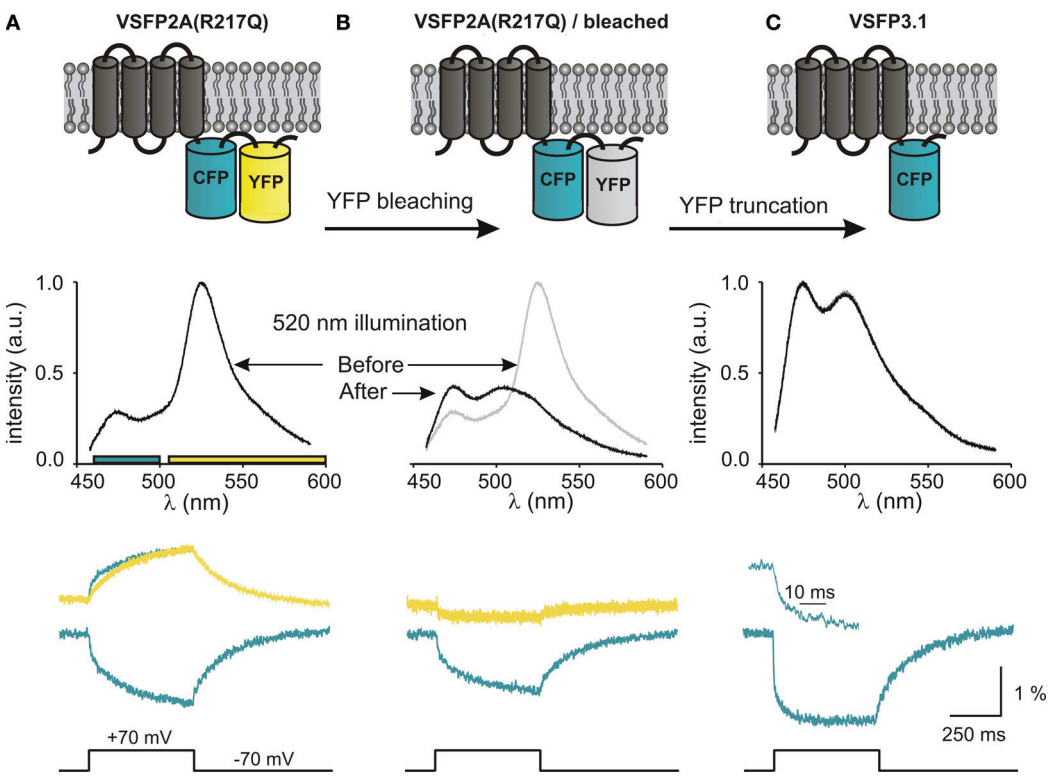

FIGURE 5 | Development of third generation voltage-sensitive fluorescent proteins. The membrane topology of VSFP2A(R217Q) **(A)**, VSFP2A(R217Q) after acceptor photobleaching **(B)** and VSFP3.1 **(C)** is illustrated in top panels. Emission spectra recorded for each constructs using 440-nm excitation light are shown below. The lower panel shows the depolarization-induced fluorescence signals recorded in the yellow and cyan channels. For VSFP2A(R217Q), a scaled mirror image of the cyan signal is shown aligned with the yellow signal; note the fast CFP component. For VSFP3.1, the top trace represents the onset of the fluorescence signal at a magnified time scale (from Lundby et al., 2008).

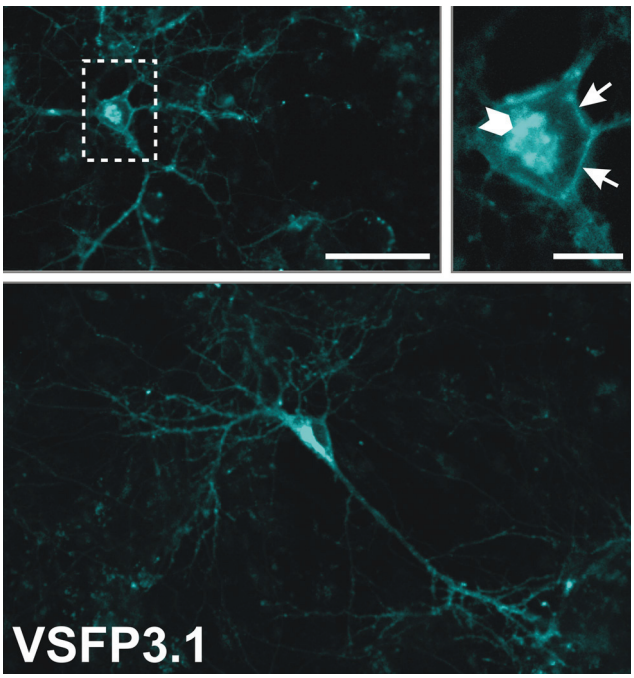

FIGURE 6 | Expression pattern of VSFP3.1 in cultured mouse hippocampal neurons. Primary hippocampal cultures were transfected with VSFP3.1 6 days after plating and confocal images were taken 6 days later. Overviews and magnified views of VSFP3.1 expression in transfected hippocampal neurons are shown. The boxed region in the left panel is shown magnified on the right panel. Arrows indicate plasma membrane expression whereas the arrowhead shows intracellular expression. Scale bar is 50 μm (upper left and lower panel) and 10 μm (upper right).

As shown in **Figure 6**, VSFP3.1 was efficiently targeted to the plasma membrane of transfected hippocampal neurons with a pattern similar to that of VSFP2.3 and VSFP2.4 (**Figure 3**). We anticipate that VSFP3.1 and its color variants will facilitate improved optical measurements of fast neuronal signals.

FUTURE DIRECTIONS

In this review, we have focused on the most advanced genetically-encoded voltage sensors reported thus far based on a fusion between the voltage-sensing domain of Ci-VSP and a FP reporter module comprising either a fluorescent protein FRET pair or a single FP.

Despite comparable amplitude and kinetics of the voltage-dependent fluorescence response of the VSFP2.1 spectral variants described in this review, each of these fluorescent probes has particular advantages when considering potential applications. For instance, VSFP2.3 is a suitable sensor for instrumentation with standard optical components since it is based on the most commonly used FRET pair. On the other hand, the spectral properties of VSFP2.4 are strongly preferable for either *in vivo* imaging due to the elimination of green/yellow autofluorescence or deep tissue imaging using two-photon excitation fluorescence microscopy. Finally, Mermaid is, in principle, a good candidate for ratiometric measurements due to the good dynamic range of the donor and acceptor fluorescence responses. However, the tendency of the FPs used in Mermaid to form bright fluorescent aggregates may severely limit the usefulness of this variant.

To our knowledge, VSFP3.1 is the fastest FP voltage sensor reported to date, exhibiting an activation time constant matching that of fast neuronal signals, which makes this single-FP voltage sensor a promising candidate for the generation of transgenic animals. However, VSFP2-type sensors have practical advantages over single color variants of the VSFP3 class. Indeed, FRET-based voltage sensors provide signals at two different colors with opposite polarity. This feature enables ratiometric measurements and thereby, at least theoretically, absolute calibration of membrane voltage. Furthermore, ratiometric measurements are less sensitive to movement artifacts. Versions like VSFP2.3 that exhibit a large baseline FRET efficacy may also be used monochromatically by exciting CFP and recording YFP fluorescence, in which case the larger separation of excitation and emission wavelengths can have practical advantages over single color variants of the VSFP3 class.

Current work aims at characterizing the performance of these voltage-sensitive fluorescent protein probes in differentiated neurons, which will undeniably constitute an important step towards the realization of an optical sensor for neuronal circuit activity. Furthermore, expression of voltage-sensitive fluorescent proteins under cell-specific promoters will allow the labeling of defined neuronal populations within assemblies composed of diverse cell types (Diez-Garcia et al., 2005, 2007; Metzger et al., 2002; Qiu et al., 2008).

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The use of lentiviral vectors and Cre/loxP to investigate the function of genes in complex behaviors

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The use of conventional knockout technologies has proved valuable for understanding the role of key genes and proteins in development, disease states, and complex behaviors. However, these strategies are limited in that they produce broad changes in gene function throughout the neuroaxis and do little to identify the effects of such changes on neural circuits thought to be involved in distinct functions. Because the molecular functions of genes often depend on the specific neuronal circuit in which they are expressed, restricting gene manipulation to specific brain regions and times may be more useful for understanding gene functions. Conditional gene manipulation strategies offer a powerful alternative. In this report we briefly describe two conditional gene strategies that are increasingly being used to investigate the role of genes in behavior – the Cre/loxP recombination system and lentiviral vectors. Next, we summarize a number of recent experiments which have used these techniques to investigate behavior after spatial and/or temporal and gene manipulation. These conditional gene targeting strategies provide useful tools to study the endogenous mechanisms underlying complex behaviors and to model disease states resulting from aberrant gene expression.

Keywords: lentivirus, gene therapy, fear, PTSD, amygdala, hippocampus, inducible knockout

INTRODUCTION

The use of conventional knockout (KO) technologies has provided valuable information in identifying the role of key genes in neural development and plasticity. While these methods have been of value, they are often accompanied by a number of limitations that can restrict their usefulness. For example, if a gene of interest is essential for development, then gene KO can lead to gross developmental abnormalities or lethality that either preclude or complicate studies on adult animals (e.g., Asada et al., 1997). Developmental defects and compensatory changes caused by mutations often make it difficult to assess whether phenotypic consequences result from the lack of normal gene expression in the adult animal or the lack of the normal expression during development. In addition, conventional methods eliminate the gene of interest in peripheral tissues as well as the central nervous system, and thus are not limited to alterations of brain molecular functions. Similarly, KO strategies produce broad changes in gene function throughout the neuroaxis and do little to identify the effects of such changes on specific brain regions or neural circuits thought to be involved in distinct functions. As a consequence, the complete deletion of a specific protein throughout the nervous system may prove ineffective toward understanding fine molecular processes in higher brain functions.

Cre/LoxP RECOMBINATION SYSTEM

To overcome these drawbacks, researchers are increasingly making use of conditional gene manipulation strategies, which have the advantage of allowing temporal and spatial deletion of a gene of interest (Gaveriaux-Ruff and Kieffer, 2007). One such approach is the Cre/loxP recombination system. The Cre/LoxP technique was first reported by Rajewsky and colleagues to KO the *pol beta* gene that encodes the DNA polymerase beta in T lymphocytes (Gu et al., 1994), but has since been utilized as a powerful and popular approach to understanding the genetic and molecular basis of higher brain functions and their role in animal behavior models. Generally, the system requires crossbreeding of two lines of mice. One line carries a transgene that encodes the *Cre recombinase* gene under the control of a cell-specific promoter. Cre recombinase is an enzyme that catalyzes the site-specific, irreversible cleavage of DNA segments flanked by unique loxP sequences. The second line carries the target gene of interest bordered by the two requisite loxP recognition sites (Sauer and Henderson, 1988; Tsien et al., 1996). Each loxP site consists of two 13-bp palindromic sequences flanking an 8-bp core sequence. They are typically located in intronic sequences bordering exon(s) of the target gene to preserve gene transcription prior to deletion. Mice homozygous for this conditional 'floxed' (*f*lanked by *lox*P) allele display a wild-type phenotype in the absence of Cre recombinase. However, crossbreeding with a Cre-expressing mouse line produces a null allele after Cre-mediated recombination.

In the Cre recombinase mouse, the spatio-temporal pattern of Cre activity can be assessed *in vivo* by using traditional immunohistochemistry or *in situ* hybridization for Cre recombinase or using sensitive Cre reporter mouse lines. Many reporter strains use different promoters to express lacZ, green fluorescent protein (GFP) or other detectible proteins in the presence of Cre recombinase (van der Neut, 1997; Lobe et al., 1999; Mao et al., 2001; Srinivas et al., 2001; Morozov et al., 2003; Morozov, 2008). It is important to note that there is great variability between the sensitivity of these reporter lines. If the reporter protein is not expressed in a particular cell-type, it does not prove that Cre is not expressed or that it would not be active on a different gene locus. Despite these limitations, this approach has proved quite successful in a large number of studies to date.

One commonly used reporter is the floxed-stop lacZ reporter mice (Soriano, 1999).These mice possess a transgene integrated into the ROSA26 locus which consists of a floxed-stop sequence upstream of a lacZ gene (Gardner et al., 1996; Takahashi et al., 2000). When these RosaLacZ mice are crossed with Cre-expressing transgenic mice, the Cre-mediated excision of the floxed-stop sequence leads to lacZ expression. The expression of LacZ in these animals serves as an extremely sensitive measure, with very low background expression levels. In these animals, Cre expression can be detected by beta galactosidase (β-gal) immunohistochemistry, utilization of the β-gal colorimetric substrate X-gal, or by *in situ* hybridization.

GENE MANIPULATION USING MICE WITH FLOXED GENES OF INTEREST CROSSED WITH Cre-EXPRESSING LINES

In recent years numerous mouse lines that carry a Cre recombinase transgene have been engineered (see, Morozov et al., 2003). Cre-expressing lines can be made both with traditional transgene technology as well as with knock-in lines that use the homologous recombination technique. In this case a Cre cassette is placed downstream of the endogenous gene promoter at the specific locus of that gene. In mice engineered using such an approach, the expression of Cre is consistent with what is known about the spatio-temporal expression pattern of the endogenous gene (e.g., Zhuang et al., 2005). A number of studies investigating learning and memory in mice have utilized the Emx1-Cre and the CaMKII-Cre transgenic knock-in lines (Guo et al., 2000; Casanova et al., 2001). Both Emx1 and CaMKII-promoter driven Cre expression cause effective deletion in the cerebral cortex, hippocampus and other forebrain regions. When these transgenic Cre-expressing mice are crossed with Cre-sensitive reporter lines, such as the Rosa26 line containing lacZ flanked by LoxP, the high-efficiency and specificity of the expression pattern is observed. When these Cre-expressing mice are crossed with such 'floxed' mice, the targeted gene is effectively deleted throughout the cerebral cortex, hippocampus and other forebrain regions that are specific to the promoter driving Cre. Although there are many advantages to these knock-in approaches, there are also several drawbacks. One limitation is the risk of haploinsufficiency when Cre recombinase replaces the gene of interest, thus leading to mice that express only a single allele of the targeted gene. One way of solving this issue has been to utilize the 'internal ribosomal entry site' (IRES) sequence, which was originally described from the picornavirus (Zhu et al., 2001). This sequence, when placed between two coding sequences of DNA allows for co-expression of both genes off of a single transcribed mRNA. Thus, a knock-in may place an IRES-Cre or other expression marker downstream of the gene of interest, which in some cases solves the issue of haplo-insufficiency allowing equivalent expression of both genes from the single endogenous promoter.

Alternatively, a line can be engineered by pronuclear injection of a fusion construct in which the Cre coding sequence is inserted downstream of a cell-specific promoter. In this approach, the transgene will be integrated into the genome randomly, thus the pattern of Cre expression, with some insertion events, may be somewhat different from that of the promoter's endogenous gene pattern. Of note, these transgenic approaches can result in more than one insertion event as well as position effects on gene expression specificity and efficiency. Homologous recombination, 'Knock-in' strategies are significantly more effortful to produce, but are generally more assured of the expected expression pattern.

With Cre transgenic lines, as with Cre knock-in lines, genetic excision occurs as soon as the promoter driving Cre expression is active and may trigger developmental compensations if the promoter is active during embryogenesis. As an example, Chhatwal et al. (2007) recently engineered a Cre-expressing line by subcloning the Cre coding sequence downstream of a promoter of the gene for cholecystokinin (CCK). The resulting plasmid (CCID) was linearized, purified and microinjected into the pronuclei of one-cell embryos, which were then implanted into pseudo-pregnant females to create a number of transgenic CCK-Cre driver mouse lines. When these animals were mated to Rosa-LacZ reporter mice, animals carrying both the CCID and Rosa-LacZ transgenes show varying expression patterns of β-gal in areas where endogenous CCK is expressed **Figure 1**. Similarly, when the neocortex-specific CCID transgenic line was crossed with a floxed-stop GFP reporter line (Mao et al., 2001; Muzumdar et al., 2007), animals carrying both transgenes show GFP expression (limited to the neocortex with no expression in other areas like the hippocampus, thalamus, striatum, hypothalamus or amygdala; **Figure 1**).

LENTIVIRAL VECTORS AS TOOLS TO RESTRICT TRANSGENE EXPRESSION

Because the molecular functions of proteins often depend on the specific neuronal circuit in which they are expressed, restricting gene manipulation to specific regions and time may be a more useful in understanding the function of genes in some complex behaviors. In such cases, the use of lentiviral vectors to deliver transgenes is a powerful tool. Lentivirus vectors integrate into both dividing and post-mitotic cells, generate little or no immune response, and express transgenes stably over several months (Naldini et al., 1996; Lai and Brady, 2002). Currently used HIV-derived lentiviruses contain only the necessary elements for gene transduction and include self-inactivating elements, which significantly improve the biosafety of vectors and greatly reduces the formation of replication competent viruses (Zufferey et al., 1998; Abordo-Adesida et al., 2005). Furthermore, in comparison to adeno-associated- and sindbis-based vectors, lentiviral vectors have a larger insert capacity. Thus, large transgenes can be subcloned into lentivirus vectors enabling the expression of genes encoding neurotransmitters (Azzouz et al., 2002), neuropeptides (Keen-Rhinehart et al., 2009), mutated receptors (Rattiner et al., 2004a; Chhatwal et al., 2006), small interfering RNAs (Tiscornia et al., 2003), and promoters for the use of transgene expression in selected populations of cells (Chhatwal et al., 2007). Lastly, lentiviral vectors, as compared to transgenic and knock-in mouse approaches, provide researchers with the choice of using rats or a variety of mouse strains at different ages. Note that age of the animal at the time of gene deletion may be particularly important in particular experimental designs or models.

For many of these reasons, the use of lentiviral vectors to deliver Cre recombinase is a useful alternative to the use of transgenic Cre-expressing mouse lines. Cre-expressing lentiviruses can be injected in a localized fashion directly into the brain of floxed mice, and the extent of the conditional genetic deletion will be restricted to the area of viral spread. For example, viral vectors

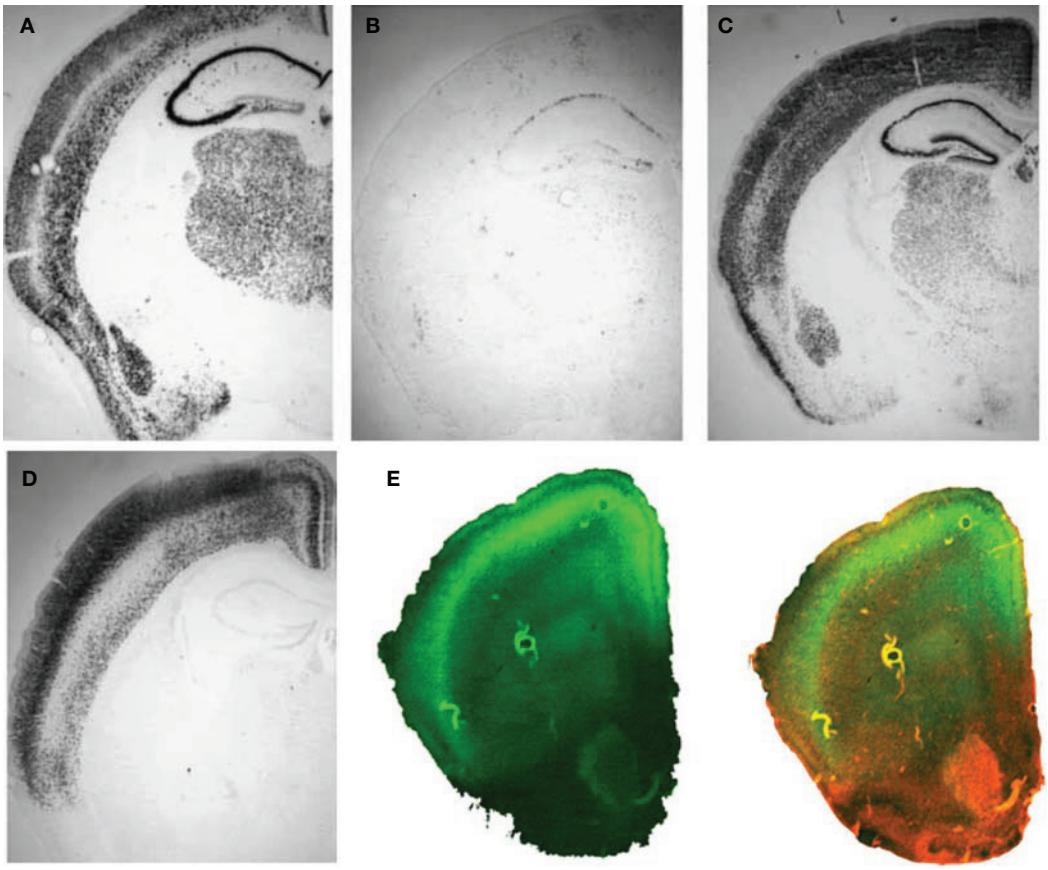

FIGURE 1 | Cre recombinase expressing transgenic mice. A DNA template in which the coding sequence for Cre recombinase was placed under the control of the CCK promoter (CCID) was used to create transgenic mouse lines. Three founder animals, with distinct transgene insertion sites, were generated and bred to RosaLacZ animals to assess Cre expression as revealed by LacZ staining. **(A)** Normal patterns of CCK mRNA expression, revealed with *in situ* hybridization for CCK mRNA. **(B)** Animals from transgene line A show low levels of Cre expression, but in appropriate CCK-specific regions. **(C)** Animals from line B relatively widespread expression, which was, with the exception of the dentate, similar to endogenous CCK mRNA expression. **(D)** Animals from line C showed high levels of Cre expression in the cortex, in a manner that was qualitatively similar to observed patterns of CCK mRNA expression but with virtually no Cre expression in hippocampus or subcortical regions. **(E)** Animals from transgenic line C were crossed with Td tomato/floxed stop GFP, to assess Cre mediated GFP expression. These mice express strong red fluorescence in all tissues and cell types examined. When bred to Cre recombinase expressing mice, the resulting offspring have the stop cassette deleted in the Cre expressing tissue, deleting Td tomato expression and allowing expression of the membrane-targeted GFP (red = Td tomato, green = eGFP, blue = DAPI; Left – GFP expression, Right – merged GFP + TdTomato expression). Panels **(A–D)** from Chhatwal et al. (2007).

used in a number of labs are derived from the lentivirus backbone pLV-CMV-GFP-U3Nhe (Naldini et al., 1996; Tiscornia et al., 2003; Jasnow et al., 2009), which allows for virally mediated expression of GFP (LV-GFP) driven by a cytomegalovirus (CMV) promoter. For manipulation of gene expression using floxed mice, a Cre-recombinase expressing viral vector (LV-Cre) can be created by replacing the GFP coding sequence with the coding sequence for Cre-recombinase (Heldt et al., 2007). In a typical experiment, mice are injected intra-cranially into specific brain areas with 1–2 µl of virus bilaterally, and allowed to recover for 7–14 days for stable integration and expression of the transgene within the host genome. **Figure 2** shows the ability of LV-Cre to efficiently remove the floxed-stop sequences upstream of lacZ in the Rosa-LacZ reporter mice (129S-Gt(ROSA)26Sortm1Sor/J, Soriano, 1999). LV-Cre injected into the striatum (**Figures 2A,B**) or CA1/dentate gyrus (DG) region of the dorsal hippocampus (**Figures 2C–E**) induces strong production of LacZ with no leakage in areas not infected with virus.

ADENO-ASSOCIATED VIRAL VECTORS

Although this review is devoted to lentiviral vector approaches, we will say a few words about adeno-associated vectors and some of the reasons why one might choose one over the other system for genetic manipulation. Generally, the lentiviral vector system utilizes a significantly larger insert size, and is thus the optimal choice when larger gene inserts, more than one gene (poly-cistronic cassettes), and longer promoters are of primary importance. Additionally, lentiviruses are somewhat easier to concentrate and there is some evidence that they express for the lifetime of the cell/animal. In contrast AAV are easier to handle (do not require same level of BSL safety due to their not being a retrovirus-based backbone), and in some cases higher titers are possible with AAV. Several manuscripts have been published recently outlining important differences between AAV and lentiviral vectors for neuroscience applications (Nathanson et al., 2009; Towne and Aebischer, 2009; van den Pol et al., 2009).

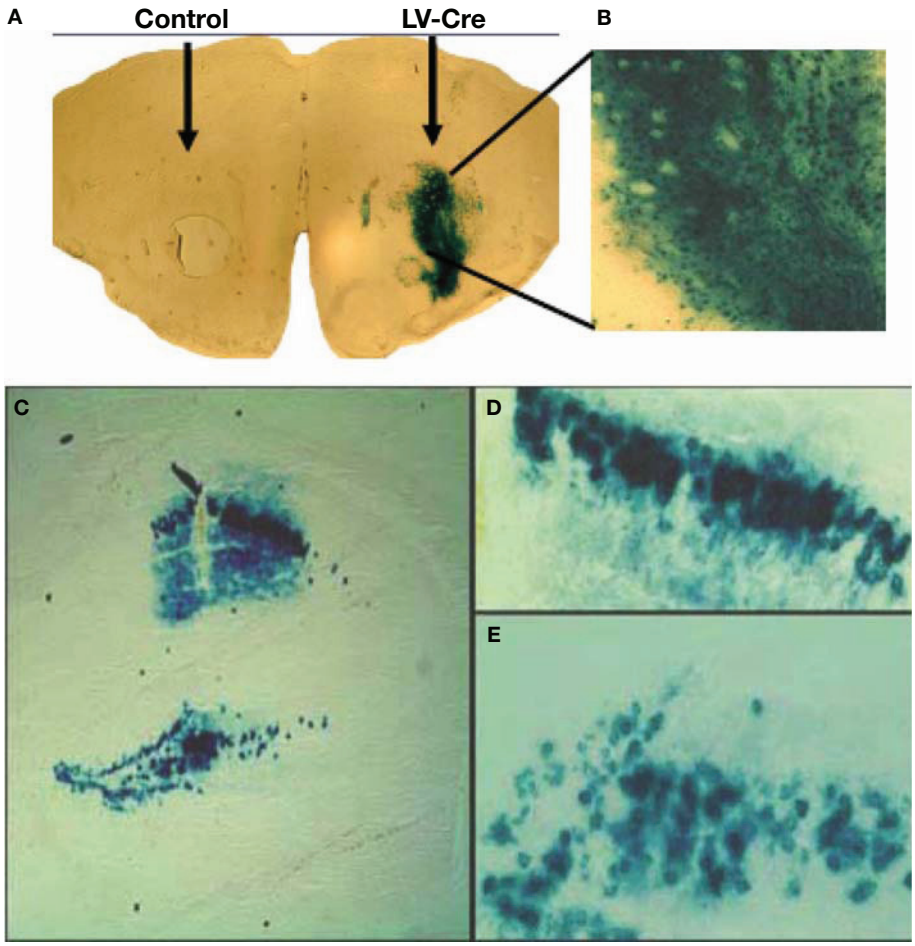

FIGURE 2 | *In vivo* validation of LV-Cre lentivirus. Robust Cre recombinase expression as labeled by LacZ when LV-Cre is injected into RosaLacZ reporter mice. Sections were processed for lacZ histochemistry with β-gal 14 days after LV-Cre injections to visualize Cre-dependent recombination *in vivo* in the **(A,B)** striatum and **(C,E)** dorsal hippocampus seen at ×10 and ×20 magnification. LV-Cre-infected cells within **(D)** CA1 and **(E)** DG, illustrating the dense β-gal and intact morphology of hippocampal neurons.

Many of the approaches discussed above have also been nicely demonstrated with AAV, along with several recent exciting innovations. For example novel combinations of Cre recombinase and channel rhodopsin (see below) have been used with AAV vectors to 'tag' neuronal populations for identification during *in vivo* electrophysiological recording (Lima et al., 2009). Another novel use of Cre in an AAV system used a modified estrogen-inducible Cre recombinase that served as an *in vivo* 'molecular switch', allowing spatial and temporal control of transgene expression, thereby potentially increasing the safety of gene therapy (Li et al., 2006). Additionally, the use of Cre-activated transgenes with AAV provide an additional assortment of possibilities for gene manipulation. For example, the 'rAAV-FLEX-*rev*' delivery approach has been demonstrated to lead to the expression of transgenes in a temporally and spatially restricted manner, using any Cre driver line as the template. (Atasoy et al., 2008). Overall, as with lentiviruses, AAV vectors allow for rapid subcloning and engineering of novel approaches to manipulate gene expression within discrete brain regions and neuronal cell types.

THE USE OF LENTIVIRUSES IN DEMONSTRATING THE ROLE OF BDNF-TrkB SYSTEM IN LEARNING AND MEMORY

Here we will illustrate several different *in vivo* uses of these vectors in approaching neuroscience questions. Lentiviruses have been particularly useful in demonstrating the role brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (TrkB) in learning and memory, including the acquisition and extinction of conditioned fear. Although its functions in the adult brain are not entirely clear, BDNF undoubtedly plays a role in development, trophic support, neural plasticity, and neuroprotection after some brain insults (Lindvall et al., 1994). There is increasing evidence that BDNF exerts acute effects on synaptic transmission and participates in long-term potentiation (LTP), an event related to memory processes (Patterson et al., 1992; Korte et al., 1995; Thoenen, 1995; McAllister, 1999).

However, understanding the role of the central BDNF-TrkB system *in vivo* within behaviorally relevant learning paradigms has been difficult (Minichiello et al., 2002). In part, this difficulty is due to the lack of specific pharmacological antagonists for the

TrkB receptor. In addition, homozygous BDNF KO mice display profound developmental abnormalities and often die prior to their third postnatal week (Ernfors et al., 1994). Heterozygous BDNF KO mice are viable but develop with roughly half of wild-type BDNF levels throughout the brain which likely leads to alterations in development that alter normal functioning (Conover and Yancopoulos, 1997). Given their time- and spatially limited gene expression characteristics, lentiviruses can be used to manipulate the BDNF-TrkB system after normal development, and thus, provide a more accurate assessment for the role of BDNF plasticity in learning and memory.

HIPPOCAMPUS-SPECIFIC DELETION OF BDNF IMPAIRS SPATIAL MEMORY AND EXTINCTION OF AVERSIVE MEMORIES

The mammalian hippocampus is the brain region containing the highest BDNF mRNA and protein levels (Conner et al., 1997). BDNF is known to have a functional role in the neuronal plasticity associated with hippocampal LTP (Korte et al., 1995). Although hippocampal circuits are quite plastic and clearly involved in learning and memory, the contribution of these circuits to the behaviors under study is often unclear. Correlational studies show that BDNF is increased in its expression following hippocampal dependent tasks (Hall et al., 2000; Mizuno et al., 2000). Genetic manipulation studies suggest that animals with decreased levels of BDNF or its receptor, TrkB, are deficient in behavioral tasks thought to be hippocampally dependent (Linnarsson et al., 1997; Minichiello et al., 1999; Gorski et al., 2003; Monteggia et al., 2004; Duman and Monteggia, 2006). However, all of these studies are limited, in that none of them examined the effects of BDNF deletion only within the hippocampus of adults while sparing BDNF in other forebrain regions. Having deletions of BDNF limited to only the hippocampus and only during a time-limited period in adulthood may also lead to a number of differences compared with the developmental and spatially broader BDNF deletions studied earlier.

To address this issue, we examined the consequence of hippocampus-specific BDNF deletion in adult animals on the acquisition and extinction of fear, object recognition memory, and a task strongly dependent on hippocampal functioning – the Morris water maze (MWM, Morris et al., 1982). Hippocampal BDNF was deleted by performing bilateral injections of LV-Cre into the dorsal hippocampus of BDNF-floxed mice (Heldt et al., 2007). Initial verification of this approach was performed with unilateral injections followed 14 days later with *in situ* hybridization for the Cre-recombinase or BDNF mRNA. **Figure 3A** reveals BDNF mRNA expression, which was specifically deleted within the hippocampus where Cre recombinase was now expressed.

In animals used for behavioral tests, both qualitative and quantitative analyses of mRNA expression levels reveal that the bilateral deletion of BDNF in the mice was largely limited to the hippocampus. **Figure 3B** shows the qualitative decrease in BDNF expression within the dorsal hippocampus following LV-Cre infection. Quantitative analyses of levels of BDNF revealed that, with reference to GFP control animals, the relative expression level of remaining BDNF within the dorsal hippocampus of LV-Cre-infected animals was 35, 30 and 45% for DG, CA1 and CA3, respectively (**Figure 3C**).

In the MWM, BDNF deletion within dorsal hippocampus caused deficits in escape latencies during acquisition as well as during probe trials when compared to the performance of control animals. BDNF hippocampal deletions also reduced the amount of time spent with the novel compared to the habituated object in the novel object recognition task (**Figures 3D,E**). Past studies indicate heterozygous and forebrain-specific BDNF KO mice likewise have deficits in MWM learning and other hippocampal dependent tasks (Mizuno et al., 2000; Gorski et al., 2003). However, these KO lines lack BDNF during development throughout cortical and subcortical areas as well as hippocampus. Together these data support the concept that BDNF expression in adults is required for normal acquisition and expression of spatial learning tasks, and that these previously reported effects were not due to developmental alterations or long-term BDNF effects on hippocampal neuronal survival or morphology.

Interestingly, hippocampal BDNF deletion also disrupted the extinction of conditioned fear without effecting the initial acquisition of fear. LV-Cre and LV-GFP mice given tone CS + footshocks during Pavlovian fear training showed equivalent levels of conditioned fear during testing. However, when groups were subsequently tested for fear over the course of next 6 days, LV-Cre mice displayed less fear extinction than controls, suggesting that hippocampal BDNF is required for the neural plasticity underlying the acquisition or consolidation of extinction memories (**Figure 4**). Impaired extinction resulting from decreased hippocampal BDNF offers new avenues for understanding emotion regulation in patients with anxiety disorders.

BDNF AND TrkB RECEPTOR INVOLVEMENT IN AMYGDALA-DEPENDENT FEAR CONDITIONING AND EXTINCTION

In addition to the hippocampus and mPFC, it is well established that fear conditioning is dependent on the basolateral amygdala (BLA) (Davis et al., 1993; Fanselow and LeDoux, 1999). Previous findings using *in situ* hybridization have revealed temporally specific increases in BDNF mRNA in the BLA after associative fear conditioning but not after exposure to an equal number of CS-alone or US alone presentations (Ressler et al., 2002; Rattiner et al., 2004a,b). Fear conditioning also results in activation of the TrkB receptor in the amygdala, as indicated by increased receptor phosphorylation during consolidation period (Rattiner et al., 2004a). In addition to the increased production of amygdala BDNF mRNA during the consolidation of fear conditioning, levels of BDNF mRNA transcripts are also increased following the extinction of conditioned fear (Chhatwal et al., 2006).

TrkB is a classical receptor tyrosine kinase, which is activated by BDNF binding, leading to receptor homodimerization, transphosphorylation and subsequent activation for downstream signaling required for neuroplasticity processes. The homodimerization of TrkB makes them particularly amenable to study with dominant-negative truncated recombinant proteins (Klein et al., 1991). Truncated TrkB receptors have extracellular domains which are identical to the full-length receptors, allowing for ligand binding, but also contain a shortened intracellular domain that inhibits BDNF signaling by blocking Trk autophosphorylation (Saarelainen et al., 2000; Haapasalo et al., 2001). Truncated TrkB receptor genes can be delivered in a spatial, temporal, and discrete manner using

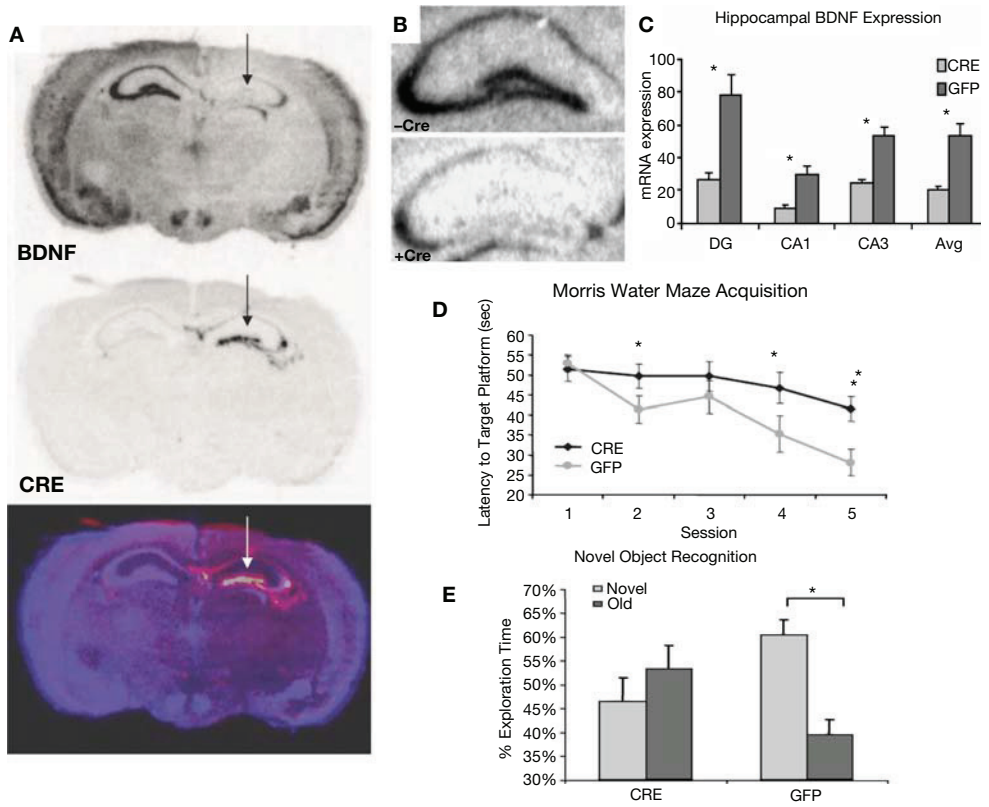

FIGURE 3 | Inducible BDNF deletion in hippocampus with Cre lentivirus. **(A)** BDNF (top) and Cre-recombinase (middle) mRNA expression visualized with *in situ* hybridization 2 weeks after LV-Cre infection into BDNF-floxed mice. The bottom figure represents a pseudocolor overlay of the two *in situ* sections, demonstrating that Cre, but not BDNF, is now expressed where BDNF was previously expressed. **(B)** Qualitative figure showing BDNF *in situ* hybridization of dorsal hippocampus following a sham injection (top, –Cre) or following LV-Cre injection (bottom, +Cre). **(C)** Relative mRNA expression in dentate gyrus (DG), and CA1 and CA3 regions and the average of all regions (Avg) of dorsal hippocampus in LV-Cre (Cre)- or LV-GFP (GFP)-infected mice. **(D)** Morris water maze acquisition, measured as the average latency to find the platform over daily sessions of training. LV-Cre-infected mice demonstrated significantly slower acquisition (impairment) compared with LV-GFP-infected controls. **(E)** Percent of time spent exploring the new vs. old object during the test day for novel object recognition. LV-GFP-infected mice spent significantly more time exploring the novel compared to the previously habituated object. The LV-Cre-infected animals did not differentiate between the two, demonstrating their impairment on this task. Panels adapted from Heldt et al. (2007).

lentiviral vectors, and in turn, these vectors can be used to evaluate the role of central BDNF-TrkB mechanisms in behaviorally relevant learning paradigms.

The use of a viral gene-delivery system to express a dominant-negative truncated TrkB receptor (TrkB.t1) has been successfully employed to examine the role of BLA TrkB receptors in the acquisition and consolidation of conditioned fear and fear extinction, as assessed by fear-potentiated startle (FPS, Chhatwal et al., 2006). For the acquisition of fear, this involved giving rats bilateral infusions of a lentivirus expressing TrkB.t1 (LV-TrkB.T1) or LV-GFP into the BLA 2 weeks before training and testing. To examine whether acquisition deficits were due to impaired expression, a second group of animals received BLA infusions of LV-TrkB.t1 or LV-GFP 4 days after training (Rattiner et al., 2004a). This ability to manipulate gene expression before vs. after the learning paradigm illustrates one of the strengths of viral vector approaches.

To confirm the applicability of these vectors *in vivo*, animals injected with LV-TrkB.t1 or LV-GFP were sacrificed after behavioral procedures. In LV-GFP infected rats, a large number of GFP-positive cells were identified in the amygdala, whereas rats infected with LV-TrkB.t1 showed a large number of TrkB.t1-positive cells in the amygdala, indicating that cells in the amygdala were successfully infected with these lentiviruses (**Figures 5A–C**). As seen in **Figure 5D**, pretraining infusion of LV-TrkB.t1 caused impaired fear learning when animals were tested 48 h after the last training session. In contrast, post-training infusion of LV-TrkB.t1 produced no significant deficits (**Figure 5E**), indicating that genetic blockade of the TrkB receptors did not prevent the expression of fear.

To examine the role of amygdala TrkB mechanisms in the formation of extinction memories, rats were fear-conditioned and matched into two groups with equivalent levels of conditioned fear before virus infusion of LV-GFP or LV-TrkB.t1 into the BLA bilaterally. When tested 2 days after extinction training, rats infected with the TrkB.t1 dominant-negative virus expressed more fear than those with the GFP virus, suggesting that blockade of the TrkB receptors prevented the retention of extinction memories (**Figure 5F**).

A separate cohort of animals was used to determine whether the extinction deficit in LV-TrkB.t1 rats was a result of the deficiencies in initial encoding or the consolidation of extinction memories. After fear-conditioned, matching and viral surgery, rats were tested for fear

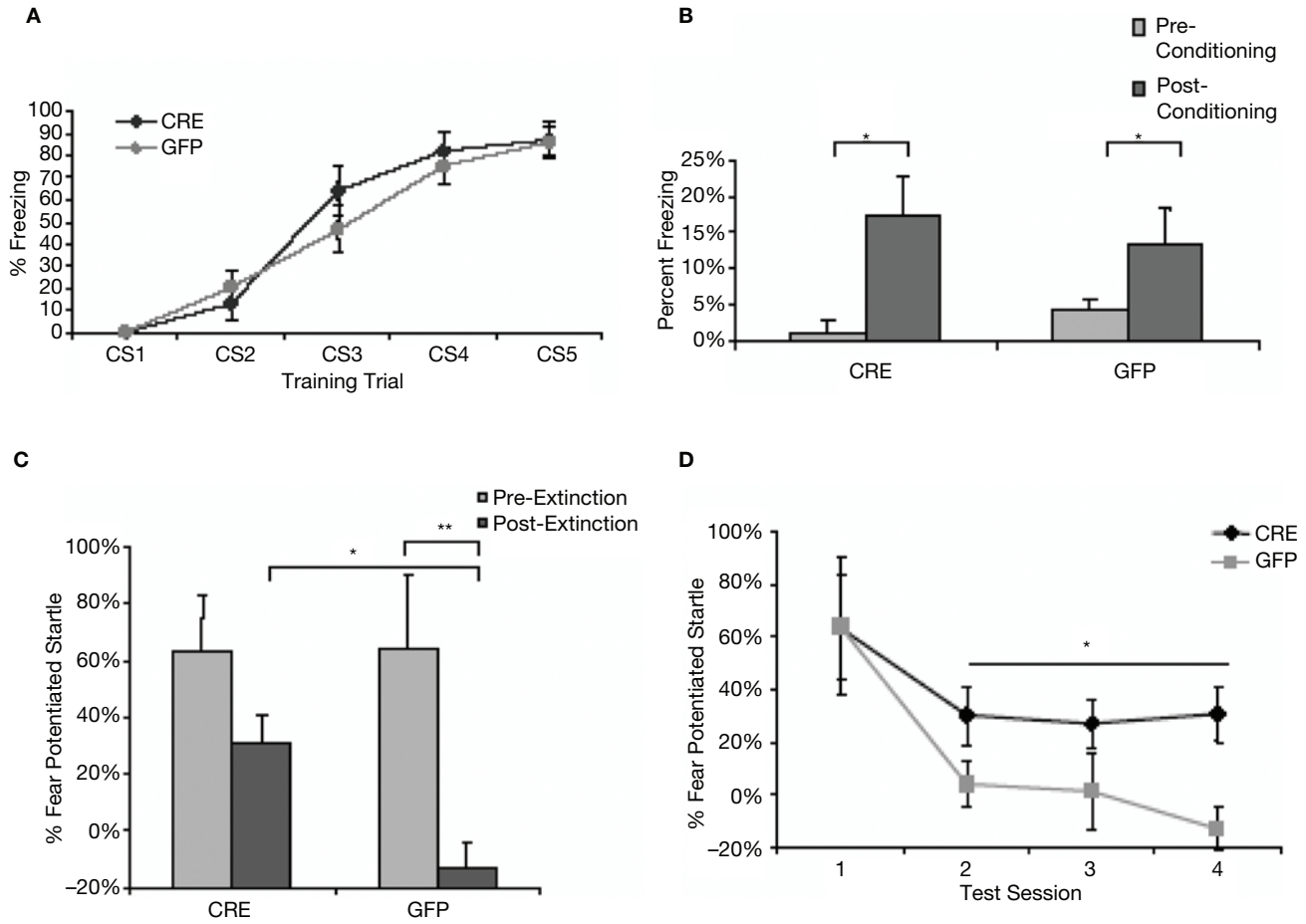

FIGURE 4 | Impaired fear extinction in mice with hippocampus-specific BDNF deletions. (A) Acquisition of cue conditioned fear, as measured with freezing after the onset of the auditory CS, during the conditioned fear acquisition session. There was no difference in acquisition of fear in animals receiving bilateral hippocampal injections of LV-Cre or LV-GFP. **(B)** Animals were tested within the same context in which training occurred for the presence of contextual fear as measured with freezing. There was no difference between the groups on level of contextual fear. **(C,D)** Extinction of conditioned fear is impaired in mice with dorsal hippocampus BDNF deletions. **(C)** Percent FPS is graphed for the first post-fear training test (pre-extinction) vs. the last test (post-extinction). Mice infected with LV-GFP demonstrate significant decreases in their level of conditioned fear as measured with %FPS compared with LV-Cre-infected mice. **(D)** Impaired extinction of fear, measured with %FPS, is stable across multiple testing sessions. Panels adapted from Heldt et al. (2007).

potentiated startle over multiple days to measure both within- and between session extinction of fear (**Figure 5G**). Testing revealed no difference between the groups in the rate of within-session extinction as observed by the decreased levels of FPS across test trials. However, rats expressing TrkB.t1 demonstrated significantly less extinction across several days of extinction testing, as compared to LV-GFP rats which showed marked extinction across days. This suggests that these rats may initially encode the extinction memory within each session but are not able to consolidate it. Together, these data suggest that the TrkB receptor is not involved in the process of within-session encoding of fear extinction, but instead is required for the normal consolidation of extinction as measured during the retention test.

EXPRESSION OF CORTICOTROPIN-RELEASING FACTOR IN THE AMYGDALA EMULATES STRESS DYSREGULATION

In addition to using lentiviral vectors to KO the gene or inhibit the protein of interest, lentiviruses can also over-express a specific protein as a means of elucidating protein function. Constitutive gene expression may be particular useful to model disease states resulting from an overactive gene or as a means of gene replacement for recovery of function experiments. An example of this application is contained in a recent study which sought to over-express corticotropin-releasing factor (CRF) in an effort to model physiological and behavioral changes observed in stress-related pathologies (Keen-Rhinehart et al., 2009). Past research suggests that CRF is upregulated in central nucleus of the amygdala (CeA) during chronic stress (Makino et al., 1999) and is related with the upregulation of adrenocorticotropic hormone release from the pituitary which is crucial for the adaptation to prolonged exposure to stressors (Herman et al., 2003). A dysregulation of this control may be involved in the development of a maladaptive response to chronic stress and may produce many of the alterations observed in several stress-induced pathologies including PTSD, anxiety, and depression (Arborelius et al., 1999; Swaab et al., 2005). Transgenic mice that over-express CRF indeed exhibit HPA dysregulation (Groenink et al., 2002). However, this approach likely produces

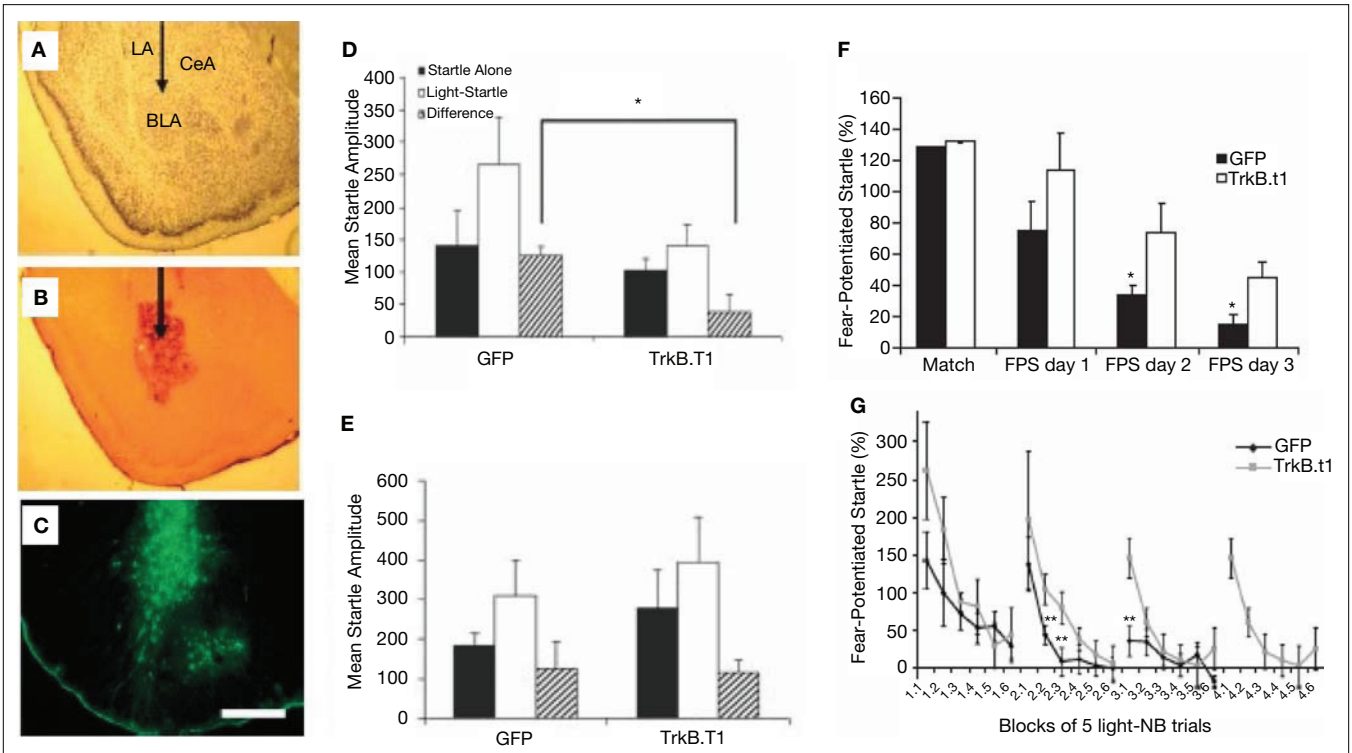

FIGURE 5 | Effect of TrkB.T1 in the amygdala on the acquisition of conditioned fear and extinction. (A–C) Histological examination of viral infection in the BLA following behavioral studies. **(A)** No amygdala damage was seen following infection as visualized with Cresyl violet staining. LA, lateral amygdala; CeA, central amygdala. **(B)** Expression of TrkB.t1 was assessed using immunocytochemistry (ICC) for a hemagglutinin (HA) epitope tag incorporated into the TrkB.t1 coding sequence. **(C)** GFP expression directly visualizing under an epifluorescence microscope (scale bar, 1 mm). Level of fear-potentiated startle (FPS) following **(D,E)** acquisition and **(F,G)** extinction of fear. **(D)** Mean startle amplitude on startle-alone trials, light-startle trials, and the difference between the two are shown for animals receiving lentivirus infusion into the amygdala. Mean difference scores of LV-TrkB.T1-infused animals were significantly lower than difference scores of LV-GFP-infused animals. **(E)** Effect of amygdala infection with LV-TrkB.T1 on the expression of fear-potentiated startle. When LV-TrkB.T1 is present during expression, but not acquisition, of fear learning there is no difference between FPS with LV-TrkB.T1 animals compared with LV-GFP animals. **(F)** Averaging across all trials, TrkB.t1-infected rats showed a deficit in extinction as compared to GFP-infected rats. **(G)** Examining extinction within the testing session suggested that the TrkB.t1-infected rats had normal within-session extinction, but lacked extinction retention across the 2-day interval between tests TrkB.t1 and GFP groups. Panels **(A–C, F,G)** adapted from Chhatwal et al. (2006) and panels **(D,E)** from Rattiner et al. (2004a).

developmental compensation within the system that does not likely mimic the consequences of region-specific increased CRF expression resulting from chronic stress. Using lentiviral vectors, Keen-Rhinehart et al. (2009) recently examined whether constitutive expression of CRF in the CeA of rats produced changes in the regulation of the HPA axis and behaviors shown previously to be indicative of chronic stress.

In this study, a lentivirus construct was engineered to over-express CRF by subcloning the CRF cDNA sequence downstream of the constitutively active CMV promoter. After injections of either the LV-CRF or LV-GFP control virus, female rats were administered a battery of tests, including the Porsolt forced swim test and the acoustic startle response which is elevated in fear and anxiety states (Davis et al., 1993). The dexamethasone (DEX) suppression test was conducted to assess the consequences of increased CRF release from CeA on glucocorticoid negative feedback.

Histological analysis after testing indicated that the CRF protein concentration was significantly elevated in the CeA of rats that received LV-CRF when compared with LV-GFP animals, indicating that the LV-CRF site-specifically increased synthesis of CRF in CeA (**Figure 6**). Prior testing revealed that LV-CRF animals showed a number of physiologic and behavioral signs typically exhibited by animals subjected to chronic stress (**Figure 7**). In the DEX suppression test, plasma corticosterone levels were significantly elevated in LV-CRF animals compared to controls by 6 h post injection, indicating that the LV-CRF animals had escaped from glucocorticoid negative feedback. As revealed in behavioral tests, the baseline acoustic startle response was significantly greater in LV-CRF compared with LV-GFP-injected rats, suggesting that basal levels of anxiety are increased in the LV-CMV-CRF group. In the Porsolt forced swim test, LV-CRF animals displayed less time attempting to escape and more immobile floating time than controls, behaviors indicative of an increased depression-like state in rats.

Together, the data from this study demonstrate that lentiviral over-expression of CRF in CeA of rats induces phenotypic behaviors reminiscent of traits associated with a state of chronic stress. Moreover, it revealed the value of using lentiviral vectors to constitutively express genes to model disease states resulting from an overactive gene.

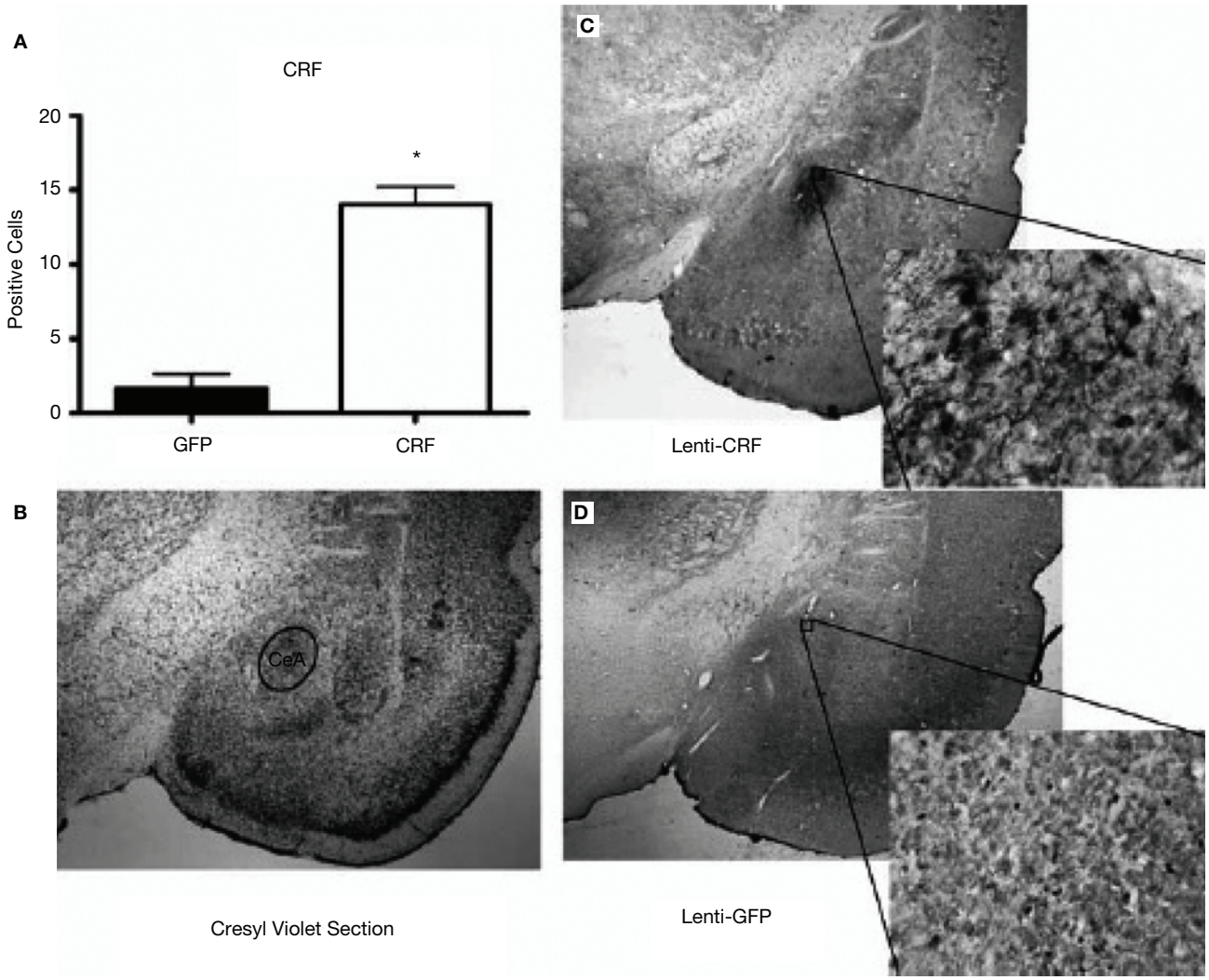

FIGURE 6 | LV-CRF injection into the central nucleus of the amygdala (CeA) significantly increased corticotrophin releasing hormone (CRF) protein production site-specifically. (A) Number of positively labeled CRF cells in CeA of LV-GFP (closed bars) and LV-CRF treated rats (open bars) determined by immunohistochemistry. **(B)** Cresyl violet stained section representing a section parallel to the sections used to quantify the number of CRF positive neurons in CeA. **(C)** Representative section with an additional ×20 magnification inset showing the effects of LV-CRF injection into CeA on the number of positively labeled CRF neurons. **(D)** Representative section with an additional ×20 magnification inset showing the amount of CRF staining observed in the control, LV-GFP-treated rats. $*P < 0.05$. Panels adapted from Keen-Rhinehart et al. (2009).

USES OF LENTIVIRAL VECTORS TO CREATE REGION AND CELL TYPE-SPECIFIC TRANSGENE EXPRESSION

An essential characteristic of most viral vectors is that they do not require a region-specific promoter. At present many lentiviruses use the constitutive CMV promoter which drives the ubiquitous expression of a downstream transgene not confined to just one subtype of cells. Thus, it is difficult to attribute whether phenotypic alterations caused by transgene transcription are the consequence of intended targeted manipulation in desired cells or unintended effects on non-target cells. This complexity highlights the value of restricting transgene expression within specific types of cells. For this reason, restricting expression to specific cell populations through the use of a cell type-specific promoter is particularly attractive in future research.

Promoters are generally defined as regions a few hundred to thousand base pairs located upstream of a gene's transcriptional initiation site; however, more distal regions and 5′ and 3′ untranslated regions (UTR), may also contain various regulatory elements that govern transcription and cell-type specificity. As such, transgenes containing full-length promoter/enhancer regions often exceed the limited insert capacity of lentiviral vectors. Thus, for lentivirus-based applications, it is essential to identify a minimal promoter region that is important for cell-type specificity which efficiently drives gene expression and is also small enough to be packaged efficiently as a viral vector.

The process of determining minimal promoter lengths usually involves the screening of several short versions of putative promoter/enhancer regions by cloning the core or minimal proximal

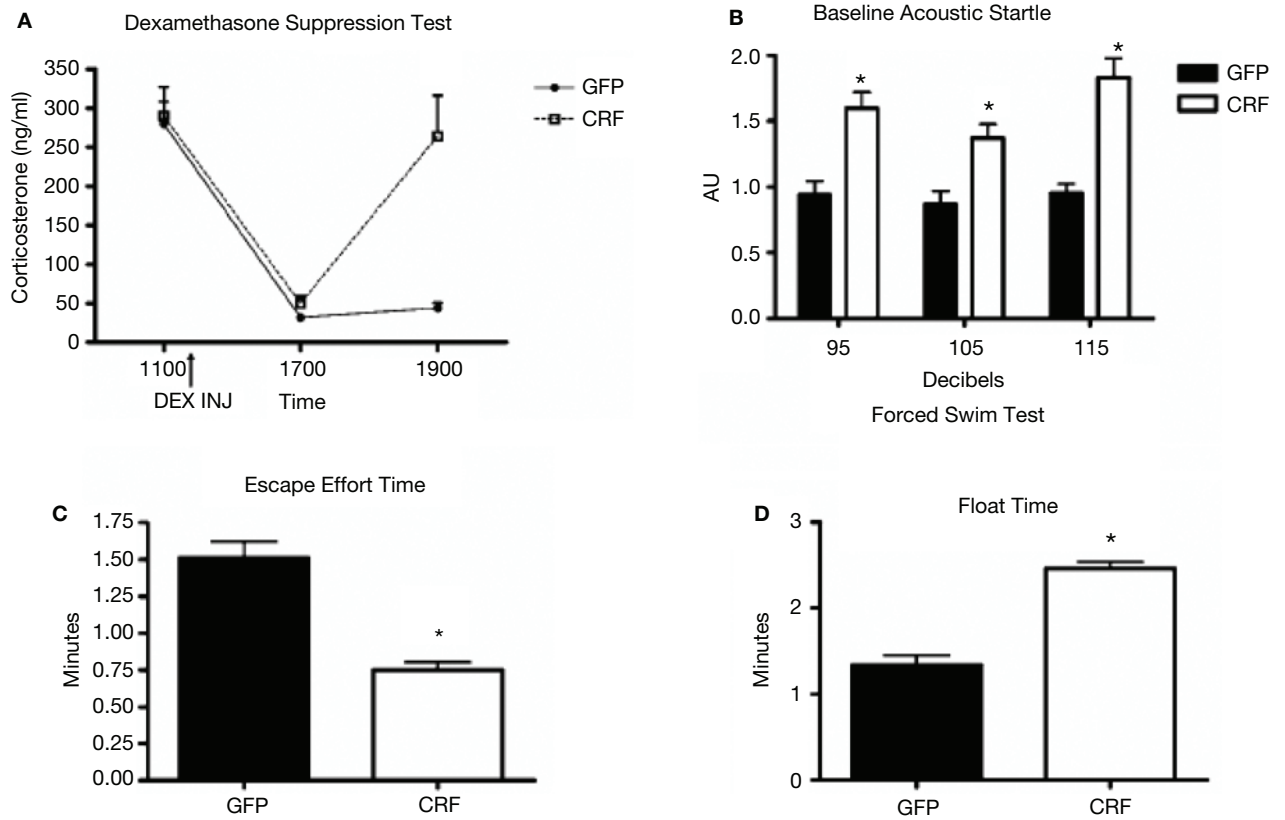

FIGURE 7 | Effect of LV-CRF injection on HPA axis feedback, anxiety-and depression-related behaviors. (A) HPA axis feedback as assessed by the dexamethasone suppression test. Corticosterone levels before and following a dexamethasone injection (shown by arrow) for LV-GFP control (closed symbol) and LV-CRF rats (open symbol). **(B–D)** LV-GFP-injected control (closed bars) and LV-CRF-injected rats (open bars). **(B)** Baseline acoustic startle response, **(C)** amount of time animals spent actively trying to escape and **(D)** and time spent floating in the forced swim test. Mean ± SEM. $*P < 0.05$. Panels adapted from Keen-Rhinehart et al. (2009).

regions of a promoter and assessing their activity and appropriate expression specificity *in vitro* and *in vivo*. An example of the *in vivo* screening process has been reported by Chhatwal et al. (2007) who recently engineered a minimal promoter of the gene for CCK, an abundant neuropeptide of significant interest in psychiatry and neuroscience (Fink et al., 1998). In this study, lentivirus constructs were engineered to express either a Cre recombinase or GFP under control of an upstream a 3-kb promoter region of the CCK gene that included 5′ UTR sequences. The resulting CCK-Cre and CCK-GFP lentiviral constructs were assessed *in vivo* by comparing the expression pattern of constructs to endogenous CCK mRNA expression after microinjection of packaged lentivirus.

Both CCK-Cre and -GFP viruses (LV-CCK-Cre, LV-CCK-GFP) were injected into the CA1 and DG regions of the hippocampus which possess a large number of CCK mRNA-positive cells located close to CCK-negative cells. In the case of LV-CCK-Cre, injections were performed on RosaLacZ mice and the resulting expression of LacZ, as detected by β-gal staining with X-gal, was co-localized with CCK mRNA by means of *in situ* hybridization. Both β-gal staining and GFP expression patterns closely paralleled CCK mRNA expression in the dentate, CA1, and fasciola cinereum regions of the hippocampus (**Figure 8**). Taken together, these observations suggest that minimal CCK promoters are capable of producing highly selective expression in the appropriate sub-population of cells. Furthermore, when used as promoters in lentivirus constructs, similar approaches will allow for *in vivo* cell-type specific expression of transgenes that are biologically relevant in the study of complex behaviors.

OTHER APPROACHES USING VIRAL VECTORS FOR GENE MANIPULATION

Many other potential uses of viral vector approaches to gene manipulation have not been discussed due to space constraints. It is important for the reader to be aware of the multitude of new approaches including gene silencing. In neuroscience, antisense oligonucleotide approaches were used with increasing frequency for about a decade (Van Oekelen et al., 2003), but there remained significant concern that the mechanism of action and the specificity of gene knockdown were unknown. In recent years, it has become increasingly appreciated that these antisense approaches likely were successful because of the previously unknown mechanisms of cellular heteronuclear RNA silencing mechanisms, which utilize the DICER complex to prevent translation or lead to targeted mRNA degradation. This field has grown exponentially in recent years, and is far beyond the scope of this brief review. Suffice it to say

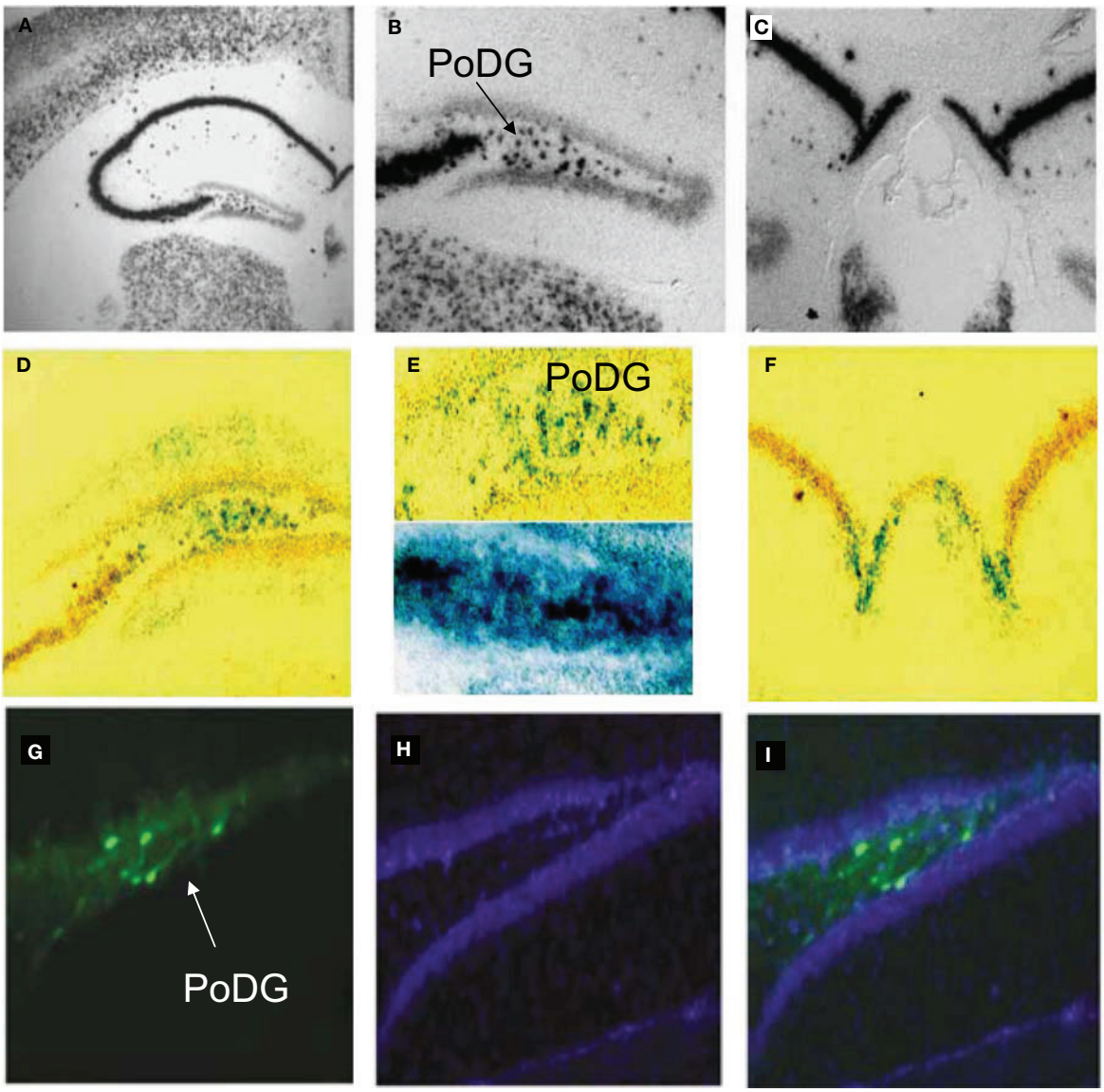

FIGURE 8 | *In vivo* validation of cell-type specific LV-CCK lentivirus. CCK mRNA expression. CCK mRNA was examined using *in situ* hybridization, demonstrating high levels of expression within the hippocampal formation [low power **(A)**, high power **(B,C)**]. **(B)** Intense CCK mRNA expression is normally present within the CA3 subfield and within the interneuron-rich region (PoDG) separating the granule cell layers of the dentate gyrus, and **(C)** in the fasciola cinereum medial to CA1. **(D)** Low power image showing LacZ expression parallels CCK mRNA expression in RosaLacZ Cre-reporter mice injected with LV-CCK-Cre. LacZ expression (blue precipitate) and CCK mRNA expression (silver grains) were assessed in the same sections. **(E)** High-power images showing a high degree of overlap in mRNA expression was observed in the polymorph layer of the dentate (high power). High and low panels depict images of low and high CCK mRNA expression in the polymorph layer of the dentate, with correspondingly low and high numbers of LacZ-positive cells. **(F)** Similar co-expression was seen in the fasciola cinereum. **(G–I)** Virus encoding CCK-GFP injected into the dentate gyrus of adult mice. **(G)** Fluorescence was assessed on sectioned, fixed tissue. **(H)** Hoechst-stained photomicrographs of the same section shown in **(G)**. **(I)** Overlays of **(G)** and **(H)**. Panels adapted from Chhatwal et al. (2007).

that small heteronuclear RNAs have been successfully encoded in lentiviral and AAV vectors in neuroscience applications to silence genes (Janas et al., 2006; Harper and Gonzalez-Alegre, 2008), and this approach will undoubtedly continue to grow rapidly.

Another area of unprecedented growth and excitement concerns light-activated ion channels which have been utilized with tremendous success by Deisseroth and colleagues (Boyden et al., 2005; Arenkiel et al., 2007). Several excellent reviews have been written about this new approach and the burgeoning field of 'optogenetics' that has resulted (Airan et al., 2007; Gradinaru et al., 2007). In brief, these approaches have shown that specific ion channels from green algae which respond to light can be expressed in the brain via both transgenic and viral vector approaches. Different channels have been identified and engineered which respond to different frequencies of light as well as produce different channel characteristics (e.g., excitation vs. inhibition). By combining localized infection with viral vectors with implanted fiber-optics, Deisseroth and colleagues have elegantly shown that they can produce cell-type specific

activation within defined neural pathways. These approaches are expanding quickly, and they have the promise of revolutionizing systems neurobiology.

In summary, viral vectors (particularly lentivirus) and Cre/loxP mediated deletion provide powerful methods to inducibly manipulate gene expression in the brain. When combined, they provide for the ability to over-express and delete genes of interest in a temporal, spatial, and cell-type specific fashion. Such inducible genetic approaches will allow for eventual understanding of the differential roles of genes in specific neural pathways which underlie the enormous complexity of brain function and behavior.

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Inducible gene manipulations in serotonergic neurons

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An impairment of the serotonergic (5-HT) system has been implicated in the etiology of many neuropsychiatric disorders. Despite the considerable genetic evidence, the exact molecular and pathophysiological mechanisms underlying this dysfunction remain largely unknown. To address the lack of instruments for the molecular dissection of gene function in serotonergic neurons we have developed a new mouse transgenic tool that allows inducible Cre-mediated recombination of genes selectively in 5-HT neurons of all raphe nuclei. In this transgenic mouse line, the tamoxifen-inducible CreERT2 recombinase is expressed under the regulatory control of the mouse tryptophan hydroxylase 2 (*Tph2*) gene locus (177 kb). Tamoxifen treatment efficiently induced recombination selectively in serotonergic neurons with minimal background activity in vehicle-treated mice. These genetic manipulations can be initiated at any desired time during embryonic development, neonatal stage or adulthood. To illustrate the versatility of this new tool, we show that Brainbow-1.0L$^{TPH2\text{-}CreERT2}$ mice display highly efficient recombination in serotonergic neurons with individual 5-HT neurons labeling with multiple distinct fluorescent colors. This labeling is well suited for visualization and tracing of serotonergic neurons and their network architecture. Finally, the applicability of TPH2-CreERT2 for loxP-flanked candidate gene manipulation is evidenced by our successful knockout induction of the ubiquitously expressed glucocorticoid-receptor exclusively in 5-HT neurons of adult mice. The TPH2-CreERT2 line will allow detailed analysis of gene function in both developing and adult serotonergic neurons.

Keywords: serotonergic, tryptophan hydroxylase 2, serotonin, Cre-transgenic, knockout, mice, Brainbow, glucocorticoid-receptor

INTRODUCTION

Serotonergic neurons receive multiple, modulatory inputs from the hypothalamic–pituitary–adrenal (HPA) axis (Harfstrand et al., 1986; Day et al., 2004) and glutamatergic, GABAergic and other monoaminergic neurons (Sodhi and Sanders-Bush, 2004). Conversely, serotonergic (5-HT) neurons project to most structures of the brain (Hensler, 2006).

Serotonergic neurons modulate physiological functions including sleep, circadian rhythm, feeding, and neuroendocrine function (Hensler, 2006) as well as complex behaviors such as aggression (Miczek et al., 2007) or anxiety (Lucki, 1998). A multitude of evidence points to serotonergic dysregulation during development and adulthood in the etiology of many psychiatric diseases (Caspi et al., 2003; Mann, 2003; Gordon and Hen, 2004; Zill et al., 2004; Carver and Miller, 2006; Miczek et al., 2007; Mossner et al., 2007; Oades, 2007; Pardo and Eberhart, 2007; Carver et al., 2008; Geyer and Vollenweider, 2008; Serretti and Mandelli, 2008).

Furthermore, 5-HT plays a key role during development, being an essential neurotransmitter for proper neuronal division, differentiation, migration and synaptogenesis (Gaspar et al., 2003). Even transient alterations in serotonin homeostasis during development cause permanent changes to adult behavior (Ansorge et al., 2004; Gross and Hen, 2004).

In order to dissect the molecular basis of the multiplex serotonergic involvement in a plethora of physiological and pathophysiological brain functions, researchers have turned to the specificity offered by gene knockout technologies in mice. Unfortunately, germline knockouts can not address the specific serotonergic function of widely expressed genes without affecting the gene in other tissues as well. Moreover, germline gene inactivation may result in a lethal phenotype or induction of compensatory, homeostatic mechanisms or pleiotropy during development. Therefore, delineation of the particular function of the serotonergic system in relationship to other neuronal systems as well as the exact developmental and adult role of genes in 5-HT neurons remains elusive.

To overcome these obstacles, we have developed a CreERT2/loxP-recombination system that allows temporal control of conditional gene manipulation specifically in serotonergic neurons. Temporal control of recombination is a prerequisite for distinguishing the developmental role of a gene from its present function during adulthood. For that reason, we took advantage of a fusion protein consisting of Cre recombinase and a mutated ligand-binding domain (LBD) of the human estrogen receptor (ER) that was developed to achieve tamoxifen dependent Cre activity (Feil et al., 1997; Indra et al., 1999).

We have chosen regulatory sequences of the tryptophan hydroxylase 2 (*Tph2*) gene which is exclusively expressed in serotonergic neurons during development and adulthood (Cote et al., 2003, 2007) to accurately direct Cre expression to 5-HT neurons of the brain. Transgenic TPH2-CreERT2 mice were generated by DNA microinjection of a modified DNA construct containing the *Tph2* open reading frame together with large upstream and downstream

flanking regions (177 kb). Breeding to three different mouse lines with loxP-flanked alleles shows reliable and efficient serotonergic neuron specific induction of recombination following tamoxifen treatment during development and adulthood and so represents a new and powerful tool for conditional gene manipulation in the serotonergic system.

MATERIALS AND METHODS

GENERATION OF TPH2-CreERT2 TRANSGENIC MICE

A 196-kb PAC (RP24-243J21, RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH) that contains the full-length mouse *Tph2* gene (107 kb) with 51 kb upstream and 19 kb downstream DNA sequences was selected for recombineering in EL250 bacteria (Lee et al., 2001). First, the kanamycin resistance gene of the pPAC4-backbone was replaced with a chloramphenicol resistance. Next, a cassette encoding a fusion protein (CreERT2) consisting of a Cre-recombinase (Cre) and a mutated LBD of the human ER (ERT2) as well as a kanamycin resistance gene flanked by two FRT sites was integrated into the ATG-start codon of the TPH2-gene. A 23-bp sequence downstream of the ATG-translation start of *Tph2* was intentionally deleted since it contained additional in-frame ATG-start sites in Exon 1 (**Figure S1** in Supplementary Material). The FRT-flanked kanamycin resistance cassette was then deleted by arabinose-induced expression of Flp recombinase. The CreERT2-modified genomic *Tph2* sequence was separated from the PAC backbone by *Not*I digestion and subsequent preparative pulse-field electrophoresis. The purified, linearized DNA was microinjected into the pronucleus of C57BL/6N mouse oocytes. Transgenic offspring (founders) were identified by PCR genotyping of tail DNA. TPH2-CreERT2 transgenic mice were always crossed with C57BL/6N mice periodically purchased from Charles River Laboratories.

The TPH2-CreERT2-transgenic mice were bred with R26R (Soriano, 1999), Brainbow1.0L (Livet et al., 2007) and GRflox (Tronche et al., 1999) mice to generate transgenic mice for recombination analysis: R26R$^{TPH2\text{-}CreERT2}$, Brainbow1.0L$^{TPH2\text{-}CreERT2}$, and GRflox/GRflox$^{TPH2\text{-}CreERT2}$. To this purpose, TPH2-CreERT2 mice were crossed with R26R and Brainbow mice to generate double transgenic R26R$^{TPH2\text{-}CreERT2}$ and Brainbow1.0L$^{TPH2\text{-}CreERT2}$ mice, respectively. To generate GRflox/GRflox$^{TPH2\text{-}CreERT2}$ mice we first crossed TPH2-CreERT2 mice with homozygous GRflox/GRflox mice. GRflox$^{TPH2\text{-}CreERT2}$ mice were then crossed again with homozygous GRflox/GRflox mice to finally generate GRflox/GRflox$^{TPH2\text{-}CreERT2}$ mice.

IN VIVO INDUCTION OF Cre-MEDIATED RECOMBINATION WITH TAMOXIFEN

Tamoxifen (Sigma) was dissolved in a corn oil/ethanol (10:1) mixture at a final concentration of 10 mg/ml. Adult R26R$^{TPH2\text{-}CreERT2}$ and GRflox/GRflox$^{TPH2\text{-}CreERT2}$ mice (8–12 weeks) were injected intraperitoneally twice daily for five consecutive days (protocol 1) with 1 mg of tamoxifen. Adult Brainbow1.0L$^{TPH2\text{-}CreERT2}$ mice (8–12 weeks) were injected intraperitoneally twice daily for one to five consecutive days. Control animals were injected with 100 µl of a corn oil/ethanol vehicle mixture (vehicle). Experimental animals for immunohistochemistry (IHC) or X-gal staining were sacrificed 5–14 days after the last injection. For recombination analysis during embryogenesis, pregnant R26R$^{TPH2\text{-}CreERT2}$ mothers were injected for four consecutive days between E15 and E18 with 1 mg tamoxifen twice daily. Offspring were delivered by C-section at E20 and raised by foster mothers. For recombination analysis in postnatal pups, lactating transgenic mothers were injected intraperitoneally for five consecutive days with either 1 mg tamoxifen twice daily (protocol 1), with 2 mg tamoxifen twice daily (protocol 2), with 3 mg tamoxifen twice daily (protocol 3) or one single injection of 1 mg tamoxifen daily (protocol 4). R26R$^{TPH2\text{-}CreERT2}$ litters from embryonal and postnatal tamoxifen injections were analyzed at P60 for β-gal expression as described below. All experimental procedures were approved by the Animal Welfare Committee (Regierungspräsidium Karlsruhe) and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

X-GAL STAINING AND IMMUNOHISTOCHEMISTRY

Transgenic mice were characterized by X-Gal staining and dual-label fluorescent IHC. The following primary antibodies were used: rabbit α-TPH2 (kindly provided by Donald M. Kuhn, 1:10000), chicken α-β-galactosidase (Abcam, 1:10000), rabbit α-GR (Santa Cruz Biotechnology, 1:1000) and mouse α-TPH1 (Sigma, 1:500-2000) antibodies. The TPH1 antibody cross-reacts with TPH2 and detects both isoenzymes. Secondary antibodies were AF488 donkey α-rabbit (Invitrogen, 1:1000) and AF488 donkey α-mouse (Invitrogen, 1:200), AF555 donkey α-rabbit (Invitrogen, 1:1000), and Cy3 donkey α-chicken (Jackson ImmunoResearch, 1:1000). Sections were examined using a Nikon C1Si-CLEM confocal laser-scanning microscope (Nikon Imaging Center, BioQuant, Heidelberg, Germany). Confocal image stacks for both channels were acquired sequentially, and projected on average using ImageJ software. For the combinatorial fluorescent expression in Brainbow1.0L$^{TPH2\text{-}CreERT2}$ mice a Leica TCS SP5 X confocal laser-scanning microscope (Leica, Mannheim, Germany) was employed. For cyan fluorescent protein (CFP) excitation a 458-nm argon line was used. Excitation of yellow fluorescent protein (YFP) and RFP was performed with a white light laser at 512 and 565 nm, respectively. Confocal image stacks for all channels were acquired sequentially, and maximally projected using LAS AF Lite software. Confocal images were not processed with Adobe Photoshop to intensify colors and accentuate hues.

STATISTICAL METHODS

Coronal slices of two to four adult R26R$^{TPH2\text{-}CreERT2}$ mice of each founder line were processed with dual-label fluorescent IHC detecting βgal and TPH2. Image stacks of all slices that showed TPH2 staining were acquired using a confocal laser-scanning microscope. The ratio of βgal+/TPH2+ neurons to all TPH2+ neurons was calculated separately for caudal, median and dorsal raphe nuclei. Confidence-bounds (CI) for recombination efficacy and background recombination in adult mice were calculated using the Clopper–Pearson method based on significance level 95.0% (Clopper and Pearson, 1934). For adult R26R$^{TPH2\text{-}CreERT2}$ mice which had been injected during E15–18, P2–6 and P12–16 (n = 3 each group) the ratio of βgal+/TPH2+ neurons to all TPH2+ neurons per group was expressed as the mean and as the range of expression.

RESULTS

GENERATION OF TPH2-CreERT2 TRANSGENIC MICE

We used the CreERT2/loxP-system which provides inducible, Cre-mediated recombination upon tamoxifen administration to generate transgenic mice which allow conditional manipulation of individual genes in serotonergic neurons at any desired time. Targeted spatial control in serotonergic neurons was achieved by using elements of the *Tph2* locus as a promoter to control Cre expression. Since precise information about the regulatory elements which govern timely and 5-HT specific TPH2 expression is not available, we used a large genomic fragment of the *Tph2* locus to drive CreERT2, thereby enhancing the probability to include most of the important regulatory components of *Tph2*. To this end, a P1-derived artificial chromosome (pPAC4) with a 177 kb genomic insert containing the entire *Tph2* gene with large additional flanking sequences (see Section "Materials and Methods") was used to generate the TPH2-CreERT2 construct. The coding sequence of the CreERT2 fusion protein was inserted in place of the ATG-start of the *Tph2* gene (**Figure 1**) by recombineering in bacteria (Lee et al., 2001). The modified genomic fragment was released from the PAC backbone and finally introduced into the mouse germline of C57BL/6N by DNA microinjection. Five TPH2-CreERT2 founders were obtained (1.21, 1.23, 1.27, 1.35 and 1.38).

EFFICIENT AND SPECIFIC RECOMBINATION IN SEROTONERGIC NEURONS OF ADULT R26R^{Tph2CreERT2} MICE

TPH2-CreERT2 founder were crossed to ROSA26 Cre reporter mice (R26R) (Soriano, 1999) to obtain R26R^{Tph2CreERT2} mice. Temporal and spatial control of tamoxifen-induced CreERT2-mediated recombination in 5-HT neurons was determined and the efficacy of serotonergic recombination in the caudal, dorsal and median raphe (MR) nuclei was calculated.

Adult R26R^{Tph2CreERT2} mice were injected twice daily with either tamoxifen (1 mg) or vehicle for five consecutive days. Animals were sacrificed for analysis 5 days after the last tamoxifen injection. X-Gal staining of tamoxifen-treated R26R^{Tph2CreERT2} mice from all lines revealed recombination in the brain stem and midbrain in regions shown to contain serotonergic somata (**Figures 2A–I**) while extraserotonergic brain regions showed no staining. Vehicle treated R26R^{Tph2CreERT2} mice showed minimal background recombination among all lines (**Figures 2J–L**). TPH2-CreERT2 lines 1.21, 1.35 and 1.38 showed the highest recombination efficiency and lowest background recombination.

Next, we performed dual-label fluorescent IHC with anti-β-galactosidase (βgal) and anti-TPH2-antibodies to investigate selectivity and efficiency of recombination in serotonergic neurons. While β-gal antibodies confirm Cre-mediated recombination in the Rosa26 locus, TPH2 antibodies detect TPH2 which is the rate-limiting enzyme in brain serotonin synthesis and solely found in serotonergic raphe nuclei. As suggested by X-gal staining, recombination occurred exclusively in serotonergic neurons of all raphe nuclei with comparable efficiency (**Figures 3A–I**). Vehicle treated mice showed only minimal background recombination (**Figures 3J–L**).

Lines 1.21 and 1.38 showed the highest recombination efficacy in serotonergic neurons when tamoxifen was injected (91 and 90%, respectively) (**Table S1** in Supplementary Material) while background recombination in vehicle treated mice was below 2% (**Table S2** in Supplementary Material). All further experiments were carried out with line 1.38.

RECOMBINATION IN SEROTONERGIC NEURONS OF R26R^{TPH2CreERT2} MICE DURING EMBRYOGENESIS AND POSTNATAL DEVELOPMENT

To investigate whether tamoxifen inducible recombination is also feasible during embryogenesis we injected pregnant mothers intraperitoneally with 1 mg tamoxifen twice daily for four consecutive days between E15 and E18. Offspring were delivered by C-section

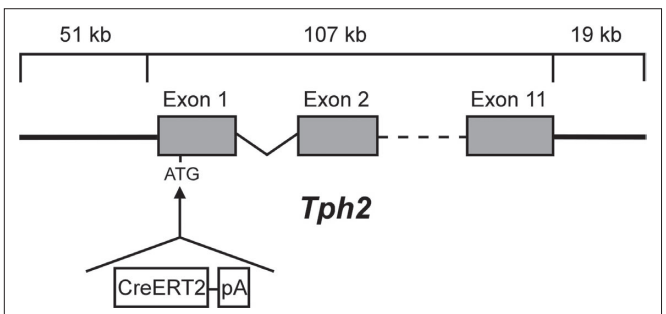

FIGURE 1 | TPH2-CreERT2 construct for DNA microinjection. A PCR-amplified CreERT2.pA cassette flanked by sequences (42 bp) homologous to the integration site of the PAC-based genomic *Tph2* locus was recombineered in *E. coli*. CreERT2.pA was inserted into the ATG-start of Exon 1 of the mouse *Tph2* gene.

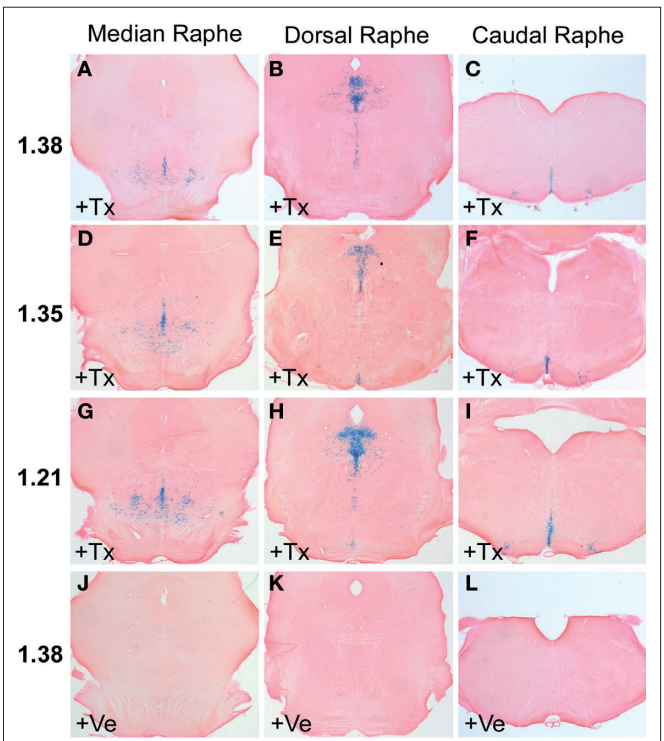

FIGURE 2 | X-Gal staining of R26R$^{TPH2-CreERT2}$ mice is restricted to the brain stem and midbrain where raphe nuclei are located. (A–C,J–L) Line 1.38. **(D–F)** Line 1.35. **(G–I)** Line 1.21. **(A–I)** Mice treated with tamoxifen (+Tx) (1 mg twice daily for 5 days) show abundant X-Gal staining at all levels of raphe nuclei. **(J–L)** Vehicle (+Ve) treated mice show nonsignificant X-Gal staining. **(A,D,G,J)** Median raphe nuclei; **(B,E,H,K)** dorsal raphe nuclei; **(C,F,I,L)** caudal raphe nuclei.

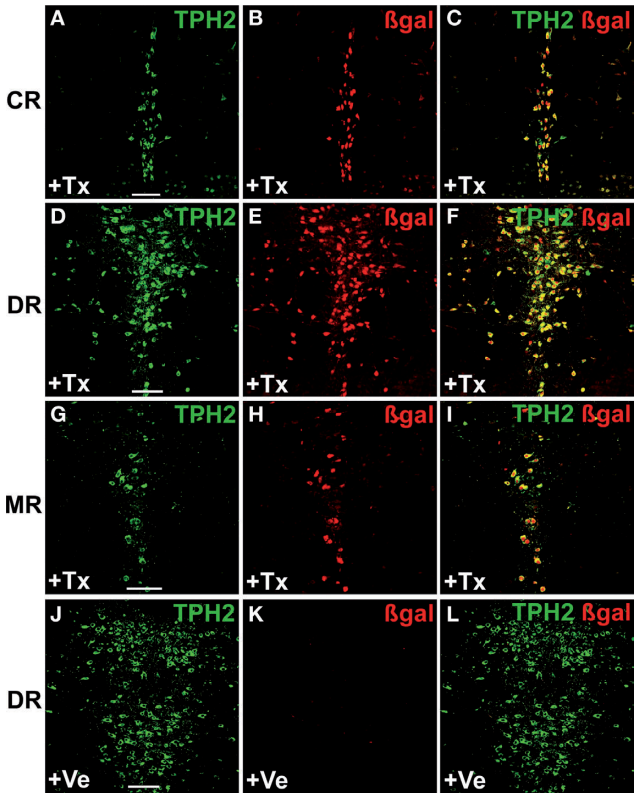

FIGURE 3 | Inducible recombination is restricted to serotonergic neurons of adult R26R$^{TPH2\text{-}CreERT2}$ mice. R26R$^{TPH2\text{-}CreERT2}$ mice of line 1.38 were injected with tamoxifen (1 mg twice daily) or vehicle for five consecutive days starting at P90. Coronal sections show dual-label fluorescence immunohistochemistry for TPH2 and β-gal in tamoxifen-treated (+Tx) **(A–I)** and vehicle-treated mice (+Ve) **(J–L)**. **(A,D,G,J)** TPH2 fluorescence; **(B,E,H,K)** β-gal fluorescence; **(C,F,I,L)** Merged images. Colocalization is visualized at the level of caudal raphe (CR) nuclei **(A-C)**, dorsal raphe (DR) nuclei **(D-F,J-L)** and median raphe nuclei (MR) **(G-I)** using confocal images. **(C,F,I)** In tamoxifen-treated mice there is extensive colocalization of β-gal and TPH2 indicating efficient recombination. **(J-L)** Vehicle-treated mice show minimal colocalization demonstrating low background recombination without tamoxifen. Scale bars: 100 µm.

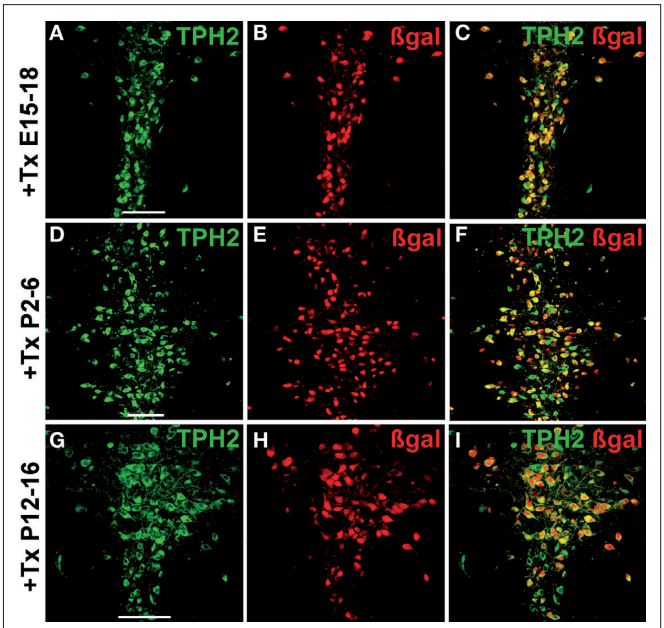

FIGURE 4 | Inducible recombination in serotonergic neurons of embryonal and postnatal R26R$^{TPH2\text{-}CreERT2}$ mice. Pregnant or lactating mothers of line 1.38 were injected with tamoxifen (+Tx). **(A–C)** One milligram twice daily at E15–18; **(D–F)** 1 mg twice daily at P2-6; **(G–I)** 3 mg twice daily at P12-16. Transgenic R26R$^{TPH2\text{-}CreERT2}$ offspring were sacrificed at P60 and analyzed with dual-label fluorescence immunohistochemistry for TPH2 and β-gal. Colocalization is visualized at the level of dorsal raphe nuclei using confocal images. **(A,D,G)** TPH2 fluorescence; **(B,E,H)** β-gal fluorescence; **(C,F,I)** merged images. Frequent colocalization of β-gal and TPH2 confirms efficient recombination during embryogenesis **(C)** and early **(F)** and later **(I)** postnatal development. Scale bars: 100 µm.

on E20 since tamoxifen treatment impairs parturition (Boyle et al., 2008; Erdmann et al., 2008). R26R$^{TPH2\text{-}CreERT2}$ offspring were sacrificed in adulthood and the efficacy and selectivity of recombination analyzed with IHC against TPH2 and β-gal. Serotonergic recombination could be efficiently induced with tamoxifen during embryogenesis (recombination efficacy 66%) (**Figures 4A–C**; **Figures S2A–C,J–L** in Supplementary Material; **Table S3** in Supplementary Material) but recombination rates were below those achieved during adulthood.

Next, we determined whether recombination during early and later postnatal development is feasible. Lactating mothers were injected intraperitoneally for five consecutive days starting at P2 and at P12, respectively. Different tamoxifen protocols were used: 1 mg tamoxifen twice daily (protocol 1), 2 mg tamoxifen twice daily (protocol 2), 3 mg tamoxifen twice daily (protocol 3) and 1 mg tamoxifen once daily (protocol 4).

Intraperitoneal injections were well tolerated by lactating mothers during the five consecutive days of tamoxifen treatment but mothers tended to abandon their offspring thereafter, in particular those treated with 2 mg × 2 mg tamoxifen during early postnatal development (P2–6). Recombination during early and later postnatal development could be successfully induced in serotonergic neurons (**Figures 4D–I**; **Figures S2D–I,M–R** in Supplementary Material) but recombination efficiency varied significantly with protocols. Tamoxifen injections twice daily (2 mg × 1 mg) led to superior recombination efficacy (68%) compared to single daily injections (1 mg × 1 mg) whereas doubling the amount of tamoxifen (2 mg × 2 mg) during early postnatal development (P2–6) did not result in better recombination efficacy than with 2 mg × 1 mg tamoxifen per day. In contrast, further increasing the amount of tamoxifen (2 mg × 3 mg) in lactating mothers with older offspring (P12–16) led to recombination efficacies (72%) comparable to those achieved during early postnatal development with 2 mg × 1 mg tamoxifen (**Table S3** in Supplementary Material).

In conclusion, significant recombination efficacies could be attained in R26R$^{TPH2\text{-}CreERT2}$ offspring from tamoxifen injected pregnant or lactating mothers but recombination rates did not reach the level of adult recombination (90%). We also noted that insubstantial recombination occurred during embryogenesis and early postnatal development in regions which do not contain 5-HT neurons in adult mice. A few β-gal-positive/TPH2-negative cells were detected in the caudal and MR nuclei, the hypothalamus and deep layers of the cortex.

INDUCIBLE RECOMBINATION IN BRAINBOW-1.0L$^{TPH2\text{-}CreERT2}$ MICE RESULTS IN COMBINATORIAL EXPRESSION OF FLUORESCENT PROTEINS WITH MULTIPLE, DISTINCT COLORS IN 5-HT NEURONS

The recently described Brainbow-1.0L transgenic mouse line (Livet et al., 2007) allows individual fluorescence labeling of living neurons. In this line, Cre-mediated recombination of loxP-flanked fluorescence transgenes leads to a switch from RFP (red fluorescent protein) expression to a stochastically determined combinatorial expression of CFP and YFP.

Double transgenic Brainbow-1.0L$^{TPH2\text{-}CreERT2}$ mice were induced with 1 mg tamoxifen twice daily for one to five consecutive days and recombination was analyzed by fluorescence emission. Confocal laser-scanning microscopy of RFP, CFP and YFP fluorescence detected the combinatorial expression of CFP and YFP leading to mosaic fluorescent labeling of serotonergic neurons (**Figure 5**; **Figure S3** in Supplementary Material). The length of tamoxifen treatment correlated with the proportion of recombined 5-HT neurons. Three days of tamoxifen treatment were sufficient to achieve maximal serotonergic recombination whereas in animals given 1–2 days of tamoxifen treatment the proportion of recombined 5-HT neurons was significantly reduced.

EFFICIENT KNOCKOUT OF THE GLUCOCORTICOID-RECEPTOR GENE IN SEROTONERGIC NEURONS OF ADULT GRflox/GRflox$^{TPH2\text{-}CreERT2}$ MICE

Both R26R and Brainbow mice are reporter mice which express fluorescent proteins or βgal following Cre-mediated recombination. To demonstrate that loxP flanked target genes can also be efficiently deleted in the adult serotonergic system we generated TPH2-CreERT2 mice that are homozygous for the loxP-flanked glucocorticoid-receptor (GR) gene (GRflox/GRflox$^{TPH2\text{-}CreERT2}$) (Tronche et al., 1999). The GR protein is ubiquitously expressed throughout the brain. In particular, 5-HT neurons show abundant GR-antibody staining. Adult mice (2 months old) were treated with tamoxifen (1 mg twice daily for 5 days) and sacrificed 14 days later. Double-immunostaining against GR and TPH revealed that 80–90% of 5-HT neurons had lost their GR-immunoreactivity whereas GR expression was unchanged in all other brain regions examined (**Figure 6**; **Figure S4** in Supplementary Material).

DISCUSSION

In this study, we describe a novel transgenic tool for conditional somatic manipulation of loxP-flanked target genes in serotonergic neurons. Using the *Tph2* genomic locus as a regulatory element to direct CreERT2 expression specifically to serotonergic neurons we show efficient, tamoxifen-inducible recombination with minimal background activity in R26R$^{TPH2\text{-}CreERT2}$ mice during development and adulthood.

Traditionally, transgenic mouse lines are generated via pronuclear injection of plasmid-derived DNA resulting in random integration of the transgene into the genome. The level and pattern of plasmid-derived transgene expression is determined both by the promotor and its integration site in the genome. Such transgenic mice show copy-number independent transgene expression in regions or cell types not associated with the original specificity of the promoter (Bronson et al., 1996; Wallace et al., 2000; Chandler et al., 2007).

Because of the large insert size of BAC/PAC clones, they can often accommodate an entire gene of interest, including long-range cis-acting regulatory elements required for correct temporal and tissue-specific expression (Chandler et al., 2007). This is especially desirable for experiments where precise transgene expression is critical, such as for promotors driving Cre-recombinase (Lee et al., 2001). These large constructs allow copy-number dependent expression of the transgene resembling the expression of the endogenous gene independent of the integration site (Schedl et al., 1993; Casanova et al., 2001; Giraldo and Montoliu, 2001; Gong et al., 2003).

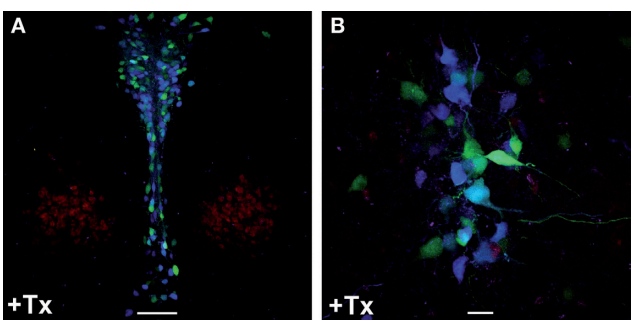

FIGURE 5 | Inducible recombination in adult Brainbow1.0L$^{TPH2\text{-}CreERT2}$ mice leads to combinatorial expression of CFP and YFP in serotonergic neurons. Tamoxifen (+Tx) (1 mg twice daily) was injected for five consecutive days starting at P90. **(A,B)** One week later, mice were sacrificed and red, cyan and yellow fluorescence emission was analyzed by confocal microscopy at the level of dorsal raphe nuclei. Stochastic recombination of loxP-flanked fluorescent genes leads to CFP and YFP expression. Depending on the ratio of CFP to YFP expression, each 5-HT neuron shows a distinct color ranging from dark blue (only CFP expression) to yellow (only YFP expression). **(B)** Fluorescence labeled neuronal projections are easily detectable. Scale bars: **(A)** 100 μm, **(B)** 20 μm.

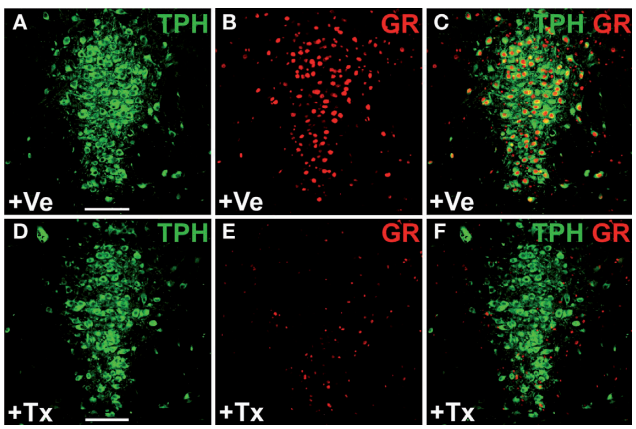

FIGURE 6 | Inducible glucocorticoid receptor (GR) knockout in serotonergic neurons of adult GRflox/GRflox$^{TPH2\text{-}CreERT2}$ mice. Tamoxifen (1 mg twice daily) or vehicle was injected for five consecutive days starting at P60. Coronal sections show dual-label fluorescence immunohistochemistry for TPH and GR in vehicle-treated (+Ve) **(A–C)** and tamoxifen-treated (+Tx) GRflox/GRflox$^{TPH2\text{-}CreERT2}$ mice **(D–F)**. Colocalization is visualized at the level of dorsal raphe nuclei using confocal images. **(A,D)** TPH fluorescence; **(B,E)** GR fluorescence; **(C,F)** merged images. **(C)** In vehicle-treated mice, GR colocalizes extensively with TPH. **(F)** GR is primarily expressed in TPH-negative neurons in tamoxifen-treated GR-knockout mice verifying the selective deletion of GR in 5-HT neurons. Scale bars: 100 μm.

In this study we were able to demonstrate that tissue-specific regulatory elements of the mouse *Tph2* locus, present on a 177 kb genomic DNA fragment and comprising the entire *Tph2* gene plus large flanking regions, reliably direct Cre expression selectively to serotonergic neurons in all five analyzed transgenic TPH2-CreERT2 lines.

To date only two mouse lines published have been shown to target loxP-flanked gene knockouts to the serotonergic system. A knockin of the Cre recombinase into the *Sert* gene has been used to achieve recombination in serotonergic neurons (Zhuang et al., 2005). However, this strategy is compromised by the fact that it results in a hemizygous *Sert* knockout. Furthermore, SERT is expressed in extraserotonergic neurons during development (Gaspar et al., 2003). Therefore, using the endogenous *Sert* locus to control Cre expression will lead to developmental recombination in neurons outside the serotonergic system. Finally, the *Sert*-Cre mouse line does not contain regulatory elements that allow temporal control of Cre-mediated recombination.

Recently, a Pet1-Cre transgenic mouse line was published (Scott et al., 2005) in which cell-type specific serotonergic gene inactivation of loxP-flanked target genes will be irrevocably initiated at E12.5 during embryogenesis. Despite its excellent characteristics to create tissue-specific embryonal knockouts in the serotonergic system, Pet1-Cre lacks the possibility of temporal control. As *Pet-1* is a key player in a transcriptional mechanism that controls embryonal 5-HT neuron differentiation (Hendricks et al., 2003) it would be of interest whether its regulatory elements are sufficient to achieve inducible CreERT2-mediated gene inactivation in the entire serotonergic system of the postnatal and adult brain as well.

TPH2 is the rate-limiting enzyme of 5-HT synthesis in the serotonergic raphe nuclei (Walther et al., 2003). In the brain, it is strongly and exclusively expressed in serotonergic neurons starting during embryogenesis around E11 and continuing throughout adult life (Cote et al., 2003, 2007). Thus, regulatory elements of the *Tph2* locus should ensure strong serotonergic Cre expression in TPH2-CreERT2 mice at any time from embryogenesis to adulthood. To test this, we induced recombination in R26R$^{\text{TPH2-CreERT2}}$ mice during embryogenesis, postnatal development and adulthood. In adult mice, tamoxifen injections twice daily for five consecutive days lead to highly efficient induction of recombination specifically in serotonergic neurons. During embryogenesis and postnatal development intraperitoneal injections to the mothers led to efficient serotonergic recombination (66–72%) in their offspring but rates were inferior to adult recombination (90%). This finding is most likely a result of the tamoxifen application route rather than of attenuated activity of *Tph2* regulatory sequences driving CreERT2 during development as expression of TPH2 commences already at E11. Moreover, we used the identical *Tph2* regulatory sequences to direct the expression of other genes to serotonergic neurons during development and found strong expression already at E15 (unpublished observation). In our opinion, these results demonstrate that ip application to the mother does not lead to sufficient tamoxifen concentrations in their offspring to achieve high recombination rates. Furthermore, tamoxifen is an active substance which inhibits ERs and stimulates progesterone receptors. In pregnant mice, tamoxifen treatment inhibits parturition (Boyle et al., 2008; Erdmann et al., 2008) which necessitated scheduled cesarean sections at E20. While behavioral effects of tamoxifen treatment have not been identified in adult mice (Vogt et al., 2008) there is no comparable data on mice available that received tamoxifen treatment during development.

Taken together, Pet1-Cre mice are preferable if embryonal serotonergic gene deletion starting at E12.5 (Scott et al., 2005) is desired as they do not require tamoxifen and convey high recombination rates. Inducible TPH2-CreERT2 mice are advantageous if recombination is to start later than E12.5. As such, TPH2-CreERT2 mice are currently the only alternative for postnatal and adult induction of serotonergic recombination. Nonetheless, further research needs to be conducted to improve tamoxifen application during development and to evaluate the biochemical, molecular and behavioral impact this treatment exerts on developing offspring.

Serotonergic recombination in Brainbow-1.0L$^{\text{TPH2-CreERT2}}$ mice (Livet et al., 2007) creates a stochastic choice of Thy1-promotor controlled expression between two different loxP-flanked fluorescence genes as a result of tandem integration of Brainbow-1.0L copies. To date, it was unclear whether the Thy1-promotor is active in 5-HT neurons. Here we show that, upon tamoxifen induction, Brainbow-1.0L$^{\text{TPH2-CreERT2}}$ mice display highly efficient recombination in 5-HT neurons. Virtually all serotonergic neurons can be labeled with combinatorial CFP and YFP expression. An advantage of the TPH2-CreERT2 system in conjunction with the Brainbow mice is the possibility of titrating the proportion of 5-HT neurons exhibiting recombination by reducing the duration of tamoxifen treatment. This should facilitate anterograde tracing experiments of single, distinctly color-labeled serotonergic cell projections by three-dimensional reconstruction from serial sections which will help to define the connectivity of individual 5-HT neurons or its cell clusters (B1-9) with other brain regions and within the raphe nuclei.

Tissue-specific recombination efficacies derived from artificial Cre reporter lines should be interpreted carefully regarding their value to predict recombination frequencies for loxP-flanked target genes (Weber et al., 2001). On this account, we substantiated our results obtained with R26R Cre reporter and Brainbow mice by inducing a selective knockout of the endogenous GR gene exclusively in 5-HT neurons of the adult mouse. With the GRflox/GRflox$^{\text{TPH2-CreERT2}}$ mice it will be possible to decipher the reciprocal interactions between the HPA axis and 5-HT neurons, systems that have been implicated together in the etiology of several pathological conditions like escalated aggression and violence or anxiety and depression (Haller et al., 2005; Miczek et al., 2007; Lanfumey et al., 2008).

The neurotransmitter serotonin plays a diverse and important role in both the developing and adult brain (Gaspar et al., 2003). With our TPH2-CreERT2 line we provide a tool for highly efficient and inducible recombination specifically in the serotonergic system. This system will allow the study of the roles of many genes in the regulation of serotonin systems and hence its role in brain development and function. Moreover, the possibility of manipulating gene expression selectively in mature serotonergic neurons allows direct molecular and behavioral effects of candidate gene deletion to be distinguished from compensatory, homeostatic mechanisms during development. In the pathology of complex neuropsychiatric diseases, serotonergic genes, in conjunction with stressors, may act at

any time from development to adult life to predispose an individual to psychiatric disorders (Caspi et al., 2002, 2003; Champoux et al., 2002). The action of perturbed serotonergic genes may even produce opposite phenotypes depending on time of action (Gross and Hen, 2004). This TPH2-CreERT2 mouse model may help to delineate time windows in which gene inactivation is crucial for the pathogenesis of neuropsychiatric disorders dependent on serotonergic abnormalities. Furthermore, varying the onset of gene manipulation during different periods of life and combining genetic manipulation with pharmacological or behavioral interventions will help to unravel gene-environment interactions that are crucial for the development or maintenance of pathological phenotypes.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/molecularneuroscience/paper/10.3389/neuro.02/024.2009/

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