Gene Targeting in the Mouse Nervous System

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Our understanding of the development, connectivity and function of the nervous system has been facilitated by gene targeting technology. Here we summarize the historic background and the current state of this experimental approach with specific regard to neuroscience research.

I. The Pioneering Experiments. Random mutagenesis is a very powerful method for elucidating gene function in simpler model organisms. Given the size of the genome and slow reproductive cycles, however, a more direct approach is required for mammalian models. This need was met in the early 1980's by establishing gene targeting in embryonic stem cells, later nicknamed mouse knockout. This technology was born at the confluence of two lines of experimentation: Firstly, the capacity of most mammalian somatic cells to carry out homologous recombination between endogenous loci and exogenous DNA was discovered. Secondly, pluripotent embryonic stem cell lines have been established that maintained the ability to intermingle with the early mouse embryo and contribute to the germline, thereby conferring heritability, even after extensive culturing in vitro**.**

The process of homologous recombination had been known to exist in yeast, and during the prophase I of meiotic cell division during gametogenesis. In somatic cells, however, this faculty was generally believed to be absent, until curious results of gene conversion in human fetal globin genes (Slightom et al., 1980) and head-to-tail concatemerizations of microinjected plasmid DNA copies in the process of genomic integration (Folger et al., 1982) strongly suggested that this mechanism is operating in any cell. Subsequently, unequivocal evidence was furnished (Folger et al., 1984; Smithies et al., 1985), opening the door for specific gene modifications in somatic mammalian cells.

In a concurrent and intellectually stimulating experimental advancement, embryonic stem (ES) cells were isolated from normal mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981).

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Unlike the previously characterized embryonic carcinoma (EC) cells, which were derived from testicular teratocarcinomas and also displayed some pluripotent features but never made germ cells (Hogan, 2007), the ES cells maintained their potential to substantially contribute to the mouse embryo after blastocyst injection (Bradley et al., 1984). ES cells could be cultured for several generations in vitro, genetically modified and subsequently introduced in the mouse germ line, which predestined them for introducing targeted the genetic modifications in the mouse as heritable traits (Capecchi, 1989b; Evans, 1989; Koller and Smithies, 1992; Robertson, 1991).

II. Mouse Strains and Stem Cells Used in Gene Knockout Technology. ES cells afford a fascinating experimental opportunity to perform in essence any conceivable genomic modification in vitro and pass it on to live, behaving animals. Presently, ES cells are defined by two criteria: (i) unlimited symmetrical proliferation in vitro (ii) pluripotent developmental potential in vivo. The capacity for sustained and complete pluripotency of these cells is critical; without the ability of the genetically modified ES cell to eventually form sperm or egg, the genetic changes would be obviously limited to a single animal. For surmounting the barrier of germline transmission, no mouse strain had a greater significance than 129. The 129 mice are genetically predisposed to develop congenital testicular germ cell tumors that resemble relatively rare pediatric germ cell tumors in humans (Oosterhuis and Looijenga, 2005). The proclivity of strain 129 to testicular teratomas was first discovered by Stevens and Little (Stevens and Little, 1954). In their original report, they showed that teratomas arise spontaneously in the testes of ~1% of 129 male mice. Although the susceptibility genes causing these tumors have nor been identified yet, it is known that an additional defect in the *Ter* locus (Stevens, 1973), affecting the *Dnd1* gene (Youngren et al., 2005), increases the incidence of testicular teratocarcinomas in the 129 genetic background up to 94% (Noguchi and Noguchi, 1985). Teratocarcinomas can be serially transplanted between mice and eventually, conditions were developed that allowed the culture of these cells, later known as EC cells, in vitro (Kahan and Ephrussi, 1970).

The 129 teratocarcinoma-derived EC cells are pluripotent and can differentiate, somewhat haphazardly, into cartilage, neural tissue, myocardium etc. Curiously, they never contribute to the tissue of origin - the germ line - after injection into a host blastocyst to generate a chimera. Further refinement of culture techniques, especially the introduction of cell feeder layers of mitotically inactivated embryonic fibroblasts, allowed Evans and Kaufman to establish a cell culture from the inner cell mass of 129 blastocyst (Evans and Kaufman, 1981). In this major

experimental breakthrough, the preimplantation blastocysts were collected at a state of developmental diapause, induced by ovariectomy, and subsequently cultured in vitro. The cell cultures derived from the cylinder-like structures had the resemblance and general growth characteristics of feeder-dependent EC cells, but unlike the tumor-derived EC cells, the embryo-derived ES cells gave rise to high-proportion of germ-line chimeras (Bradley et al., 1984). This success immediately turned the 129-derived stem cells into the vehicle of choice for genomic modifications in the mouse (Johnson et al., 1989; Koller et al., 1989; Robertson et al., 1986; Schwartzberg et al., 1989; Thomas and Capecchi, 1990; Thompson et al., 1989; Zijlstra et al., 1989).

As a result, the vast majority of mice genetically modified by gene targeting thus far have a portion of129 genome in their genetic background. Unfortunately, 129 mice are poor breeders, and have abnormal anatomy (Wahlsten et al., 2006), immunology (McVicar et al., 2002), and behavior (Crawley et al., 1997). Genetic variability among more than half a dozen 129 substrains, and as many ES cell lines (e.g., EK.CCE, AB1, AB2.2, D3, HM1, J1, mEMS32, R1) is also considerable and has been a confounding factor in interpretation of the resulting phenotypes (Simpson et al., 1997). One illuminating example is the strain's tendency to develop corpus callosum defects that erroneously implicated the *Emx1* homeobox gene in the formation of this major axon bundle (Qiu et al., 1996; Yoshida et al., 1997). Although the *Emx1* mutants manifested acalllosal phenotype in the 129 background, after 10 generations of backcrossing with C57BL/6 this defect was reversed and all $Emx1^{-/-}$ mice developed corpus callosum normally (Guo et al., 2000). Therefore, caution should be exercised whenever neuroanatomical or behavioral phenotype is evaluated in animals with a high share of 129. Backcrossing to a more robust strain is typically carried out as a corrective measure. It is, however, very time consuming; in order to achieve >99% congenicity, backcrossing for 10 generations is necessary, which requires about 3 years. It can also be very costly; Speed Congenic projects guided by genome-wide DNA maker scans can achieve a similar level of congenicity in 5-6 generations, but the associated cost is high. Even more disturbingly, genetic context close to the targeted gene (~10 cM) is always that of the stem cell, even after extensive backcrossing (Hospital, 2001). If strain-specific cis-regulatory mutations exist, expression of the targeted gene will always reflect the 129 pattern.

Neuro-behavioral researchers have been gradually shifting the focus towards ES cells derived from other genetic strains, mainly the C57BL/6 strain that was developed by Clarence Cook Little in 1921. There are numerous reasons why C57BL/6J is widely used. Since it was the first strain to have its genome completely sequenced, comprehensive genetic resources are widely accessible. Most notably, high-quality bacterial artificial chromosome (BAC) libraries have been constructed (Osoegawa et al., 2000), end-sequenced (Zhao et al., 2001), and aligned against the mouse genome by the UCSC genome browser (Zweig et al., 2008). These publicly available resources have reduced the time required for the identification and acquisition of isogenic DNA for the construction of targeting vectors to minutes and days, respectively. From the anatomical and behavioral point of view, C57BL/6J mice suffer from age related hearing loss 1 (*Ahl*) due to mutations in the *Cdh23* gene, with an onset after 10 months of age (Johnson et al., 2000). The C57BL/6J strain is also mutant for nicotinamide nucleotide transhydrogenase (*Nnt*), which contributes to glucose intolerance, resulting in mild to moderate hyperglycemia and hyperinsulinemia, and susceptibility to diet-induced obesity (the C57BL/6N substrain carries the wild type allele for this locus). Curiously, C67BL/6J mice prefer 10% ethanol to water (Fuller, 1964). Other traits include a high incidence of microphthalmia, low bone density, increased incidence of hydrocephalus, as well as hair loss associated with overgrooming and barbing. Yet, good longevity, fertility, low susceptibility to tumors and good performance on cognitive and learning tasks make C57B/6 a very useful model for neurobiology studies that can be interpreted in the context of a large body of research.

Although 129 ES cells consistently outperform other mouse strains in terms of their potential to colonize developing embryos, their culture vigor and relative tolerance to genetic background of recipient blastocysts, many C57BL/6 ES lines have demonstrated a surprisingly high germ line transmission and good breeding performance in chimeras with otherwise low (10-40%) coat color contribution (Seong et al., 2004). Certain C57BL/6 ES cell lines (Bruce4, BL/6-III) have been available for almost two decades (Kontgen et al., 1993; Ledermann and Burki, 1991) and are still in productive use despite of reports on their genetic heterozygosity and aneuploidy (Hughes et al., 2007). It commonly requires more attempts to generate chimeric mice with C57BL/6 ES cells, and therefore, hybrid ES cells from 129 x C57BL/6 F1 crosses have been introduced to circumvent this problem (George et al., 2007). These hybrid ES cells (termed G4) are capable of generating 100% ES cell-derived animals if combined with electrofusion-induced

tetraploid carrier embryos (tetraploid embryo complementation assay) (George et al., 2007). However, this approach compromises the purity of the genetic background and, hence, the pursuit of robust C57BL/6 ES lines continues (Keskintepe et al., 2007). With optimized culturing techniques, high rates of germline transmission have been obtained using all three major methodological approaches−blastocyst injection, tetraploid complementation and co-culture aggregation with diploid 8-cell embryos with several new C57BL/6 ES lines (Tanimoto et al., 2008). Importantly, the C57BL/6 strain had been chosen for the production of mutant alleles by large-scale international knockout programs, such as KOMP or EUCOMM (Collins et al., 2007b). More recently, JM8 embryonic stem cells of C57BL/6N origin have been developed that appear to meet the strict requirements for these ambitious projects (Pettitt et al., 2009).

III. Gene Targeting in Other Rodent Species. The serendipity of the presence of teratomas in the 129 strain uncovered the path towards gene targeting in the mouse, which until recently has been the sole mammal amenable to this protocol. However, in order to better appreciate the causality of gene expression in nervous system specialization, particularly in the evolutionary context, it will be necessary to extend this approach to other mammalian models. New discoveries in the field of molecular and developmental biology carry a promise of achieving this goal in the near future. Remarkably, standard laboratory mice are in fact recombinant strains with unequal contributions from all three major subspecies of *Mus musculus* (*M. m. domesticus, M. m. musculus* and *M. m. castaneus*) which undergo spontaneous genetic exchanges at the boundary of their natural geographic ranges (Galtier et al., 2004). The closest relative to *M. musculus* is the North African *Mus spretus*, that is only cross-fertile with *M. musculus* through the female lineage. Inbred strains of *M. spretus* have been established and *M. spretus* x *M. musculus* crosses had been very instrumental during the pre-genomic era in unambiguously mapping hundreds of genes by haplotype analysis, owing to the 2% genome sequence divergence between the two species. Moreover, strong phenotypes observed in *M. spretus* that are related to various immunological and behavioral aspects potentially expand the phenotypic polymorphism available in laboratory rodents (Dejager et al., 2009). Germline-competent ES cells have been established from *M. spretus* (SPRET/Ei) x C57BL/6J hybrid (Hochepied et al., 2004), providing access to gene targeting in this valuable model. Analogous approaches will likely be adaptable for other mouse species capable of generating F1 hybrids with *M. musculus.*

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Given the advantage of size and physiological accessibility, the rat is one of the premier animal models for neuroscience, but its experimental potential has been limited by the lack of the key reagent: germline-competent ES cells. This situation is changing rapidly now, although it took almost 25 years to overcome the case-specific strategies developed for the 129 strain of mice. The recent work on ES cells has made it clear that in order to maintain the ground state of "stemness" in ES cells, it is essential to repress several key pathways towards differentiation – particularly those governed by fibroblast growth factor (FGF) and glycogen synthase kinase 3 (GSK3) signaling (Ying et al., 2008). Effectors of these signaling pathways are ubiquitously present in fetal calf serum, a common component of ES cell media, which has been used along with the leukemia inhibitory factor (LIF) and bone morphogenetic proteins (BMP) to promote ES cell proliferation and self-renewal. The 129 strain and some other murine ES cells can endure these conditions, but as it turned out, rat and presumably other mammalian ES cells absolutely require additional active repression of GSK3 and mitogen-activated protein kinase (MEK/ERK) activities, which would otherwise respond to extrinsic and autocrine FGFs and drive the cells towards commitment. By supplementing the serum-free ES media with specific small molecule inhibitors of GSK3 and MEK/ERK pathways, authentic rat ES cells have been isolated and their germline potential has been confirmed (Buehr et al., 2008; Li et al., 2008). Undoubtedly, this discovery heralds a new era for the rat model in experimental neuroscience. Moreover, induced pluripotent stem (iPS) cells have been derived from adult rat fibroblasts using the newly established technique of genetic reprogramming with a cocktail of four transcription factors (Oct4, Sox2 c-Myc and Klf4) (Li et al., 2009; Liao et al., 2009). Although germline competence of rat iPS cells has not been demonstrated yet, it can be expected in the near future, promising to further expand availability of genetic targeting to particular strains of rats or mice and potentially to other species.

IV. Strategies to Specific Gene Inactivation. To specifically modify the eukaryotic genome, one has to introduce foreign DNA with extensive sequence homology, termed targeting vectors, into the cell nucleus. Initially, this was done by adding crystals of calcium phosphate with co-precipitated plasmid DNA to the cultured cells (Graham and van der Eb, 1973). However, this approach has a very low efficiency–approximately one in one million cells. This deficiency was dramatically improved by microinjecting DNA directly to the cell nucleus through glass micropipettes (Capecchi, 1980), producing targeted recombinants at a frequency of

about 1 per 1,000 injected cells or better (Thomas et al., 1986). Until today, this technique remains unsurpassed regarding the success rate of the desired DNA modifications, which is probably related to an optimal (limited) number of exogenous DNA molecules per cell. Because microinjection is laborious and difficult to automate, it was eventually replaced by other mass delivery methods, out of which electroporation approximates best the ideal method. In our laboratory, one to ten million ES cells in one milliliter of the buffer are electroporated with fifty micrograms of linearized targeting vector and plated onto six ten-centimeter plates with confluent irradiated feeder cells for selection. Drug-resistant cells give rise to clones that are further expanded and analyzed.

Once introduced into the nucleus, the exogenous DNA molecule frequently becomes integrated in a host's chromosome by one of the two principal mechanisms: either by a non-specific integration that involves random chromosome breakage and non-homologous end-joining (NHEJ), or by homologous recombination (HR) that utilizes alignment of extensive sequence homology between the recipient DNA molecule and exogenous DNA. The NHEJ process is of vital importance since it is believed that a single unrepaired DNA break can induce cell death (Rich et al., 2000). The double-stranded end repair involves two Ku70/Ku80 complexes, two DNA-PKcs molecules and is repaired by the DNA ligase IV/XRCC4 complex (Lieber, 2010; Wyman and Kanaar, 2006). The homologous recombination employs the activities of *Rad51* (the mammalian homologue of *RecA*) as well as *Rad52*, *Rad54* and tumor suppressor genes *Brca1* and *Brca2* (San Filippo et al., 2008). This mechanism is essential for DNA repair, preservation of DNA replication fork, telomere maintenance, and chromosome segregation in meiosis I. Since it normally uses sister chromatids as homologous templates, this mechanism is mainly available during the S and G2 phases of the cell cycle. The relative proportion between NHEJ and HR activities determines the success of specific gene targeting event. Surprisingly, the principal barrier to efficient gene targeting is not the low frequency of HR, but rather the high frequency of NHEJ.

Indeed, the unexpected finding that homologous recombination can occur with substantial frequency between the exogenous DNA and endogenous mammalian chromosomes turned gene targeting in embryonic stem cells into a powerful genetic tool (Capecchi, 1989a; Smithies et al., 1985; Thomas and Capecchi, 1987). Soon it was established that a key parameter affecting the frequency of legitimate homologous integration is the length and quality of the

regions of sequence homology. Extending the length of the homology arms up to about 15 kb consistently increases the frequency of homologous recombination (Deng and Capecchi, 1992; Scheerer and Adair, 1994; Shulman et al., 1990), whereas shortening the homology under 1 kb decreases the targeting efficiency precipitously (Thomas et al., 1992). Similarly, the degree of sequence identity plays a role in properly aligning the homologous regions and 97% or greater identity is necessary for maximum efficiency, stressing the importance of using isogenic DNA for the construction of targeting vectors, especially in the intronic and intergenic regions. Two basic types of gene targeting vehicles have been devised, sequence insertion vectors (ends-in) and sequence replacement vectors (ends-out)**.** Both techniques have specific uses. For example, sequence insertion vectors have been employed to generate gene duplications to study gene dosage effects during development (Boulet and Capecchi, 2002). The sequence replacement vectors have a comparable targeting efficiency, are more intuitive, and are more suitable for most common applications. They constitute a principal targeting approach today. Very few genes provide the opportunity to directly select for their loss of function, which was a convenient feature of initial targeting experiments in the *Hprt* locus counter-selected with 6-thioguanine (Doetschman et al., 1987; Doetschman et al., 1988). Gene conversion events are rare (approximately 1 in 1,000,000 cells) and therefore, they have to be enriched using a suitable positive selection marker. The amino 3'-glycosyl phosphotransferase (*neo*) gene in a combination with the neomycin analog G418 (geneticin) is a highly effective and most commonly used selection, whereas the puromycine and hygromycine selection systems are also effective, but tend to be harsher to the cells.

The targeting frequency remains the least predictable variable of genetic manipulations. The incidence of successful homologous recombination in a specific locus varies widely (~0.01 to 40%), and it is widely believed that it reflects ongoing transcription activity in the particular locus of the ES cell host. Great efforts have been directed towards improving this limiting step across the genome, with partial success (Vasquez et al., 2001). Invariably, linearization of the targeting vector prior to electroporation improves the targeting frequency, presumably by exposing free DNA ends. However, further treatment of the DNA ends, such as addition of dideoxynucleotides or using enzymatic conversion to leave single-strand tails, has not yielded a substantial practical advantage. Similarly, interfering with individual components of the NHEJ or HR pathways to suppress non-specific integration of targeting vectors and enhance homologous

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recombination has so far proved inefficient or deleterious to the pluripotency of ES cells. This is exemplified by the virus-induced chicken B cell line DT40 that displays 10-100% targeting efficiency without any selection (Buerstedde and Takeda, 1991),. On the other hand, a commonly used mechanism that consistently increases the percentage of correctly targeted clones several-fold among the pool of G418-resistant colonies is negative selection. This strategy is mostly used in conjunction with the sequence replacement vectors and relies on the inclusion of a conditional toxin gene in the targeting vector outside the homology regions. In case of illegitimate integration, the herpes virus thymidine kinase HSVTK1 gene and subsequent gancyclovir treatment (Mansour et al., 1988) or diphtheria toxin DT gene (Yagi et al., 1993) eliminate the illegitimate integrations. If homologous strand exchange takes place, the negative selection gene does not integrate and the clone survives.

Several alternative strategies improving the gene targeting productivity deserve special mention. First, gene trapping greatly increases the percentage of successfully targeted clones. The gene trapping method, also known as promoter trapping, hijacks the endogenous transcription activity of the targeted locus to express a promoter-less selection marker included in the targeting vector (Chen and Soriano, 2003; Joyner et al., 1992; Sedivy and Dutriaux, 1999; Stanford et al., 2001). This refinement eliminates the majority of false positive clones that normally result from random intergenic insertion in a standard experiment, because they fail to express the selection gene. If regions of specific DNA homology are included in the gene trap vector, gene targeting of the selected locus will be highly enriched. However, one drawback of this technique is that the gene of interest must be transcriptionally active in embryonic stem cells, which is unfortunately not true for many important genes functioning in the adult nervous system. More recently, new gene trap technology has emerged, based on a DNA transposon from cabbage looper moth *Trichoplusia ni*. This mobile element, named *piggyBac* (Fraser et al., 1996), was found to be highly active in vertebrate cells (Ding et al., 2005). It is precisely excised and integrated into TTAA sites through the activity of *piggyBac* transposase. Importantly, new integration sites have a high preference for the first intron of transcription units (Ding et al., 2005). Hence, with appropriately modified *piggyBac*, random mutagenesis can be accomplished *in vivo* without any selection (Wu et al., 2007). Other transposon systems have been employed to study mammalian genomes, such as the *Sleeping Beauty* transposon recovered from salmonid fish (Ivics et al., 1997), that exhibits a stronger tendency for local “hopping”.

Second**,** targeting with bacterial chromosomes (BACs) harboring extensive DNA homology has been introduced (Valenzuela et al., 2003; Yang and Seed, 2003). Standard plasmid DNA-based targeting vectors usually accommodate up to about 20 kb of sequence homology. In contrast, BACs have a ten-fold greater capacity and the vector is maintained at 1-2 copies per *E. coli* cell, which improves the stability of recombinant mammalian DNA. Moreover, partly because of the low copy number, BACs can be predictably modified by homologous recombination, directly in the prokaryotic host (Muyrers et al., 2001; Muyrers et al., 1999; Zhang et al., 1998; Zhang et al., 2000), enabling the construction of versatile targeting vectors in a short time (Fu et al., 2010; Lee et al., 2001; Testa et al., 2003). This adaptation of homologous recombination in *E. coli* is often referred to as Red/ET cloning or recombineering (Copeland et al., 2001), and it is based on precise regulation of lambda phage (or Rac prophage) recombinogenic genes (*bet*, *gam* and *exo*) as well as several positive and negative selection markers (Warming et al., 2005) to seamlessly join heterologous DNA fragments using as little as 45-55 bp of sequence homology. This affords a much-needed technological breakthrough that lessens the burden of targeting vector preparation. The task has traditionally relied on restriction enzyme digestion, PCR, and ligation of DNA fragments. This sequence used to take months, whereas recombineering reduces the time required to a week or two and can be applied to any size vectors (Wu et al., 2008). As previously mentioned, BAC clones carrying a gene of interest can be identified with the UCSC genome browser (Zweig et al., 2008) and obtained from several sources (ATCC, BACPAC Resources) that greatly facilitate the preparatory work. However, aside from several unique advantages, such the possibility to target several distant loci with one targeting vector (Testa et al., 2003) or targeted replacement of very large genes (Capecchi et al. unpublished data) the large-size targeting vectors did not dramatically enhance the targeting efficiency (Valenzuela et al., 2003). BACs are more difficult to handle and prepare in large quantities. Unequivocal verification of a correct homologous recombination event is also more complex. Extensive homology arms make the standard Southern blotting procedure with external hybridization probes nearly impossible, and alternative methods must be used (quantitative real-time PCR or FISH to determine the copy number of the DNA in question (Valenzuela et al., 2003; Yang and Seed, 2003)).

Third, zinc finger nucleases are changing the ways gene targeting can be implemented. In molecular biology's vocabulary, zinc fingers are 30-amino-acid sequence repeats containing two

invariant pairs of cysteins and histidines (C2H2) structurally stabilized with one Zn^{2+} ion. They were first recognized in the transcription factor IIIA (TFIIIA) from frog oocytes (Klug, 2010). Evolutionarily, this domain has proven to be a very successful molecular design, as it is present in 3% of all genes of the human genome. Zinc finger proteins bind DNA by virtue of the variable region of each zinc finger that recognizes three specific base pairs of DNA. Linear clusters of zinc fingers bind consecutive triplets (Pabo et al., 2001), and three to four fingers provide a sufficient range to recognize 9-12 base pairs with a good specificity. Moreover, variable portions have been thoroughly diversified by mutagenesis and zinc fingers have been isolated that bind most of the 64 possible triplets (Dreier et al., 2001; Dreier et al., 2005; Liu et al., 2002; Segal et al., 1999). Utilizing these biochemical properties of zinc fingers, Zinc Finger Nucleases (ZFNs) have been engineered as protein fusions between several designer zinc finger modules and a non-specific cleavage domain derived from the type II restriction enzyme *Fok*I (Kim et al., 1996). Since the *Fok*I cleavage domain must dimerize to cleave DNA, ZFNs are designed and applied as heterodimers. The unique advantage of these artificial protein hybrids is that they can be rationally designed to introduce double-strand breaks to specific sequences in pre-selected loci. As a consequence, cellular DNA repair pathways are strongly activated, stimulating gene targeting in a given locus 100- to 10,000 fold (Durai et al., 2005; Porteus and Carroll, 2005), which is perhaps the greatest targeting frequency boost presently achievable. The repair can occur by either NHEJ or HR pathways. Repair mediated through NHEJ is error prone, often leading to frame-shift mutations and consequently mutant alleles of the targeted gene. With ZFNs, knockout animals have been obtained in models not easily amenable to genetic modifications, including zebrafish (Meng et al., 2008). ZFNs can be introduced in ES cells, iPS cells or even early stage rat embryos (Geurts et al., 2009) to achieve gene disruption. If a targeting vector is co-introduced that is homologous to the target locus on both sides of the double-strand break, the site can be repaired by homology-directed repair, which allows specific genetic replacements and insertions of exogenous sequences (Hockemeyer et al., 2009). Considering the high, selection-independent modification efficiency in target loci, ZFN-based approaches are bound to rapidly expand the range of rat knockouts available and move us closer to clinical applications aspiring to correct inborn mutations in adult stem cells (Lombardo et al., 2007).

Using these technologies, thousands of specific mouse genes have been disrupted, providing an increasingly detailed model of mammalian brain development and function. During this phase, the selection of genes to target was exclusively determined by individual research groups pursuing their specific interests. Inevitably, a coordinated international effort to systematically mutate all protein-coding mouse genes has become a logistical necessity (Collins et al., 2007b). In 2007, the International Knockout Mouse Consortium (IKMC, http://www.knockoutmouse.org) was established to curate the progress summary of four participating programs: the Knockout Mouse Project (KOMP) (Austin et al., 2004), the European Conditional Mouse Mutagenesis Program (EUCOMM) (Friedel et al., 2007), the North American Conditional Mouse Mutagenesis Project (NorCOMM), and the Texas A&M Institute for Genomic Medicine (TIGM) (Collins et al., 2007a). IKMC is based in the Jackson Laboratory, Bar Harbor, ME, and is maintained under the auspices of the Mouse Genome Database (Bult et al., 2009). As of March 2011, according to the information provided by IKMC, nearly 16,000 genes (out of ~25,000 registered) have been mutated in ES cells, employing a variety of strategies including gene trapping and conditional mutagenesis that will be discussed in the next section. Only a fraction of these cell lines has been made into mouse lines, but virtually all major neuroreceptors and developmentally important transcription factors have been mutated and their phenotypes have been studied at some extent. However, a more precise understanding of specific gene functions will continue to require case-by-case customized targeting approaches to address experimental questions ranging form assessing point mutations in animal models of human diseases (Zeiher et al., 1995) to evaluating functional equivalence of evolutionary related genes (Greer et al., 2000; Tvrdik and Capecchi, 2006). Predictably, large-scale and small-scale gene targeting strategies will coexist, and their complementary strengths will benefit the diversity of neuroscience research.

V. Conditional Gene Inactivation and Genetic Labeling in the Nervous System.

Numerous genes that are involved in the patterning and function of the nervous system also play essential roles in the embryonic development. If targeted inactivation of such a gene halts embryogenesis, studies on its adult brain functions are impossible. One illuminating example is the case of *Fgf8*, which is absolutely required for gastrulation. As a result, the *Fgf8* mouse mutant embryos die on day 8.5. However, conditional mutagenesis demonstrated that Fgf8 plays additional essential roles in areas such as limb outgrowth (Lewandoski et al., 2000; Moon and

Capecchi, 2000). In the nervous system, *Fgf8* signaling is required for the formation of the midbrain/hindbrain boundary (isthmus) (Sato and Joyner, 2009) or for the patterning of the telencephalon (Storm et al., 2006).

Standard conditional mutagenesis techniques in the mouse utilize the exquisitely specific interaction between the site-specific Cre recombinase and its cognate loxP sequences. As first demonstrated by Sauer and Henderson (Sauer and Henderson, 1988), the bacteriophage P1-derived Cre (causes recombination) is highly active in mammalian cells and low expression levels are sufficient to mediate recombination between two 34-base-pair long loxP sites. Each loxP consists of two 13 base-pair palindromic arms and one 8-base-pair asymmetric spacer, which determines the orientation. Recombination between two loxP sites in direct orientation leads to an excision of the intervening sequence; inverted orientation results in perpetual inversions of the intervening sequence. Moreover, lox sites with mutant asymmetric spacers have been identified, such as lox2272, which can mediate recombination between themselves with a high efficiency, but not between mutant and wild type loxP (Langer et al., 2002; Lee and Saito, 1998). These heterologous lox sites are used in parallel with wild type loxP sites if several alternative recombination events are desired, for example in the Brainbow mouse (Livet et al., 2007), or to achieve an irreversible inversion of the intervening sequence (Schnutgen et al., 2003).

In order to generate a conditional ("floxed") allele, at least two cognate loxP sequences (in the same orientation) have to be introduced by homologous recombination in the genome. This process is governed by same gene targeting principles that were mentioned previously, with some additional considerations. Importantly, the loxP sites have to be engineered in the targeting vector to flank an essential exon in the gene. At the same time, the placement of the loxP sites must not elicit any adverse effects on gene activity before the recombination and that is usually accomplished by choosing insertion points in the flanking introns. Meeting both requirements can be occasionally challenging, such as in the case of very large or highly conserved genes with dense regulatory elements. In the targeted ES clone, it is essential to verify the presence of both the proximal loxP site (usually near the *neo* selection marker) and the distal loxP site, which becomes co-integrated at about half the frequency of the *neo* marker. Recombination between widely spaced loxP sites (>10,000 base pairs), or loxP sites embedded in heterochromatin requires greater intracellular concentration of Cre. However, with a sufficiently strong Cre

expression, such as that in the HPRT-knockin mouse (Tang et al., 2002), very large intra-chromosomal deletions, or even interchromosomal recombinations and syntenic replacements can be achieved (Wallace et al., 2007; Wu et al., 2007).

The spatial precision of a conditional mutagenesis is determined by the expression pattern of Cre that is either governed by an endogenous gene or by a randomly integrated exogenous promoter. Cre insertions in endogenous loci (gene replacements or "knock-ins") reliably follow the pattern of the driver gene expression. One of two strategies is usually selected: Cre is either inserted in the exon 1 of the gene of interest, or it is "appended" in the last, untranslated exon of the gene, using a viral element known as internal ribosomal entry site (IRES). This latter design is very reliable and results in a bicistronic, independent translation of two separate proteins, the endogenous gene product and Cre. The former approach makes it possible to study a cell lineage that is mutant for the gene, if the animal carries the Cre replacement in both alleles. However, targeted approaches require a significant investment of time and effort. To accelerate access to a greater variety of Cre lines for the neuroscience community, the GENSAT Project at the Rockefeller University streamlined a method to generate Cre lines of mice using bacterial artificial chromosomes (BACs) (Gong et al., 2010). Hundreds of these lines, many of which have been characterized (http://www.gensat.org), enable researchers to genetically modify various regions of the mouse brain with good specificity.

In one cluster of applications, conditional Cre/loxP recombination is used to turn on expression of a reporter gene, rather than delete a conditional allele of a gene of interest. This is usually achieved by inserting a loxP-flanked transcriptional stop/polyadenylation signal inside a ubiquitously active gene, to disrupt its expression. Cre removes the transcriptional block and enables synthesis of a reporter molecule. Among several implementations of this basic principle, one stands out for its reliability and wide-spread use; this is the use of a conditional lacZ reporter gene, encoding beta-galactosidase, in the endogenous ROSA26 locus (Soriano, 1999). Beta-galactosidase forms blue precipitate from colorless X-gal substrate and this enzymatic reaction has no background activity in mouse nervous system. ROSA26 was identified by gene trapping experiments in embryonic stem cells, which revealed that this gene has a low-to-moderate but nearly ubiquitous expression, and that its gene product is dispensable in development. One or two copies of the gene are lost in each experiment using genetic tools targeted in this locus. The phenotype of ROSA26 mutant is very mild, but unfortunately it is poorly understood since the

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gene function is not well characterized. Similar to lacZ, the ROSA26 gene has been used to express, in a Cre-dependent fashion, hundreds of genes of interest.

Albeit rather weak in certain glia, ROSA26 is expressed throughout the entire nervous system across the adult life span. As a result, even transient expression of Cre becomes permanently recorded in the genome of the reporter cell and subsequently in the entire clonal population derived from this cell. Hence, this genetic technique reveals, in a cumulative fashion, the extent and fate of specific cell lineages derived from the expression domain of a certain gene. This information is important for an understanding of morphogenesis and function in normal organ development and disease, and cannot be easily attained by other methods. However, if the Cre-labeled gene has several waves of expression associated with multiple developmental roles, this method will not differentiate the staining derived from individual stages. Because of this, numerous efforts have been made to impose temporal control over Cre recombination. Currently, the most widely used system takes advantage of a protein fusion with a member of the nuclear hormone family of intracellular receptors, the estrogen receptor (ER). In the absence of estrogen receptor ligands, the CreER fusion is localized in the cytoplasm, thereby preventing Cre from mediating recombination. ER-specific ligands cause a translocation of the protein fusion to the nucleus and subsequent loxP recombination by Cre. Through several rounds of optimization, three amino acid substitutions were introduced in the ER moiety in a way that alters its specificity from endogenous estradiol to tamoxifen (Feil et al., 1996; Feil et al., 1997). Thus, a single intraperitoneal injection of tamoxifen can elicit loxP recombination in CreER expressing cells within hours of the administration. Unfortunately, tamoxifen also has unwanted biological effects, often leading to pregnancy loss in embryonic studies. Moreover, this approach has to balance two opposing requirements, one to minimize background leakage and another to maximize recombination after induction. Illegitimate recombination in the absence of tamoxifen can be improved by joining the ER moiety on either end of Cre (Matsuda and Cepko, 2007), but the induction efficiency never approaches 100% and CreER-mediated recombination is typically mosaic. The active metabolite causing nuclear translocation is actually 4-hydroxytamoxifen, which is converted from tamoxifen in the liver. Synthetic 4-hydroxytamoxifen is much more costly but its use is warranted if it can be localized, such as in the case of stereotaxic injections in the mouse brain (Weber et al., 2001). Analogous to ER, progesterone receptor (PR) CrePR fusions have been developed that can be induced by the antiprogesteron RU486 (mifepristone).

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CrePR inserted in the tau locus has been shown to induce recombinase activity in neurons in a RU486-dependent fashion (Hashimoto et al., 2008).

The great impact of Cre/lox technology has stimulated a search for alternative site-specific recombinases with distinct sequence specificities. FLP, isolated from the 2-micron circle of Saccharomyces cerevisiae, is the second most common recombinase used in mouse genetics (Dymecki, 1996). FLP binds to FRT, its cognate sequence that is similar in size and structure to loxP. FLP/FRT operates similarly to Cre/lox, but both systems show no mutual cross-reactivity. FLP applications have included excision of residual *neo* markers by breeding with FLP deleter mouse (Rodriguez et al., 2000), genetic labeling of FLP-dependent lineage (Dymecki and Tomasiewicz, 1998), and precise intersectional manipulations of the brainstem in conjunction with Cre/lox system (Kim et al., 2009). Initial studies were compromised by very low activity of the yeast FLP in mammalian cells due to thermolability of the wild type protein. A more stable FLPe was engineered by cycling mutagenesis using error-prone PCR and DNA shuffling (Buchholz et al., 1998) and its activity in mammalian cells was further increased five fold in the FLPo version by codon optimization (Kranz et al., 2010; Raymond and Soriano, 2007). Dre, a more recently discovered recombinase that shares many similarities with Cre, interacts with a distinct recognition target termed rox (Sauer and McDermott, 2004). The Dre/rox system appears to be as effective as Cre/lox (Anastassiadis et al., 2009), and could become an ideal complement to Cre in the toolbox of intersectional genetics with or without temporal control. Lastly, PhiC31 deserves a special mention as it has been used in genome engineering application (Belteki et al., 2003) and optimized for expression in the mouse (Raymond and Soriano, 2007). PhiC31 belongs to a distinctive family of large serine site-specific recombinases that mediate directional recombination between two asymmetrical sites attP and attB. PhiC31 recombination is more prone to error than the tyrosine family of recombinases represented by Cre, Dre and FLP. However, PhiC31 proved useful in removing residual *neo* selection markers from complex alleles (Sangiorgi et al., 2008). The use of site-specific recombinases has had a significant impact on modern neuroanatomy and is also discussed in the Chapter 3.

In its most common implementation, site-specific recombination is irreversible and causes a permanent activation or inactivation of a gene of interest. However, in many experimental paradigms it is desirable to toggle between the On-State and Off-State with regard to a specific gene function. The most widely used technology meeting this requirement is the Tet

regulatory system developed by Herman Bujard's group (Gossen and Bujard, 1992). The Tet system is a binary system consisting of two components, a synthetic transcription factor based on tetracycline-controlled repressor from Escherichia coli Tn10 transposon, and a composite promoter containing a cytomegalovirus (CMV) minimal promoter linked to multiple tetracycline operator (tetO) DNA binding sites. The promoter has been progressively improved by empirical modifications from the original Ptet-1 to a low noise Ptet-14 (aka P_{tight}) to the currently best performing third generation Ptet-T6 (Loew et al.). This promoter displays minimal background activity while maintaining a near maximum of inducibility over 5 orders of magnitude. Transcription of the Ptet-T6 promoter can be controlled with several tetracycline-dependent transcription factors that have been manipulated to function in different modes. The DNA-binding domain of the Tet-OFF (tTA) (Urlinger et al., 2000) transactivator binds the tetO site in the absence of tetracycline, whereas Tet-On (rtTA) (Zhou et al., 2006) binds DNA in the presence of tetracycline. The DNA-binding moiety is typically fused to the VP16 transactivation domain from herpes simplex virus that stimulates transcription of the adjacent gene. However, either DNA domain can be fused to a transcription silencer domain, such as the Kruppel-associated box (KRAB) domain of the Kid-1 zinc finger protein, which results in silencing of transcription initiation within 3,000 base pairs (Freundlieb et al., 1999). Together, Tet system provides exceptional versatility to genetic induction or silencing of genes of interest in the nervous system. Although the Tet system was originally developed using tetracycline as an antibiotic, doxycycline proved to be a more efficient and better-tolerated inducing antibiotic; concentrations of doxycycline as low as 5-10 ng/ml are effective with Tet-Off as well as the latest generation of Tet-On (Zhou et al., 2006). This level of sensitivity enables efficacious gene induction in the brain. Transactivators can be introduced by gene targeting or by random transgenesis and specific brain expression can be can be further increased by combining the Tet system with Cre/lox. By using a combination of these systems, Nakashiba et al. (Nakashiba et al., 2009; Nakashiba et al., 2008) have been able to achieve CA3-specific inducible gene expression of tetanus toxin leading to the inhibition of synaptic vesicle exocytosis and block of neurotransmission. This genetic technology allowed the authors to provide new insight in the role of CA3 in learning and memory consolidation.

VI. Genetic Tools for Functional Dissection of Neuronal Circuits. The isolation and expression of the gene for Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria*

ushered a new era of *in vivo* imaging of live neurons (Chalfie et al., 1994). On the heels of this discovery, searches for additional fluorescent proteins resulted in the identification of a new families of genes, such as those from non-bioluminiscent red mushroom coral *Discosoma* (Matz et al., 1999). Initially, the use of wild type fluorescent proteins in mammalian cells was limited by technical problems, including dim fluorescence, poor photo stability and folding at 37°C, inconvenient excitation spectra, and cellular toxicity due to aggregation, especially prominent in coral-derived proteins. Largely due to the efforts of Roger Tsien and colleagues, fluorescent proteins have been optimized for brightness, emission spectra, and stability and have become the most widely used tracer molecules (Shaner et al., 2005). In addition to the optimized green fluorescent protein (EGFP), tdTomato, an especially bright head-to-tail dimer of the original DsRed protein, has gained popularity. For example, Cre reporter mice with the tdTomato tracer, generated at the Allen Institute for Brain Science (Madisen et al., 2010), provide exceptionally bright and detailed picture of neuronal anatomy, in both live and fixed tissue (**Figure**). In addition, emission spectra of several fluorescent protein reporters have been extended to the near-infrared region, which allow deeper *in vivo* imaging (Shcherbo et al., 2010; Shu et al., 2009). However, the utility of these tools for brain imaging remains to be investigated.

If the density of Cre-positive cells is too high, fluorescent tracing may results in a poorly differentiated signal in which individual cells are difficult to distinguish. To overcome this deficiency, Jeff Lichtman's and Joshua Sanes's laboratories designed a technology to express random combinations of different color fluorescence reporters with Cre/lox technology (Livet et al., 2007). Depending on a specific design (Brainbow 1 or Brainbow 2), a combination of up to four different fluorescent proteins is re-shuffled by the use of compatible and/or heterologous loxP sites positioned in between the genes. Cre makes alternative use of these loxP sites and stochastically determines which fluorescent protein is juxtaposed to the promoter. If multiple copies of Brainbow cassettes are integrated in the genome, about 100 individual color hues can be distinguished. This technology provides an unprecedented possibility to uniquely identify and follow neuronal projections over long distances (Livet et al., 2007).

Another powerful strategy using fluorescent tracing, named Mosaic Analysis with Double Markers (MADM) was developed by Liqun Luo's laboratory (Zong et al., 2005). They have introduced, by gene targeting, Green-loxP-Red and Red-loxP-Green chimeric fluorescent proteins at an equivalent locus in two homologous chromosomes. The chimeric proteins are

colorless. But in the process of mitosis, directed Cre expression can mediate recombination between two different chromosomes, which will result in the reconstitution of fluorescent proteins. An opportune segregation will result in daughter cells of two distinct colors, Green-loxP-Green and Red-loxP-Red. If the progenitor cell harbors a heterozygous mutation on the telomeric side of the green marker, the green daughter cell will inherit both mutations and will be mutant, whereas the red daughter cell can serve as an internal heterozygous control. The fate of the green mutant cell progeny can be then precisely followed in the context of normal brain development. This method will strongly facilitate the task of unraveling the roles of individual genes in brain development and functioning.

Genetically encoded reporters have also improved the sensitivity and specificity of several principal *in vivo* brain imaging techniques, including magnetic resonance imaging (MRI), positron emission tomography (PET) or optical imaging (OI). For example, endogenous expression of either human heavy chain of ferritin or bacterial iron transporter from *Aquaspirillum magnetotacticum* (magA) has been shown to improve MRI visualization of cells positive for these reporters by accumulating iron intracellularly (Cohen et al., 2007; Zurkiya et al., 2008). More recently, a nonmetallic, lysine rich–protein (LRP) reporter has been developed which conveys similar contrasting benefits using so-called chemical-exchange saturation transfer (MRI-CEST) that has less adverse effects on cellular metabolism (Gilad et al., 2007). For PET imaging, the herpes simplex virus type 1 thymidine kinase (HSV1-tk) is most common reporter gene used in conjunction with a series of radiolabeled substrates (Min and Gambhir, 2008). Optical imaging uses relatively inexpensive CCD cameras to detect bioluminescent signal generated by the firefly or *Renilla* luciferases following administration of the substrate luciferin. Remarkably, neural activity in the barrel cortex of anesthetized mice was recorded with luciferase driven by the promoter of immediate early gene *c-fos* (Wada et al., 2010). The special and temporal resolution of these methods is unfortunately rather poor due to their inherent technical limitations.

Great advances in imaging of neural activity were recently made with genetically encoded biosensors based on optimized fluorescent proteins. Of particular importance is the monitoring of calcium transients that are widely used as a proxy for neuron activity. Genetically encoded calcium indicators (GECIs) have been dramatically improved in the past few years, exploiting several alternative strategies. The first design utilized pH-sensitive variant of green

fluorescent protein to measure loss of protons associated with synaptic vesicle fusion and neurotransmitter release, termed synapto-pHluorin. Although this tool did not allow monitoring the activity at individual synapses, it was very instrumental in deciphering odor-induced patterns of glomerular activation in the olfactory bulb (Bozza et al., 2004; Miesenbock et al., 1998). A next generation approach has employed Förster resonance energy transfer (FRET) between cyan and yellow fluorescent proteins that are connected through a linker consisting of calmodulin (CaM) and CaM-binding peptide of myosin light-chain kinase (M13). In the presence of calcium, M13 binds CaM and bends the flexible linker, bringing the fluorescent proteins closer together and causing energy transfer from cyan to yellow. Remarkable sensitivity and dynamic range has been achieved in several variants of these so-called Yellow Cameleons (Horikawa et al., 2010; Nagai et al., 2004). More recently, a particularly efficient calcium sensor has been engineered from a single green fluorescent protein. This strategy relies on circularly permuted GFP: The original N and C termini are fused and new termini are generated in the middle of the protein, one joined with CaM and the other with M13 (Baird et al., 1999; Nakai et al., 2001). To reflect its modular structure, this calcium indicator was named G-CaM-P. Calcium stimulates dimerization of CaM and M13, which in turn stabilizes the chromophore in the centre of the GFP barrel and increases its intrinsic fluorescence by several-fold. The newly optimized variant GCaMP3 shows robust fluorescence responses to calcium transients and faster kinetics than previous constructs (Tian et al., 2009). (**Figure**) Using powerful deep tissue imaging with two photon excitation (Svoboda and Yasuda, 2006), Dombeck et al. were able to monitor the activity of place neurons in the CA1 region of the hippocampus in mice navigating through a virtual maze (Dombeck et al., 2010). Together with rapidly improving genetically targeted voltage-sensitive fluorescent proteins (Akemann et al., 2010), these new sensors will enable a new level of performance for monitoring the activity of large neuronal assemblies in freely behaving animals.

Ultimately, gene targeting provides means to specifically manipulate neuronal activity in behaving experimental models. Numerous attempts have been made to dissect the role of neuronal circuits with endogenously expressed effectors, ranging from complete genetic ablation with Cre-dependent diphtheria toxin (Wu et al., 2006) to more subtle alterations of neural functions with e.g. other naturally occurring neurotoxins, heterologous ligand-gated channels (Li et al., 2002), or designer G protein coupled receptors (Rogan and Roth, 2011). For example, the

previously mentioned tetanus toxin gene has been used in conjunction with Cre- and FLPe-dependent intersectional genetics to probe the role of rhombomere-specific groups of brainstem neurons in serotonin- modulated behaviors (Kim et al., 2009). Inducible expression of genetically encoded conotoxins (from cone snail venoms) or spider agatoxins has been used to specifically inhibit $Ca_v2.1$- and $Ca_v2.2$-mediated calcium currents in dopaminergic and nociceptive modulatory circuits (Auer et al., 2010). However, the kinetics of these approaches is generally slow and does not allow experimental interrogation of dynamic neural networks. This deficiency has been addressed by the introduction of a toolbox of light-gated ion channels collectively referred to as optogenetics (Deisseroth, 2011). Although the phenomenon of light-activated channels directly controlling the flow of ions across the plasma membrane was known for decades, its usefulness for precise control of neuronal activity had not been generally accepted until a landmark report was published in 2005 (Boyden et al., 2005). Two classes of light-sensitive opsins have been harnessed for expression in mammalian cells–activating (depolarizing) channelrhodopsins, and silencing (hyperpolarizing) halorhodopsins and bacteriorhodopsins. Channelrhodopsin-2 (ChR2) from green alga *Chlamydomonas reinhardtii*, a sodium channel-controlling gene, is the most widely used opsin that causes membrane depolarization when exposed to blue light. The protein function has been thoroughly exploited by random mutagenesis, yielding variants with a faster spiking pattern (ChETA) and a greater light sensitivity (CatCh) (Gunaydin et al., 2010; Kleinlogel et al., 2011). Among hyperpolarizing opsins, which are yellow light-driven chloride or proton pumps, the halorhodopsin eNpHR3.0 (Gradinaru et al., 2010) or archaerhodopsin-3 (Arch) from *Halorubrum sodomense* (Chow et al., 2010) are particularly useful for *in vivo* experiments. Successful attempts have even been made to create protein chimeras between bovine retina rhodopsin and adrenergic G protein coupled receptors, to control distinct signaling pathways (intracellular calcium concentration or cyclic AMP) in response to light (Airan et al., 2009). Using a combination of viral and transgenic techniques, optogenetic tools have been used in freely moving animals to begin elucidating the circuits underpinning complex behaviors and disorders such as conditioned fear, depression anxiety or aggression (Covington et al., 2010; Haubensak et al., 2010; Lin et al., 2011; Tsai et al., 2009; Tye et al., 2011).

In synergy with further technological breakthroughs in the physics of two photon excitation (Grewe et al., 2010), biochemistry of light-controlled protein interaction, such as split

Cre dimerization (Kennedy et al., 2010) or cell biology of virus dependent mono-trans-synaptic labeling techniques (Miyamichi et al., 2010), gene targeting will continue to play an important role in providing a precise spatial and temporal platform for increasingly sophisticated experiments to unravel how information is represented and processed in different neural assemblies of the brain.

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FIGURE. Genetically encoded fluorescent reporters used in studies of brain development and function. (A–D) Enhanced green fluorescent protein (EGFP) has been used in countless application to illuminate live cells of interest. In this example, a tauEGFP protein fusion was knocked in the endogenous Hoxb1 locus via internal ribosomal entry site (IRES). This genetic modification allows direct visualization of Hoxb1-governed processes in the mouse embryo, including the specification of the rhombomere 4 (r4) in the developing hindbrain and outgrowth of the facial motor nerve (7n). The tauGFP reporter is enriched in the axons by virtue of its interaction with microtubules. E denotes the day of gestation. Modified from Tvrdik and Capecchi (Tvrdik and Capecchi, 2006). (E) Red fluorescent protein variants, such as tdTomato engineered from the original DsRed prototype, are superior neuronal tracers. Avery bright tdTomato Cre reporter mouse line was developed by the Allen Institute for Brain Science (Madisen et al., 2010). In the crosses with the Hoxb8-IRES-Cre driver, which is expressed in the spinal cord, ascending spinal projections in the brain can be directly imaged by confocal microscopy in fresh or fixed sagittal sections, including the most rostral spinothalamic tract (ST). (F) In addition, Hoxb8 is expressed in the definitive hematopoietic system (Chen et al., 2010). As a result, many cortical microglia are also distinctly labeled and readily identifiable with this pancytoplasmic tdTomato reporter. (G) Using a different fluorescent reporter line, in which ubiquitously expressed plasma membrane-associated tdTomato becomes specifically replaced with membrane-bound EGFP in Cre-expressing cells (Muzumdar et al., 2007). Early postnatal infiltration of Hoxb8-positive microglia through the blood vessels into brain parenchyma can be directly observed with two photon imaging. P indicates postnatal age in days. (H–K) Most recently, circularly permuted GFP has been transformed into a very sensitive calcium indicator dubbed GCaMP3, which enables monitoring of neural activity in live brain tissue (Tian et al., 2009). In these rat brain slices, a GCaMP3 gene was incorporated in the DNA of rat embryonic brain cells using piggyBac transposase and in utero electroporations. After pharmacological stimulation, transfected neurons and astrocytes in the cortex show robust two photon signal changes reflecting calcium transients. Two photon imaging was performed by Mike Economo, the GCaMP3 data is courtesy of John White's and Karen Wilcox's labs, University of Utah.

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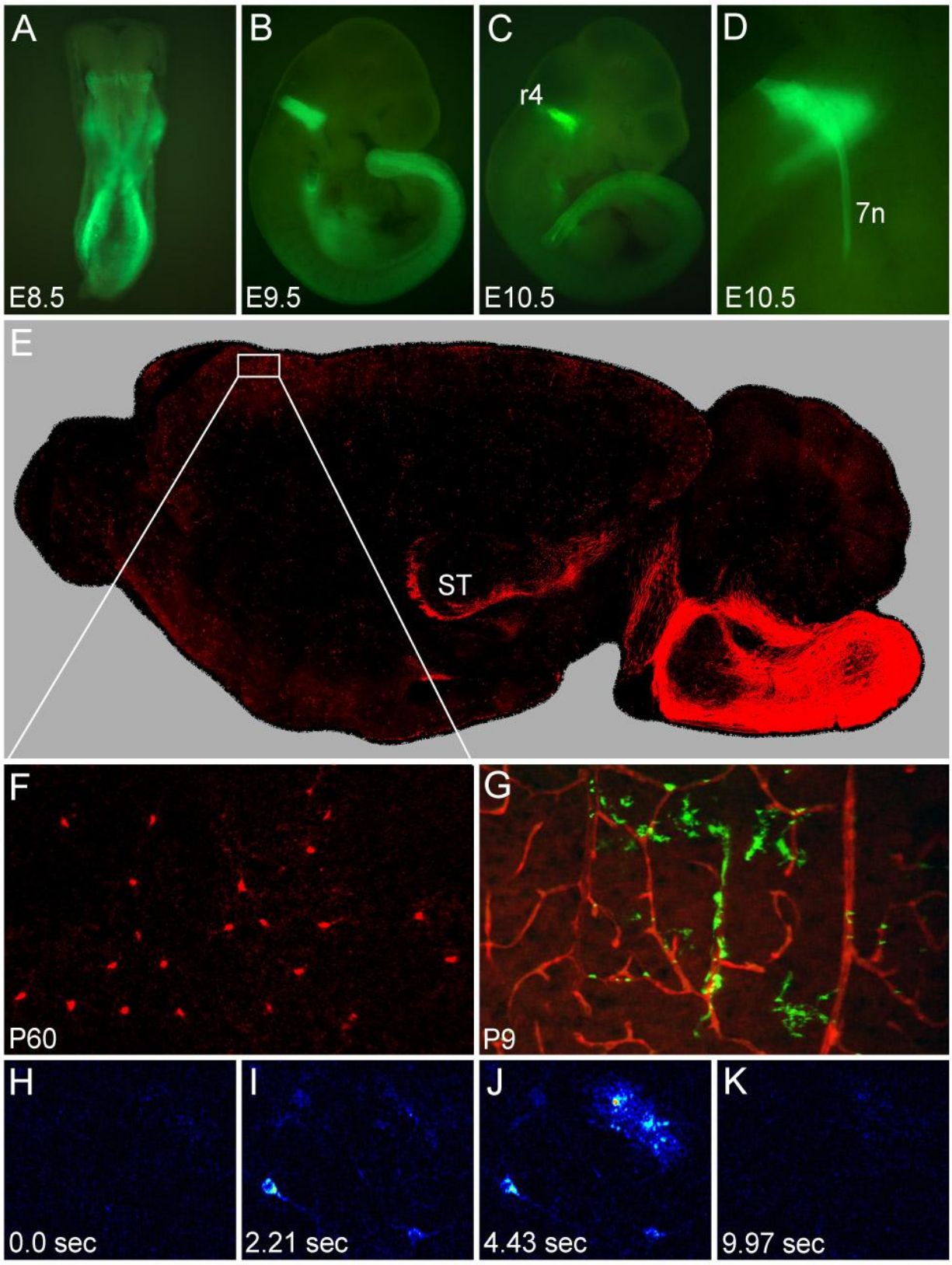

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