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Directing neuron-specific transgene expression in the mouse CNS

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Recent advances in molecular genetics have produced many novel strategies for directing the expression of both functional and regulatory elements in transgenic mice. With the application of such approaches, the specific populations that comprise CNS networks can be both visualized and manipulated. Transgenic methods now range from the use of specific enhancer elements and large genomic regions assembled using BACs and PACs, to the use of gene targeting to a specific locus. In addition, the advent of transactivators and site-specific recombinases has provided unprecedented spatial and temporal control for directing expression in the CNS using a combination of appropriate alleles. As a result, the promise of being able to use transgenics to target specific neuronal populations is now being realized.

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Introduction

Fundamental to gaining an understanding of the development and function of the nervous system is the ability to target and manipulate specific populations of neurons in a reproducible manner. With such tools available, one will be able to visualize a single neuron *in vivo*, alter its gene function, determine its afferent and efferent connections, control its activity, and ultimately understand its contribution to a neural network. Although the use of transgenic approaches to direct gene expression in the nervous system is now in its third decade, recent advances are only now approaching a level of specificity that will make a systematic analysis of neural circuits possible. Here, we review recent advances that are bringing this goal closer to fruition.

Classical methods for manipulating the central nervous system

The importance of manipulating the central nervous system (CNS) *in vivo* has been long recognized, but until recently non-genetic approaches have predominated. Although strategies such as cellular transplantation, viral infection, anterograde and retrograde tracing methods have provided powerful methods for studying CNS development and function, their lack of reproducibility severely limits their usefulness. For example, wheat germ agglutinin (WGA), a plant lectin, has classically been used as a trans-synaptic tracer. However, and not surprisingly, targeting injections of this tracer to specific CNS structures is quite challenging, and during early development or in the case of small nuclei impossible. This limitation can, however, be overcome with transgenic approaches. Specifically, the DNA coding sequence for WGA can be placed under the control of appropriate regulatory elements to form a transgenic construct [1]. This construct can in turn be used to generate transgenic mice, in which this transgene is stably inserted into the genome. In such mice, WGA will be transcribed and translated within specific populations of the CNS in a precise spatial and temporal manner [1]. Both the adaptation of classical methods and the advent of new tools have opened up unprecedented opportunity for manipulating the CNS [2]. Bearing in mind space limitations, we provide a table of recently discovered functional components that can be driven with transgenes to provide a sample of the many tools that are on the horizon (Table 1).

Achieving cell-type specific gene expression using transgenic approaches

Transgenes, in their various forms, offer a broad spectrum of approaches to direct gene expression. In achieving the goal of cell-type specific transgene expression in the nervous system, the approach employed becomes a trade off among cell type specificity, the cost and time of transgenic production, and the breeding complexity involved when using multiple transgenic alleles simultaneously. Here we compare the benefits and limitations of three approaches: conventional, knock-in gene targeting, and BAC/PAC transgenic strategies.

In its simplest form, transgenics is the use of enhancer elements and a minimal core promoter to direct the gene expression of a desired cDNA linked to a polyadenylation signal cassette (see glossary). This minimalist approach takes advantage of the fact that gene expression in different cell types is generally controlled by multiple *cis* acting regulatory elements (see glossary). By identifying the enhancer responsible for the expression of a gene in the subset of cells of interest, one can, in principle, direct effective expression of a gene in this population with this enhancer sequence using a conventional

Glossary

Allelic effect: The same transgenic construct can have quite different patterns or levels of expression in two specific alleles as a result of their being in distinct genomic loci.

Bicistronic: When two protein products are generated from a single RNA transcript.

***Cis* elements:** DNA sequences residing in the vicinity of a target DNA region.

Concatemerization: The tendency of DNA constructs to form long repetitive sequences.

Haploinsufficiency: When a phenotype results from the lowered level of gene expression due to the loss of one allele.

Homologous recombination: Recombination events between stretches of identical DNA sequences. Often used for targeting constructs of interest to a specific locus.

Inducible transactivator: A factor that can direct expression by directly binding to its target recognition site. It can be silenced (tTA) or activated (rtTA) in the presence of a suitable ligand (Doxycycline in the case of tTA and rtTA).

Insulator sequence: Insulator sequences act as a barrier to regulatory elements in the chromosomal DNA flanking the insertion site.

Intersectional methods: This refers to genetic methods in which two conditions must be satisfied for the expression of a reporter, generally a combination of Cre and Flpe mediated recombination [14,15••], see also Figure 1a.

LacZ: The bacterially derived enzyme ß-galactosidase encoded by the LacZ gene is a commonly used reporter, which can be visualized using a simple histochemical reaction

Leaky: Recent unpublished observations suggest that some transcriptional read-through can occur in existing stop-cassettes resulting in expression of reporters in the absence of recombination. (Dymecki S, pers comm).

Polyadenylation signal cassette: DNA sequence that directs the addition of a string of adenosine nucleotides to the end of the transcribed mRNA sequence (i.e. polyadenylation). Polyadenylation serves to increase the stability and translation of messenger RNAs.

Site-specific recombinase: A protein that mediates deletion or inversion of a segment of DNA that is flanked at each end by identical or inverted recognition sequences, respectively.

transgenic approach. For example, although the intermediate filament *Nestin* gene is expressed in multiple tissue types, including neural progenitors, muscle and testis, the second intronic region of *Nestin* is neural-specific and in combination with a minimal promoter sequence has been used effectively to direct specific expression in CNS progenitors [3]. The small size of the construct usually leads through concatemerization (see glossary) to transgenic alleles with high copy number and correspondingly to high levels of expression in the resulting transgenic mice. Albeit this at times comes at the cost of ectopic expression, due to either the integration site (probably as a result of synergistic interactions with local chromosomal control elements) or the absence of appropriate *cis*-acting elements. To minimize the allelic effect, an insulator sequence (see glossary) can be used [4] to increase the number of founder mice with appropriate expression [5].

Although perhaps beyond the detail of interest to the casual reader, a number of nuances in transgene construct design have been found to enhance expression in mouse transgenics. For instance, usually at least one intronic sequence is necessary in the construct to acquire sufficient mRNA for translation, as splicing is coupled to polyadenylation and the cytosolic transport pathway. For this reason, an artificially made chimeric intron sequence is often included in transgenic constructs [6]. In addition, the 3′ untranslated sequence (UTR), which includes a polyadenylation signal cassette, greatly affects the levels of mRNA and protein production from a given transgene [7].

Variations in transgene expression in different founder lines generated from the same transgenic construct can prove quite useful. For instance, when the expression observed in specific transgenic lines occurs in unique populations, such as a subset of the cells the gene is normally expressed in. For example, GAD67, an enzyme important for GABA synthesis, is expressed in all cortical GABAergic interneurons. The Agmon group [8] generated transgenic mice by using a 10 kb promoter region of GAD67 to drive enhanced green fluorescent protein (EGFP). Using this approach, they were fortunate enough to establish two independent founder lines with mutually exclusive expression patterns of EGFP in subsets of GABAergic cortical interneurons.

Unfortunately in the great majority of cases in which one wishes to attain high levels of expression in specific neural populations, a simple strong enhancer element has not been identified. In lieu of this, two basic strategies have been used with varying success. One is to target the gene locus of interest and replace it with a transgenic construct using homologous recombination (see glossary). Not surprisingly, given that this provides the transgene with most, if not all, of the intrinsic machinery of the endogenous gene, the pattern of transgene expression normally closely resembles that of the endogenous gene. However, the loss of one copy of the original gene can be problematic because of haploinsufficiency (see glossary). One way to circumvent this problem is to make the locus bicistronic (see glossary) instead of ablating the endogenous gene, through the addition of an internal ribosome entry site (IRES) sequence followed by the transgene of interest. IRES sequences enable the generation of two protein products from a single transcript. The *encephalomyocarditis* virus-derived IRES sequence is the most efficient one to date and hence the one most commonly used. In this IRES sequence, major translation of the second sequence occurs at the 11th AUG (in fact when the second coding region is not fused to this AUG the IRES works less efficiently). The generation of two protein products from a single transcript usually results in lower levels of expression of both proteins compared with that normally produced at the endogenous locus (particularly the protein encoded by the cistron following the IRES sequence). Another possible strategy for acquiring two protein products from a single transcript is the use of the short self-cleaving 2A peptide sequence [9] as a

Table 1

Name	Origin	Function	Induction	References
Fluorescent proteins				[42,43]
GFP families	Hydrozoa or Anthozoa	Fluorescent	Varies	[42]
FlAsH, ReAsH	Tetracysteine peptide tag	Inducible fluorescent	FlAsH/ReAsH-EDT2	[44]
Fluorescent physiological indicators				[42,43,45,46]
VSFP1	Voltage sensitive fluorescent protein Kv2.1-CFP-YFP fusion (FRET)	Detects K^+ influx	No	[47]
Clomeleon	Chloride sensitive-YFP (FRET)	Detects Cl^- influx	No	[48]
Split Cameleon	CFP-Calmodulin M13-YFP (FRET)	Detects Ca^{2+} signaling	No	[49]
Synapto-pHluorin	VAMP2/Synaptobrevin-pH sensitive GFP mutant fusion	Indicator of vesicle exocytosis	No	[50,51]
hVOS (hybrid voltage sensor)	Dipicrylamine (synthetic voltage-sensing molecule) combined with farnesylated-EGFP	FRET based voltage detection fast response and recovery (0.5 ms)	No	[52]
Trans-synaptic tracers				
WGA	Plant lectin	Anterograde tracer	No	[1,15••]
GFP-TTC	Tetanus neurotoxin C terminal fragment-EGFP fusion	Retrograde tracer	No	[53,54]
Activity modulators				[2]
Kir2.1	Human Inwardly rectifying potassium channel	Inhibits evoked and spontaneous activity	No	[55]
Tethered-peptide toxin	Bungarotoxins or conotoxins –Lynx1 GPI anchor fusion	Antagonizes nicotinic receptor subunits or $Na_V1.2$, $Ca_V2.2$ channels	No	[56]
GluCl	Nematode Glutamate-gated chloride channels	$\alpha + \beta$ subunit produces inhibition with Cl^- conductance	Ivermectin	[57,58]
Allatostatin receptor	*Drosophila* G-protein coupled receptor	Inwardly rectifying K channels	Allatostatin	[59•,60,61]
VAMP/Syb MIST	VAMP2/synaptobrevin-FK506 binding protein (F36V) fusion	Inactivates synaptic transmission	Rapamycin or FK506	[62]
RO4	Rat rhodopsin 4	Hyperpolarization	Light	[63]
ChR2	Green algae channel rhodopsin 2	Excitation	Light	[63]
H- and D-SPARK	Shaker K^+ channel-photoisomerizable azobenzene-channel blocker fusion (D-SPARK: mutation in pore of the channel)	Photoswitchable current injection H-SPARK: K^+ efflux hyperpolarization D-SPARK: Na^+ influx depolarization	380 nm on 500 nm off	[64,65]
LiGluR	Ionotropic glutamate receptor 6 (L439C)-photoisomerizable azobenzene-agonist fusion	Photoswitchable current injection K^+ efflux, Na^+ and Ca^{2+} influx depolarization	380nm on 500 nm off	[66]
Transactivators				[23]
GAL4	Yeast GAL4	Activates UAS	No	[40]
tTA and rtTA	*Escherichia coli* Repressor of tetracycline resistance operon	Activates TRE	Doxycycline-off (tTA) Doxycycline-on (rtTA)	[41]

A compilation of functional elements that can be genetically encoded and used in transgenic mice. This list is not comprehensive and represents only a sample of those that are presently available. More details on fluorescent physiological indicators can be found in [42,45]. Abbreviations: CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; TRE, tetracycline responsive element; UAS, GAL4 upstream activating sequence; YFP, yellow fluorescent protein.

linker separating the two genes of interest. In this instance, a single fusion protein is first generated and then automatically cleaved. However, transgenic mouse lines have yet to be generated using this method. Other than the time and cost associated with this targeting strategy, this approach is often limited by the levels of transgene expression that can be obtained. At best, one attains a level of expression of the transgene similar to that of the original gene, and this in some cases is insufficient for carrying out functional studies.

The alternative to gene targeting is to use a large fragment of mouse genomic DNA. Bacterial or P1 phagemid-based artificial chromosomal (BAC, PAC) systems are currently widely used as vectors for constructing large transgenes, as they can hold large fragments of DNA (BAC: up to 300 kb, PAC: 130–150 kb) stably and are relatively easy to handle [10]. BACs are more frequently used for generating transgenic lines than PACs because of their capacity to accommodate larger DNA fragments. Assembly of a transgene using this method is achieved by homologous recombination in bacteria with an artificial chromosome [11], in the hope that most of the *cis* regulatory element resides in the general vicinity of the gene of interest. In bacteria, homologous recombination can be quickly achieved using relatively short flanking arms (50–500 bp, see [11] for review) to facilitate construction of targeting vectors. After assembly, transgenes are introduced into recipient mice on the basis of random integration into the genome. Although their large size tends to limit transgene concatemerization and hence copy number [10], this approach can reliably be used to overexpress transgenes. The downside of this, of course, is that genes in the region of the BAC/PAC are unavoidably overexpressed in these transgenic mice. This limitation is balanced by the convenience of using BAC and PAC fragments, and the fact that they can be used to generate a large number of transgenic lines quickly. This is illustrated by GENSAT (Gene Expression Nervous System Atlas), a large-scale BAC transgenic project [12]. The GENSAT database catalogs the many transgenic mouse lines generated by this project using BAC clone constructs. It also reports the EGFP expression patterns in the nervous system observed within these BAC transgenic mice.

Use of binary systems to target specific neuronal populations

It is unknown whether all or even most neuronal populations in the CNS can be uniquely identified by their expression of a single gene or cell-type specific enhancer. Moreover, some of the best candidates of cell-specific genes are expressed only transiently during development. This suggests that binary systems in which genetic fate mapping approaches can be used might be essential to a systematic approach for targeting the breadth of neuronal cell types that make up the CNS. This approach combines the use of a so-called 'driver line' and a 'reporter line'. In the driver line, the expression of a site-specific recombinase (see glossary) is placed under the control of a specific genetic locus. The expression of the recombinase is, therefore, restricted to the population of cells in which this locus is actively transcribed. The P1 bacteriophage-derived Cre and a temperature insensitive variant of the *Saccharomyces cerevisiae*-derived Flp, Flpe [13], mediate directed recombination at loxP and FRT sites, respectively. Cre and Flpe thus provide two alternative methods for directing site-specific recombination [14,15••,16].

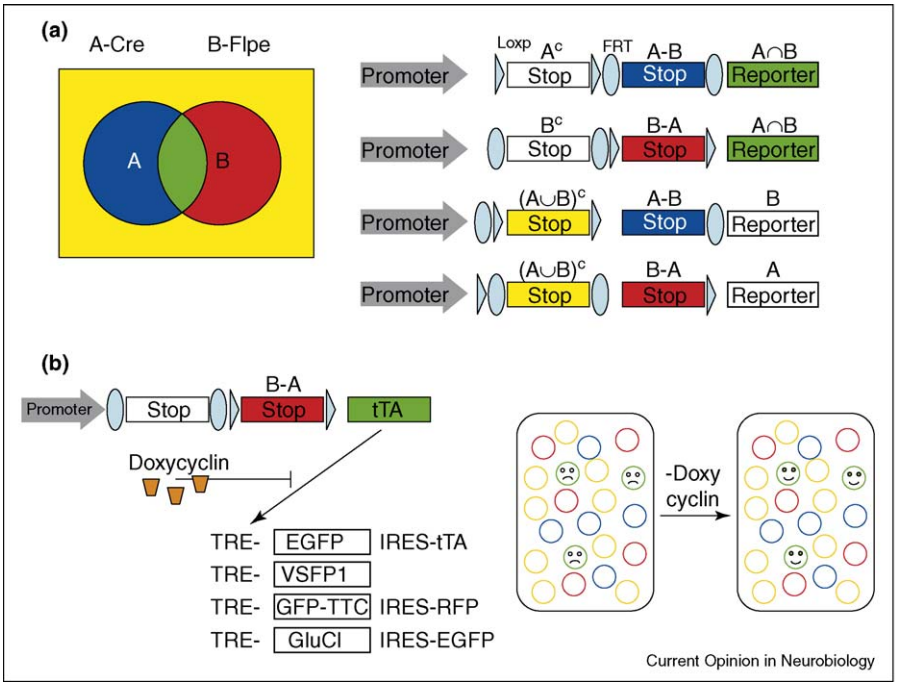
Visualization of the targeted population is achieved through the use of a reporter line that is permanently activated by coexpression of the driver line. This is generally a ubiquitously activate genetic locus such as *ROSA26*, in which expression of a reporter gene, such as EGFP or LacZ (see glossary), is conditionally dependent on the prior expression of a recombinase [17–19]. This is achieved by the presence of a recombinase-recognition sequence flanked stop cassette, that otherwise precludes expression of the reporter. As existing stop cassettes have a tendency to be 'leaky' (see glossary), the search for tighter stop cassettes is worth considering. Possibilities for the construction of tighter stop cassettes include the use of elements involved in RNA polymerase II termination [20], or the inclusion of chromatin modification elements, such as insulators [4] or a scaffold/matrix attachment region (SMAR) [21].

Recently, binary approaches have become more sophisticated with the use of intersectional methods (see glossary) that enable the retrospective labeling of neuronal populations on the basis of their expression of two genes, even when the expression of those genes are not temporally coincident. For example, one might use such methods to label the subset of a population that expresses gene X during development and gene Y only after the cell has become mature. This approach provides a powerful method for targeting gene expression to specific neuronal populations [14,15••,22]. The drawback, of course, is that to achieve this a minimum of three transgenic alleles must be crossed onto a single animal [23].

Intersectional methods rely on reporter strains in which the expression of a reporter is contingent on some combination of recombinase expression within the targeted cell population. This approach enables cells to be targeted on the basis, in effect, of genetic algebra (i.e. the expression or lack of expression of two genes, see Figure 1a for possible intersectional strategies). Depending on the design of the reporter line, different target populations can be identified. For example, one can track cells that express two different genes at some point during their lifetime (Cre^{on}-$Flpe^{on}$), one gene but not another (Cre^{on}-$Flpe^{off}$ or Cre^{off}-$Flpe^{on}$) or neither of the two genes (Cre^{off}-$Flpe^{off}$).

Inducible forms of both the Cre and the Flpe recombinases have been engineered that enable the specific timing of gene expression to be assessed. This has been accomplished by fusing either Cre or Flpe to both a binding domain for HSP90 and either an estrogen (ER) or a progesterone (PR) binding site that has been mutated so that they can bind to synthetic exogenous ligands but not to the endogenous ligands [24–28]. As a result the recombinases are only active when the exogenous ligands (tamoxifen for ER or RU486 for PR) are administered. In the case of CreER (which is the only one that has to date been

Figure 1



Intersectional strategies and functional manipulation of neurons using a ternary system. **(a)** A schematic of populations marked by the combination of two driver lines, A-Cre and B-Flpe. Different subpopulations such as, A∩B:green, A-B:blue, B-A:red, $(A \cup B)^C$:yellow can be selectively labeled by applying an intersectional strategy with the use of different constructs of dual reporter lines with stop cassettes flanked by loxp or FRT sites. By including reporters in each of the stop cassettes, a subpopulation can be selectively identified within a larger population. Set symbols ∩, intersection; ∪, union; X^C, complement; −, difference. **(b)** A dual reporter construct driving tTA in the population A∩B: green, as shown in (a). tTA can be used for the expression of an open-ended variety of functional elements under regulation of TRE, such as a fluorescent protein (EGFP), an activity indicator (VSFP1), an anterograde trans-synaptic tracer (GFP-TTC) and an activity modulator (GluCl). By making the construct bicistronic, one can also visualize the affected cell population (IRES-EGFP) or even boost the expression level of the functional modulator (IRES-tTA).

rigorously examined), upon ligand administration, activation is initiated at 6 hours and peaks between 12 and 24 hours post-induction [25,29•]. An alternative approach that has yet to be tested in transgenic animals is to use 'fragment complementation', in which the recombinase can only function when two halves of the protein are brought together either by simple co-expression or inducibly by systemic introduction of a ligand linker, such as rapamycin [30,31]. Alternatively, a number of groups have explored the use of inducible promoters. For example, an interferon-responsive promoter [32] or the transactivator (see glossary) tTA used in conjunction with TRE-Cre [33] (see Table 1). Using inducible forms of recombinase for genetic fate mapping enables the specific timing of gene expression in cell populations to be used as a means to target unique populations. Indeed, as neuronal populations often express genes at specific times during their maturation, this can be particularly useful [29•,34]. For instance, because many neuronal populations express members of the basic-helix-loop-helix (bHLH) family of genes transiently, marking when a neuron expresses bHLH genes can indicate its involvement in the specification of a given neuronal subtype [35•]. Another strength of inducible recombinase systems is that they can be used to generate mosaicism (which enables the sampling of a subset of the target population). This is particularly useful in contexts in which conditional alleles are used for loss-of-function (LOF) analysis. Mosaic LOF enables us to study the effect of genes at the cellular level, when the global removal of a specific gene results in distortion or removal of entire neural structures. It also provides the means to control precisely the timing and cell type in which gene removal occurs.

Given that binary methods rely on the fidelity of the reporter allele, considerable thought should be given to the characteristics of the reporter line used. The foremost consideration concerns the temporal and spatial specificity of the reporter locus activity. For instance, the *ROSA26* locus that is frequently used in the generation of reporter lines [14,15••,17–19, 36••] is widely considered to be ubiquitous. However, it is evident that the level of activation of this locus decreases postnatally, and is marginal in specific cell types, including glia [37,38]. Even in circumstances in which the reporter locus is identical, detection of different reporters can vary significantly because of many factors, including fixation and method

of visualization. For instance, ß-galactosidase can be visualized either histochemically or through immunocytochemistry, and depending on optimization can yield varying results. Hence, one needs to be aware that comparing the populations visualized by different reporters or the same reporter visualized using different methods is dependent on many factors and must be optimized on a case by case basis. Moreover, the utility of a given reporter for *in vivo* methods can be limiting if it cannot be detected without enhancement (e.g. EGFP can often be weak or undetectable without immunocytochemical visualization). Equally important is the recombination efficiency of the reporter line. Our own comparisons have revealed that when a particular inducible Cre driver line is used with two different reporter lines (the *ROSA26* LacZ reporter line [18] versus the *Z/EG* EGFP reporter line [39]) the recombination efficiency can vary significantly (G Miyoshi, G Fishell, unpublished). This could be due to the accessibility of the Cre to the locus and/or the different size of the stop cassettes used in each reporter. Hence, depending on the characteristics of the reporter line, the population of cells labeled using particular driver lines can differ.

Binary to ternary system

Although genetic fate mapping can provide a wealth of information concerning the genetic and developmental origins of specific neural populations, equally intriguing is the potential this approach has for studying and manipulating neurons *in vivo*. To achieve this, one can imagine substituting any number of proteins in place of a simple marker within the reporter allele to monitor neuronal activity, trace circuitry or silence or activate neuronal populations (see Table 1). However, although feasible, it would be extremely time consuming and expensive to target such a wide array of constructs into a single locus. We suggest the use of a ternary system, in which the growing number of Cre and Flpe driver lines provide the input and dictate the target specificity, whereas the output comprises simple-to-generate transgenic lines that express functional components under the regulation of a transactivator, such as GAL4 or (r)tTA (see Table 1) [23,40,41]. Linking the input and output layers is a middle layer reporter, in which the recombination of an intersectional allele results in the expression of an inducible transactivator, such as tTA or rtTA [36••] (see Figure 1b for this system). Such an approach would be widely adaptable to a near-endless variety of applications. Another strength of a ternary transgenic approach is that it can be readily combined with other genetic tools, such as viral or electroporation vectors. Each component of this ternary system could potentially be contained within either type of vector and introduced into transgenic mice at the desired time point. Once a strategy to label a target population is identified through the intersectional use of driver and reporter lines, different aspects of this population could be studied by breeding in different output lines. Moreover, as new output alleles are generated they could be readily adapted to existing ternary systems.

Conclusions

It is apparent that transgenic approaches will provide the tools to revolutionize the study of the mouse nervous system. To facilitate this effort, the current trend towards sharing mouse alleles must be accelerated and encouraged throughout the mouse research community. In addition, for the potential of these approaches to be fully realized, developmental neurobiologists focused on molecular genetic approaches and system neurobiologists who are adept at the functional analysis of neural circuits need to establish better cross-disciplinary communication. Certainly the promise for such efforts to expand our understanding of the nervous system enormously should provide a sufficient incentive to do so.

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