Published in final edited form as:

J Neurochem. 2016 October ; 139(Suppl 2): 24–57. doi:10.1111/jnc.13629.

Gene regulation and genetics in neurochemistry, past to future

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Abstract

Ask any neuroscientist to name the most profound discoveries in the field in the past 60 years, and at or near the top of the list will be a phenomenon or technique related to genes and their expression. Indeed, our understanding of genetics and gene regulation has ushered in whole new systems of knowledge and new empirical approaches, many of which could not have even been imagined prior to the molecular biology boon of recent decades. Neurochemistry, in the classic sense, intersects with these concepts in the manifestation of neuropeptides, obviously dependent upon the central dogma (the established rules by which DNA sequence is eventually converted into protein primary structure) not only for their conformation but also for their levels and locales of expression. But, expanding these considerations to non-peptide neurotransmitters illustrates how gene regulatory events impact neurochemistry in a much broader sense, extending beyond the neurochemicals that translate electrical signals into chemical ones in the synapse, to also include every aspect of neural development, structure, function, and pathology. From the beginning, the mutability – yet relative stability – of genes and their expression patterns were recognized as potential substrates for some of the most intriguing phenomena in neurobiology – those instances of plasticity required for learning and memory. Near-heretical speculation was offered in the idea that perhaps the very sequence of the genome was altered to encode memories. A fascinating component of the intervening progress includes evidence that the central dogma is not nearly as rigid and consistent as we once thought. And this mutability extends to the potential to manipulate that code for both experimental and clinical purposes.

Keywords

Central Dogma; chromosomal instability; epigenetics; history of medicine; next-gen sequencing; transcription

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Gene transcription

Transcription factors

Soon after the structure of DNA had been determined, in both generalized terms of the helix and specific terms of individual gene sequences, it became clear that an understanding of how genes are regulated would be at least as exciting and important. Because of their relative simplicity and potential for rapid amplification to biochemical quantities, prokaryotic genomes provided many of the early discoveries, such as the fact that certain primary genes could influence the activity of other secondary genes physically distant in the genome. Systematic quests for the agents of this '*trans*-acting' phenomenon soon determined that proteins, expressed by the primary genes, conducted some of the most important *trans* effects, often by binding specific and predictable sequences within the secondary genes. These DNA-binding sites of transcription factors are responsible for a great many of the identified 'cis-acting' elements, sequences within a given gene that control its own expression. Once bound to a *cis* element, a transcription factor typically acts to recruit the RNA polymerases necessary for transcription. These and related phenomena were soon demonstrated in eukaryotes, as well, where the complexity of chromatin structure (more below) afforded interactions of transcription factors not only with RNA polymerase II, for instance, but also with histone acetyl transferases and other proteins of the 'transcriptosome' complex. Early studies on the role of gene regulation in ontological events meant that a great many hormones, growth factors, and similar agents were found to activate transcription factors critical for development, including that of the nervous system. But, some of the most intriguing roles for transcriptional regulation in the CNS related to neurotransmission and synaptic plasticity, and it was not long before gene regulatory events were invoked to explain these and other aspects of neurochemistry.

AP-1—Curran and colleagues (Morgan *et al.* 1987; Shin *et al.* 1990) created quite a stir when they presented evidence that neuronal activity was associated with induction of the c-Fos protooncogene under disease models of epileptiform states. Perhaps, more consequential was the demonstration of c-Fos induction as a consequence of physiological changes in neuronal activity, including trans-synaptic induction in second-, third-, and perhaps fourth-order neurons in a circuit (Hunt *et al.* 1987; Sagar et *al.* 1988). This opened up the potential to use c-Fos elevation as an index of connectivity. Similar forms of regulation were eventually discovered for other members of the c-Fos family, such as FosB (Chen et *al.* 1995). Protooncogenes, the normal versions of genes that can foster neoplasia when mutated, had been the subject of intense scrutiny in the early 1980s as essential players in mitosis and tissue pattern development. But, just as the neurotrophin nerve growth factor was found to share tyrosine phosphorylation and other signal transduction elements with mitogens such as epidermal growth factor, parallels in those transduction pathways were found to extend to transcription factors and gene regulation. Still, it was somewhat novel at the time to consider that ion channels and other mediators of rapid neurotransmission could intersect with the same pathways.

The transcription factor formed by c-Fos is AP-1, a heterodimer of a Fos with a member of the c-Jun family. Although the regulation of c-Fos and its contribution to AP-1 is nearly

always dependent upon changes in quantity, the Jun proteins were found to be regulated by growth factors and cytokines primarily via rapid phosphorylation, namely by Jun N-terminal kinase (JNK) (Derijard *et al.* 1994). However, many neural phenomena in which Jun-family proteins participate appear to require induced expression, similar to that for c-Fos (Morgan and Curran 1988; Sonnenberg *et al.* 1989; Oo *et al.* 1999). Jun has been connected to a host of biological phenomena in neurons, with particular emphasis on roles in neuroplasticity and death from trophic factor withdrawal (Raivich 2008).

Of particular relevance to AP-1 and its modulation by JNK are the many aspects of neuroinflammation in which that kinase participates. Cytokines, chemokines, and pathogen-/danger-associated molecular pattern ligands often activate JNK. Indeed, an impact of such immune-related stimuli on neurochemistry is manifest in the induction of the serine racemase gene (*Srr*) by JNK activation of JunB (Wu and Barger 2004). This induction of *Srr* by a JunB-containing AP-1 may even involve activation of an alternative transcription-initiation site (Figs 1 and 2). The resulting elevation of serine racemase in activated microglia is responsible for their production of D-serine, the most abundant and relevant agonist of the 'glycineB' site of the NMDA receptor in the forebrain (Van Horn *et al.* 2013). In addition to potentially contributing to excitotoxicity, the elevated tone for NMDA-receptor activity could create transient, less drastic perturbation of neurotransmission. The role of JNK in neuroinflammation is covered more thoroughly in other contributions to this special issue (Feinstein 2016).

CREB—The correlation of Fos and Jun factors with neurophysiology and neurochemical stimulation found rational explanations in the connections that were being established at the time between such protooncogenes, mitogens, and calcium. Many growth factor receptors are coupled to activation of phospholipase C, resulting in production of inositol trisphosphate, which activates release of calcium from stores in the endoplasmic reticulum. Considerable evidence indicated that cell-surface calcium channels are often activated too, in part to provide calcium for refilling the ER stores. It was soon determined that a major component of the activity-dependent induction of Fos was secondary to the activation of cyclic AMP-responsive element-binding (CREB) protein. CREB is activated by phosphorylation, and the first relevant kinase identified was cAMP-activated kinase (PKA). But, it was eventually determined that calcium/calmodulin-activated kinase II (CaMKII) could perform this role as well. And given the critical contribution that calcium makes to neurotransmission, it is not surprising that neurochemists led the charge in exploration of this aspect of CREB regulation. First came colocalization of phospho-CREB and CaMKII, which was reported in a retinal system in which elevation of calcium was shown to mimic photostimulation in the elevation of both CREB phosphorylation and c-Fos expression (Yoshida *et al.* 1995). Soon, calcium-dependent activation of CREB came to be recognized as an important connection between neurochemical signals that elevate intracellular calcium, such as glutamate receptors, and gene regulatory events (Dash *et al.* 1991; Sheng *et al.* 1991; Fukuchi *et al.* 2014).

Of course, discoveries of CREB activation by CaMKII does not mean that PKA is irrelevant to CREB in the nervous system. As would be predicted by the array of neurotransmitters and other neuroactive agents coupled to cAMP production, the PKA-CREB axis is involved in a

host of neural events, such as induction of the tyrosine hydroxylase gene (Piech-Dumas *et al.* 2001). Indeed, the respective reliance on calcium or cAMP may be specific to brain region and/or cell type (Moore *et al.* 1996). In some cases, cAMP- and calcium-mediated pathways may cooperate to effect a super-induction of CREB-dependent transcription (Hansen *et al.* 2004). There is even evidence that the capacity for calcium-dependent activation of CREB reflects a permissive state that is established through the integration of neuronal activity patterns by PKA activation over time (Pokorska *et al.* 2003). (This concept is discussed at greater length below.)

CREB is involved in important non-neuronal events in the nervous system too, such as glial differentiation and reactivity. Inflammation-related stimuli activate the CREB-Fos axis in primary glial cultures (Simi *et al.* 2005). Several agents impacting oligodendrocyte differentiation rely on CREB (Sato-Bigbee *et al.* 1999). Astrocytic expression of proenkephalin appears to involve a phosphorylation of CREB by a Src-related kinase (Kobierski *et al.* 1999).

NFκB family—Once the floodgates of neurochemical-transcriptional interactions had been opened, investigators began to test many different transcription factors for roles in the nervous system. One family that appeared intriguing was that contained under the rubric 'nuclear factor κ light chain enhancer of activated B cells' NFκB. Canonical NFκB, most commonly comprising a heterodimer of NFKB1/p50 with RelA/p65, was known to dwell in the cytoplasm until rapid activation is effected via liberation from a tonic inhibitor IκB when the latter is targeted for degradation by the ubiquitin/proteasome system. This seemed an ideal system for conveying signals from post-synaptic compartments to the neuronal nucleus to effect the gene regulatory aspects of neuroplasticity. In a classic example of confirmation bias, a spate of publications reported activation of NFκB in cultured neurons by glutamate receptor agonists and other neurochemical stimuli. Most of these paradigms appear to have been confounded by artifacts of glial contamination of the cultures or supraphysiological over-expression of active NFκB subunits via transfection or viral transduction (reviewed in Mao *et al.* 2009). Studies performed in intact animals or acute tissue slices have often correlated neuronal activity or neuropathology with active NFκB in homogenized tissue extracts (Cardenas *et al.* 2000; Nakai *et al.* 2000; Madrigal *et al.* 2001; Hu *et al.* 2005). But these paradigms, of course, include the potential for contributions from glia, endothelial cells, etc.

For the purposes of understanding transcriptional regulation in post-mitotic neurons, one can almost dismiss out-of-hand NFκB studies that were performed in 'neuronal' cell lines derived from neuroblastomas or other tumor cells, since shown to be poor representatives of this and other aspects of differentiated neurons. Nevertheless, NFκB has important roles in proliferative activity and survival properties in tumors; so, its activity may persist and even contribute to neuroblastoma. This is implied by preclinical studies being conducted with the NFκB inhibitor bortezomib, which appears to hold some promise in the treatment of neuroblastoma (Sholler *et al.* 2013), as it has proved to be useful in treatment of other cancers.

Regardless of the problems with early studies on glutamatergic regulation of NFκB, evidence exists to suggest that the protein subunits have some natural biological role in neurons. For instance, NFκB ablation or inhibition inhibits neurite outgrowth and synapse formation (Imielski *et al.* 2012; Saleh *et al.* 2013; Su *et al.* 2013). It is possible that these involve some mechanism other than activation of gene transcription. For instance, NFκB can physically interact with glucocorticoid receptors, inhibiting their transcriptional effects (Fig. 1). It is noteworthy that RelA/p65 has been documented to translocate from the cytosol to the nucleus even in neurons where it does not activate RNA transcription (Barger *et al.* 2005), similar to its behavior in other paradigms (Mukaida *et al.* 1994; Brostjan *et al.* 1996; Ray *et al.* 1997; Harant *et al.* 1998; Wang and Baldwin 1998). Nevertheless, more sophisticated techniques have provided evidence for some amount of NFκB activity in neurons. In particular, a Cre/Lox system has been employed to deplete RelA/p65 from neurons, and this was documented to reduce the expression of a luciferase reporter gene containing a NFκB-responsive promoter (Boersma *et al.* 2011). In the end, there is ample evidence that NFκB proteins – while present in CNS neurons – fail to participate in robust gene transcription in these cells (Jarosinski *et al.* 2001; Srinivasan *et al.* 2004; Saha and Pahan 2007; Mao *et al.* 2009; Lian *et al.* 2012, 2015; Listwak *et al.* 2013; Dvoriantchikova and Ivanov 2014). Such might be the expectation for a family of proteins extensively documented to participate in self-sacrificial inflammatory states, such as the surface expression of major histocompatibility complex I proteins (Drew *et al.* 1993; Rall *et al.* 1995). Unusual aspects of this system in neurons apparently extend to NFκB-inducing kinase, which participates in non-canonical activation of the transcription factor in other cell types, yet appears to inhibit NFκB in post-mitotic neurons (Mao *et al.* 2016).

Roles for NFκB are very well established in glia. In astrocytes, it is activated by amyloid β-peptide (Aβ) and lipopolysaccharide (LPS) (Dodel *et al.* 1999), mediating the latter's induction of glucose 6-phosphate dehydrogenase (Garcia-Nogales *et al.* 1999) and glucose transporter 3 (Cidad *et al.* 2001), and it mediates the induction of interleukin 6 by bradykinin (Schwaninger *et al.* 1999) and of glial fibrillary acidic protein by interleukin 1 (Krohn *et al.* 1999). Indeed, the contributions of NFκB to these and other aspects of the innate immune system, particularly in microglia (Laflamme and Rivest 1999; Bruce-Keller *et al.* 2001; Nicolini *et al.* 2001; Liu *et al.* 2005), make this transcription factor family extremely important to every aspect of neuroinflammation. Anti-inflammatory drugs such as aspirin and ibuprofen even owe a portion of their effectiveness to 'off-target', yet serendipitous inhibition of NFκB (Kopp and Ghosh 1994; Scheuren *et al.* 1998).

Sp1 family—One of the first eukaryotic transcription factors ever identified was Specific protein 1 or, as it is known universally now, Sp1. Indeed, this appears to have been the first eukaryotic transcription factor for which the gene was cloned. Eventually, other family members were isolated and cloned, including Sp2, −3, and 4; but, Sp1 was considered to be the ubiquitous family member among mammalian tissue and cell types. Indeed, there appeared to be a considerable amount of Sp1 in neurons, as it was identified as the factor occupying a substantial subset of NFκB target sequences under the neuronal conditions of paucity for the latter. Sp1 has a somewhat broad specificity for sites that can be said to generally possess high GC content, and most NFκB target sequences are GC-rich. DNA

sequences containing optimal Sp1-binding sites were found to efficiently compete for binding to the proteins that were found constitutively interacting with these NFκB sequences in nuclear extracts from CNS neurons. However, antibodies to Sp1 did not appear to interact with these proteins, and they were eventually determined to be a mixture of Sp3 and Sp4 (Mao *et al.* 2002). Indeed, this is one of the few settings in which Sp4 is present. Indeed, Sp1 was found to be essentially replaced by Sp4 during neuronal differentiation (Mao *et al.* 2009; Milagre *et al.* 2012). The significance is not entirely clear; but, Sp4 is generally less active as a transcriptional activator, and many Sp1-induced genes are active in cell proliferation. This suggests that Sp4 replaces Sp1 to suppress mitotic genes in neurons, which will no longer need them and, in fact, may suffer from their expression (Wang *et al.* 2009).

The Sp3 and −4 DNA-binding activity in neurons is vulnerable to cytosolic calcium overloads. In neurons treated with abusive levels of glutamate-receptor agonists, Sp3 and −4 DNA binding is diminished, and the remaining activity has a faster migration consistent with proteolysis (Mao *et al.* 2002). This effect can be blocked with calcium chelator or inhibitors of the calpain proteases. Thus, some portion of the calcium-dependent phenomenon of excitotoxicity may involve degradation of Sp3 and −4. This appears to lead to liberation of at least one Sp1-induced mitotic gene (Mao *et al.* 2009). It remains to be determined whether or not this contributes to excitotoxicity via the documented ability of mitotic signals to foster apoptosis in neurons (Liu and Greene 2001).

Nuclear receptors—Many steroid hormones and other small, lipophilic molecules bind and modulate proteins that physically interact with DNA and components of the transcriptosome essentially as transcription factors. Estrogen receptors provide a classic example of such ligand-dependent transcription factor (Gruber *et al.* 2002). Other, similar factors are the glucocorticoid/mineralocorticoid receptors, thyroid hormone receptor (T_3R), retinoid-activated receptor, 9-cis retinoic acid receptor (RXR), liver X receptors (LXR), and peroxisome proliferator-activated receptors (PPAR). A considerable amount of the progress made in the past 60 years on the actions of these ligand-receptor pairs relates to their interactions with other components of the nuclear transcriptional control mechanisms. In particular, several members of the LXR and RXR families act via corepressors for other transcription factors such as nuclear receptor corepressor (N-CoR) and silent mediator of retinoic acid receptor and thyroid receptor (Perissi *et al.* 1999; Hu *et al.* 2003). RXR and GC modulate the actions of the nuclear orphan receptor Nurr1 in neurons (Wallen-Mackenzie *et al.* 2003; Carpentier *et al.* 2008).

Roles for nuclear receptors in cellular differentiation and other aspects of development were predicted by groundbreaking studies on the ontological effects of retinoic acid, intensified to a focus on individual genes regulated by retinoids (Suzuki *et al.* 1995; Cheung *et al.* 1997). It was only a matter of time before the nuclear receptors for these retinoids were invoked to explain their effects on such genes (Nikcevic *et al.* 2008; Murakami *et al.* 2010), as well as on broader phenomena such as hippocampal neuron morphology (Liu *et al.* 2008), astrogliogenesis (Faigle *et al.* 2008), and control of inflammatory states (van Neerven *et al.* 2010).

The nuclear receptors are particularly important to neuroinflammation. There is an extensive literature on the antiinflammatory effects of estrogens (Giatti *et al.* 2012; Petrone *et al.* 2014). The PPAR family generally contributes to antiinflammatory effects when activated by ligands such as prostaglandin J2 (Xu *et al.* 2008). An LXR agonist also attenuates inflammation in an experimental model of spinal cord injury (Paterniti *et al.* 2010).

The LXR and retinoid-activated receptor/RXR factors and their interactions have gained substantial interest in the field of Alzheimer's disease, due in part to the role for lipid homeostasis in that disease implied by apolipoprotein E genetics. When activated by ligand, LXRα and -β induce the expression of ATP-binding cassette transporters (especially, A1 and G1) and other gene products involved in the transport of cholesterol and other lipids. A natural agonist of LXRs, 24S-hydroxycholesterol, creates conditions that facilitate transport of cholesterol – in the context of lipoprotein particles – from individual cells in culture (Burns *et al.* 2006) and into the CSF *in vivo* (Fujiyoshi *et al.* 2007), presumably shifting the gradient from the brain parenchyma. This may explain, in part, why LXR-knockout mice exhibit degenerative accumulations of cholesterol in the CNS (Wang *et al.* 2002). RXRs participate in the homeostasis of lipids and Ab in the brain as well, as illustrated by the ability of RXR agonist bexarotene to reduce (at least, temporarily) amyloid burden in Aβ-overproducing models of Alzheimer's disease (Cramer *et al.* 2012).

Transcription factors in neuroplasticity—From the earliest applications of molecular biology to neuroscience, investigators hypothesized about the potential for genes and their activity to serve as a physicochemical substrate for long-term changes such as those required for learning and memory. Although an individual protein or small-molecule metabolite may have a half-life of only seconds or hours, the expression patterns of genes obviously represent a form of stability that lasts a lifetime. Mechanistically, the capacity for transcription factors to integrate neurophysiological signals may depend on a difference in the natural rhythms created by the relative kinetics of these two modes of activity. The kinetics of biochemical events regulating transcription factors – protein phosphorylation, acetylation, ubiquitination, degradation, etc. – are generally slower than most electrochemical events that mediate neurotransmission. Therefore, for those transcription factors that are impacted by the ions and signaling cascades set in motion by neurotransmitter receptors, a volley of depolarizations can induce post-translational modifications more rapidly than they can be reversed, generating a summation or 'recording' of the activity, e.g. through nuclear translocation or transcription of second-order transcription factors such as Fos (Fig. 2). The possibility that such modifications can be converted to a somewhat irreversible form by epigenetic mechanisms could reinforce the stabilization of the record.

One undesirable form of neuroplasticity is drug addiction, and AP-1 and the Fras are impacted in these paradigms. Drugs of abuse such as opiates and cocaine induce an acute elevation of c-Fos and other Fras such as FosB, Fra-1, Fra-2, and DFosB. But, chronic addiction models produce attenuation of these responses, mirroring many other aspects of drug dependency; the acute Fras are replaced, however, by the 'chronic Fras' (Laorden *et al.* 2002; Larson *et al.* 2010; Nunez *et al.* 2010), which now appear to be somewhat longer alternative splice variants of the conventional 33-kDa ΔFosB (Chen *et al.* 1997). In a chronic

methamphetamine-exposure model, the activation of AP-1 attenuated in second and third exposures, and this was attributable to a recalcitrance of c-Jun rather than its Fos partner (Ishihara *et al.* 1996). In keeping with evidence that some of the engrained activity patterns responsible for addition also underlie chronic pain, ΔFosB is induced in a carrageenan-induced model (Luis-Delgado *et al.* 2006).

Epigenetic gene regulation

DNA methylation—The chromosomal DNA of all known species of life undergoes chemical modification in the form of post-replicative methylation (Billen 1968; Lark 1968; Kalousek and Morris 1969; Kappler 1970), including such covalent modification of mitochondrial DNA. DNA methylation occurs most often on the cytosine of a C-G pair (usually referred to as 'CpG' in this context), but guanosine is occasionally methylated. Unique to mature CNS neurons and certain stem cell populations is the methylation of cytosines outside of CpG pairs (Xie *et al.* 2012; Lister *et al.* 2013; Varley *et al.* 2013).

DNA methylation suppresses transcription, as first evinced by Venner and Reinert (1973). In the simplest interpretation, the conformational changes in DNA brought about by this modification interfere with the binding of proteins such as transcription factors. However, this explanation is likely incomplete, as several heavily methylated genes are transcribed at a high rate, especially if methyl-CpG-binding proteins are absent. The latter information suggests that it is protein-protein competition that squelches the interactions of the transcriptosome with methylated DNA. This relationship may represent a two-way street: evidence suggests that transcriptionally active genes are less readily targeted by methyltransferase enzymes (Brandeis *et al.* 1994; Mummaneni *et al.* 1998), possibly through sheer steric competition (see 'molecular momentum', below). This is particularly likely for the Sp1 family of transcription factors, as their consensus binding sites are all GC-rich.

The inactivation of genes by methylation is a primary means of epigenetic stability. Several key methyltransferases make it their business to methylate the opposite strand at sites where DNA is hemimethylated; in this way, methylation patterns are perpetuated after DNA replication creates the pairing of a methylated strand with a nascent, unmethylated strand. DNA methylation was first recognized as mechanism to confer gene imprinting only 28 years ago (Reik *et al.* 1987; Sapienza *et al.* 1987), though this inheritance phenomenon – expression of a locus restricted to either the maternally or the paternally derived copy – has been recognized since the 1920s (Boycott and Diver 1923; Sturtevant 1923; Boycott *et al.* 1930).

Although DNA methylation is considered a means of perpetuating gene expression patterns, it is nevertheless pliable. Methyltransferases rely on methyl donors such as *S*-adenosylmethioine that are cyclically renewed from dietary one-carbon sources. The ability to alter epigenetic gene regulation via environmental and behavioral nuances is well supported by the reliance of DNA methylation on dietary levels of folate, an important contributor to the maintenance of *S*-adenosylmethionine. Deficiencies in vitamin B12 can also compromise methytransferases, many of which use B12 as a cofactor. This is one of the risks associated with alcohol abuse, which often gives rise to B12 deficiencies (Kruman and Fowler 2014). Issues of blood–brain barrier permeability create nuances for the brain's

utilization of this one-carbon cycle (Long *et al.* 1989). Failure to maintain proper methylation patterns also seems to be a predictable and efficacious consequence of aging (Horvath 2013; Keleshian *et al.* 2013), whether through stochastic loss of fidelity in replication or through some other impact of the aging process on the relevant enzymes.

Dietary folate and its impact on DNA methylation is illustrated in an interesting phenomenon that unites neurological mechanisms with something as esoteric as hair color. The *Agouti* gene product is a peptide antagonist of melanocortin receptors and thereby suppresses melanin content in hair. In strains of mice bearing the dominant *Agouti* allele *lethal yellow* (A^y), the long-terminal repeat of a retrotransposon has been inserted in the promoter region of the *Agouti* gene, causing ectopic expression of the agouti peptide. This results in antagonism of the MC4R receptor in the hypothalamus, leading to hyperphagia, obesity, insulin resistance, and a shortened life span, as well as a yellow coat color (Hidaka *et al.* 2001). However, hypermethylation of the A^y promoter can be forced via a high-folate diet. This suppresses expression of agouti peptide and mitigates the phenotype (Wolff *et al.* 1998).

The importance of DNA methylation for neural systems is also exemplified by Rett syndrome, which begins postnatally as an autistic-spectrum disorder in girls that progress to profound motor and sensory disability, seizures, cognitive impairment, and a somewhat shortened life span. The disorder is caused by mutations in methyl-CpG-binding protein 2 (MeCP2) (Amir *et al.* 1999) (though this is something of a misnomer, as the protein binds any methyl-cytosine). The gene for MeCP2 resides on the X chromosome, but it is mutated sporadically in most cases, defying an X-linked inheritance pattern; Rett syndrome is observed exclusively in females, as males with a MeCP2 mutation are so severely affected as to die shortly after birth. MeCP2 effects widespread changes in gene expression patterns (Mellen *et al.* 2012), and loss of function even at adult ages causes disability similar to that of Rett (McGraw *et al.* 2011), further underscoring the rather unique dependency of the CNS on gene regulation through DNA methylation.

Chromatin structure—A key distinction between prokaryotic and eukaryotic genomes is the presence of higher level structural components such as histones. Histones have been known to biologists since the work of Albrecht Kossel in the late 19th century. But, it was only in the 1980s that Michael Grunstein found that histones were able to suppress gene transcription (Han *et al.* 1987; Grunstein 1990), and a true understanding of the critical role they play in gene regulation would await demonstrations by Vincent Allfrey in the 1990s that this inhibition was dynamic and modifiable (Vidali *et al.* 1978; Prior *et al.* 1983), ushering in a key aspect of the modern theory of epigenetic mechanisms. It is now recognized that the steric, conformational interactions of chromosomal DNA with histones critically influence the accessibility of that DNA to the transcriptosome and that this DNA-histone interaction is tightly regulated through post-translational modification of the proteins. In recognition of its profound role in transcriptional regulation, the regulation of histones and their interactions with DNA have been dubbed the Histone Code (Jenuwein and Allis 2001).

Histones and their modification—Over half a dozen types of modifications of histone residues have been identified, including acetylation, phosphorylation, methylation, mono-ubiquitination, ADP-ribosylation, citrullination, and SUMOylation. Acetylation is commonly associated with a reduction in the affinity of the histone for DNA, thereby dissolving this association and making the DNA more accessible for transcription. This acetylation is conducted by histone acetyltransferases, generally considered an important component of the transcriptosome complex. Their action is opposed by the histone deacetylases, critical players in gene silencing (Anekonda and Reddy 2006). The other modifications of histone are not as universal in their outcome as acetylation. Methylation and ubiquitination of specific residues on specific histones can either promote or inhibit transcription (Kouzarides 2007). Indeed, contextual fear learning is associated with two separate histone methylation events, each of which has an opposite effect on transcription (Gupta *et al.* 2010). Likewise, phosphorylation events can either facilitate or inhibit acetylation, and thus transcription (Rossetto *et al.* 2012).

Histone modification and cellular differentiation—Early in the investigation of these mechanisms, it was determined that silencing the expression of specific genes via tight association of DNA with histones is a key element of cellular differentiation. Neurons, of course, are often considered among the most highly differentiated cells in the vertebrate body plan. However, CNS neurons are known for their relatively high euchromatin-to-heterochromatin ratio, manifest as a large nucleus with rather pallid staining by nucleophiles. Indeed, the Nissl substance in neuronal cytosol often accrues denser staining than does the nucleus. Euchromatin is recognized as being the template of active transcription, thus marking neurons as the most diverse transcript venue. This is consistent with the findings that (i) histone acetylation levels are higher among neurons than glia and (ii) neuronal differentiation of neural progenitor cells is fostered by an HDAC inhibitor (Hsieh *et al.* 2004; Yellajoshyula *et al.* 2011). Thus, it would appear that neurons are distinct from most other cell types in their requirement for relatively high levels of histone acetylation during (and after) differentiation.

The neuronal histone code is largely attributable to a master regulatory factor known as RE1-silencing transcription factor (REST; AKA neuron-restrictive silencer factor, NRSF). Originally identified as a protein that silences neuronal genes in mature non-neuronal cell types, REST is now known to play a critical role in the maintenance of a tenuous neuronal potential through the transition of neural stem cell to mature neuron (Ballas *et al.* 2005). Key to this discussion is the manner in which REST exerts its effects: The protein exerts transcriptional repression via recruitment of histone deacetylases, G9a histone methyltransferase, and heterochromatin protein-1 (HP-1) to specific genes in nonneuronal and undifferentiated cells. This results in cell type-appropriate expression of individual genes critical for the differentiated phenotype of important neuronal classes, such as the NR1 component of the NMDA receptor (Bai *et al.* 2003).

Translational impacts of the histone code—Diseases from cancer to epilepsy are associated with gene expression changes that appear to arise from aberrations in histone modification. Generally speaking, deacetylation of histones is important for the quiescence

of proinflammatory genes expected in a state of health. Microglial activation is dramatically influenced by modulators of histone acetylation, for instance (Suuronen *et al.* 2003). Accordingly, hyperinflammatory conditions are generally associated with histone acetylation (Suuronen *et al.* 2005; Ma *et al.* 2010; Forgione and Tropepe 2012; Machado-Filho *et al.* 2014), presumably because the genes for cytokines and their receptors are particularly sensitive to histone deacetylation. To the extent that macromolecular synthesis is required for apoptosis and other forms of programmed cell death, histone modification appears critical to these phenomena as well (Boutillier *et al.* 2003; Ryu *et al.* 2005; Yang *et al.* 2011; Koriyama *et al.* 2014; Feng *et al.* 2015). In addition, the relevant enzymes, such as HDACs, can interact with non-histone proteins such as Tau in a disease-relevant manner (Ding *et al.* 2008; Perez *et al.* 2009).

Histone modification is a pharmacologically tractable modality. Current drugs impact global histone acetylation and thus one might expect that they would be limited to conditions in which the primary defect is one that alters the histone code universally. However, butyrate has been used successfully to treat a seizure condition resulting from inheritance of a glycosylphosphatidylinositol deficiency (Almeida *et al.* 2007).

If specificity beyond the general histones is required clinically, it may be afforded by targeting less universal proteins. REST is typically restrained in the cytosol of neurons, and this appears to fail in Huntington's disease. A drug-discovery project has identified compounds that have shown preliminary success in a screen aimed at restoring the cytosolic retention of REST in Huntington's (Conforti *et al.* 2013).

The nuclear envelope—The condensation of DNA around histones is not the only means by which nuclear structure influences transcription. Considerable evidence indicates that interaction of chromatin with the nuclear envelop also makes an impact (Gay and Foiani 2015). The lamins, nuclear envelope proteins that contribute to this structure, appear to be negatively impacted by ethanol and thereby participate in the untoward effects of ethanol on astrocyte gene expression (Marin *et al.* 2008). An intriguing array of signal transduction enzymes – phospholipases, kinases, GTPases, ubiquitin ligases/proteasomes, and the like – have been localized to the cellular nucleus in recent years. The lamins appear to participate in regulating these localizations (Garcia Del Cano *et al.* 2014; Koliou *et al.* 2016).

Epigenetics in neuroplasticity—In addition to the medium-/long-term integration of neural activity that transcription factors mediate (above), some aspects of gene regulation also appear to be critical for the integration of experiences and exposures that can be passed transgenerationally from parent to offspring (or even grandoffspring). It has become increasingly clear that epigenetic control over gene expression is malleable, and impacts on the epigenetic status of chromosomes in an individual' s gametes can change patterns and propensities for gene expression in his/her progeny. Natural and pharmaceutical agonists of PPARγ can create an acute leptin resistance (Hosoi *et al.* 2015); this and similar conditions have often been found to convey in a heritably vertical manner from one generation to the next via epigenetic mechanisms (Masuyama and Hiramatsu 2012). As might be expected, given the connections between such metabolic disturbances and inflammation, one finds a high potential for epigenetic transmission of the inflammatory state. Tumor necrosis factor is

more readily elevated by 6-hydroxydopamine in the substantial nigra of rats that had been exposed to LPS *in utero* (Ling *et al*. 2004), and microglia generally express more proinflammatory genes when obtained from animals that had been exposed to LPS *in utero* (Cao *et al*. 2015). Such a priming toward inflammation has been associated with a decline in global CpG methylation (O'Brien *et al*. 2014).

One possible mechanism for the ability of epigenetic phenomenon to integrate experience and have transgenerational conveyance is a sort of 'molecular momentum'. Intuitively, it would seem that a transcriptionally active promoter may be less accessible to DNA methyltransferases, MeCPs, or even histones; the transcriptosome may simply exert steric hindrance on these enzymes locally (Fig. 3). Whether or not this is true, more precise molecular mechanisms have been identified (Jones 2012). Regardless of the mechanism, 'molecular momentum' is consistent with the finding that transcriptional quiescence is achieved prior to DNA methylation (You *et al*. 2011); and genes that are highly expressed early in the life span – e.g. *in utero* – tend to continue in high expression in later life stages. It is easy to envision a pregnant dam's environment or behavior making an impact on the conditions that prevail *in utero*. The vertical transmission of 'molecular momentum' from parent to progeny outside of pregnancy, however, is more demanding; it would require that the transcriptionally active state be extended to the gamete.

Post-transcriptional regulation

As the beginning of new macromolecular synthesis, gene transcription is a logical control point. But, the decades since the discovery of mRNA have seen the elucidation of a host of subsequent events that control the rate at which genes become proteins. In fact, several elements of these post-transcriptional mechanisms involve genes that exert an impact on phenotype without ever being converted into proteins. Only about 20 percent of eukaryotic transcription is devoted to mRNA (Kapranov *et al*. 2007). The other genomic sequences that participate in expression encode the 'non-coding' RNAs, and beyond the well-known requisite players rRNA and tRNA are several categories that have complex and intriguing modulatory functions, such as microRNA (miRNA), piwi-interacting RNA, small nuclear RNA, and long non-coding RNA. Many non-coding RNAs are still poorly understood and will likely provide fascinating work for molecular biologists – including some neurochemists – throughout the next 60 years. In addition to those processes that depend on non-coding RNA, there are several post-transcriptional controls that are manifest through conventional enzymology and signal transduction. These are no-less important and are increasingly invoked to explain translational aspects of neurochemistry from disease etiology to the aging process.

Control of translation

RNA-binding proteins—A practical understanding of post-transcriptional processing, even in its most pedestrian forms, was only possible after the discovery of RNA-binding proteins that are involved in steps such as exon splicing, polyadenylation, and nuclear export. RNA-binding proteins, however, also participate in modulating mRNA translation rates, stability, and subcellular localization. Some of these processes are generalized to

common elements, e.g. the poly(A)-binding protein which regulates translation rates and stability via interactions with the poly(A) tail of most messages (Bernstein and Ross 1989). Somewhat more interesting are those that interact with discrete sequences in specific mRNAs. Many of these interactions depend upon the formation of RNA secondary structure, such as a stem-loop motif. One of the most instructive classes of structures exemplifying these phenomena are the iron-responsive elements that participate in regulation of proteins involved in iron handling. Ferritin and the transferrin receptor are markedly regulated at the translational level by iron levels, and this occurs via iron-binding proteins that specifically interact with a stem-loop structure in the 5′ untranslated region (UTR) of the ferritin and transferrin receptor mRNAs (Hu and Connor 1996). A similar structure and its interaction with iron-regulatory RNA-binding proteins have also been connected to translational regulation of the amyloid precursor protein by transition metals and, interestingly, by the proinflammatory cytokine interleukin-1 (Rogers *et al.* 1999). Though a specific binding protein has not been identified, a stem-loop structure in the 3′ UTR of brain-derived neurotrophic factor (BDNF) mRNA is critically involved in a phenomenon by which the message, particularly specific splice variants, is stabilized by neurophysiological activity (Fukuchi and Tsuda 2010).

Musashi is an RNA-binding protein of particular interest to neurobiologists (MacNicol *et al.* 2008). Maintenance of neural stem cell populations appears to depend upon musashi, and its expression continues into mature astrocytes (Sakakibara and Okano 1997). However, musashi expression levels in the subgranular zone of the dentate gyrus – one of the few areas of adult neurogenesis in the CNS – are correlated with continued expression of neuronal markers in mature dentate granule neurons, illustrating the importance of this RNA-binding protein for maintaining neuropotency. Choline acetyltransferase levels, in particular, wane along with musashi in Alzheimer's disease progression (Perry *et al.* 2012).

Phosphorylation of ribosomal proteins—Much of what has been learned in recent decades about the translational regulation RNA-binding proteins exert involves their interaction with proteins that play critical roles in the initiation and progression of translation at the ribosome, such as eukaryotic initiation factors (eIF) 2α, 4A, and 4G. Modulation of such proteins and their function can also be mediated by post-translational modifications such as phosphorylation. Several of the initiation factors interact with the 5′ 7-methyl-guanosine cap of mRNA, so modification of these proteins and their function alters protein translation globally. Because some mRNAs can be translated independently of the 5′ cap, phosphorylation of these eIFs can change the mix of proteins being synthesized rather than shutting down translation universally (Thakor and Holcik 2012). Nevertheless, phosphorylation of eIF2α is effected by several stress-related kinases, and the general inhibition of protein synthesis that results can culminate in either energy conservation or – if it is too extensive and lasts too long – cell death. Thus, eIF2α phosphorylation has been connected to the neurodegenerative effects of ischemia, Aβ, and calcium overload (DeGracia *et al.* 1996; Althausen *et al.* 2001; Kumar *et al.* 2001; Chang *et al.* 2002).

Moderate and reversible changes in protein translation are an appropriate response to limitations in the supply of energy or amino acids. Thus, glucose deprivation typically activates phosphorylation of eIF2α, interfering with the critical action of eIF2α in

facilitating the ternary complex of an mRNA's 'Start' ATG with the ribosome and Met-tRNA. In addition, phospho-eIF2α appears to inhibit (Rajesh *et al.* 2015) a master regulator that serves to coordinate energy availability with an array of cellular functions from macromolecular synthesis to mitosis to apoptosis: 'mammalian target of rapamycin' (mTOR), so named for its relationship with the first TORs, which were isolated from yeast in the early 1990s. This lynchpin of cell energetics, mTOR, was isolated from mammalian cells a couple of years later. Now recognized to function within a unit comprising important cofactors, more useful characterizations parse mTOR into complexes 1 and 2 (mTORC1 and −2). These moieties have become the focus of intense scrutiny. The lifespan extension properties of caloric restriction appear to involve mTORs, particularly their inhibition; this scheme is consistent with the activation of mTORC1 by insulin/insulin-like growth factor signaling, which is itself generally inhibited under caloric restriction and genetic alterations that extend lifespan (Johnson *et al.* 2013). In addition, recent research suggests that mTOR inhibition may mimic starvation in a way that activates autophagy (Roscic *et al.* 2011); to the extent that neurodegeneration involves protein aggregates, stimulation of autophagy is considered a promising therapeutic approach for preventing their accumulation.

A major target of the mTORC1 axis is ribosomal protein S6. Its immediate upstream regulators are members of the S6 kinase family, which includes two major branches: p90rsk, a.k.a. MAPK-activated protein kinase-1, and p70rsk, a.k.a. S6-H1 kinase. The latter also phosphorylates and thereby inactivates eEF2-kinase, leading to a reduction in the levels of phospho-eEF2 and a resultant increase in general translation rate (Wang et al. 2001). But, the more direct actions of S6 kinases and their target S6 itself appear to be somewhat more restricted to the '5′ terminal oligopyrimidine (TOP)' RNAs, those that have a polypyrimidine stretch near the 5′ end and encode proteins that orchestrate responses to nutrients (e.g. glucose/insulin) and (hyper-)trophic signals such as insulin-like growth factor, nerve growth factor (NGF), and BDNF (Matsuda *et al.* 1986; Cahill and Perlman 1991; Ishizuka *et al.* 2013). And the examination of the consequences of S6 phosphorylation for those hypothalamic circuits regulating appetite, activity, and glucose disposal accounts for a great deal of the attention devoted to S6 and its kinases in recent years (Ono 2009; Xia *et al.* 2012).

One can be forgiven for becoming confused about the value and role(s) of mTOR in health and disease. Activation of mTOR has been associated with post-conditioning neuroprotection in models of brain ischemia (Xie *et al.* 2013), but the remarkable resistance of hibernating squirrels to ischemia has been attributed to their pre-emptive inhibition of mTOR (Miyake *et al.* 2015). Forsoklin-induced long-term potentiation (LTP) in the hippocampal CA1 depends upon mTOR (Gobert *et al.* 2008), but mTORC1 appears critically involved in the circuitry reinforcement that facilitates drug dependency (Neasta *et al.* 2014). As with other instances of 'antagonistic pleiotropy', there are benefits and disadvantages to mTOR activation; the ultimate conclusion is dependent upon both context (Is there adequate nutrition available to embark on a hypertrophic or neuroplastic cellular endeavor?) and perspective (Is the robust function of an individual cell moot in comparison to the health of the entire organism?) (Fig. 4).

ER stress—Many of the players active in modulating protein translation as a response to energy depletion and toxins also participate in conveying the translation machinery stresses in the endoplasmic reticulum. The unfolded protein response (UPR) is a stereotypical series of events set in motion by denaturation of nascent proteins in the ER as a consequence of heat, over-expression, or failures in glycosylation (Paschen and Frandsen 2001). Though it includes nuclear events aimed at transcriptional regulation, the UPR exerts a rapid and marked inhibition of protein translation, a reprieve that gives chaperones and other protective mechanisms a chance to clear the backlog and potential aggregation that may arise in the ER. Chief among the mechanisms executing this inhibition of translation is protein kinase RNA-like ER kinase, which acts as a sensor of misfolding in the ER and thereafter inactivates eIF2α (Harding *et al.* 1999).

Many stressful and toxic circumstances interfere with proper progression of proteins through the ER, and therefore the list of conditions under which UPR is active has continued to grow. Elements of this program have been detected after traumatic brain injury (Nakagawa *et al.* 2000; Paschen *et al.* 2004), cerebral ischemia (Kumar *et al.* 2001; Llorente *et al.* 2013), 6-hydroxydopamine lesions (Holtz *et al.* 2006), and even sleep deprivation (Naidoo *et al.* 2005). Indeed, neurodegeneration models that include aggregating proteins – e.g. α-synuclein transgenesis (Zagrodnick and Kaufner 1990), parkin knockout (Wang *et al.* 2008a), parkin-associated endothelin receptor-like receptor overexpression (Kubota *et al.* 2006), and TDP-43 over-expression (Suzuki and Matsuoka 2012) – have an obvious connection to the UPR. Curiously, however, peroxidative stress has been reported to antagonize the UPR (Paschen *et al.* 2001). Also reported to be inert or inhibitory for the UPR are Tau-related neurofibrillary pathologies (Spatara and Robinson 2010; Liu *et al.* 2012b); this might be predicted based on the location of Tau filaments outside the ER secretory pathway. Nevertheless, conflicting evidence exists, suggesting that it may be sufficient for Tau to impinge on the ER externally (Nijholt *et al.* 2012; Abisambra *et al.* 2013).

Type-2 diabetes mellitus (T2DM) and the associated insulin resistance seen in prodromal phases have been connected to the UPR of late. This may simply reflect the fact that insulin resistance compromises glucose transporter function, starving the intracellular environment of hexoses; this would likely result in inadequate glycosylation of ER proteins and thereby activate the UPR. However, evidence suggests that a somewhat more complex chain of events involving fatty acid metabolism could be at work. Non-esterified free fatty acids released from adipose appear capable of activating the UPR (Kawasaki *et al.* 2012). Activation of the UPR by such lipids may be detrimental to any tissue, compromising the health and function of pancreatic β-cells (Kharroubi *et al.* 2004; Karaskov *et al.* 2006), hepatocytes (Pfaffenbach *et al.* 2010; Achard and Laybutt 2012), or even adipocytes themselves (Miller *et al.* 2007; Basseri *et al.* 2009; Han *et al.* 2013). Some evidence indicates that the UPR exerts its most nefarious effects when activated within the hypothalamic nuclei that control appetite, activity levels, and glucose disposal (Kozuka *et al.* 2012; Cragle and Baldini 2014). But exercise, which prevents many of the untoward effects of high-fat diets in mice, actually elevates markers of the UPR in the hypothalamus (Kim *et al.* 2010). In this vein, it is important to recognize that the UPR evolved as an adaptive

program. Whether it contributes to pathology appears to depend on degree of activation and the weighing of cytotoxicity versus systemic derangement; in this sense, it is somewhat analogous to the Janus-faced characterization of mTOR.

Subcellular localization of mRNA

Dendritic targeting—Another important development in the maturation of molecular biology – one with a unique relevance to neurobiology – is the discovery that the mRNA products of specific genes are differentially trafficked to subcellular niches for localized translation. Oswald Steward led the way in the early demonstrations of this phenomenon, first documenting the presence of polyribosomes (beads on the 'string' of an mRNA undergoing rapid translation) at the base of dendritic spines (Steward and Levy 1982), illuminating the possibility of protein translation that might contribute to – or be influenced by – synaptic activity. But, the real significance was realized a few years later when Steward and his colleagues demonstrated specific transport of individual mRNAs (Kleiman *et al.* 1990; Eberwine *et al.* 2001). Dendrite-specific mRNAs were also found to be enriched in synaptosomes (Rao and Steward 1993), paving the way for utilizing this sort of preparation to explore activity-dependent changes in translation. At the turn of the century, elements of the transport machinery that couple mRNA to microtubule-dependent transport mechanisms were elucidated (Ohashi *et al.* 2000). The *cis* elements within the mRNA sequences that underwent specific transport were identified soon after (Miller *et al.* 2002; Chen *et al.* 2003).

The implications of targeted delivery of specific mRNAs to specific synapses created immediate excitement in the field. It is true that synaptic plasticity involves, to some extent, localized delivery of the ultimate gene products: proteins themselves. Indeed, the targeting of an mRNA to a specific subcellular locale is mediated by proteins that interact with specific sequence elements in the message (Ohashi *et al.* 2000; Miller *et al.* 2002; Chen *et al.* 2003). However, localized translation provides the capacity for much more rapid changes in the neurotransmitter receptors (Conti *et al.* 1994; Eberwine *et al.* 2001), cytoskeletal elements (Kremerskothen *et al.* 2006; Barker-Haliski *et al.* 2012), kinases (Wang *et al.* 2008b), and other critical elements of synaptic plasticity (Huang *et al.* 2004; Duning *et al.* 2008). Dendritic transport of proteins that might be needed to establish and/or maintain a potentiated synapse would be expected to take place at ~ 15 μm per hour. By contrast, translation can occur at a rate of 6–10 amino acids per second, stamping out the 905-aa GRIK1, for instance, in just a couple of minutes.

Axonal translation—For several decades dogma held that the axon was a peculiar subcellular domain from which translation was essentially excluded. In fact, Steward's hypothesis about targeted mRNA delivery to dendritic spines was lent considerable support by the inability to detect mRNA delivery to axons (Davis *et al.* 1987). Like so many rules, however, this one has its exceptions. And the acknowledgment of axonal protein translation need not have awaited the 21st century. Localized synthesis of acetylcholinesterase and other proteins in mammalian axons was demonstrated only a few years after the christening of the *Journal of Neurochemistry* (Koenig 1961) and was elaborated upon throughout the 1960s (Koenig 1967a,b). Axonal translation appears to be particularly important during regeneration from injury or axotomy (Koenig and Adams 1982; Perry *et al.* 1983).

Eventually, synaptosome preparations, combined with the modern advent of sensitive proteomic approaches, were also used to uncover proteins synthesized within presynaptic boutons (Jimenez *et al.* 2002). Telomere repeat-binding factor 2 was recently determined to play an important role in facilitating axonal transport of specific mRNAs, a process antagonized by fragile X mental retardation protein (Zhang *et al.* 2015).

MicroRNA

For well over half of the 60 years that the *Journal of Neurochemistry* has been extant investigators have succeeded in manipulating the expression of specific genes by introducing into cells RNA containing the reverse-complement of the targeted genes' mRNA (Stephenson and Zamecnik 1978; Zamecnik and Stephenson 1978; Izant and Weintraub 1984). This 'antisense' approach was one example of several phenomena in which suppression of the levels of an ultimate protein product was effected through naturally occurring or empirically induced RNA hybridization, referred to variously as 'quelling', 'post-transcriptional gene silencing', and 'co-suppression of gene expression'. After many years of attempting to optimize the antisense technique by modifying nucleotides' structure for the sake of stability or more efficiently introducing it into the cell' s interior, a key discovery was made by Andrew Fire and Craig Mello when they found that double-stranded RNA worked much more efficiently – substoichiometrically, in fact – compared to single-stranded RNA (Fire *et al.* 1998).

Fire and Mello shared the 2006 Nobel Prize for showing us (i) that RNAi was mediated most efficiently by dsRNA and (ii) how to harness RNA interference in an empirically practical way. Some viewed their award as an affront to the investigators who discovered some years earlier the natural phenomenon underlying these effects, such as the normal endogenous production of *lin-4* in *C. elegans* (Lee *et al.* 1993; Wightman *et al.* 1993) or of antisense RNA for myelin basic protein (Okano *et al.* 1991). But, whether it was scientific synergism or just good timing, Fire & Mello (Fire *et al.* 1998) kicked off a spate of studies that eventually uncovered shared mechanisms uniting empirical manipulation of gene expression and the underlying natural phenomena that the Nobel laureates unwittingly mimicked.

That underlying natural system is now appreciated as a critical form of post-transcriptional regulation of the stability and translatability of mRNA. It is dependent upon noncoding RNA that is initially transcribed from the nuclear genome as a stem-loop structure wherein the stem is highly homologous to one or more mRNA sequences. These non-coding transcripts are referred to as microRNA (miRNA). They are transcribed primarily by RNA polymerase II and often reside within the introns of other, mRNA-encoding genes. In animals, the complementary regions with which they hybridize are typically in the 3′ UTR of mRNAs. Key to understanding the function of miRNA is an appreciation for the multiprotein complex within which it is ultimately processed, resides, and functions. This RNA-induced silencing complex contains one RNase termed 'dicer' that prepares the dsRNA by cleaving it into 21–25 basepair fragments. One strand of these oligomers is subsequently combined with the remaining protein subunits of the RNA-induced silencing complex, including a second RNase of the Argonaute family, which is the component that can ultimately cleave the mRNA target. This cleavage of the mRNA actually happens only rarely, however, typically

requiring a perfect or near-perfect match between the miRNA and the mRNA. More often, the miRNA has several mismatched nucleotides and the resulting hybridization merely inhibits translation. It is perhaps worth noting here that the term 'siRNA' was coined by Elbashir *et al.* (2001) when they confirmed the work of Hamilton and Baulcombe (1999), Hammond *et al.* (2000), and of Zamore *et al.* (2000) showing that larger dsRNA molecules were processed to active, 22-b.p. fragments.

Besides inhibition of translation (with or without mRNA degradation), miRNA can have other effects on gene expression. In some cases, miRNA can suppress transcription via impacts on DNA methylation or histone modification at genomic sites (Bayne and Allshire 2005; Guil and Esteller 2009); in very rare cases, miRNA can even participate in elevation of transcription rates (Li *et al.* 2006).

Deviations from the central dogma

If the exception proves the rule, the central dogma is perhaps strengthened by several instances in which the straightforward relationship between gene, mRNA, and protein expression is altered in ways that were initially shocking.

Selenocysteine—From *Archaea* to mammals, the same set of 20 amino acids is supplied for protein translation by designated tRNA molecules that recognize corresponding mRNA codons. It was something of a surprise when Thressa Stadtman and colleagues reported the presence of a novel amino acid, selenocysteine, in certain proteins (Cone *et al.* 1976). In these cases, a selenium-containing selenol group takes the place of the sulfur-containing thiol that would otherwise constitute the R- group of cysteine. Often, these selenocysteine proteins are enzymes involved in reduction–oxidation reactions such as glutathione peroxidase, explaining the requirement for selenium as a micronutrient. The biochemical world was in for an even greater surprise when August Böck and coworkers – initially working in bacteria – determined that the thiol of this novel amino acid was not replaced with a selenol moiety through post-translational modification of an intact protein but rather through the cotranslational utilization of a novel tRNA ($tRNA^{Sec}$) that is coupled to extant selenocysteine and acts at the site of a UGA 'stop' codon (Leinfelder *et al.* 1988). Part of the secret by which this mechanism takes place lies in a specific sequence – a *cis* element – lying just 3′ to the relevant UGA. The $tRNA^{Sec}$ has an unusually long acceptor arm, the stem-loop structure that interacts with elongation factors. The unique acceptor arm of the tRNASec eschews the typical elongation factor (eEF1A in eukaryotes), favoring instead eEFSec as it interacts with the 3′ *cis* element (reviewed in Chen and Berry 2003).

RNA editing—Another surprising exception to the central dogma is the modification of mRNA sequences after transcription and routine processing. RNA editing was first reported in the mRNA of mitochondria in the mid-1980s (Benne *et al.* 1986); eventually, mitochondrial tRNA was found to be edited, as well (Janke and Paabo 1993). But along the way, rather profound consequences were discovered for editing of a nuclear-encoded mRNA that dramatically impacts neurophysiology. From 1991 to 1993, Peter Seeburg and colleagues published a series of articles outlining an editing of the mRNA for three ionic glutamate receptors whereby 1–3 codons in the mRNA were altered from the genomic DNA

sequence at incomplete rates, resulting in heterogeneity in the ultimate protein sequences (Sommer *et al.* 1991; Higuchi *et al.* 1993; Kohler *et al.* 1993). Eventually, the laboratories of Tom Maniatis, Richard Axel, and Ron Emeson got into the act, elucidating some of the mechanisms, e.g. adenosine conversion into inosine, through which the codon is recognized by a tRNA anticodon distinct from that which its genomic sequence would dictate. This editing and the resulting amino acid change can result in markedly altered ion permeability of the channel, to calcium, for instance (Kohler *et al.* 1993).

One of the most consequential changes rendered by editing is in the mRNA for the GluR2 AMPA-receptor subunit (the gene for which is now designated *GRIA2*). The editing of the so-called Q/R site in this protein greatly diminishes calcium permeability of the channels in which it participates (Hollmann *et al.* 1991); thus, calcium conductance is rare in a-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors because of the widespread expression – and editing – of the GluR2 subunit. One exception is the lower motor neurons of the spinal cord, a considerable proportion of which have calcium permeability because of incomplete *GRIA2* mRNA editing, a status that may explain some of the selective vulnerability of these cells in motor neuron diseases (Takuma *et al.* 1999; Greig *et al.* 2000; Kawahara *et al.* 2003). Stresses such as ischemia (Peng *et al.* 2006) or chronic depolarization (Condorelli *et al.* 1993) can reduce the expression or editing rate of *GRIA2*, and these conditions have been invoked to explain some instances of excitotoxicity, even in glial cell types (Yoshioka *et al.* 1995).

Another RNA-editing event is embedded in the unfolded protein response introduced above. One of the first sensors of ER stress is inositol-requiring protein-1 (IRE1), and it participates in the unusual function of executing on-demand, extranuclear mRNA splicing of a single substrate: the mRNA for X-box binding protein-1 (XBP1); IRE1 cleaves the XBP1 mRNA, and the catalytic subunit of the tRNA ligase complex (RTCB) ligates it back together (Ron and Walter 2007; Jurkin *et al.* 2014). Once updated in this manner, the new XBP1 mRNA is translated into a transcription factor that induces several other components of the UPR.

Genetic instability

RNA is believed to be older than DNA, which is derived from the former through dehydroxylation of the 2′ ribose carbon; yet, the latter is the substrate for encoding biological blueprints. This seems to be because DNA is more stable, the dehydroxylation having removed the potential for alkaline conditions to instigate a nucleophilic attack by the 2′ hydroxyl on the 3′ bond and thus break the phosphate backbone of an RNA polymer. But the relative stability of DNA is only that: relative. The structure and sequence of our chromosomes is subject to change. Of course, we know this happens through the accidents of mutagenesis; the process of evolution depends on that. What has been surprising is the series of discoveries demonstrating the extent to which the genome is modified, expanded, and abbreviated in a programmatic manner throughout the development of an individual organism. And few tissues exhibit these events, or are affected by them, to the extent seen in the brain.

Structural variations of the genome include substantial differences (i.e. greater than the single-nucleotide polymorphisms and similarly discrete differences) that exist between individuals but are consistent throughout the body (or, at least, as consistent as any other genomic element). This includes copy number variations (CNVs) that comprise duplication or deletion of sequences that can be several megabases in size (Martin *et al.* 2015).

In addition to such somatically consistent deviations from the norm, there are somatic mutations and even somatic CNVs in individual tissues or cell types, creating a genomic mosaicism that we are only now beginning to appreciate (Fig. 5). Mosaic variation of chromatin in neurons was documented in invertebrates beginning around 1970 (Coggeshall *et al.* 1970; Manfredi Romanini *et al.* 1973). A few investigators reported similar findings in vertebrates, indeed even humans (Brodskij and Kusc 1962; Herman and Lapham 1968; Lapham 1968; Mares *et al.* 1973). But, these reports went largely unappreciated, in part because of skepticism about the precision of the methods available at that time (Swartz and Bhatnagar 1981). More sophisticated techniques and instrumentation were applied in the 21st century to confirm 'constitutional' tetraploidy of neurons in the chicken retina (Morillo *et al.* 2010) and the mouse retina and brain (Lopez-Sanchez and Frade 2013).

In addition to the evidence that whole genomes are duplicated to tetraploidy in a few cells in the brain, there are signs of more piecemeal hyperdiploidy in neurons. Thomas Arendt and others used a sequencing-independent measurement of DNA content per cell (slide-based cytometry) and found that approximately 11% of neurofilament-positive cells had greater than 2N chromatin. Eighteen months later, Fred Gage and colleagues showed that a considerable number of neurons in the human frontal cortex have CNVs at least as large as 1 megabase (McConnell *et al.* 2013). An additional finding reported by Arendt and coworkers is that the proportion of cells with these CNVs declines with aging. One interpretation is that the CNVs are maladaptive; so, these cells die sooner than the others or cause the individual bearers to develop neurodegenerative diseases (which would have removed these individuals from the pool of neurologically healthy specimens used for the study).

A quarter century ago, Huntington Potter proposed the very novel hypothesis that Alzheimer's disease is a consequence of mosaic Down's syndrome, i.e. aneuploidy for Chromosome 21 in a cell-by-cell basis in the brain (Potter 1991). Over the years, his laboratory and others have produced evidence that supports this idea, including an elevated degree of aneuploidy or hyperdiploidy in Alzheimer brain tissue, i.e. DNA allelic counts that are greater than 2N (Potter 1991; Yang *et al.* 2001; Rehen *et al.* 2005; Iourov *et al.* 2009); the Potter laboratory has also produced evidence that mutated forms of presenilin that give rise to Alzheimer's can interfere with proper mitotic segregation of chromosomes, which might facilitate aneuploidies (Li *et al.* 1997). Frade and López-Sánchez have more recently proposed that Alzheimer's disease may involve simply an expansion of the frequency with which cortical neurons normally exhibit constitutional tetraploidy (Frade and Lopez-Sanchez 2010). However, other evidence argues against this hypothesis (Westra *et al.* 2009). Arendt and colleagues posit that a pathological process distinct from what is considered constitutional tetraploidy gives rise to the CNV mosaicism observed in pathological states such as Alzheimer's (Mosch *et al.* 2007).

Though there is no evidence that it contributes to the mosaic CNVs discussed above, a startling phenomenon recently observed in CNS neurons further contributes to the impression that these cells are quite different from any other in the body. Lennart Mucke's laboratory reported that double-strand breaks are routinely generated in mouse neurons through normal physiological activity, including that which accompanies exploring a novel environment (Suberbielle *et al.* 2013). Though spurred by normal learning and memory correlates, the breaks were more extensive and persistent in an amyloid precursor protein-transgenic mouse model. Long-term potentiation is associated with the elevation of reactive oxygen species (Klann *et al.* 1998), and so were some of the conditions that fostered double-strand breaks in neurons; this would be consistent with the well-characterized ability of oxidative stress to effect DNA damage. It was somewhat surprising that antioxidants did not impact the generation of double-strand breaks in Mucke's study. Though DNA damage is certainly stressful, only a single observable repair focus was present in about 99% of the cells showing breaks in this study, and they seemed to recover well over the following 24-h period.

Owing to the error-prone mechanism that predisposes them to disease mechanisms, tri- and hexanucleotide repeats often exhibit somatic mosaicism. Such variety across different tissues and organs is well established in the CAG repeat of huntingtin (Telenius *et al.* 1994). Mosaicism has also been reported in the trinucleotide repeats relevant to spinocerebellar ataxias, where it is seen across peripheral tissues, as well as across the brain regions (Tanaka *et al.* 1996; Hashida *et al.* 1997; Ito *et al.* 1998). Mosaicism is also seen in the length of the GAA repeat expansion across various brain regions in Friedreich' s ataxia (Montermini *et al.* 1997). Though the repeat size did not appear to correlate with neuropathological findings in the various regions, mosaicism was invoked to explain dramatic differences in the clinical presentation of two siblings who showed similar repeat counts in the DNA obtained from their leukocytes (Klopstock *et al.* 1999). Mosaicism also appears to explain a rare occurrence of Rett syndrome (above) in a male; in hair and blood samples, the affected individual had a mixture of wild-type and mutated *MECP2* genes though he had only one X chromosome (Topcu *et al.* 2002). Nevertheless, mosaicism of hexanucleotide repeats in *C9orf72* does not appear to contribute to the genotypic profile of amyotrophic lateral sclerosis (ALS) (Pamphlett *et al.* 2013).

In addition to mosaicism within an individual' s genome, there are well-documented cases of cellular chimeras among dizygotic ('fraternal') twins that happen to be monochorionic. This results from the two chorions fusing because of proximity in the uterus, and it evidently allows blood (and apparently stem cells) to intermingle between the two individuals. This phenomenon is more common in pregnancies that are the product of *in vitro* fertilization (Williams *et al.* 2004; Miura and Niikawa 2005). Thus, the rise in frequency of such assisted reproductive technology may have implications for genetic diseases, with regard to both the potential 'partial' manifestation of the phenotype and the complications for genetic screening from a single cell type (which may not manifest the same proportion of chimerism or mosaicism as that occurring in the more relevant cell or tissue type).

Dividends of molecular technology

Our understanding of molecular aspects of biology, particularly those relevant to the central dogma, has benefited tremendously from technical advances. The ability to sequence and synthesize polynucleotides, to manipulate DNA sequences *in vitro* and even *in situ,* and to precisely quantify specific sequences has allowed advances that could not have been dreamt 60 years ago. And the boon this technology has provided to basic science may be surpassed someday by the benefits to human health provided by the utilization of these methods in translational medicine.

Polymerase chain reaction

The impact of RNA editing on glutamatergic activity (above) gained considerable heft from a better understanding of the subunit composition of functional receptors, and this was dramatically aided by the ability to analyze the mRNA levels of individual receptor subunits in a single cell. It is difficult to imagine this and a great many other advances, coming to pass without the technological watershed provided by invention of the polymerase chain reaction (PCR). This technique now underlies the state-of-the-art techniques for quantification of mRNA, forensic investigations, several site-specific mutagenesis strategies, the power of laser-capture microscopy, and much of the efficiency of next-generation sequencing (below). In addition to its common utilization in quantitative analysis of RNA levels, the structural constraints of PCR also make it useful for qualitative assessments of polynucleotide structure, such as the relative positions of gene elements or the nature of transcripts and splice variants (Fig. 6).

PCR also serves as one of the murkiest and contentious episodes in the history of scientific discovery. Volumes have been written about this story and its inherent controversies. However, a retrospective such as this has at least some duty to highlight a few historical aspects of the saga. Kary Mullis won the 1993 Nobel Prize in Chemistry for his role in developing PCR, but the team that first reported the concept – 14 years earlier – was led by a 1968 Nobel laureate Har Gobind Khorana. In 1971, Khorana and his postdoc Kjell Kleppe first outlined a strategy for amplifying DNA by cyclical polymerase reactions running off terminal primers (Kleppe *et al.* 1971); colleagues remember the pair presenting this technique at major scientific conferences of the day. However, the potential utility of this procedure was not realized at the time. In part, this was because of the fact that a new bolus of DNA polymerase had to be resupplied at each cycle of amplification because each cycle requires heating to a relatively high temperature to melt the nascent strands apart and allow access of the primers; this denatured and inactivated the DNA polymerase. Consequently, Kleppe and Khorana only managed a four-fold amplification. Mullis reckoned that this could be extended by several orders of magnitude, and thankfully he was right. He and his colleagues also sold the scientific community on the technique by explicating some of the utilitarian dividends it would reap. In perhaps the earliest report focused on the procedure itself, they explained (or demonstrated) the power of PCR to essentially isolate a sequence from a complex mixture, to attach useful tags such as restriction sites, to analyze RNA via the reverse-transcriptase corollary, and to synthesize relatively large novel sequences entirely

from synthetic oligonucleotides by stepwise progression of consecutive primers that extended their overlapping 3′ ends in cyclical amplifications (Mullis *et al.* 1986).

Randall Saiki and Henry Erlich, colleagues of Mullis at Cetus Corporation, were keen to apply the technique to one of their primary interests, genotyping of human disease loci. Their first report, a method for diagnosing sickle cell anemia (Saiki *et al.* 1985), actually made it to press before the initial explanation of PCR cited above. Saiki is also credited by most for the momentous brainchild of using a heat-stable polymerase for PCR (Saiki *et al.* 1988). It was this innovation that seemed to have captured the imagination of industry, and soon thereafter automated thermocyclers (e.g. US Patent 5 616 301) were being sold to laboratories all over the world, relieving scientists of the drudgery of manually moving tubes between various temperature baths.

In a sense, the advance that allowed PCR to have its profound impact on biological sciences began in the 19th century with Pierre Miquel's reports of 'thermophilic' bacteria that could thrive at 72°C (Miquel 1888). Nearly, a century later, Thomas D. Brock and Hudson Freeze reported a new species of thermophilic bacterium which they named *Thermus aquaticus* (Brock and Freeze 1969). It was this bacterium, discovered in the Lower Geyser Basin of Yellowstone National Park, that Saiki and colleagues would employ to solve the only real limitation of Mullis' s vision. As a key enzyme of a bacterium that could obviously replicate its genome at high temperatures, the *T. aquaticus* (*T.aq.*) DNA polymerase was stable at the melting temperature of PCR and thus well suited for application to this procedure.

As mentioned above, one of the earliest major advances that PCR afforded neurochemistry was the ability to determine receptor subunits and channels responsible for the actions of neurotransmitters. This relied on the ambitious vision of Jean Rossier and others who dreamed it might be possible to patch-clamp a neuron, record from it, and then extract the cytosolic contents through the patch pipette for reverse-transcriptase PCR of the cellular RNA (Lambolez *et al.* 1992). Clues about glutamate receptor distribution, for instance, had been provided a few years earlier through studies by Sakmann and Seeburg, who demonstrated through *in situ* hybridization that certain receptor subunits, such as 'flip' and 'flop' splice variants were exclusively expressed in single - sometimes adjacent - cells (Sommer *et al.* 1990). However, a true understanding of the way subunits were combined into a functional receptor required comprehensive determination of all the subunits which were in a cell that exhibited specific conductance properties and ligand specificity. After glutamate receptors, the approach was applied to the subunit composition of GABA and other receptors (Santi *et al.* 1994). More recently, these efforts benefited from the application of techniques that utilize internal standards to make possible an absolute quantification of the number of individual *molecules* (± 10) of a given subunit (Tsuzuki *et al.* 2001)!

DNA sequencing

Virtually all of molecular biology has been developed in the past 60 years, making a comprehensive discussion of its progress during this period an onerous task. This retrospective has therefore focused primarily on those advancements with a particular relevance to neurochemistry. Nearly, none of this progress could have taken place without the ability to determine the sequence of polynucleotides. But, most biological scientists

working today have been well schooled in the two traditional, workhorse methods of DNA sequencing: ‘Maxam-Gilbert’ and ‘Sanger’. So, these (generally obsolete) methods will be passed over to devote space to the mind-boggling potential that next-generation sequencing provides via its speed and power.

Next-generation sequencing—A few different modes have been used in the next-generation (‘next-gen’) sequencing boon: sequencing-by-hybridization (SBH), sequencing-by-synthesis (SBS), and sequencing-by-ligation. Next-gen SBS is similar to the traditional Sanger method: in that, it actually reports the sequence of a DNA polymerase reaction executed on a primer that is complementary to the template DNA supplied from the ‘unknown’ sample. In both Sanger and next-gen SBS, only one nucleotide (dATP, dCTP, dGTP, or dTTP) is offered per ligation reaction, and whether or not it is ligated to the primer is determined because of the fact that it is labeled with radioactivity (Sanger only) or fluorescence (utilized by commercial systems currently marketed by Illumina and Roche), or by the reaction’ s effect on pH (utilized by the Ion Torrent system). Although the Sanger method utilizes only one polymerase reaction per strand (and therefore requires much more input DNA), next-gen SBS is reiterative, supplying each nucleotide in successive reactions, creating an ever-growing complementary strand and recording whether or not the nucleotide offered in each round is conjugated or not. Because it utilizes these reiterative cycles, and often involves an initial PCR step to amplify the input DNA, next-gen SBS paradigms can be performed with as little as 50 ng of input DNA. For fluorescence detection, the reiterative cycle format is possible through photobleaching of the fluorescent tag after each polymerase reaction so that each successive round can be assayed against low background. The Ion Torrent system relies on acidification resulting from liberation of a proton when the primer’s 3′ hydroxyl executes nucleophilic attack on the α-phosphate of a nucleoside triphosphate. The liberation from a photobleaching step and other issues of sensitivity allow the Torrent to run considerably faster than the light-based sequencers.

SBH approaches have found a solid utility in specialized types of genotyping and are gaining in popularity for more yeoman work as well. The application for which hybridization seems most needed is in the identification of CNVs (above), especially the moderate-to-large regions of the genome that appear to have been duplicated not only during evolution but also during diversification of the human diaspora. Often, these duplications are so large and so similar to the original sequence that they can scarcely be detected by conventional sequencing approaches. Except for heterozygosity that may not be consistent with 2N allelic distributions, the results of SBS sequencing are so myopic that they report essentially no difference between the sequence of 200 base pairs in the p arm of Chromosome 17 and the same sequence within a 1.4-megabase duplicate lying next door in some individuals. SBH – in particular, comparative genomic hybridization arrays – can quantify the number of copies hybridizing to its templates; therefore, it is one of the few techniques that can readily detect CNVs.

RNA-Seq—One of the most powerful dividends of next-gen sequencing is its application to quantitative gene expression analysis. Early strategies for gaining a somewhat comprehensive view of the changes in gene expression across two or more conditions relied

on burdensome and technically tricky methods such as differential display (Shirvan *et al.* 1997; Toki et al. 1998; Chen et al. 1999; Liu et al. 1999; Yagita *et al.* 1999; Yamashita *et al.* 1999) and subtractive hybridization (Walker and Sevarino 1995; Gould *et al.* 2000; Leypoldt *et al.* 2001; Ftouh *et al.* 2005; Boucquey *et al.* 2006). Enormous gains in throughput and quantitative accuracy were made when microarray screening was developed (Ang *et al.* 2001; Chun *et al.* 2001; Grunblatt *et al.* 2001; Yoshihara *et al.* 2002). Nevertheless, arrays must be constructed in advance; they are inherently limited to the 'known universe' of expressed sequences. The human genome is estimated to express protein-encoding mRNA from only about 19 000 genes, and microarrays can accommodate as many as 480 000 'features' (spots) per microscope slide. It is usually considered necessary to include multiple sequences for each gene, primarily for the sake of confirmation. But, this could be achieved at a level of 25 sequences for each gene on an 8 × 60 000 microarray.

Nevertheless, next-gen sequencing offers the advantage of potentially pulling novel sequences out of a specimen. And the true beauty is that the sequence is not merely tabbed qualitatively, it can be identified entirely on the basis of a quantitative distinction it exhibits between two treatment groups, between disease and healthy tissue, or indeed between any two (or more) sets of conditions. Use of next-gen sequencing for RNA screening is often called 'RNA- seq', but a somewhat more formal name is 'whole-transcriptome shotgun sequencing'. This technology is somewhat better at quantitation than are microarrays, especially at the high and low ends of the abundance scale. In addition, quantitative comparisons in microarrays are limited by differences in affinity for each target-probe hybrid, a handicap that RNA-seq transcends. It should also be noted that an investigator is dependent upon commercial enterprises or other third parties to select the best probes, and this trust has proved to be misplaced occasionally. Finally, the ability to discover novel splice variants and other modifications should not be overlooked. It has been argued that RNA-seq is uniquely qualified to overcome challenges inherent in analyzing expression patterns in complex human neurological disorders (Sutherland *et al.* 2011).

Human genome project and HapMap—The political will and financial investment made in sequencing the human genome represents a commitment that rivals the endeavor to put a man on the moon; the payoff may have been even more significant in practical terms. Although much is made of the limitations on understanding the contributions of genes without elucidation of their regulation, discoveries related to the roles that genetic variations play in human biology and disease have been dramatically accelerated by sequencing even a single genome. Almost as important has been the delineation of genetic haplotypes – the various 'Chinese menu' combinations of polymorphisms that predominate in the population – which reached a milestone in 2005 with public release of Phase I of the International HapMap Project (International HapMap Consortium, 2005). This release marked a 'data freeze' that included genotypic information about at least one common single-nucleotide polymorphism (SNP) for every five kilo-bases in all populations under study. The combination of these two undertakings has made possible the tremendous progress of genome-wide association studies (GWAS) and their application to neurological disorders. As inarguably the most complex organ of the body, the brain and its functional auxiliaries is subject to the most complex genetic circumstances of health and disease. GWAS approaches

are perhaps best suited to discovering quantitative trait loci that summate in 'sporadic' instances of complex diseases such as Alzheimer's disease or bipolar disorder (Baum *et al.* 2008; Liu *et al.* 2012a; Lambert *et al.* 2013), but GWAS are also important for identification of genes that influence age of onset, severity, and rate of progression for diseases known to arise from discrete, highly penetrant genetic loci (Finch *et al.* 2011; Soler-Lopez *et al.* 2011; Vass *et al.* 2011).

There are numerous examples of neurological disorders that have yielded to insights gained from genomic profiling of normal and abnormal brain function (Tsuji 2013). It was traditional genetic analysis that provided mechanistic insights, and the potential for familial screening, regarding Huntington' s disease (The Huntington' s Disease Collaborative Research Group, 1993). However, next-gen approaches have proved their usefulness with a spate of discoveries. Mutations in over 50 genes have been associated with Charcot-Marie-Tooth, and at least one, identified through a next-generation (exome-sequencing) approach, had been missed by conventional Sanger sequencing (Landoure *et al.* 2012). Spinocerebellar ataxia, another condition with marked locus heterogeneity, was also mechanistically unraveled by exome sequencing. Two compound heterozygous mutations in the GLB1 gene were found to confer recessive juvenile-onset GM1 gangliosidosis (Pierson et al. 2012). Of course, the true hope is that genetic understanding can lead to therapeutic innovation. One striking example of the power of genetic discovery comes from the treatment of obesity, a disease we did not even perceive as neurological until we came to understand, largely through basic studies on the molecular physiology and genetics of rodents, that the primary site of action for the satiety hormone leptin is in the hypothalamus (Harvey 2007). Humans with a loss-of-function mutation in leptin, while rare, have benefited tremendously (Paz-Filho et al. 2011).

Transgenesis

Perhaps, no other advance in molecular biology has captured the imagination of the layperson like transgenesis, the technical ability to rationally and specifically manipulate the genome of any living thing. The genetic makeup of other species has been manipulated by selective breeding and other aspects of domestication for approximately 20 000 years of human history. Experimentalists have occasionally accelerated this process with chemical mutagenesis. However, the power inherent in directing the genetic modification as it is done in transgenic plants and animals obviously renders this objective into another dimension. The lay public immediately comprehends the potential that transgenesis holds for curing genetic diseases or leveraging the efficiency of food production. It is worth noting that a great many inborn errors of metabolism, typically resulting from loss-of-function mutations in an anabolic or catabolic enzyme, manifest as neurological disorders. Thus, the concept of supplying a functional gene through transgenic techniques has particular resonance with those who have a concerned with neurological disease. However, neurochemists and other scientists have probably been just as captivated by the profound impact such genetic modification has as an experimental independent variable for answering difficult biological questions.

Terminology—The lay press sometimes presumes that ‘transgenic’ always refers to trans-species insertion of a gene, which it is not, of course. But, scientists are guilty of creating confusion in nomenclature, as well. It seems that one routinely encounters non-standard uses of the term ‘knock-in’, for instance. Convention dictates that this term be reserved for genetic transfers resulting from homologous recombination, but investigators often use it when working with a randomly inserted transgene if it is combined with knockout of the homolog. The field would be done a great service if all publications and presentations on genetically modified mice were prepared after consultation of standards prepared by the International Committee on Standardized Genetic Nomenclature for Mice (Montoliu and Whitelaw 2011). Nevertheless, there remain substantial differences in the conventions for genetic nomenclature across different species. Today, a great many projects benefit from utilization of multiple models, many transcending entire phyla; thus, it seems that a universal convention for genetic nomenclature might be in order.

Germline transgenics—The earliest germline transgenic animals were created by injection of viral – and later, plasmid – DNA directly into blastocytes or eggs, typically targeting the male pronucleus. A recombination event at this stage enhances the chances that gametes will be included among the cells that incorporate the transgene (though, as a result of the potential for epichro-mosomal replication of concatemerized transgenes, only 20–30% of founder animals are true mosaics). It also results in a founder that is of a pure strain. The other major approach is stable transfection of a cultured embryonic stem (ES) cell line, followed by injection of the transfectants into a blastocyst. Because the ES cells may or may not differentiate into germ cells in the resulting chimeric mouse, founder lines are somewhat less likely to propagate the transgene. In addition, the vast majority of transgenics produced in this manner rely on ES cells from the Sv129 strain of mice due their efficiency in this application. Introduction of the transgene into another strain – either as the initial blastocyst recipient or through interbreeding of a germline transgenic – creates an animal that is genetically mosaic for more than just the transgene. Though backcrossing to the desired strain for experimentation for six generations is conventionally considered sufficient, even this can permit artifacts; particularly if a phenotypically potent gene is in linkage disequilibrium with the transgene. This and related issues have been highlighted as particularly problematic for neurobehavioral studies (Gerlai 1996). There are a few other approaches to creating germline transgenics, including viral transduction of blastocysts or using sperm as vectors, but these represent a very small fraction of the total lines created.

Knockouts and other instances of homologous recombination—The availability of ES cells has greatly facilitated experimental objectives that require homologous recombination. In straightforward transgenics, the novel gene integrates at site in the genome outside the control of the investigator. However, there are situations in which the genetic modification must be site-specific or ‘targeted’. This is a key element of gene ablation, commonly known as ‘knockout’. Such targeted deletional mutagenesis is most commonly accomplished by replacing at least part of a gene with a selectable marker such as a drug-resistance gene. This has the practical advantage of allowing enrichment for cells in which integration has taken place, a considerable percentage of which will be correctly

inserted into the gene of interest via sequences flanking the Neo^R gene that are homologous to the targeted gene.

In some cases, a more subtle mutation is desired; e.g. a point mutation. In this case, a missense mutation or other small change can be included in the homologous flanking arm of mutagenic construct. It is usually necessary to remove, after the homologous recombination, the selectable marker gene (e.g. Neo^R) that intervenes between the mutated homologous flanking arm and the wild-type flanking arm. This is often accomplished via Cre/Lox technology (below), such that a residual of the mutagenesis remains: a single *loxP* site. If the mutagenic construct is designed such that the selectable marker (and its attendant *loxP* sites) is placed in an intron, there should be no significant consequence for the gene's expression or function other than the intended subtle mutation.

A tremendous boon to *in situ* site-specific mutagenesis has been provided by 'CRISPR' technology (van der Oost 2013; de Souza 2013). The details are too complicated to thoroughly describe here, but this methodology utilizes an RNA guide to seek out a specific position in the chromosomal DNA and target it for attack by an endonuclease (Cas9). The resulting double-stranded break is ligated back together by DNA-repair mechanisms that will usually generate small deletions, resulting in frame-shift mutations that are often deleterious for the targeted gene. Thus, CRISPR is typically used for knockout objectives. However, other types of mutation are possible with the inclusion of a DNA repair template. This construct, introduced along with the guide RNA and Cas9 expression vector, is analogous to the homologous recombination constructs described above; in that, it has two flanking sequences homologous to the gene of interest and straddling the mutation. The latter is incorporated by the naturally occurring, endogenous 'homology-directed repair' system operating on the chromosome following a Cas9 cut. This sort of subtle, site-specific mutation is best accomplished with a slightly modified version of Cas9 that lacks one of its nuclease sites and thereby produces only single-strand breaks; this greatly reduces non-homologous recombination.

Conditional mutation and inducible expression—A high proportion of neurologically relevant genes cause developmental lethality when ablated. This may be related to the intersection of physiological requirements with important behaviors, such as suckling, and neurological control of other systems, as in the case of respiration. For this and other reasons, it is often desirable to restrict a genetic modification spatially (e.g. to a specific cell type) or temporally (e.g. postnatal). A variety of approaches have been developed for this purpose, applicable to either ablation ('conditional knockout') or over-expression.

Some of the earliest strategies for creating precise control of a genetic modification involved inducible transgenes. A system dependent upon the ecdysone receptor (EcR) was used rather commonly in the late 1990s and was based on supplying a nuclear hormone receptor from insects (actually, a highly engineered derivative of that EcR) along with a transgene whose promoter had a *cis* element targeted by EcR (No *et al.* 1996). Expression of the transgene could be induced by administering a synthetic ecdysone to transgenic mice (or cell cultures) carrying both elements. The other major systems used for such inducible expression are

those utilizing tetracycline-responsive trans-acting factors (Gossen and Bujard 1992; Furth *et al.* 1994; Gossen *et al.* 1995); they predated EcR systems by a couple of years and has persisted in more widespread use since. The original approach utilized a tetracycline-binding factor (tTA) that associates with its target DNA element and activates transcription until ligand (a tetracycline, most commonly, doxycycline) is supplied; accordingly, it is termed 'Tet- Off™'. Later, mutations were discovered that reversed the actions of tTA such that administration of doxycycline induces the gene in question, creating a system dubbed 'TetOn™' (Gossen *et al.* 1995). In all of these systems, the requirement for an auxillary transgene expressing EcR or tTA creates an additional level of control. The timing of expression can be dictated by administration of the chemical inducer or suppressor, and the distribution of expression can be controlled by placing the auxillary transgene under a cell type-specific promoter, such as the CaMKII promoter for expression in forebrain neurons (Engel *et al.* 2006). Spatial restrictions that are independent of cell type are sometimes effected by injection of a viral vector carrying tTA, for instance, under a universal promoter (Bahi *et al.* 2005). Of course, when the objective is the *reduction* of a specific gene product, the inducible transgene can be an RNAi construct (i.e. expressing a short-hairpin RNA) (Bahi *et al.* 2005).

While inducible expression of RNAi is one means to suppress expression of a gene of interest, it is often more desirable to completely remove the gene in a specific cell type or developmental stage. This is now accomplished most commonly via the Cre/Lox approach, pioneered by Brain Sauer (Sauer and Henderson 1988; Lakso *et al.* 1992). Like the systems for inducible transgenes (above), Cre/Lox involves transgenic modification not only of the gene of interest but also the introduction of an auxillary protein not present in wild-type eukaryotes: the Cre recombinase, in this case. This enzyme cuts and religates DNA sequences of the *loxP* motif; when two *loxP* sites are placed on either side of a particular chromosomal region (a practice that has come to be called 'floxing') Cre will cut both sites and religate, excising the intervening chromosomal region. It has been demonstrated empirically that this technique can be effective (albeit with somewhat compromised efficiency) for excising DNA stretches as long as two centimorgans (~ 4 Mb) (Zheng *et al.* 2000). Just as with EcR and rTA, tissue-specific promoters can restrict expression of Cre spatially and developmentally. In addition, constructs expressing a Cre transgene fused to an estrogen receptor ligand-binding domain (Cre^{ER}) is used to exert temporal control on the Cre activity (Metzger *et al.* 1995). The modified Cre^{ER} enzyme is only active in the presence of an estrogen analog such as tamoxifen. This can be used to overcome one oft-overlooked problem of germline transmission of the Cre: temporary activity arising in a 'cell-type-specific' promoter at unique developmental nodes. A case in point is the expression of glial fibrillary acidic protein in neural stem cells. Driving Cre from a glial fibrillary acidic protein (GFAP) promoter with the intent to restrict a gene excision to astrocytes would be folly unless the Cre^{ER} construct is used in conjunction with carefully timed tamoxifen administration.

Viral transduction

One of the earliest approaches for the manipulation of genes in intact organisms entailed infection with genetically altered viruses. Indeed, one might say that Mother Nature beat us

to it! There is essentially a continuum of gene transfer evident in the natural history. From the transient infection and expression of virally encoded genes, to retroviruses that insert their genomes into the host' s during a lysogenic phase, to retrotransposons that become stabilized and get passed vertically through reproduction, one can say that viral vectors have been shuffling genes around for eons. There are now many reputable studies providing evidence that DNA has been passed between species as disparate as reptiles and mammals for millions of years (Piskurek and Okada 2007; Thomas *et al.* 2010; Walsh *et al.* 2013). Thus, it is only natural that scientists and clinicians came to apply this strategy rationally to their métier.

Modified viruses were first used to express foreign genes in mammalian cells in 1976 (Goff and Berg 1976; Nussbaum *et al.* 1976). A key development was rendering the source virus replication deficient; propagation in cell cultures for the purposes of producing a sufficient titer is achieved with the assistance of a co-infecting 'helper virus' that supplies the missing components of the vector. Although conventional plasmid transfections are very inefficient in non-replicating cells (perhaps due to a need for the nuclear membrane breakdown that mitosis effects), viruses proved quite capable of transducing post-mitotic cells such as neurons. Several viruses with favorable traits for gene transduction also happen to have significant neurotropism. One of the first used for neuroscience studies was an attenuated herpes simplex 1. Early vectors derived from herpes simplex 1 showed early success in cultured neurons, which they infected readily and directed in the robust expression of important proteins (Geller and Breakefield 1988; Geller *et al.* 1995; Ho *et al.* 1995; Fink *et al.* 1997). But, these vectors were more problematic *in vivo,* where they suffered frequent inactivation and tended to promote a prohibitively strong immune reaction (Fink and Glorioso 1997). Adenoviruses and adeno-associated viruses (AAV) were being developed concurrently (Fritz *et al.* 1997; Robert *et al.* 1997; McFarland *et al.* 2009), and the latter seem to have surpassed these other first-generation vectors, largely by virtue of their ability to fly under the radar regarding the host immune system (McFarland *et al.* 2009).

Recombinant AAV (rAAV) vectors are derived from a naturally occurring parvovirus first isolated in 1966 in studies of pathogens in the respiratory tract (Hoggan *et al.* 1966). Regarding the initial hurdles of making viral vectors replication deficient, AAV almost seems to have been designed for this application, as even in the wild form it is not capable of replicating autonomously (hence its description as an adenovirus-associated virus). But, despite their other favorable properties for neuronal gene expression, rAAVs have a significant limitation on the size of the novel genetic material they can carry – the maximum of foreign DNA is approximately 4.4 kilobases. Lentiviruses and other similar retroviruses can solve this problem, with insertion capacities on the order of 10.5 kilobases. This larger size comes at a cost, however, the infectious particle is also large, and this limits the spread of lentiviruses in tissues somewhat. Moreover, side effects of retroviral vectors are somewhat unpredictable because of their insertion at multiple sites in the genome; destruction of a tumor-suppressor locus or transcriptional activation of an oncogene is quite common, with the potential result being an iatrogenic cancer. It had been thought that rAAV-based vectors were free from this concern, though the native wild-type AAVs are capable of insertion. But, even rAAV vectors are now known to integrate oncogenically at a rate that may be unacceptable (Valdmanis *et al.* 2012). Considerable hope is placed on the idea that

nanotechnology may help solve some of the most persistent problems of viral vectors by modifying – perhaps even replacing – viral envelop components with artificial bioactive polymers (Dodds *et al.* 1999; Shea and Houchin 2004).

Applications

Experimental implementation—Transgenesis of genes has, of course, made a dramatic impact on empirical biology. The ability to add or remove a gene, or replace it with mutated versions, has meant the world for our understanding of neurochemistry and other subdisciplines. And beyond testing the role of individual proteins, such manipulations have helped to elucidate networks through which genes impact one another in a systemic manner. Indeed, network (or 'pathway') analysis is now a major computational endeavor in expression analysis outputs such as those provided by microarrays and RNA-seq (Crawford *et al.* 2006; Boulaire *et al.* 2009; Host *et al.* 2010; Loke *et al.* 2013). Nevertheless, some of the dividends of gene shuttling were novel techniques that probably could not have been imagined, much less implemented, prior to the age of transgenesis.

One important innovation has been the use of viral vectors for transsynaptic tracing of neuronal connectivity. Horseradish peroxidase and its conjugates with lectins have been used to trace axon projections since the 1970s (Kristensson *et al.* 1971; Trojanowski *et al.* 1981). This technique was even considered as a drug-delivery method for some time (Haschke *et al.* 1980). The drug in question was an antiviral one, which is somewhat ironic considering that the biggest advance in circuitry tracing in the past decade has used viruses as the tracer. The primary advantage of viral vectors for axonal tracing is the improved capability for transsynaptic propagation. Though wheat-germ-agglutinin and tetanus- or cholera-toxin conjugates of horseradish peroxidase have limited utility as transsynaptic tracers, they dilute quickly and can rarely be used beyond single synapses. Though the strains used are somewhat attenuated, these viruses can replicate; so, they can propagate after they cross a synapse, thus maintaining signal strength. The earliest uses of viruses used more-or-less native pseudorabies virus (PRV) and visualized by immunostaining (Spencer *et al.* 1990), followed by recombinant PRV containing β-galactosidase (Loewy *et al.* 1991). But, the approach gained popularity when sensitivity and versatility were enhanced with the introduction of green-fluorescent protein and its color variants into the viruses (Maskos *et al.* 2002).

Versatility of this technique is one of its assets. Despite the power of transsynaptic tracing, it is sometimes desirable to restrict the labeling. Restriction to a single cell can be achieved by use of a replication-deficient virus (Wu *et al.* 2014). And it is even possible to engineer a virus that will stop after crossing just a single synapse (Wickersham et *al.2007*). The latter is achieved by supplying a missing replication component within only the single original cell (or cell type); this can be provided from another virus or from a specifically expressed transgene (Weible *et al.* 2010). It is also possible to include or exclude spread through other types of connections, e.g. gap junctions. Many viral tracers will spread via such non-synaptic sites, but the 'challenge-virus standard'-derived strains of rabies virus and the Bartha strain of PRV, travel only across synapses (Ekstrand *et al.2008*). In the right hands, viral transsynaptic tracing can reveal novel information about circuitry that has been missed

by other anatomical approaches. But like so many scientific methods, this technology is also prone to the effect of the observer (McCarthy *et al.* 2009).

Also benefiting from the transgenic expression of fluorescent proteins is the 'Brainbow' mouse (Livet *et al.* 2007). This technology utilizes the Cre/Lox system to produce a quasi-random distribution of neurons labeled with different colors of fluorescent proteins. The effect is produced by introducing a compound transgene that contains a coding region (CDS) for green-fluorescent protein, its red variant, its yellow variant, etc., separated by distinct and – this is key – incompatible *loxP* sites. By placing a compatible partner of each of these *loxP* sites in a small region outside the entire protein coding region, the investigators allow Cre (driven by a neuron-specific promoter) to stochastically choose any compatible pair and thereby excise any intervening fluorescent protein CDS(s) – and the other *loxP* sites – in any given neuron. The recombination event also removes a transcriptional 'Stop' signal blocking the remaining fluorescent protein(s) CDS(s). Because the other *loxP* sites have been removed, no other Cre-mediated deletions can occur, locking that cell into its fated color for the rest of its life. More than one fluorescent CDS may remain, however, creating as many as 90 distinguishable combinations!

Similar, but chromatically simpler, approaches are utilized for neuronal fate mapping. A single fluorescent protein reporter gene (mCherry is a favorite) is introduced, again, squelched by a transcriptional block (e.g. a Neo^R coding region followed by robust polyadenylation signals) that is flanked by *loxP* sites. This can be combined with a Cre construct driven by a promoter specific to a stage in differentiation, such doublecortin *(Dcx).* If the Cre is also the estrogen-dependent variety (Cre^{ER}, above), tamoxifen can be administered in a pulse-chase paradigm to time stamp a population of neurons that were neural stem cells or neuroblasts at the time of tamoxifen treatment. In addition to *Dcx*, there are promoters specific to the progenitors of specific neuron subtypes, e.g. *Nkx* and *Dlx* homeodomain genes are transiently expressed in neuroprogenitors giving rise to specific GABAergic interneurons (Fogarty *et al.* 2007; Taniguchi 2014). The utility of this sort of approach lies in the fact that a marker (e.g. mCherry) can be permanently activated after the transient activation of a developmental- or lineage-specific gene promoter, thus providing versatility that liberates the investigator from a need to find promoters that are active in throughout the life span of a specific cell type.

Light can be used not only to report a cell's location and lineage but also to control neurophysiological activity. 'Optogenetics' is the term most commonly used for technologies that manipulate a neuron's electrophysiological activity via illumination of a light-sensitive, transgenically introduced ion channel. Channelrhodopsin, for instance, is a cation channel discovered in the alga *Chlamydomonas reinhardtii.* Transgenically expressing it ectopically in specific neurons of an animal allows rather precise regulation of the cell' s firing rate; it was first used to manipulate animal behavior by Lima and Miesenböck in 2005 (Lima and Miesenbock 2005) and had gained recognition as a revolutionary technology by 2010 (Crick 1999; News Staff, 2010). Optogenetic in mammals was extended by the use (and improvement) of optical fibers to deliver light to deeper brain structures (Aravanis *et al.* 2007; Sparta *et al.* 2012; Pisanello *et al.* 2014). Most recently, the reciprocal relationship

between ‘opto-’and ‘genetics’ has taken a new twist with the advent of a photoactivatable Cas9 for use in CRISPR applications (Nihongaki et al. 2015).

An alternative to optogenetics is chemogenetics. Research questions that require long-term control or structures that may be vulnerable to the invasive procedures required for delivery of light can be accessed with pharmacology. Intersection of a toxin with transgenic expression of the enzyme that mediates the toxicity – e.g. ganciclovir/thymidine kinase or etronidazole/nitroreductase – has been used for many years to effect a time- and location-specific elimination of specific cell populations. But, more recently, investigators have been interested in manipulating the electrophysiological activity of specific neuronal populations rather than killing them. ‘Designer receptor exclusively activated by designer drugs’ and ‘receptor activated solely by a synthetic ligand’ are terms used in this application. Although optogenetic methods mostly commonly utilize an ion channel as the controllable transgene, Designer receptor exclusively activated by designer drugs/receptor activated solely by a synthetic ligand use metabotropic receptors (Redfern et al. 1999).

Translational implementation—As exciting as a new technical tool may be to the experimentalist, one cannot forget that gene therapy has always been one of the primary objectives of molecular biology.

Despite a great many promising studies in preclinical animal models, gene therapy has yet to make a major impact on human health care. And among the success stories, very few can claim inroads regarding treatment of neurological disorders. As hinted above, lysosomal storage diseases and other inborn errors of metabolism appear to represent one of our best chances for cracking into this difficult therapeutic modality. The very first attempt to apply gene therapy to a lysosomal strorage disease in humans was a viral-vector strategy directed at infantile neuronal ceroid lipofuscinosis, whose victims suffer a loss-of-function mutation of palmitoyl-protein thioesterase 1 (*PPT1*). An initial attempt to resupply palmitoyl-protein thioesterase with an AAV2 elicited positive outcomes in a few trial participants, but nearly half developed humoral immune responses that mitigated the beneficial effects (Worgall *et al.* 2008). So, when some of the same investigators joined a team designing a gene therapy trial against mucopolysaccharidosis type IIIA, they incorporated the application of immunosuppressive drugs before and after delivery of the viral gene therapy. In this case, the AAV2-mediated gene delivery seemed to be well tolerated, with only mild reactions being reported. And while the study was not designed to assess efficacy, there were promising trends toward positive outcomes (Tardieu *et al.* 2014).

A fairly recent study documented a slightly more validated success in metachromatic leukodystrophy by starting early (Biffi *et al.* 2013). Three toddlers were identified by genetics and biochemical markers to be at risk for were treated prior to clinical symptoms. Autologous stem cell transplants were conducted using hematopoietic stem cells virally transduced *ex vivo* with a functional gene for arylsulfatase A, the enzyme lacking in this disorder. After returning the stem cells to the participants, high enzyme expression was detected in many hematopoietic lineages and in cerebrospinal fluid of the recipients. At and beyond the age when the participants would have been expected to present with neurological

symptoms, none were observed. As of April of 2015, an expanded Phase I/II trial had begun with 20 participants.

The antisense RNA work mentioned above (see Post-transcriptional regulation) has been a very useful tool for exploring the roles of individual gene products in a biological system. But, at least one human disease has shown us the promise that such approaches can hold for clinical application. Spinal muscular atrophy (SMA) is a genetic disorder that arises when one copy of the 'survival (of) motor neuron 1′ gene (SMN1) suffers a deletion or other loss-of-function mutation, leaving lower motor neurons to rely on the backup, SMN2 (Cartegni and Krainer 2002). Although the specific biochemical functions of the SMNs is not entirely clear (they seem to be involved in synthesis and maintenance of small nuclear ribonucleoproteins or snRNPs), they are crucial for the viability of motor neurons. SMN2 differs from SMN1 in a single nucleotide change that reduces the efficiency of an RNA splicing modulation, resulting in far fewer mRNA molecules that contain Exon 7, which in turn generates a predominance of C-terminally truncated protein that consequently has an abbreviated half-life. In 2003, Adrian Krainer and Luca Cartegni showed that it was possible to shift the splicing of SMN2 to include Exon 7 more frequently by introducing a nucleotide-peptide chimeric molecule that would (i) hybridize near the Exon 7 splice acceptor site of SMN2 and (ii) introduce a peptide sequence that would recruit the splice enhancer machinery (Cartegni and Krainer 2003). Later, the Krainer laboratory was able to effect the same outcome with antisense RNAs that mask splicing silencer sequences in two of the SMN2 introns (Hua *et al.* 2008). Krainer and Kua teamed up with investigators at Genzyme and Ionis (nee Isis) Pharmaceuticals in 2011 to show that these antisense approaches promote survival in a preclinical mouse model of SMA (Passini *et al.* 2011). Ionis Pharmaceuticals is currently recruiting participants for Phase-3 clinical trials of similar antisense therapies. This body of work required not only a practical understanding of the SMA disease process but also a detailed understanding of the mechanisms of RNA splicing gleaned from thousands of basic science studies.

The next 60 years...

The breadth and scope of discovery in the field of molecular neurobiology in a little over half a century is as difficult to grasp as it has been to summarize here. What advances might be accomplished by 2076 can hardly be fathomed, as discoveries tend to open new vistas and reveal new questions and necessities for both technical innovation and conceptual progress. It is predictable, however, that trends we see now will continue in their arc. For instance, the speed, sensitivity, and accuracy of DNA (and RNA) sequencing seems to continue to advance, perhaps in parallel with Moore's law of computational processing. The bioinformatics boon of the past decade may find a fertile field of specialization in 'neuroinformatics' (Amari *et al.* 2002). A priority has been placed on substantially enhancing our understanding of brain connectivity by policy makers in the US and Europe (Waldrop 2012; Insel *et al.* 2013); President Obama's BRAIN Initiative has been compared to the Human Genome Project, and they may be related by more than analogy. To the extent that every aspect of biology relies on the structure and expression of genomes, these initiatives must incorporate the tools and concepts of molecular biology to gain their full

potential. Virally mediated transsynaptic tracing and chemogenetics are just a couple of examples of the assistance gene jockeys can provide to these efforts.

A great deal of hope continues to be placed in the concept of 'personalized medicine'. Implicit in this objective is the individuality in our disease predilections and drug responses conveyed by the distinctions in our genomes. And just as exome analysis of a tumor may provide tailor-made strategies for attacking it, the molecular biology behind each individual case of schizophrenia or autism may be the secret to unlocking successful treatments of *persons,* vis-àvis 'problems'. Rational drug design already seeks to fashion drugs on the basis of conformationally detailed understanding of their targets; it is reasonable to suppose that this may one day extend to the design of a specific drug for a specific case.

Regardless of where the genes of our brains and the brains of our geneticists take us in the next six decades, there is one likelihood that can be expressed with a great deal of confidence: *The Journal of Neurochemistry* will be here to celebrate 120 years of disseminating the latest and greatest progress in our field!

Acknowledgements and conflict of interest disclosure

I appreciate technical and conceptual inputs to Fig. 6 by Deborly Wade. Supported by the National Institutes of Health (R03AG043784). No competing interests to disclose.

Abbreviations used:

CaMKII	calcium/calmodulin-activated kinase II
CREB	cyclic AMP-responsive element binding
JNK	Jun N-terminal kinase
LPS	lipopolysaccharide
NFκB	nuclear factor κ light chain enhancer of activated B cells
NRSF	AKA neuron-restrictive silencer factor
PKA	cAMP-activated kinase
REST	RE1-Silencing Transcription factor

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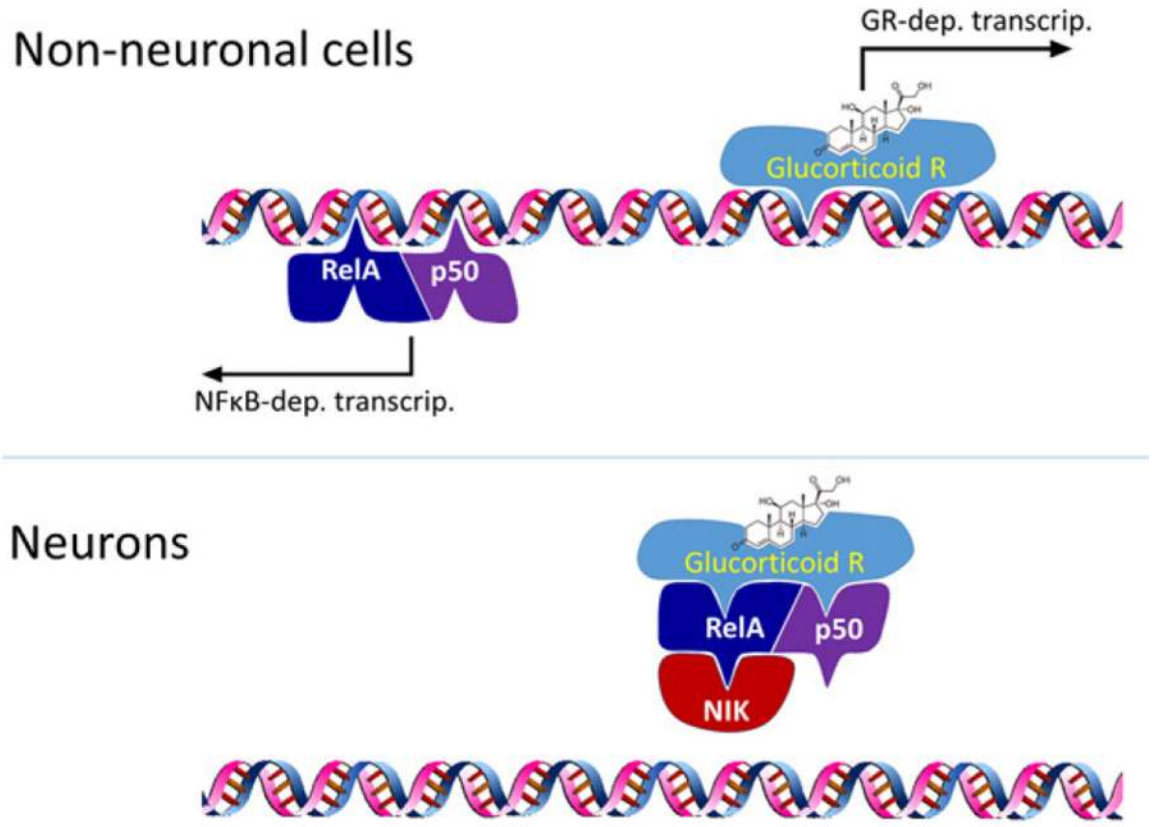

Fig. 1.

Hypothetical alternative actions of NFκB in neuronal nuclei. Though DNA binding by NFκB is scant in mature CNS neurons, it has been documented to undergo nuclear translocation in these cells, and its expression can have biological effects therein. The hypothesis is depicted that activation of the NFκB pathway in neurons may result in protein-protein interactions in the nucleus of neurons that could indirectly affect gene expression via an influence on other transcription factors such as the glucocorticoid receptors. NFκB-inducing kinase was recently found to participate in the inhibition of NFκB in neurons.

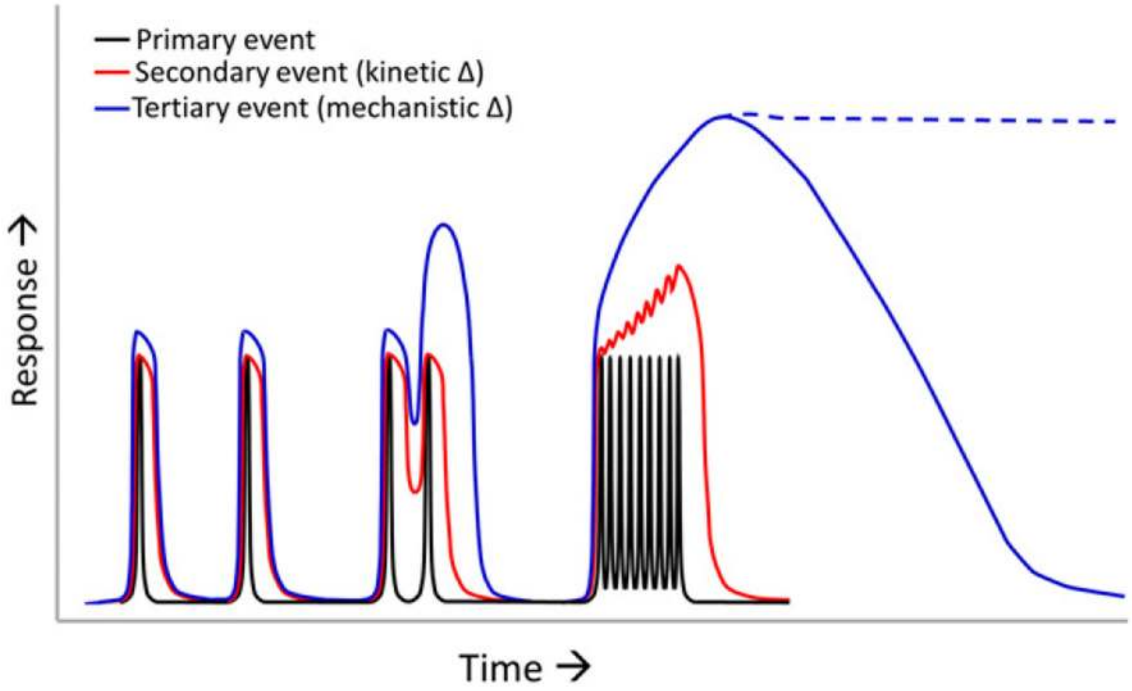

Fig. 2.

Integration of effects by progressive signal transduction. Most neuroscientists are accustomed to interpreting electrophysiological events (e.g. depolarization spikes) that summate in an additive fashion. This type of integration is often manifest in a chain of events for which the secondary response simply has longer decay kinetics, such that a subsequent primary event can arrive before the secondary event has time to dissipate. For instance, opening of a ligand-gated sodium channel can evoke conductance as a primary event; the resulting depolarization can open a calcium channel as a secondary event, which may exhibit a difference in *kinetics*, e.g. have a longer mean open time. Tertiary signaling events may differ not only in kinetics but also in *mechanism*, perhaps catalytically extrapolating the effect size; e.g. activation of a calcium-calmodulin kinase. In this way, a quantitative difference becomes a qualitative one. Similar to these ionic integrations, gene regulation events can act as molecular switches that convert a quantitative change, e.g. repeatedly reaching the activity threshold to activate CREB, to a qualitative one, e.g. a level of CREB-dependent transcription that changes histone acetylation. These can even become semipermanent changes (dashed line), e.g. gene activity levels that change DNA methylation patterns via ‘molecular momentum’ (below).

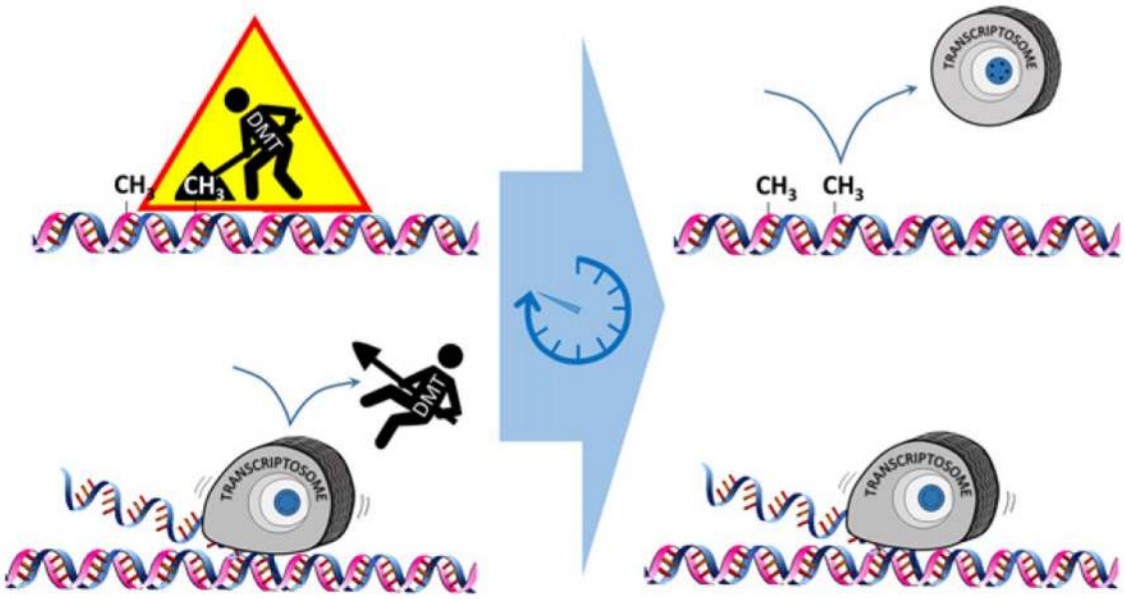

Fig. 3.

Molecular momentum. The rich get richer, and active genes tend to stay active. Perhaps, by a process as simple as steric interference by RNA polymerase and its accompanying proteins, the transcriptional process appears to inhibit DNA methyltransferases (DMT) from modifying an active gene. If this persists over the passage of time, particularly through a developmental stage that is especially plastic, the gene may remain hypomethylated into adulthood, thus adapting the individual to express higher levels of the gene. Similar events may impact other epigenetic processes, such as histone modification. The consequences may be adaptive, e.g. elevating appetite for a scarce nutrient, or maladaptive, e.g. sensitizing the inflammatory genes to aberrant activation.

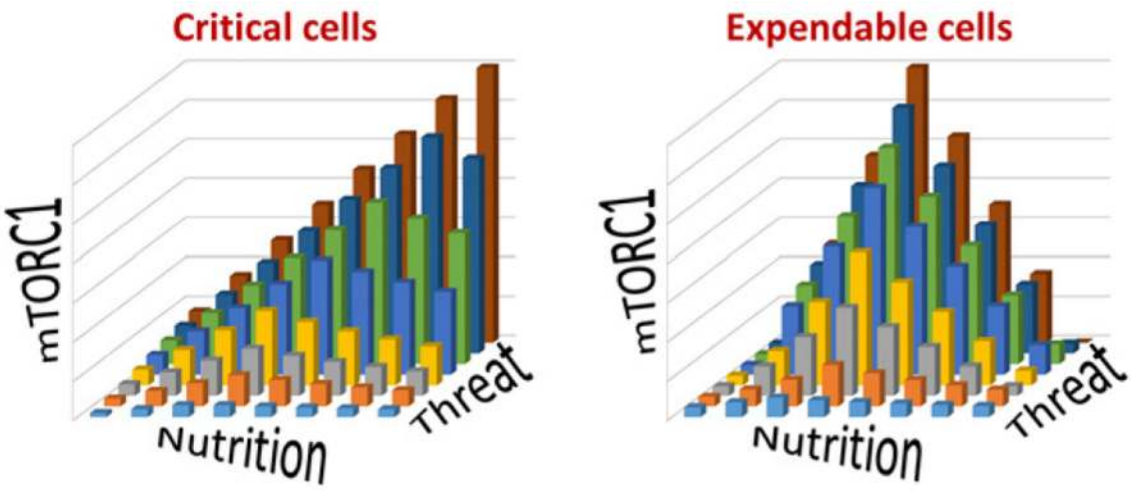

Fig. 4.

Theoretical consequences of mTORC1 activation for various cell types. On the front, (x)-axis is the availability of food, increasing left to right. On the receding (y)-axis is the challenge or threat to survival an individual cell may face, increasing front to back. On the elevation, (z)-axis is the optimal activity of mTORC1. Critical cells may be essential neurons in a simple invertebrate or, in the extreme, the entire individual in the case of a unicellular species; expendable cells are those that may be sacrificed because of a high natural renewal rate, for instance. Benefits to life span are generally seen after inhibition of mTORC1 (e.g. with rapamycin) in many species, including yeast. But, this may be limited to circumstances with little stress; life span may benefit from neuroprotection, for instance, that results from elevated protein synthesis and other consequences of mTORC1 activity in stressful situations. This is difficult to engage when nutrition is limited. Perhaps, more to the point, excessive food can lead to shortening of life span –as well as detrimental effects along the way – unless mTORC1 is inhibited. It is possible that benefits to the intact organism may arise from inhibition of mTORC1 in less critical cells, even when these expendable cells are challenged with stressors.

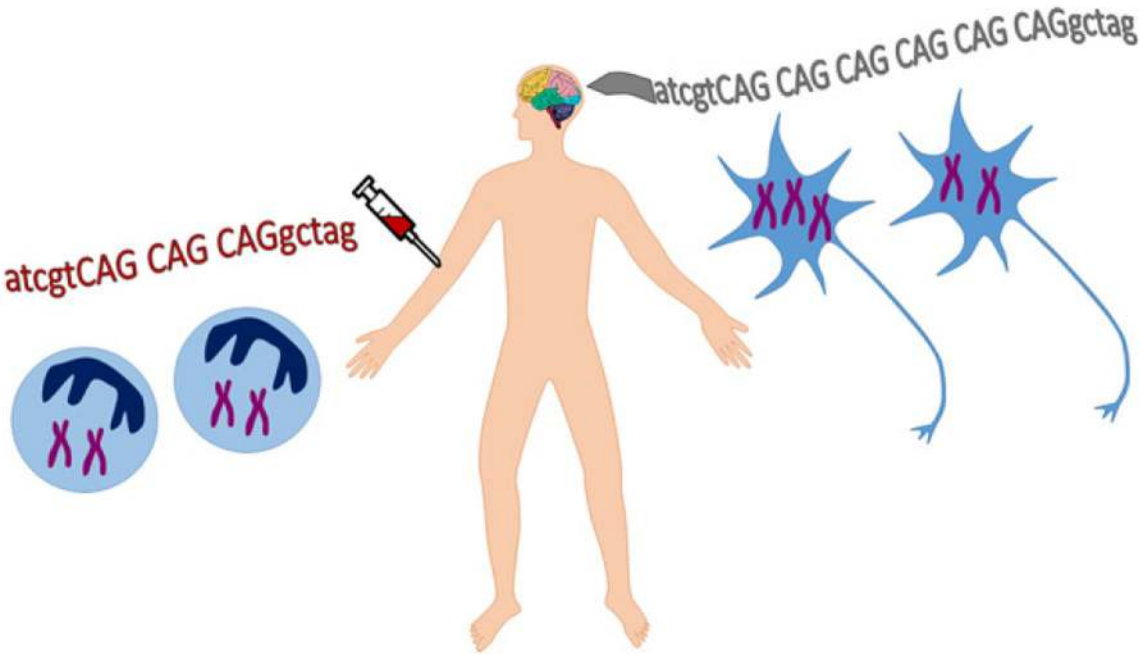

Fig. 5.

Somatic mosaicism and peripheral-central variations. Genetic variation appears to be particularly abundant in the CNS. Copy number variants, extension of tri-or hexanucleotide repeats, and aneuploidy of entire chromosomes that are limited to this compartment may cause disease or less consequential effects in the nervous system while remaining cryptic to DNA sequence analyses that are performed on blood or other peripheral tissue specimens.

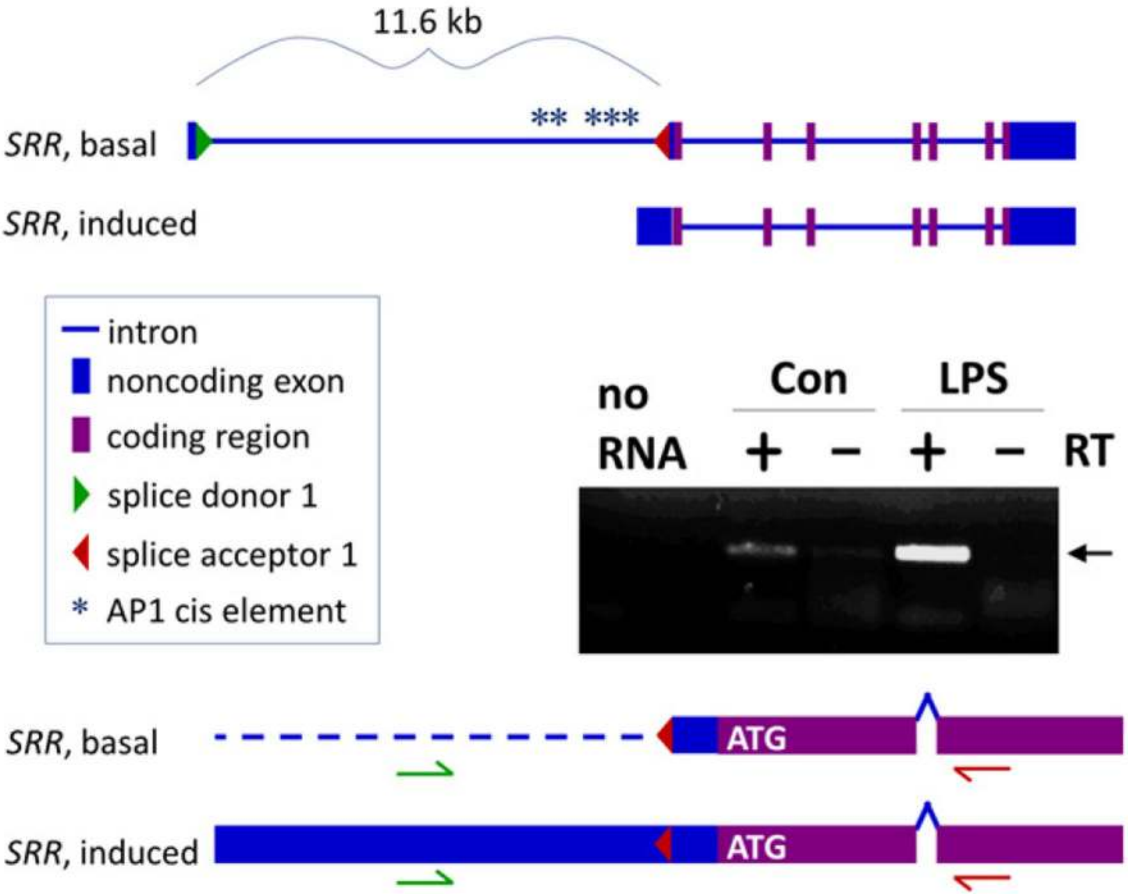

Fig. 6.

PCR demonstrates alternative transcription of the serine racemase gene. Organization of the human serine racemase (*SRR*) gene is shown; the canonical transcript produced basally, with the full-length Intron 1 is at top. LPS was previously found to induce expression in microglia via a cluster of AP-1 transcription factor binding sites found just 5′ to the translational start site, and an alternative transcript was postulated to result from a nearby alternative transcriptional start site. The potential mRNA species are diagrammed at the bottom, with the dashed region representing the canonical Intron 1 and the coding region in purple. RT-PCR reactions were run with an upstream primer (green) that should be omitted from the mature mRNA by splicing out of Intron 1; this region may be maintained, however, in the alternative transcript because of the removal of the Intron-1 splice donor. The downstream primer (red) was placed in Exon 3 to exclude hnRNA. Microglia treated with LPS were compared to controls, and the RT-PCR results indicate the presence of a product consistent with the alternative transcript, particularly elevated in LPS-treated cells.