Published in final edited form as:

Nat Rev Neurosci. ; 13(2): 94–106. doi:10.1038/nrn3154.

Activity-dependent neurotransmitter respecification

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Abstract

For many years it has been assumed that the identity of the transmitters expressed by neurons is stable and unchanging. Recent work, however, shows that electrical activity can respecify neurotransmitter expression during development and in the mature nervous system, and an understanding is emerging of the molecular mechanisms underlying activity-dependent transmitter respecification. Changes in postsynaptic neurotransmitter receptor expression accompany and match changes in transmitter specification, thus enabling synaptic transmission. The functional roles of neurotransmitter respecification are beginning to be understood and appear to involve homeostatic synaptic regulation, which in turn influences behaviour. Activation of this novel form of plasticity by sensorimotor stimuli may provide clinical benefits.

Neurotransmitters are part of the core machinery expressed by all neurons, and genetic programmes specify the default expression of neurotransmitters at very early stages of development. Although until fairly recently it was believed that neurotransmitters are fixed, compelling evidence has accumulated to indicate that neurotransmitter specification can be changed at later developmental stages by electrical activity, intercellular interactions and secreted factors via alterations in the expression of genes encoding transcription factors. Over the past decade or so, electrical activity in particular has emerged as an important, previously little-recognized way in which the early development of the nervous system is regulated and adapts to changes in the environment.

It has been known for some time that neurotransmitters such as GABA and glutamate are expressed and released at early stages of development. They can act as paracrine factors and can exert morphogenic actions, regulating neuronal proliferation and migration. Their role in embryonic neuronal differentiation has been less clear, but recent findings, particularly from studies of embryonic *Xenopus laevis*, suggest that activity-dependent respecification from the embryonic to the mature pattern of neurotransmitter expression — which begins when neuronal differentiation starts — is also dependent on the paracrine action of

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Competing interests statement

The author declares no competing financial interests.

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neurotransmitters (FIG. 1). Prior to synapse formation, intrinsic calcium-dependent electrical activity driven by such local factors within the embryonic nervous system is important. After synapses have been established, extrinsic signals that change this electrical activity become decisive. Importantly, neurotransmitter respecification is a way in which the composition and function of the developing nervous system are modified in response to both the internal and external milieu. It seems to have a dual role, both in context-dependent maturation of the nervous system and maintenance of homeostasis in developing and mature circuits.

Activity-dependent neurotransmitter respecification can involve either inducing or eliminating expression of excitatory or inhibitory transmitters or switching between excitatory and inhibitory transmitters. The capacity for multidirectional change enables neurotransmitter respecification to exert a homeostatic effect on levels of activity within neuronal circuits. Avoiding excessive electrical activity and maintaining excitability within a particular range of operation seem to be essential for correct neuronal development and mature function. Superabundance of excitation or inhibition, not balanced by inhibitory or excitatory forces, could render the nervous system unresponsive to further stimulation. This leads to the prediction that neurons in circuits that receive sustained high levels of activity may undergo activity-dependent neurotransmitter respecification as a way of maintaining responsiveness.

It is not yet clear precisely which neurons undergo neurotransmitter respecification, but it seems that not all neurons exhibit these changes. For example, because it is essential to retain reliable sensory input and motor output, changes in the transmitters of sensory and motor neurons seem to be unlikely. The attention of most research in this area has focused on interneurons. As might be expected from corrections in the balance of excitation and inhibition, when this occurs after synapse formation there is a corresponding correction or homeostasis of behaviour.

Many of the key advances in our understanding of activity-dependent neurotransmitter respecification have been made possible because of the accessibility of the developing *X. laevis* nervous system and the use of cultured developing spinal neurons in studies investigating the role of calcium-dependent electrical activity. In this amphibian, as in the fly, fish, chick and mouse, embryonic action potentials express a substantial inward calcium current, the function of which was elusive. It is now known that these action potentials are first driven by paracrine signals and are later generated synaptically during normal development; clues to their function have been provided by experimental perturbations that suppress or enhance this activity.

In this Review, I first discuss the evidence for activity-dependent transmitter respecification in the immature nervous system and the molecular mechanisms by which it is achieved. I next examine the cases in which transmitter receptor expression has been shown to change and match the change in transmitter specification and describe changes in behaviour driven by activity-dependent transmitter respecification during development. I then consider the evidence for activity-dependent changes in transmitter specification in the mature nervous

system and, finally, I explore potential clinical implications of activity-dependent transmitter respecification.

Activity-dependent transmitter respecification

Early studies

The embryonic nervous system of *X. laevis* has been particularly useful for investigation of activity-dependent transmitter respecification, because before the formation of synaptic connections, the emergence of electrical excitability and the appearance of GABA expression develop at similar rates in spinal neurons both in culture and *in vivo*^{1–5} (BOX 1). *In vivo*, these neurons begin generating calcium-dependent action potentials at the time of closure of the neural tube, triggered by paracrine signals^{1,3,6}. Early experiments using cultured neurons first characterized the patterns of spontaneous calcium spikes, and their manipulation was discovered to result in changes in the numbers of neurons expressing GABA. The developmental appearance (in both protein and transcript form) of the GABA-synthesizing enzyme 67 kDa glutamic acid decarboxylase (GAD67; also known as GAD1) was later found to parallel the appearance of GABA expression. Increasing calcium spike frequency led to increases in the percentage of GABAergic neurons and increasing numbers of GAD67 transcripts^{7,8}. Crucially, these early findings suggested that a frequency code drives GABA respecification in these neurons, and thus motivated the analysis of neurotransmitter respecification *in vivo*.

Box 1

Development of electrical excitability

Neurons become excitable at early stages of embryonic development, often before synapse formation, which suggests roles for action potentials before signalling in circuits. In many cases the action potential is initially long in duration and involves a substantial inward calcium current; repolarizing outward potassium currents are small. These impulses become briefer in duration as outward potassium currents increase later on in development, and inward sodium currents become more prominent as well^{1,94–99}. During adult neurogenesis, the differentiation of electrical excitability is similar to that during embryonic development¹⁰⁰.

Importantly, embryonic calcium influx can trigger calcium-induced calcium release and enable the generation of spontaneous, endogenous, brief elevations of intracellular calcium before synapse formation; these calcium spikes are driven by paracrine signals and can be imaged with fluorescent indicators of the cytoplasmic calcium concentration. When calcium spikes occur in single neurons and are not propagated, they are candidates for the regulation of unique neuronal phenotypes. The persistence of voltage-gated calcium channels in mature neurons makes it likely that the roles of calcium spikes in transmitter specification are not restricted to the developing nervous system.

Recognition of the calcium dependence of early forms of electrical excitability in *Xenopus laevis* embryos prompted investigation of the effects of blocking calcium elevation on neuronal differentiation^{2,7,101–105}. It was found that before synapse formation, the frequency of calcium spikes regulates the numbers of neurons expressing

different transmitters both *in vitro* and *in vivo*^{7,9,13}, and it has since been discovered that sensory stimulation regulates calcium-dependent transmitter specification *in vivo*¹⁴.

Neurotransmitter respecification before synapse formation and its role in homeostasis

As noted above, plasticity of neurotransmitter identity could endow the nervous system with a mechanism to maintain the essential balance of excitation and inhibition, although the possibility has not received much consideration by the research community. To determine the role of spontaneous calcium spiking and frequency coding in transmitter respecification, activity was monitored in different classes of neurons in the embryonic *X. laevis* neural tube during an early period of development starting before synapse formation. Spike activity was then suppressed or enhanced either pharmacologically or by misexpressing inward rectifier potassium channels (Kir channels) or voltage-gated sodium channels (Nav channels) to determine the role of such activity in transmitter respecification. These experiments were designed to mimic the alteration of calcium spiking that could be caused by natural variation in expression of paracrine factors. During the period before synapse formation, suppression of calcium spiking led to increases in the numbers of neurons expressing the excitatory transmitters glutamate and acetylcholine (ACh) and decreases in the numbers of neurons expressing the inhibitory transmitters GABA and glycine. Enhancing calcium spike incidence and frequency produced the opposite result⁹. Transmitter switching (such as the replacement of an excitatory transmitter by an inhibitory one) and transmitter superposition (such as the addition of an inhibitory transmitter to an excitatory one) could be induced (FIG. 2a). Furthermore, when developmentally equivalent neurons were examined in culture to further evaluate frequency coding, similar results were obtained: the number of neurons expressing excitatory transmitters was inversely related to the frequency of imposed calcium spikes. Transmitters expressed following alterations in calcium spike activity were functionally released, indicating a potential functional relevance. These results suggested that neurotransmitter respecification before synapse formation is a homeostatic process in which alteration of excitability leads to a compensatory alteration in the expression of neurotransmitters, as if to maintain levels of excitability around a set point. This process is similar in concept to homeostatic resetting of synaptic strengths^{10,11} and electrical excitability¹², which provide negative feedback to balance the positive feedback of Hebbian plasticity. Just as synaptic strengthening and weakening can be generated both pre- and postsynaptically (for example, in long-term potentiation (LTP) and long-term depression (LTD)), homeostatic plasticity can take place pre- as well as postsynaptically (with transmitter respecification and synaptic scaling)^{9–11}.

A homeostatic role for neurotransmitter respecification was also found in the *X. laevis* hindbrain, where calcium spiking shapes expression of the neuromodulator 5-hydroxytryptamine (5-HT; also known as serotonin). Manipulating this spontaneous activity by ion channel misexpression led to changes in the number of serotonergic neurons in the raphe¹³. Suppression of activity generated an increase in the number of neurons expressing tryptophan hydroxylase (TPH), the synthetic enzyme for 5-HT, as well as 5-HT itself; enhancement of activity produced the opposite result. These changes in the number of serotonergic neurons could not be accounted for by differences in neuronal proliferation or

by changes in the large-scale patterning of the nervous system. Serotonergic innervation of motor neurons is excitatory, and therefore the recruitment of serotonergic neurons by reduction of calcium spike activity is homeostatic.

These results were interesting for several reasons. First, the demonstration of activity-dependent neurotransmitter respecification *in vivo* meant that the process could no longer be dismissed as a curious artefact of cell culture. Second, electrical activity was shown — somewhat surprisingly — to have a role in neurotransmitter specification at a stage when differentiation was thought to be driven by genetic programmes. Moreover, neurotransmitter respecification seemed to promote homeostatic equilibrium of excitability by changing which neurotransmitters neurons synthesized and released. This suggests that this equilibrium is important for normal development of the nervous system, which has the important implication that perturbations of embryonic activity with drugs could alter the composition and function of the nervous system by disturbing crucial homeostatic regulation of excitability.

Neurotransmitter respecification after synapse formation and its role in behavioural adaptation

Activity-dependent transmitter respecification also occurs *in vivo* later in development, after synapse — and circuit — formation, and can be driven by natural sensory stimuli. Dopaminergic neurons in the ventral suprachiasmatic nucleus (vSCN) of the hypothalamus of *X. laevis* larvae regulate skin pigmentation that provides camouflage for the animals. The activation of retinal ganglion cells by light causes neurons in the vSCN to release dopamine, which activates D2 receptors on melanotrope cells and inhibits the release of melanocyte-stimulating hormone; in turn, melanocytes reduce their display of melanin and embryos appear pale. The reverse takes place in the dark. Ion channel misexpression led to sustained increases or decreases in calcium spike activity and changes in expression of tyrosine hydroxylase immunoreactivity in many regions throughout the CNS^{14,15}. By contrast, illumination of dark-exposed animals for a period as brief as 2 hours stimulated expression of tyrosine hydroxylase and dopamine only in the activated circuit — in neuropeptide Y (NPY)-immunoreactive and GABA-immunoreactive neurons that are located in an annulus surrounding the core neurons of the vSCN that already express tyrosine hydroxylase¹⁴ (FIG. 2b). Because dopaminergic innervation of melanotrope cells is inhibitory, recruitment of dopaminergic neurons in response to increased excitation of retinal ganglion cells by light is homeostatic. This light-induced transmitter respecification was blocked by local application of tetrodotoxin or introduction of the calcium chelator BAPTA (which suggests that it depends on sodium- and calcium-dependent electrical activity), and the process was reversible when larvae were subsequently kept in the dark. The results indicate that sensory stimulation induces transmitter respecification in this model — that is, the expression of dopamine by NPY-immunoreactive neurons. Importantly, the findings reveal that neurotransmitter respecification is a key mechanism by which *X. laevis* embryos adapt to their environment to enhance survival. There have been only a few studies of this process in the developing mammalian nervous system, including a demonstration that blockade of sensory input to the neonatal rat olfactory bulb reduces the number of juxtglomerular neurons expressing tyrosine hydroxylase without affecting their expression of GABA¹⁶.

These results suggest a selective activity-dependent loss of dopamine while GABA expression is spared.

Taken together, these findings demonstrate that activity-dependent transmitter respecification in *X. laevis* is a robust process, occurring both before and after synapse formation and for a range of different neurotransmitters (BOX 2). The change in expression of neurotransmitters induced by changes in sensory stimulation at stages after synapse formation has led to the concept of ‘reserve pool neurons’ — neurons that already express one or more transmitters and are wired into circuits, but can be recruited to express a different transmitter or lose a transmitter that is already expressed in response to changes in their activity¹⁷. They share afferent and efferent innervation with what can be considered ‘core neurons’ but, in the absence of changes in activity, they express different transmitters and have different functions. They seem to be ‘in reserve’, ready and waiting to be induced to adjust their complement of transmitters and rewire existing circuitry through novel neurotransmission. If they are the second- or third-order neurons in the activated circuit, the impact of the change may be linked to a particular sensory input. Alternatively, if respecification occurs in higher-order neurons that have received many other inputs, the change would be likely to reflect the summation of multiple contributions.

Box 2

Looking back: how was it missed?

With hindsight, one may wonder how activity-dependent neurotransmitter respecification escaped detection for so long. At the time of the discovery of chemical synaptic transmission and the award of the Nobel Prize to Otto Loewi and Sir Henry Dale, particular neurotransmitters were linked to particular classes of neurons on the basis of pharmacological evidence. As a result, it became natural to think of transmitter phenotype as a fixed component of neuronal identity. This view was later reinforced by the demonstration that transmitter phenotype could be altered by knocking out or misexpressing transcription factors that regulate the expression of genes that are essential for neurotransmitter expression. Genetically determined features were thought to be constant, and these findings led to the implicit conclusion that activity played little or no part in determination of this phenotype. The dogma that neurotransmitter identity is fixed and immutable may also have made investigation of its plasticity seem irrelevant.

However, several observations could have triggered an earlier appreciation of this process. The demonstration of calcium-dependent switching from cholinergic to adrenergic phenotype in cultured superior cervical ganglion neurons¹⁰⁶ was not followed up, perhaps because endogenous calcium elevations had not yet been described and rapid and simple immunocytochemical assays for transmitter synthetic enzymes were not yet available. The discovery that more than 80% of motor neurons in nerve-muscle cultures from *Xenopus laevis* embryos were cholinergic^{102,107} — far higher than *in vivo*¹⁰⁸ — could have sounded an alarm. Given the absence of neurogenesis or prominent cell death *in vitro*, this mismatch could have triggered further investigation. We now understand

that the constancy of transmitter phenotype can reflect the constancy of the electrical activity of the neurons in which it is evaluated.

Regulation of development by cascades of gene expression is accompanied by expression of developmentally transient phenotypes, but they have been considered to be peripheral to regulation of sequences of development. Changes in transmitter specification driven by calcium-dependent electrical activity either before or following synapse formation, in conjunction with the regulation of other aspects of development by the expression of other phenotypes (such as the regulation of neuronal migration and proliferation by paracrine action of neurotransmitters), has led to a greater appreciation of the role of 'phenotypic checkpoints' in neuronal development¹⁸. The effect of blockading the expression of phenotypic checkpoints, including developmentally intermediate phenotypes such as calcium-dependent electrical activity, indicates that these checkpoints are necessary for normal differentiation.

Neurotransmitter co-expression has been recognized for more than three decades^{19–22}, and many neurons have now been found to express more than one neurotransmitter²³. Their functional role was demonstrated clearly for mammalian spinal motor neurons, each axon of which provides exclusively cholinergic innervation of neuromuscular junctions, while each collateral axon provides combined glutamatergic and cholinergic innervation of Renshaw interneurons^{24,25}. Such dual transmitter expression at axon terminals has been suggested to occur at other CNS synapses^{26–29}. What is the relationship of transmitter co-expression to transmitter switching? One possibility is that transmitter co-expression is a normal part of compartmentalized neuronal physiology. Conversely, transmitter co-expression may reflect a midpoint in transition from expression of one transmitter to another or result from a collapse of the process that switches from one transmitter to another. Analysis of the molecular mechanisms by which co-expression and switching are established should clarify the nature of the connection between them.

Molecular mechanisms for respecification

As described above, neurotransmitters can be respecified both before and after synapse formation, and molecular mechanisms that regulate this process have now been elucidated. Voltage-gated calcium channels are expressed (BOX 1) and seem to be crucial. Various factors trigger their activation to generate different patterns of calcium-dependent activity that are then transduced into biochemical signals. These signals in turn engage expression of transcription factors that alter the expression of genes encoding transmitter synthetic enzymes and transmitter transporters to drive transmitter respecification. Prior to synapse formation, neurons seem to be susceptible to these changes only during a brief sensitive period of development.

Molecular triggers of calcium signalling

Observations of spontaneous activity in neurons in the developing nervous system have prompted investigations of the mechanisms by which it is initiated. GABA and glutamate are ectopically expressed promiscuously in the embryonic *X. laevis* nervous system before

synapse formation (including by neurons that do not express them at later stages) and regulate calcium spiking in spinal neurons through paracrine action⁶. Blocking GABAergic or glutamatergic signalling via metabotropic receptors and protein kinase A (PKA) and PKC reduces the incidence of calcium spiking and results in homeostatic neurotransmitter respecification, favouring an increase in excitability as the number of glutamatergic and cholinergic neurons increases and the number of GABAergic and glycinergic neurons decreases.

Regulation of sodium pump activity also plays a part in regulating calcium spiking in embryonic *X. laevis* neurons: during neurulation, upregulation of the $\beta 3$ subunit increases pump activity, which is necessary for calcium spiking to appear³⁰. The emergence of calcium spiking is likely to be due to hyperpolarization of the membrane potential, because a reduction in calcium spiking observed when expression of the $\beta 3$ subunit is suppressed is mimicked by sustained depolarization with KCl. Decreased sodium pump expression and reduced spiking reduces expression of *Orail*, which encodes the pore-forming subunit of a store-operated calcium channel that is involved in the generation of intracellular calcium stores. Given that the amplitude of calcium spikes depends on calcium release from stores, calcium spiking seems to autoregulate itself in a similar way to the self-regulation of calcium spiking in the embryonic mouse cortex³¹. Whether paracrine action of neurotransmitters and regulation of pump activity act in series or in different neurons remains to be investigated.

However, the pattern of calcium spiking varies along the dorsoventral axis of the embryonic spinal cord, and investigations of the underlying mechanisms have suggested a role for Sonic hedgehog (SHH). It is well known that a ventral-to-dorsal gradient of SHH specifies the pattern of motor neurons and interneurons^{32–34}. More recently, SHH has been found to generate a similar gradient of calcium spiking in the embryonic *X. laevis* spinal cord, with greater incidence and frequency on the ventral surface than on the dorsal surface³⁵. Suppression and enhancement of SHH activity and calcium spiking alter the expression of GABA homeostatically. The effect of SHH on calcium spiking in embryonic *X. laevis* spinal neurons is exerted through its co-receptor Smoothened, G protein activation of phospholipase C and generation of inositol triphosphate (IP₃). The activation of IP₃-operated calcium stores and activation of transient receptor potential channel 1 (TRPC1) and voltage-gated calcium channels leads to increases in calcium spike activity. Thus, signalling by the morphogen SHH is an important trigger of calcium signalling that drives neurotransmitter respecification.

Signal transduction downstream of calcium elevations

After calcium signalling has been triggered, it must be transduced, and it is typically amplified to implement changes in gene expression or post-translational protein modification. There have been numerous studies of the transduction mechanisms by which different patterns of electrical activity are converted into biochemical signals that drive various aspects of plasticity in the nervous system. However, most of them have focused on briefer signals that are generated at higher frequencies than those of the calcium spikes

under discussion here^{36–38}, and their relevance to activity-dependent neurotransmitter respecification remains to be determined.

Analysis of calcium-dependent regulation of tyrosine hydroxylase expression in cultured rat sensory ganglion neurons demonstrated that the signal transduction pathway depends on the form of calcium entry. The effect of depolarization with KCl and activation of L-type calcium channels was prevented by blockade of the mitogen-activated protein kinase (MAPK) pathway but was unaffected by blocking PKA or PKC, whereas the effect of patterned stimulation at 5 Hz and activation of N-type calcium channels was suppressed by blocking PKA or PKC but not by blocking the MAPK pathway^{39,40}. These results suggest a convergence of the molecular mechanisms activated by chronic and phasic stimulation to cause an increase in the number of dopamine neurons.

The signal transduction pathways for neurotransmitter respecification in other types of neurons are different, however. In contrast to the above example, the use of pharmacological inhibitors demonstrated that both PKA and PKC, as well as the extracellular signal-regulated kinases MEK and ERK, were required for the increase in the proportion of embryonic rat intestine tyrosine hydroxylase-immunoreactive neurons induced by activation of L-type calcium channels⁴¹. In cultured rat hypothalamic neurons, components of the signal transduction cascade underlying the switch from glutamatergic to cholinergic status following blockade of NMDA glutamate receptors or L-type voltage-gated calcium channels have been identified as calcium/calmodulin-dependent protein kinase type II (CaMKII), CaMKIV, PKC, PKA, MAPK, cyclic AMP-responsive element-binding protein (CREB) and nuclear factor- κ B (NF- κ B)^{42–44}. It will be interesting to establish the epistatic relationships of these components. At this point it seems likely that there is no consensus calcium-dependent signalling pathway that is used by all neurons, potentially reflecting the absence of a consensus pattern of calcium signals that engage neurotransmitter respecification.

Regulation of transcription

Induction of neurotransmitter respecification by signal transduction pathways activated by calcium spiking requires changes in gene expression. Transcriptional regulation of neurotransmitter specification has been investigated extensively in the worm, fly, chick and mouse. These studies have shown that mutation of genes encoding different transcription factors can lead to either loss or gain of expression of particular neurotransmitters^{45,46}. Similarly, ectopic expression of diverse transcription factors can lead to either gain or loss of transmitter expression^{47,48}. It is clear that transcriptional regulation is central to neurotransmitter specification and that the regulatory pathways vary widely.

The interaction of calcium signalling and gene expression has been investigated in cultured rat sensory petrosal ganglion neurons, where the timing of expression of paired mesoderm homeobox protein 2A (PHOX2A) and PHOX2B transcription factors is tightly linked to the ability of depolarization to increase the number of tyrosine hydroxylase-expressing neurons⁴⁹, suggesting a crucial period of susceptibility. Stimulation of activity in these neurons caused similar increases in the numbers of neurons expressing the transcription factors and tyrosine hydroxylase — and these proteins were expressed at much higher levels than occur naturally *in vivo*. These results suggest the existence of a reserve pool of neurons

that are available for activity-dependent tyrosine hydroxylase expression. Physiological stimulation by hypoxia *in vivo* (which mimics challenges during embryogenesis) also induced tyrosine hydroxylase expression solely in cells expressing PHOX2A and PHOX2B. The results demonstrate that in sensory petrosal ganglion neurons, the expression of specific transcription factors creates a window of plasticity that enables the regulation of the number of dopaminergic neurons by calcium signalling.

Activity-dependent homeostatic switching between glutamate and GABA transmitters in the embryonic *X. laevis* spinal cord before synapse formation is regulated by the calcium spike-dependent state of phosphorylation of the c-Jun transcription factor. In chick and mouse dorsal spinal cord, the T-cell leukaemia homeobox 3 (*TLX3*) and ladybird homeobox 1 (*LBX1*) genes act as molecular switches; misexpression of *TLX3* leads to an increase in the number of glutamatergic neurons and a reduction in the number of GABAergic neurons. Furthermore, mutation of *TLX3* leads to a reduction in the number of glutamatergic neurons and an increase in the number of GABAergic neurons^{50,51}. *tlx3* also functions as a switch in the embryonic *X. laevis* spinal cord, and simultaneous manipulations of *tlx3* expression and calcium spike activity demonstrate that expression of this gene is activity-dependent⁵². The effect of activity is mediated by a variant cAMP response element (CRE) in the *tlx3* promoter to which c-Jun binds. Knockdown and overexpression of c-Jun regulate the number of glutamatergic and GABAergic neurons. Calcium spiking alters the phosphorylation status of c-Jun, and mutating specific residues of c-Jun prevents the effects of calcium spike activity on glutamate and GABA specification (FIG. 3a). These results demonstrate a mechanism by which calcium spike activity and gene expression interact to drive neuronal differentiation.

Activity-dependent specification of 5-HT in neurons of the raphe of *X. laevis* embryos is achieved by regulation of expression of LIM homeobox transcription factor 1 β (*lmx1b*)¹³. Suppression and enhancement of calcium spiking regulate the numbers of neurons expressing *lmx1b* and TPH; the expression of *nkx2.2*, a transcription factor upstream of *lmx1b*, is not affected. Directly manipulating *lmx1b* expression alters the number of TPH-immunoreactive neurons (FIG. 3b). These experiments reveal another link between endogenous electrical activity and an intrinsic genetic pathway.

Neurons in different dopaminergic brain nuclei of *X. laevis* larvae respond to different extents to alterations in calcium spiking achieved by misexpression of Kir or Nav channels¹⁵. In the vSCN, the state of development of different subpopulations of neurons — as identified by position, transmitter expression and transcription factor expression — correlates with different patterns of spontaneous activity. These subpopulations also respond differently to perturbations of activity, and dopamine expression seems to be induced most easily in spontaneously active neurons expressing GABA and the *nurr1* (also known as *nr4a2*), *lim1* (also known as *lhx1*) or *lim2* transcription factors. In response to stimulation by light, the recruitment of newly dopaminergic neurons from a reserve pool of NPY neurons in the annular region of the vSCN is associated with increases in their expression of tyrosine hydroxylase, the dopamine transporter, the vesicular monoamine transporter and dopamine immunoreactivity. Moreover, tyrosine hydroxylase transcripts are upregulated at the same time¹⁴, which is consistent with the expression of a cassette of genes devoted to

dopaminergic function⁵³. Decreasing light exposure reduces the expression of tyrosine hydroxylase transcripts in neurons both in the core and the annulus of the vSCN. It will be interesting to determine the effect of these sensory stimuli on the expression or activity of transcription factors upstream of tyrosine hydroxylase, both in reserve pool neurons and in neurons already expressing it. Identification of the points of convergence of electrical activity with genetic programmes may provide opportunities for molecular interventions to drive changes in neurotransmitter specification that have therapeutic benefits.

Activity-dependent transmitter–receptor matching

Information transfer at chemical synapses requires the release of transmitter molecules from the presynaptic nerve terminal and binding to their cognate receptors on postsynaptic cells. Activity-dependent changes in transmitter specification would be physiologically irrelevant without corresponding changes in postsynaptic receptor expression, because mismatches between transmitters and receptors would block synaptic transmission. Intriguingly, however, corresponding changes in receptor expression that enable synaptic function have been revealed by studies of the identity of transmitter receptor populations following changes in transmitters, suggesting some form of pre- to postsynaptic communication.

The vertebrate neuromuscular junction is the classical cholinergic synapse at which ACh is released and binds to ACh receptors, leading to the depolarization of muscle cells. Strikingly, embryonic trunk muscle cells of *X. laevis* larvae express receptors for ACh, glutamate, GABA and glycine before innervation by motor neurons⁵⁴, and nicotinic ACh receptors, AMPA receptors, NMDA receptors, GABA_A receptors and glycine receptors can be detected in these muscle cells in both transcript and protein form. Although counterintuitive, this multiplicity of neurotransmitter receptor expression may have arisen evolutionarily as a simple way to ensure transmitter–receptor matching. Glutamate receptors become stabilized at many invertebrate neuromuscular junctions where glutamate is the transmitter, and ACh receptors become stabilized at vertebrate junctions that use ACh.

In embryonic spinal neurons, altering neuronal calcium spike activity to change the neurotransmitters expressed in spinal neurons⁹ changes the expression of the corresponding receptor proteins in muscle cells⁵⁴. An increase in calcium spike frequency that induces an increase in numbers of GABAergic and glycinergic neurons leads to a matching upregulation of GABA_A and glycine receptors. Conversely, suppressing calcium spiking stimulates upregulation of ACh-, AMPA- and NMDA-class glutamate receptors associated with the increase in numbers of neurons expressing these transmitters. Patch clamp recordings from muscle cells in these larvae reveal the presence of miniature postsynaptic currents generated by glutamate, GABA and glycine, as well as ACh (FIG. 4a). Thus, activity-dependent changes in transmitter expression are presumed to lead to stabilization, clustering and enhanced synthesis of the appropriate transmitter receptors. Activity ensures formation of functional synapses, and the level of activity determines the class of synapse. This process seems to involve the action of diffusible factors that may include the transmitters as well as other components, such as trophic factors^{54–57}. The results are consistent with the demonstration of glutamatergic synapses by classically cholinergic neonatal mouse motor neurons^{24,25}.

Activity-dependent recruitment of dopaminergic neurons in the annular region of the vSCN of the larval *X. laevis* hypothalamus (as described above) provides another case of transmitter–receptor matching. These neurons initially express NPY and GABA but not dopamine, and no D2 dopamine receptors are detected beneath their nerve terminals on the melanotrope cells. However, following induction of tyrosine hydroxylase and dopamine expression in these neurons by exposure of larvae to light, D2 receptor immunoreactivity is detected on the melanotrope cells that they innervate¹⁴ (FIG. 4b). The mechanism by which D2 receptors are upregulated is unknown, but it may involve signalling via a low density of receptors that are not detectable immunocytochemically. It is reassuring to see that a physiologically driven change in neurotransmitter specification is accompanied by a corresponding change in specification of the cognate transmitter receptors.

It has been known for some time that transmitter receptor expression can be modulated by electrical activity^{58–60}. The more recent demonstrations of activity-dependent transmitter–receptor matching described above raise the fascinating possibility that these changes in receptor expression are also the consequence of transmitter respecification. The increase in numbers of cholinergic cultured hypothalamic neurons induced by NMDA receptor blockade is prevented by the blockade of ACh receptors, suggesting a requirement for ACh receptor function for cholinergic upregulation and retrograde regulation of activity-dependent transmitter specification⁴⁴. More work is needed to determine the mechanism of anterograde and retrograde transynaptic signalling in the specification of neurotransmitters and their receptors.

Regulation of behaviour by respecification

Most animal behaviours have multigenic determinants: changes in the expression of single genes typically have little detectable effect on the phenotype, although there are a number of notable exceptions. Therefore, it has been interesting to discover that neurons expressing a novel neurotransmitter can drive appropriate behaviours at early stages of development.

Camouflage behaviour, as achieved by changes in epithelial pigmentation, is a survival asset for young amphibia during their vulnerable larval stage. The retinohypothalamic pathway regulates skin pigmentation via the dopaminergic neurons of the vSCN of larval *X. laevis*, as described above. The recruitment of additional dopaminergic neurons enhances the light sensitivity of changes in pigmentation, and larvae ‘white-adapt’ (adapt to increases in illumination) more rapidly and to a greater extent, suggesting that induction of dopamine expression confers a behavioural advantage¹⁴. Elimination of dopaminergic neurons in the SCN abolishes changes in pigmentation in response to changes in ambient illumination. When these animals are exposed to illumination for several hours, new dopamine expression is induced in neurons previously expressing NPY and GABA. These neurons are able to drive changes in pigmentation that are blocked by sulpiride, the D2 dopamine receptor blocker, confirming the role of dopaminergic signalling by the neurons respecified to express dopamine in the retinohypothalamic pathway that regulates camouflage (FIG. 5a).

Swimming behaviour is also essential for amphibian larvae to escape predation. Serotonergic neurons in the raphe innervate motor neurons and modulate swimming

activity⁶¹. Their effect on this behaviour has been analysed in several ways. Electrophysiological recording of impulse activity from ventral roots allows the identification of bursts of activity that are correlated with swimming episodes, referred to as fictive swimming. The duration of swimming episodes is shortened by 5-HT and lengthened by blockade of 5-HT_{1A} receptors. Misexpression of ion channels results in changes in the number of TPH-immunoreactive serotonergic neurons, which in turn alters the duration of swimming episodes: larger numbers of serotonergic neurons yield shorter durations, and smaller numbers give rise to longer durations¹³. Because the effects of ion channel expression were widespread throughout the embryo, a morpholino (antisense construct) to the *lmx1b* transcription factor (which has been shown to be necessary for TPH and 5-HT expression) was focally electroporated into the presumptive raphe. This intervention reduced the number of serotonergic neurons, increased the duration of swimming episodes in recordings of fictive swimming from anesthetized animals and increased the duration of swimming episodes of freely behaving animals (FIG. 5b). Overexpression of *lmx1b* produced the opposite effects. It will be of interest to determine the physiological stimuli that regulate *lmx1b* expression.

These findings put activity-dependent transmitter specification on a functional map for the developing nervous system. Given that the structure and function of the nervous system track changes in the environment and activity-dependent neurotransmitter specification seems to be reversible, it seems likely that activity-dependent changes in transmitter identity are a mechanism that enables behaviour to change flexibly. Furthermore, drugs that interfere with normal patterns of activity at various stages of development may lead to abnormal behaviours mediated by altered expression of neurotransmitters.

Respecification in the mature nervous system

Evidence for transmitter respecification in the adult has been available for many years but has received surprisingly little attention, perhaps because reduction of activity was found to lead to homeostatic loss of expression of inhibitory transmitters, making it difficult to measure a response. Demonstration of activity-dependent specification of a novel transmitter — a gain of function — was achieved only relatively recently. Although this has sparked some renewed interest in this area, the extent to which transmitters can be respecified in the adult nervous system remains to be determined, and the functional consequences of this plasticity are largely unexplored.

Early research in adult rodents showed that blockade of sensory input to the olfactory bulb reversibly reduces the number of juxtaglomerular neurons expressing tyrosine hydroxylase without affecting their expression of GABA^{62,63}, and blockade of ACh receptors in adult frog optic tectum reduces the number of neurons expressing substance P⁶⁴. Clear evidence for transmitter switching in the adult brain comes from more recent studies examining mossy fibre innervation of hippocampal CA3 pyramidal neurons by granule cells in the rat^{65,66}. These synapses are normally GABAergic at early stages of development⁶⁷ but become glutamatergic in the adult. Physiological stimulation of the dentate gyrus in the adult leads to a gain of function: the appearance of GABAergic inhibitory synaptic potentials (however, see REF. 68).

Remarkable early studies in primates demonstrated that the number of GABAergic neurons in the visual cortex of young adult monkeys is regulated by activity^{69,70}. GABA and GAD expression were reduced in animals following intraocular tetrodotoxin injection or eyelid suture, and GABA expression reappeared when activity was restored. Consistent with these observations, the dorsolateral prefrontal cortex of patients with schizophrenia has decreased activity⁷¹ and also shows a decrease in the number of neurons expressing GAD mRNA and protein relative to controls without a significant reduction in the number of cells. This suggests that reduced activity led to downregulation of the GABAergic phenotype⁷².

Thus, although less extensively examined in the mature nervous system than during its development, activity-dependent transmitter respecification also occurs in the adult brain. It will be of considerable interest to determine how widespread this process is in the mature nervous system.

Perhaps unsurprisingly, transmitter–receptor matching seems to occur as well. Grafts of the distal stump of transected motor nerves into the lateral white matter of the adult rat spinal cord led to functional reinnervation after several months. Vesicular glutamate transporters were detected in the innervating nerve, and AMPA receptor subunits were detected on the target muscle underneath the innervating nerve. This indicates that adult muscle retains the capability for glutamatergic innervation, identifying a remarkable plasticity for this normally hardwired synapse⁷³. It is reasonable to expect that GABAergic innervation of adult vertebrate neuromuscular junctions is also achievable — as naturally observed at some invertebrate neuromuscular junctions.

Potential clinical implications

Many neurological disorders involve altered levels of neurotransmitters and transmitter receptors. The loss of dopaminergic neurons in Parkinson's disease^{74,75} results in too little dopamine, the loss of cholinergic neurons in Alzheimer's disease^{76,77} results in too little ACh, and fragile X mental retardation results from too few AMPA glutamate receptors^{78,79}.

Extremes of activity-dependent transmitter specification may contribute to these disorders, and seasonal affective disorder (SAD) is a likely candidate. Individuals living at high latitudes are subject to long nights during winter months and correspondingly long days during the summer. During the winter, some subjects suffer from depression, which resolves in the absence of treatment when the days grow longer again⁸⁰. SAD can be treated pharmacologically with fluoxetine or just as successfully with phototherapy — exposure to bright white light for half an hour each day⁸¹. The mechanism by which phototherapy exerts its effects is not known, but the retinohypothalamic tract studied in larval *X. laevis* is present in primates as well, and light exposure may enable the induction of newly dopaminergic neurons.

A potentially negative consequence of activity-dependent transmitter respecification occurs when seizure activity leads to the release of GABA from normally glutamatergic mossy fibres projecting from the dentate gyrus to the CA3 area of the hippocampus⁸². Following seizures, neurons in the dentate gyrus release GABA and tonically inhibit beta/gamma (20–24 Hz) oscillations in the CA3 area that are thought to be involved in encoding and retrieval

of memory⁸³. The result may be to suppress storage of information in the hippocampus, and this could be a mechanism underlying post-seizure amnesia.

In either case, the success of phototherapy and the possibility of an underlying mechanism involving the recruitment of a reserve pool of neurons suggest the opportunity of harnessing activity-dependent transmitter respecification for the treatment of other neurological disorders. Because respecification can be generated by sensory stimulation, it is non-invasive, and because it hijacks existing circuitry, the onset of efficacy may be relatively quick¹⁷. In Parkinson's disease, the dopaminergic neurons in the pars compacta of the substantia nigra degenerate, and dopaminergic innervation of the striatum is lost⁷⁴. Interestingly, lesioning dopaminergic neurons in the pars compacta of macaque monkeys and mice can lead to the appearance of newly dopaminergic neurons via a phenotypic switch^{84,85}, although it is not known whether these neurons are incorporated into functional circuits. However, in Parkinson's disease models, the neurons of the pars reticulata of the substantia nigra are unaffected⁸⁶. These neurons receive afferent inputs that are similar to those found in the pars compacta⁸⁷, and they also project their efferent axons to targets in the striatum that are similar to those projected to by the pars compacta^{88,89}; thus, these neurons constitute a reserve pool with circuitry parallel to that of the degenerating pars compacta. These neurons are GABAergic⁹⁰, suggesting that they may be preferentially inducible to dopaminergic status in an activity-dependent manner¹⁵. Mounting evidence indicates that dance therapy benefits patients with Parkinson's disease⁹¹; it is intriguing to think that the activation of sensory and motor cortical inputs to the pars reticulata of the substantia nigra might recruit newly dopaminergic neurons. Expression of glutamate transporters in dopaminergic neurons of the substantia nigra raises the possibility that glutamate co-transmission has a role in the development and function of dopaminergic circuits^{92,93}.

Conclusions and future directions

Activity-dependent neurotransmitter respecification constitutes an addition to the portfolio of plasticity mechanisms in the nervous system, in addition to changes in synaptic strength, synapse numbers and neuronal excitability. Although much remains to be learned, this process endows the nervous system with further flexibility to respond to the environment. The fact that visual input can respecify neurotransmitter expression both during development and in the adult raises the possibility that different sensory stimuli drive changes in transmitter identity in different neuronal populations, either contributing to cognitive disorders or mediating therapeutic benefits.

The homeostatic nature of activity-dependent transmitter respecification raises the possibility that it contributes to sleep, which is a major form of homeostasis. The high frequency of neuronal activity during a day may change the identity of the transmitters expressed in neuronal populations, converting some excitatory neurons to inhibitory status; low frequency activity during sleep could then return these inhibitory neurons to excitatory status. Sleep would thus be necessary to reset the capability for activity-dependent homeostatic transmitter plasticity; this hypothesis has yet to be tested.

The identification of activity-dependent neurotransmitter respecification raises a number of questions. Which of the different forms have a critical period? The duration of this plasticity is restricted in the embryonic spinal cord^{6,9} for the stimuli that were tested. However, the process extends to later stages of development in the brain^{13,14} and is reversible in the larval *X. laevis* hypothalamus, in which it tracks changes in the environment. We now know that sensory stimulation can lead to activity-dependent neurotransmitter respecification, but does motor activity do something similar? Does activity-dependent neurotransmitter respecification have a role in LTP, learning and memory? What are the molecular markers that identify reserve pool neurons? And what is the machinery that enables some neurons to change their neurotransmitters but prevents other neurons from doing so? Answers to these questions will provide many insights and opportunities.

Acknowledgments

I thank L. Borodinsky, D. Dulcis and M. Demarque for their comments and suggestions for the manuscript. N.C.S. is supported by grants NS15918 and NS57690 from the US National Institutes of Health.

Glossary

Activity-dependent neurotransmitter respecification	Expression or loss of a transmitter or switch between expression of one transmitter and another in response to changes in activity
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Figure 1. Developmental context for activity-dependent neurotransmitter respecification

Early development involves sequential but overlapping phases of neuronal proliferation, migration and differentiation. Spontaneous activity appears before synapse formation that enables sensory inputs. Both forms of excitability generate calcium signals that drive changes in neurotransmitter identity.

Figure 2. Activity-dependent transmitter respecification in neurons distinguished by molecular markers of neuronal identity in the developing *Xenopus laevis* spinal cord and hypothalamus

a | In the spinal cord of controls, sensory Rohon–Beard neurons express *hnk1* (also known as *chst10*) and are glutamatergic, whereas motor neurons express *lim3* (also known as *lhx3*) and are cholinergic. Increases in calcium spiking by misexpression of voltage-gated sodium channels (Nav channels) lead to the appearance of GABA and glycine (Gly) in some *hnk1* and *lim3* neurons without changes in the numbers of these cells. By contrast, decreases in calcium spiking by misexpression of inward rectifier potassium channels (Kir channels) lead to increased numbers of glutamatergic and cholinergic neurons without changes in the numbers of *hnk1* and *lim3* neurons. **b** | In the hypothalamus of controls kept in the dark, interneurons in the core of the ventral suprachiasmatic nucleus express dopamine, whereas those in the surrounding annulus express neuropeptide Y (NPY). Increases in calcium spiking by exposure of animals to bright light lead to co-expression of dopamine with NPY in annular neurons without changes in the number of NPY neurons. Subsequent decreases in calcium spiking in the dark lead to loss of dopamine expression in annular and core neurons. Figure is modified, with permission, from REF. 9 © (2004) and REF. 14 © (2008) Macmillan Publishers Ltd. All rights reserved. ACh, acetylcholine; Glu, glutamate.

Figure 3. Molecular mechanisms of activity-dependent transmitter respecification in the *Xenopus laevis* CNS

a | Integration of activity-dependent and genetic specification of glutamate versus GABA by the c-Jun transcription factor in the spinal cord. Calcium spike frequency enhancement (through voltage-gated sodium channels (Nav channels)) and calcium spike suppression (through inward rectifier potassium channels (Kir channels)) modulate the phosphorylation of c-Jun, which regulates transcription of T-cell leukaemia homeobox 3 (*tlx3*) by binding to a cyclic AMP response element in the *tlx3* promoter. Expression of *tlx3* results in selection of a glutamatergic fate over the GABAergic fate in the dorsal spinal cord. **b** | Combination of activity-dependent and genetic specification of 5-hydroxytryptamine (5-HT) by LIM homeobox transcription factor 1 β (*lmx1b*) in the hindbrain. The enhancement and suppression of calcium spike frequency cause a decrease and increase, respectively, in the incidence of expression of *lmx1b*. The *lmx1b* transcription factor regulates the expression of tryptophan hydroxylase (TPH), which catalyses 5-HT synthesis. Figure is modified, with permission, from REF. 13 © (2010) Elsevier and REF. 52 © (2010) Macmillan Publishers Ltd. All rights reserved. shh, Sonic hedgehog.

Figure 4. Transmitter receptor expression matches activity-dependent transmitter respecification in *Xenopus laevis* larvae

a | Under conditions of normal spontaneous calcium spike activity, spinal motor neurons express acetylcholine (ACh), and miniature postsynaptic currents recorded from trunk muscle cells are cholinergic. When calcium spiking is suppressed, motor neurons express glutamate as well as ACh, and postsynaptic currents mediated by NMDA receptors (NMDARs) and AMPA receptors (AMPARs) are recorded. When calcium spiking is enhanced, motor neurons express GABA and glycine (Gly) as well as ACh, and postsynaptic currents mediated by GABA and glycine are recorded. **b** | In the dark with low levels of calcium spiking, neuropeptide Y receptors (NPYRs) but not dopamine D2 receptors (D2Rs) are detected on the melanotrope cells innervated by NPY annular neurons of the ventral suprachiasmatic nucleus. In the light with high levels of calcium spiking, both NPY and

tyrosine hydroxylase (which indicates the synthesis of dopamine) are expressed in annular neurons, and both NPYRs and D2Rs are observed on the melanotrope cells that they innervate. Figure is modified, with permission, from REF. 109 © (2008) Royal Society and REF. 14 © (2008) Macmillan Publishers Ltd. All rights reserved. Glu, glutamate.

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Figure 5. Activity-dependent changes in transmitter expression regulate behaviours in *Xenopus laevis* larvae

a | Camouflage is regulated by a neural circuit that includes the dopaminergic neurons in the ventral suprachiasmatic nucleus (vSCN); control animals kept in the light appear pale, whereas animals kept in the dark appear dark. Selective ablation of these dopaminergic neurons with a dopaminergic neurotoxin, MPTP, prevents animals from changing their pigmentation in response to light; recruitment of newly dopaminergic neurons rescues camouflage behaviour. **b** | Swim duration is regulated by a circuit that includes the serotonergic neurons in the raphe. Control animals swim once around a small well in response to sensory stimulation. Animals make several circles around the well following reduction in the number of serotonergic neurons by misexpression of voltage-gated sodium channels, introduction of a LIM homeobox transcription factor 1 β (lmx1b) morpholino or blockade of 5-hydroxytryptamine 1A (5-HT_{1A}) receptors. Animals manage only half a circumference following an increase in the number of serotonergic neurons by misexpression of inward rectifier potassium channels or addition of 5-HT (serotonin). Figure is modified, with permission, from REF. 13 © (2010) Elsevier and REF. 14 © (2008) Macmillan Publishers Ltd. All rights reserved.