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Review

Advances in Bioluminescence Imaging for Assessment of Developmental Neurotoxicity

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The developing brain establishes mature neural circuits through processes such as neuronal differentiation and synapse formation. However, these processes are susceptible to disruption by external factors, including exposure to environmental chemicals. These disruptions have been associated with neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD). This underscores the increasing significance of developmental neurotoxicity (DNT) assessment. However, conventional DNT testing methods are extremely costly and time-consuming, making broad *in vivo* coverage of the large number of chemicals in use impractical. Therefore, there is an increasing international demand for more efficient, quantitative screening approaches. Bioluminescence imaging (BLI) using reporter transgenic (Tg) mice enables the noninvasive visualization of gene expression and signal transduction in living animals and has been widely applied in fields such as oncology and regenerative medicine. This review highlights recent advances in the application of reporter Tg mice to assess DNT and explores their potential applications in DNT screening, chemical risk assessment, and future research on developmental neurotoxicity.

Key words bioluminescence imaging, developmental neurotoxicity, reporter transgenic mice, Syn-Rep mouse, Synapsin I

INTRODUCTION

Bioluminescence imaging (BLI) is a powerful method that enables the real-time, noninvasive visualization of gene expression and signal transduction in living organisms. Using the luminescent enzymes, luciferases (Luc), derived from bioluminescent organisms (e.g., the firefly *Photinus pyralis*) and their substrates, this technique generates optical signals without external excitation light. Owing to its high sensitivity, quantitative capability, and suitability for longitudinal tracking of *in vivo* events within the same individual, BLI has been applied in diverse fields such as oncology, regenerative medicine, infectious diseases, and pharmacokinetics.[1–4)]

In contrast, the use of BLI in toxicology remains limited. Although there are reports evaluating endocrine-disrupting activity[5–7)] and oxidative stress responses,[8–10)] the overall body of work is still scarce. In particular, endpoints in nonclinical assessments of toxicological effects on developing offspring—namely developmental and reproductive toxicology (DART) and developmental neurotoxicity (DNT)—are governed by multiple stage-specific, time-dependent processes (e.g., progenitor differentiation, migration, synapse formation and circuit maturation) that unfold continuously during development, with diverse sites and windows of chemical action. Consequently, observed changes often represent a mixture of effects from different stages rather than a single event. Given these complexities, BLI's noninvasive, within-subject longitudinal readouts hold potential to advance both nonclinical toxicology assessments of chemical effects on developing offspring and the mechanistic elucidation of those effects.

In this context, we developed a reporter transgenic (Tg) mouse, the Syn-Rep mouse, to visualize the effects of chemical exposure on neuronal differentiation processes in the developing brain *in vivo*.[11)] In this review, we first outline the characteristics of BLI technology and the current status of DNT assessment. We then discuss potential applications of BLI technology to DNT assessment and future directions, including our own studies.

CURRENT STATUS OF DEVELOPMENTAL NEUROTOXICITY AND ITS RISK MANAGEMENT

Our living environment contains a wide variety of chemicals, including pharmaceuticals, pesticides, food additives, and industrial compounds. While these substances support the conveniences of modern society, concerns have been raised about their long-term effects on human health and ecosystems.

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The increasing prevalence of neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD), has attracted international attention in recent years, and exposure to environmental/industrial chemicals is suspected to be one of the contributing factors.[12]

During development (the fetal and early postnatal stages), the brain forms mature neural networks through processes such as proliferation, differentiation, migration, and synapse formation. These processes are highly dynamic, and even minor environmental changes may exert long-term effects on neural circuit construction and functional development. Therefore, accurately evaluating the effects of chemical exposure on the developing brain requires capturing brain changes at each developmental stage. The effects of chemicals on the developing brain are collectively referred to as DNT, a concept that encompasses chemically induced disruptions of neuronal differentiation and synapse formation during brain development. Once DNT occurs, its effects are often difficult to reverse and may manifest as behavioral or cognitive abnormalities that persist over extended periods. The mechanisms underlying DNT are considered to be multistage, time-dependent, and variable depending on the chemical. Thus, accurately identifying such diverse effects is necessary in DNT assessment.

Currently, DNT assessment is conducted based on international standard tests, such as the Organisation for Economic Co-operation and Development (OECD) Test Guideline 426. These tests primarily consist of behavioral assessments and histopathological analyses. However, these tests require an extremely high experimental burden, costing approximately US$ 0.5–1.5 million per chemical and taking more than a year to complete.[13–15] Consequently, it is practically impossible to perform DNT testing on all chemicals. In fact, less than 0.2% of all chemicals have been evaluated.[16] Furthermore, the information obtained from these tests mainly concerns behavioral abnormalities and morphological changes, which makes it difficult to quantitatively capture brain alterations during development.

Given these limitations, there is a growing need to establish new methods that can evaluate developmental brain changes noninvasively and longitudinally while minimizing the burden on animals. Imaging techniques, such as BLI, which enable the quantitative visualization of brain development in living animals, are expected to complement existing test methods as next-generation DNT assessment approaches.

IN VIVO IMAGING USING REPORTER TRANSGENIC MICE

Reporter Tg mice are genetically engineered to express a reporter gene under the control of a defined promoter region. This reporter system allows noninvasive, spatiotemporal monitoring of biological processes and has been applied across diverse areas of life science research.

Two major classes of reporter proteins are commonly used: fluorescent proteins, such as green fluorescent protein (GFP), and bioluminescent enzymes, such as Luc. Fluorescent proteins enable high-resolution imaging of subcellular localization and morphological changes using fluorescence or confocal microscopy. Although external excitation light is required for *in vivo* applications, running costs can be kept relatively low once the appropriate instrumentation and measurement conditions are in place. However, significant detection challenges exist *in vivo*: wavelengths below ~600 nm are strongly absorbed and scattered by endogenous chromophores, such as hemoglobin and melanin, resulting in poor tissue penetration. Additionally, the excitation and emission bands of many fluorescent proteins (including GFP) overlap with tissue autofluorescence, further complicating quantitative analysis of fluorescence signals *in vivo*. Thus, effective detection is generally restricted to superficial tissues, surgically exposed tissues, window preparations, or probes shifted to the near-infrared, and the overall applicability remains limited.[17]

In contrast, BLI uses Luc in the presence of its substrate (e.g., D-luciferin), oxygen, adenosine triphosphate (ATP), and magnesium (Mg^{2+}) to generate light without external illumination, where the emitted photon arises from the radiative decay of an excited-state emitter (e.g., excited-state oxyluciferin in the firefly Luc/D-luciferin system) (Fig. 1). For the firefly Luc/D-luciferin system, oxyluciferin emission typically peaks near 560–570 nm, but the spectrum extends beyond 600 nm—depending on microenvironmental factors and the Luc/substrate pairing—thereby improving tissue transmittance relative to shorter-wavelength fluorescence. Coupled with the intrinsically low background owing to the absence of excitation light, BLI achieves a high signal-to-noise ratio and excellent quantitative sensitivity for *in vivo* detection.[2–4] While there are practical considerations—namely, the need to administer the substrate and account for tissue distribution depending on the dose and route—these do not detract from the above advantages. These advantages make BLI particularly suitable for longitudinal measurements in living animals. Consequently, BLI is

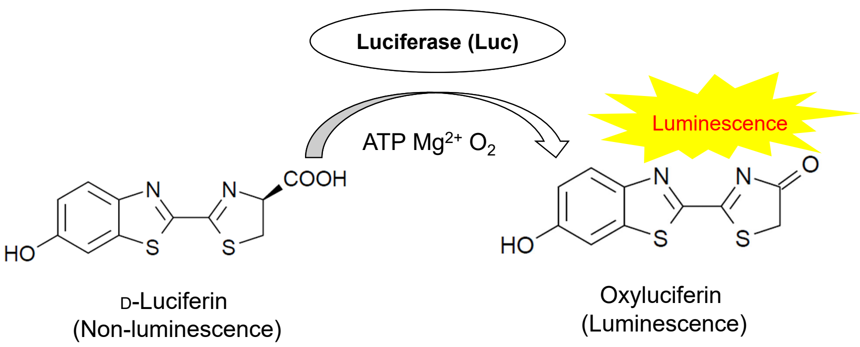

Fig. 1. Firefly Luciferase Biochemistry

Bioluminescent reaction catalyzed by firefly Luc. Upon systemic administration of D-luciferin, firefly Luc converts the substrate in the presence of ATP, Mg^{2+}, and O_2 to electronically excited oxyluciferin, which relaxes radiatively to emit a photon (hν).

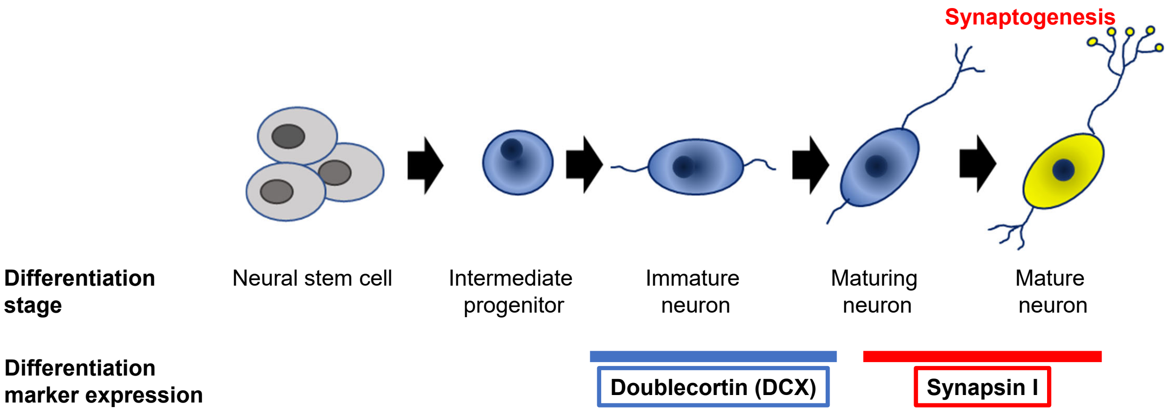

Fig. 2. Temporal Expression of DCX and Synapsin I Across a Simplified Neuronal Differentiation Sequence

Schematic of neuronal maturation condensed into five stages—neural stem cell → intermediate (transit-amplifying) progenitor → immature neuron → maturing neuron (synaptogenesis/circuit integration) → mature neuron—with overlaid expression profiles of DCX and Synapsin I. Here, the immature neuron category encompasses neuroblasts/early postmitotic migrating neurons. DCX is high in the immature neuron stage and declines as neurons enter the maturing stage, becoming largely absent in mature neurons. Synapsin I is minimal/absent in stem/progenitor stages, rises with the onset of synaptogenesis during the maturing stage, and remains enriched at presynaptic terminals in mature neurons. Stage boundaries are approximate, and timing may vary by brain region and species.

widely used in diverse *in vivo* studies, including tumor, inflammation, regeneration, and drug efficacy research.[1)]

LONGITUDINAL BIOLUMINESCENCE REPORTER IMAGING FOR DEVELOPMENTAL NEUROTOXICITY ASSESSMENT

Prior Studies on *In Vivo* Bioluminescence Imaging of Neurogenesis in the Developing Brain This subsection summarizes prior studies that applied BLI to noninvasively and longitudinally visualize the developing brain. Doublecortin (DCX), a microtubule-associated protein, is widely used as a marker of immature neurons—namely neuroblasts and immediately postmitotic neurons—in humans and rodents (Fig. 2). In rodents, DCX is strongly expressed throughout embryonic cortical neurogenesis, particularly in neurons migrating from the intermediate zone (IZ) to the cortical plate (CP), as well as in neurons undergoing tangential migration originating in the basal ganglia.[18)] After birth, DCX expression in the cerebral cortex rapidly diminishes; nevertheless, it persists selectively in immature neurons within neurogenic niches—the subventricular zone (SVZ) with its rostral migratory stream to the olfactory bulb (RMS/OB)—and in the subgranular zone of the dentate gyrus (DG/SGZ). Notably, in some regions such as the piriform cortex, DCX can be retained by mature neurons generated during development; in such exceptions, DCX positivity does not necessarily indicate recent neurogenesis.[19)]

Leveraging these properties of DCX, Tg mice expressing Luc under the control of the human DCX promoter (DCX-promo-Luc) were generated, establishing a system for longitudinal detection of endogenous neurogenesis by BLI.[20,21)] In these mice, brain bioluminescence increases from embryonic day 14.5 (E14.5) onward, with strong signals observed in neonatal periventricular regions, cortex, olfactory bulb, and cerebellum. In adults, bioluminescence arising from the olfactory bulb and the SVZ is also detected; moreover, age-dependent declines in signal—reflecting reduced baseline neurogenesis—have been longitudinally tracked in the same animals. Together, these findings demonstrate that the temporal progression of neurogenesis can be traced noninvasively *in vivo*.

Furthermore, this system has been used to fate-map transplanted neural progenitor cells. When neural progenitor cells derived from DCX-promo-Luc mice were transplanted into the lateral ventricle, BLI revealed a redistribution of the signal toward the olfactory bulb, peaking 6–12 days post-transplantation and subsequently declining, consistent with migration and settlement. To examine the relationship between this promoter-driven bioluminescent signal and neuronal maturation at the cellular level, histological analyses were performed. Immunohistochemical analyses combining BrdU labeling of proliferating cells with identification of the mature neuronal marker NeuN confirmed the transition from young to mature neurons.[20)] In addition, this system has been applied to pathophysiological studies in brain disease models, such as longitudinal analysis of neurogenesis in a stroke model.[22)]

In summary, BLI using Tg reporter mice can provide noninvasive, longitudinal, and quantitative visualization of neurogenesis-related events in developing and adult brains. To date, however, there have been no reports of direct toxicological applications, including DNT, using DCX-promo-Luc Tg mice. Importantly, because DCX expression is transient and confined primarily to immature neurons, the DCX-promo-Luc approach is restricted to the neurogenesis–early differentiation window. Consequently, the indicator effectively disappears as neurons mature, precluding direct assessment of later neurodevelopmental stages such as synaptogenesis, circuit maturation, and activity-dependent plasticity. This renders the model unsuitable as a stand-alone framework for DNT assessment and underscores the need for complementary indicators of later-stage differentiation.

Generation and Characterization of the Syn-Rep Mouse In the developing brain, a series of processes proceeds in a stage-specific and interdependent manner. These processes include the proliferation, differentiation, and migration of neural progenitors; axonal and dendritic outgrowth; synapse formation; activity-dependent maturation; synaptic pruning; and ultimately, myelination. Because each step has its own window of vulnerability, exogenous chemicals can disrupt circuit formation and function at multiple stages. Thus, DNT is not reducible to a single event; rather, it should be understood as a multistage disruption spanning from early progenitor differentiation and migration to circuit maturation and plasticity. Although stage-specific abnormalities should theoretically be evaluated at each developmental step, the strong interdependence and timing constraints of these processes make continuous, stage-by-stage monitoring with separate readouts impractical from an experimental-design perspective. From a regulatory perspective, current test guidelines position behavioral assays as definitive tests that capture integrated neurodevelopmental outcomes. Aligned with this principle, a practical approach is to adopt a single imaging indicator that reflects the overall developmental attainment of the differentiation process, measured noninvasively and longitudinally in the same animal. This approach aggregates multistage influences into a single output, enabling longitudinal tracking of developmental attainment and allowing early identification of upstream perturbations as delayed attainment or attenuated signal, thereby helping to minimize false negatives and improve screening efficiency.

As a complementary strategy, we focused on Synapsin I, a neuron-specific phosphoprotein that tethers synaptic vesicles to presynaptic terminals. Its expression increases in postmitotic neurons after the immature stages marked by DCX and persists at presynaptic terminals from synaptogenesis into adulthood (Fig. 2).[23] Thus, Synapsin I can serve as an indicator that reflects outcomes from later differentiation through network maturation, complementing the early-stage information provided by DCX. Furthermore, the rat Synapsin I promoter has been used to drive neuron-specific expression: selective activity in differentiated neurons was demonstrated in Cre recombinase–expressing transgenic mice,[24] and this promoter has since been widely adopted to generate neuron-specific conditional knockouts, supporting research in neurodevelopment, neurotransmission, plasticity, and behavior.[25–27] Accordingly, the Synapsin I promoter is regarded as a de facto standard neuron-specific regulatory element for transgene control.

Leveraging these properties, we generated a reporter Tg mouse (Syn-Rep mouse) that expresses Luc2 (codon-optimized *Photinus pyralis* luciferase) under the rat Synapsin I promoter, enabling noninvasive *in vivo* visualization of neuronal maturation and synaptogenesis (Fig. 3).[11] Assays of Luc activity across tissues in adult animals showed the highest activity in the cerebral cortex, with little or no activity in nonneural organs, confirming brain-selective reporter expression in this model. Furthermore, longitudinal BLI from the early postnatal period into adulthood revealed a characteristic trajectory: head-region bioluminescence was detectable immediately after birth, peaked within several days, declined to roughly one-tenth of the peak by weaning, and then stabilized at a low level. This pattern parallels the generally described time course of synapse numbers in the human brain,[28] indicating that Syn-Rep bioluminescence dynamics reflect the progress of synaptic assembly and, by extension, the overall attainment of the neurodevelopmental differentiation process *in vivo*. Taken together, Syn-Rep mouse enables noninvasive, quantitative, longitudinal assessment of events from late differentiation through network maturation. As an intermediate indicator consistent with the guideline principle of capturing integrated neurodevelopmental outcomes, it represents a useful model for DNT assessment.

Validation of the Syn-Rep Mouse as a DNT Assessment System In reproductive and developmental toxicity testing, decreases in body weight can occur as a systemic effect. Because reduced body weight can influence brain develop-

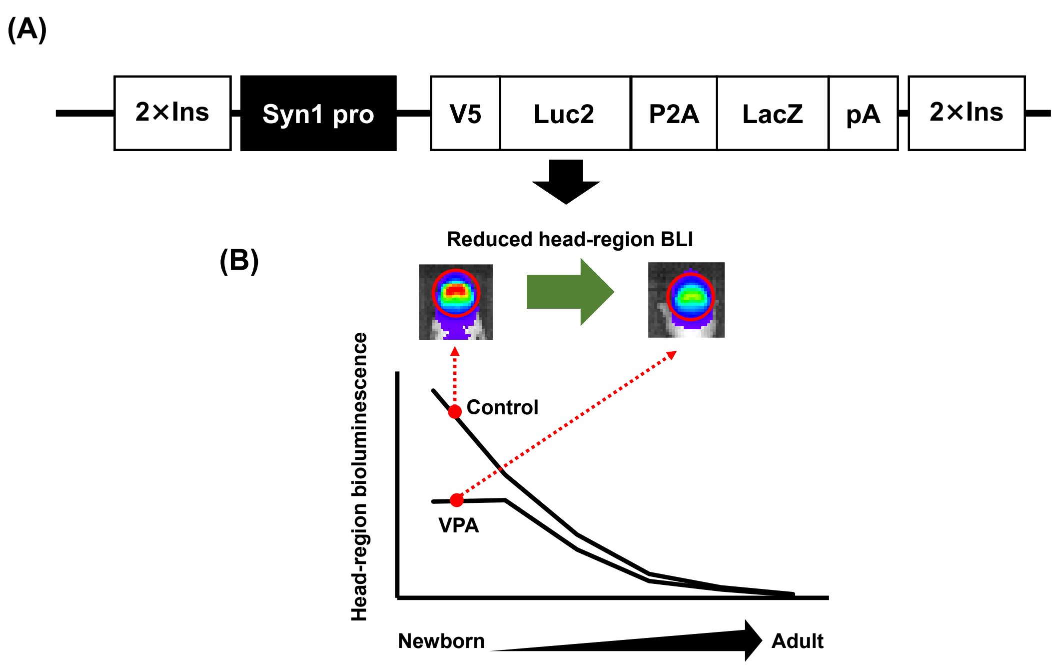

Fig. 3. Syn-Rep Transgene Construct and Longitudinal BLI of Syn-Rep Offspring Following Prenatal VPA Exposure

(A) Schematic of the Syn-Rep reporter cassette. The rat Synapsin I promoter (Syn1 pro) drives an N-terminal V5-tagged Luc2 (Promega's fourth-generation, codon-optimized *Photinus pyralis* luciferase). The C-terminus of Luc2 is followed by a self-cleaving P2A peptide and LacZ, then a polyadenylation signal (pA), enabling bicistronic expression of Luc2–V5 and LacZ from a single transcript. To minimize position effects and heterochromatin silencing, the entire cassette is flanked at the 5' and 3' ends by two GC-rich core fragments of the chicken β-globin insulator (Ins).

(B) Pregnant Syn-Rep dams were administered VPA under conditions known to elicit ASD-like phenotypes.[11,29–31] Offspring were imaged repeatedly by BLI from the early postnatal period through adulthood. Relative head-region bioluminescence is shown. Across the time course, the VPA-exposed group exhibited consistently lower head-region bioluminescence than unexposed controls.

ment, it is often difficult—particularly when relying on behavioral endpoints—to distinguish brain-specific toxicity from nonspecific consequences of growth retardation. Therefore, we examined the extent to which bioluminescence intensity in the brains of Syn-Rep mice is influenced by variability in body weight and maturation status within the physiological range. During the developmental period from postnatal day 4–16, analysis of the relationship between head bioluminescence intensity and body weight revealed no significant correlation.[11] Collectively, these findings suggest that, within the physiological range examined, head-region bioluminescence in Syn-Rep mice may serve as a relatively independent indicator of neurodevelopmental progression, with minimal apparent influence from mild variability in body weight or maturation.

Building on this feature, we evaluated whether Syn-Rep mice can detect the effects of a known DNT-inducing substance. Valproic acid (VPA), a widely used antiepileptic drug, has been reported to increase the risk of neurodevelopmental disorders, such as ASD and intellectual disability, in children when taken during pregnancy.[29] Prenatal exposure to VPA in mice is also known to induce ASD-like behavioral changes,[30] and VPA is commonly used in toxicology as a positive control for DNT.[31] Thus, we administered VPA to pregnant Syn-Rep mice under conditions known to elicit ASD-like phenotypes.[11] We then longitudinally quantified head-region bioluminescence in the offspring by BLI from the early postnatal period through adulthood (Fig. 3). Across the time course, the VPA-exposed group showed consistently lower head-region bioluminescence than unexposed controls. Furthermore, histopathological analyses of the cerebral cortex at developmental and adult stages revealed a reduction in neuronal cell number in the VPA-exposed group. These histological findings support the interpretation that the observed decrease in bioluminescence reflects delayed neuronal differentiation and synaptogenesis. Taken together, these results demonstrate that BLI using Syn-Rep mice is an effective, noninvasive, quantitative tool for capturing the effects of chemicals on the developing brain.

CONCLUSIONS

In this review, we outlined the current status and challenges of DNT assessment, and the potential of BLI with reporter Tg mice as a complementary approach. Internationally standardized DNT tests are time-consuming and costly, and in practice, only a limited number of chemicals have been evaluated. Furthermore, conventional methods centered on behavioral testing and histopathology have inherent difficulty in directly capturing dynamic neurodevelopmental processes, such as neuronal differentiation and synaptogenesis. In contrast, BLI with Syn-Rep mice enables the noninvasive, quantitative, longitudinal visualization of these processes *in vivo*. In addition, the bioluminescent signal is relatively insensitive within the physiological range examined (e.g., body weight, maturation status), supporting its use as an indicator of neurodevelopmental progression. These features suggest that Syn-Rep-based BLI could serve as a prescreening system prior to guideline DNT tests, helping to efficiently prioritize chemicals. When combined with established behavioral and histopathological evaluations, this approach may contribute to a more comprehensive and quantitative understanding of how chemicals impact neurodevelopment. Accurately detecting and managing chemicals that adversely affect children's neurodevelopment may help reduce the risk of neurodevelopmental disorders and support a safer, more sustainable society. We hope this approach will contribute meaningfully to these goals.

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Conflict of interest The authors declare no conflict of interest.

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