Neurotoxicology 71 (2019) 52–59

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Bioluminescence imaging of *Arc* expression in mouse brain under acute and chronic exposure to pesticides

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ARTICLE INFO

Keywords:
Pesticide
Neurotoxicity
Arc
Bioluminescence imaging
Neuronal-activity-dependent gene expression

ABSTRACT

Exposure to pesticides can induce neurobehavioral effects in rodents, as well as in other mammals, including humans. However, the effects of the toxicity of pesticides on the central nervous system (CNS) remain largely unclear. The expression of the activity-regulated cytoskeleton-associated protein gene (*Arc*) is induced in a neuronal-activity-dependent manner and is implicated in synaptic and experience-dependent plasticity. We previously developed *Arc*-promoter-driven luciferase transgenic (Tg) mouse strains to monitor the neuronal-activity-dependent gene expression under physiological and pathological conditions *in vivo*. In this study, we examined the effect of acute administration of four different pesticides (deltamethrin, glufosinate, methylcarbaryl, and imidacloprid) on neuronal activity using *Arc-Luc* Tg mice. The change in the bioluminescence signal in mouse brain upon treatment with deltamethrin and glufosinate occurred more slowly than that of kainic acid, a potent neuroexcitatory amino acid agonist. These two pesticides also caused convulsive responses in adult *Arc-Luc* Tg mice. In the case of glufosinate, we detected the long-term upregulation of bioluminescence signal intensity of *Arc-Luc* over 24 h after the treatment. Furthermore, we observed greater changes of bioluminescence signal in adults than in juveniles, and a lower incidence of convulsions at the juvenile stage. In contrast to the acute treatment, we detected a decrease of bioluminescence signal after low-dose chronic treatment with glufosinate, without neuronal overexcitation. From these results, we suggest that *Arc-Luc* Tg mice are useful for assessing the acute and chronic effects of pesticides on the CNS.

1. Introduction

Pesticides are widely used for improving the efficiency of agricultural yield and for enabling the prolonged storage of crops. They are typically expected to act on specific targets such as micro-organisms, insects, or plants. However, exposure to pesticides is potentially associated with health risks, which is an increasing focus of public concern (https://www.epa.gov/pesticide-worker-safety). Epidemiological studies have suggested that the main adverse health effects of pesticides involve the nervous system (Kamel and Hoppin, 2004). In addition, toxicological vulnerability in the perinatal and childhood periods and the risk of developing neurodegenerative disease are attracting increasing interest (Roberts and Karr, 2012). Thus, more in-depth information on the effects of exposure to pesticides on the central nervous system (CNS) is required to protect human health.

Neurotoxicity is an adverse effect of xenobiotics including pesticides on structure and function of the nervous system (Tilson et al., 1995). Histopathological and behavioral evaluations in laboratory animals have been used as complementary components to assess the risk of neurotoxicity associated with exposure to pesticides. With representative biomarkers such as glial fibrillary acidic protein or neuropathy target esterase, neuropathological changes provide insight into the state of cells for estimating the worsening states induced by exposure to pesticides. However, even using these biomarkers, it is difficult to estimate the neurobehavioral alterations in many cases (Moser, 2011). To obtain a better understanding of the neuronal substrates of behavior related to exposure to pesticides, it is crucial to integrate the molecular/cellular events and behavioral findings.

Neuronal activity regulates the remodeling of synaptic connectivity through, in part, the induction of the neuronal activity-regulated genes (Flavell and Greenberg, 2008; West and Greenberg, 2011). Several studies have suggested that exposure to pesticides is one of the environmental factors affecting diverse components of the synapse function (Harrill et al., 2008; Kimura-Kuroda et al., 2016; Lee et al., 2015). Considering the implications of neuronal activity-dependent processing for neurological outcomes, and the advantages of reporter mice in

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https://doi.org/10.1016/j.neuro.2018.12.003
Received 19 June 2018; Received in revised form 27 November 2018; Accepted 13 December 2018
Available online 15 December 2018

toxicological analysis (Boverhof et al., 2011), sensitive and quantitative monitoring of neuronal activity in *in vivo* models should accelerate our elucidation of the effects of single or repeated exposure to pesticides on the CNS.

The activity-regulated cytoskeleton-associated protein gene (*Arc*) is rapidly transcribed in response to neuronal inputs (Link et al., 1995; Lyford et al., 1995). The transcripts of Arc, in a form of being ready for translation (Na et al., 2016), are distributed to neuronal dendrites in activated neurons and surrounding cells (Pastuzyn et al., 2018). Arc is considered to be one of the key proteins in neurobiology because of its roles in the regulation of spine morphology through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor endocytosis (Peebles et al., 2010), neurodevelopment and maintenance of neuronal networks (Mikuni et al., 2013), and cognitive function through neuronal-activity-dependent synaptic development (Evert and Greenberg, 2013; West and Greenberg, 2011). Furthermore, recent studies also suggest that Arc is a possible therapeutic target for neural plasticity and disease (Jenks et al., 2017; Mandel-Brehm et al., 2015; Zhang et al., 2015). We have developed reporter transgenic (Tg) mouse strains expressing the firefly luciferase (*Luc*) gene under regulation of the *Arc* gene promoter (Izumi et al., 2011, 2017). These *Arc-Luc* Tg mice enable the non-invasive detection and quantification of changes in neuronal activity in the mouse brain throughout the lifespan.

Based on this background, we decided to examine the usefulness of our Tg mouse strains for monitoring the effect of pesticides on the CNS. We selected four different representative pesticides classified as a pyrethroid [deltamethrin (DM)], an organophosphate [glufosinate ammonium (GLA)], a carbamate [methylcarbaryl (NAC)], and a neonicotinoid [imidacloprid (IMI)] as subjects of our investigation (Casida and Durkin, 2013). By tracking spatiotemporal changes in the bioluminescence signal of *Arc-Luc* Tg mice, we detected the acute induction of Arc-Luc by DM and GLA treatments in the adult and juvenile stages. In addition, we also characterized the specific behavioral changes associated with each pesticide in the mice. We found that our reporter mice are useful for monitoring long-term effects upon chronic treatment with low-dose DM or GLA. This study shows that the *Arc-Luc* Tg mice will be valuable for the evaluation of the cumulative effects of pesticides on neuronal activity-dependent processes in the CNS.

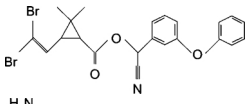
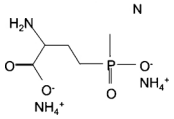
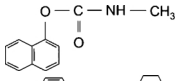
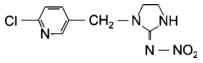
2. Materials and methods

2.1. Chemicals

Kainic acid (KA) was purchased from TOCRIS. Glufosinate ammonium (GLA), deltamethrin (DM), methylcarbaryl (NAC), and imidacloprid (IMI) were purchased from Wako. In the acute treatment study, KA and GLA were dissolved in saline. DM, NAC, and IMI were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). For the chronic treatment study, DM was suspended in corn-oil and GLA was dissolved in saline.

To examine the acute effect of pesticide administration on Arc-Luc expression, adult *Arc-Luc* Tg mice (Izumi et al., 2011) were intraperitoneally (i.p.) injected with above described four pesticides (DM, GLA, NAC, and IMI), also shown in Table 1 (n = 3 for each group in Fig. 1). Animals for negative control group received the same volumes of vehicle, 10 mL of saline/kg BW (n = 3) or 1 mL of DMSO/kg BW (n = 3), as a single i.p. injection. The positive control group was injected with 25 mg of KA/kg BW (n = 3), which is an excitatory amino acid receptor agonist selective for KA-type glutamate receptor and induces Arc expression. *Arc-Luc* Tg hairless (HL) mice (Izumi et al., 2017) at 4 weeks of age (4 W) and 8 W were i.p. injected with DM (n = 4 for 4 W, n = 3 for 8 W in Fig. 2) or GLA (n = 3 for each age in Fig. 3). The dose of each pesticide for acute treatment was determined on the basis of previous reports (Baron et al., 1964; Chao and Casida, 1997; Elwan et al., 2006; Izumi et al., 2011; Matsumura et al., 2001; Takasaki et al., 2013; Tomizawa et al., 2001). Bioluminescence signals were measured before (at 0 h) and after the treatment (at 3 and 6 h) in parallel with the observation of animal behavior. The generalized convulsions were evaluated on the basis of kainate-induced seizures (Morrison et al., 1996, Supplemental Table 1). The signs of salivation were graded using the scoring system by Anand et al. (2006) (Supplemental Table 2).

Table 1
Structure and dose of pesticides used for acute exposure.

Compound	Structure	Dose (mg/kg)
Deltamethrin (DM)		3.5
Glufosinate ammonium (GLA)		80
Methylcarbaryl (NAC)		35
Imidacloprid (IMI)		40

For the chronic treatment from postnatal day 28 (P28) to P56 of *Arc-Luc* Tg HL mice, DM was suspended in corn-oil and was orally (p.o.) administered at a dose of 3 mg/kg BW every other day (n = 6). GLA was dissolved in saline and was injected i.p. at a dose of 8 mg/kg BW every day (n = 10). Negative control mice for DM (n = 5) were p.o. administered corn-oil and negative control mice for GLA (n = 8) were i.p. injected saline, respectively. Then, Bioluminescence imaging (BLI) was performed every 7 days (at P28, 35, 42, 49 and P56).

2.2. Animals

Adult (3–6 months of age) *Arc-Luc* Tg mice were used in the acute treatment study (Fig. 1). Juvenile (4 W) and young adult (8 W) *Arc-Luc* Tg mice were used for acute and chronic treatment studies (Figs. 2–5,). In addition to the above-mentioned animals, juvenile (4–5 W) and adult (> 8 W) wild-type mice were subjected to the examination of convulsions associated with GLA (Fig. 3C). All the animals were maintained under standard conditions (12 h light/12 h dark cycle with lights on at 8:00 a.m., room temperature at 22 ± 2 °C) at the Laboratory Animal Resource Center of the University of Toyama. Homozygous Tg mice were used in acute study, and heterozygous Tg mice were used in chronic study. The *Arc-Luc* Tg mice are available to the research community upon request.

The experimental procedures in this study conformed to the guidelines for the care and use of laboratory animals of the University of Toyama (Authorization Nos. A2011 MED-3, A2015 MED-28). This study complies with the ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines. All possible efforts were made to reduce the number of animals studied and to avoid their suffering. Because BLI is a high quantitative and reproducible method, we used minimal number of animals repeatedly to evaluate statistically.

2.3. In vivo imaging

BLI was performed as described previously (Izumi et al., 2011, 2017; Mori et al., 2017). Briefly, mice were anesthetized by the inhalation of isoflurane (1.5% in air) and i.p. injected with D-luciferin (AAT Bioquest, Sunnyvale, CA, USA) dissolved in phosphate-buffered saline (PBS, pH 7.4) at 150 mg/kg BW. Ten minutes after luciferin injection (5 mL of solution/kg BW), bioluminescence signals were repeatedly measured for 30–60 s with 4 × 4 binning using an *in vivo* imaging system (Clairvivo OPT; Shimadzu Co., Kyoto, Japan). As Fig. 3

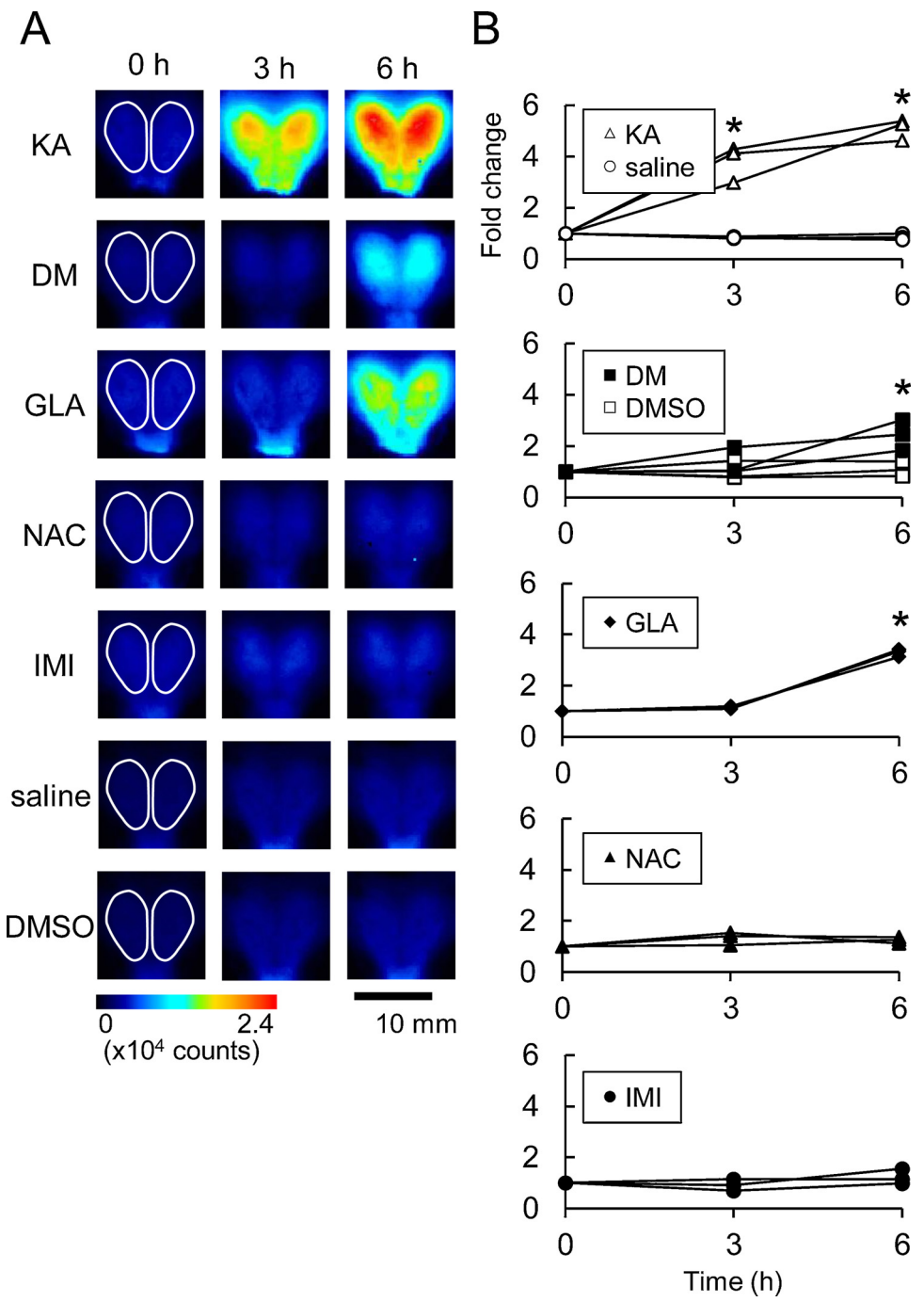


Fig. 1. Non-invasive *in vivo* assessment of *Arc-Luc* induction by acute treatment with pesticides.

A. Bioluminescence signals of *Arc-Luc* Tg mice treated with four kinds of pesticide. BLI of adult mice was performed before (at 0 h) and after (at 3 and 6 h) acute treatment with the respective pesticides (DM, GLA, NAC, and IMI). KA was used as positive control in this experiment. Animals in negative control group were treated with same volume of vehicle (saline or DMSO). Regions of interest (ROIs) in the forebrain are indicated as white circles in images at 0 h. Bioluminescence signals detected with the photon counter are pseudocolored from 0 to 24,000 (counts). *Scale bar*, 10 mm.

B. Fold changes in bioluminescence signals after acute treatment with pesticides. Four kinds of pesticide were injected in each group (n = 3, each), while KA was used in the positive control group (n = 3). Fold changes in bioluminescence signals are shown relative to the basal level (signal intensity at 0 h) for each mouse studied. Data from KA-, GLA-treated group were compared with those of the saline group (n = 3), and DM-, NAC-, IMI-treated group were compared with DMSO group (n = 3) at the same BLI points. Each line is a different animal. *$p < 0.05$; two-way ANOVA with Tukey-Kramer post hoc test.

shows, for the monitoring of the acute GLA treatment study for 24 h, an osmotic pump (Durect Co., Cupertino, CA, USA) filled with 100 μL of 700 mM luciferin solution was subcutaneously (s.c.) implanted into the backs of mice. Photon intensity was calculated from bioluminescence images by region of interest (ROI) analysis fusing NIH ImageJ. Background bioluminescence signal intensity from wild-type mice was subtracted from all the values. The data are expressed as the mean number of photons per pixel (count) in the ROI.

2.4. Statistical analysis

Two-way repeated measures analysis of ANOVA was applied to examine the statistical significance of differences between groups, followed by post hoc comparison using Tukey-Kramer. Fisher's exact test (2 × 2) was performed to determine whether adults and juveniles differed significantly in the incidence of convulsions after acute GLA treatment. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Arc-Luc induction in adult mouse brain by acute treatment of pesticides

Fig. 1 shows representative images and fold changes after the administration of each pesticide. An increase of the bioluminescence signal in the positive control group was detected in the posterior regions of the forebrain 3 h after KA injection and the signals spread throughout the forebrain. Treatment with KA elevated the signal intensity up to 5.1-fold at 6 h. At 6 h after injection, the DM- and GLA-treated groups showed gradual increases in bioluminescence signal up to 2.4- and 3.2-fold relative to that at baseline, respectively. The groups

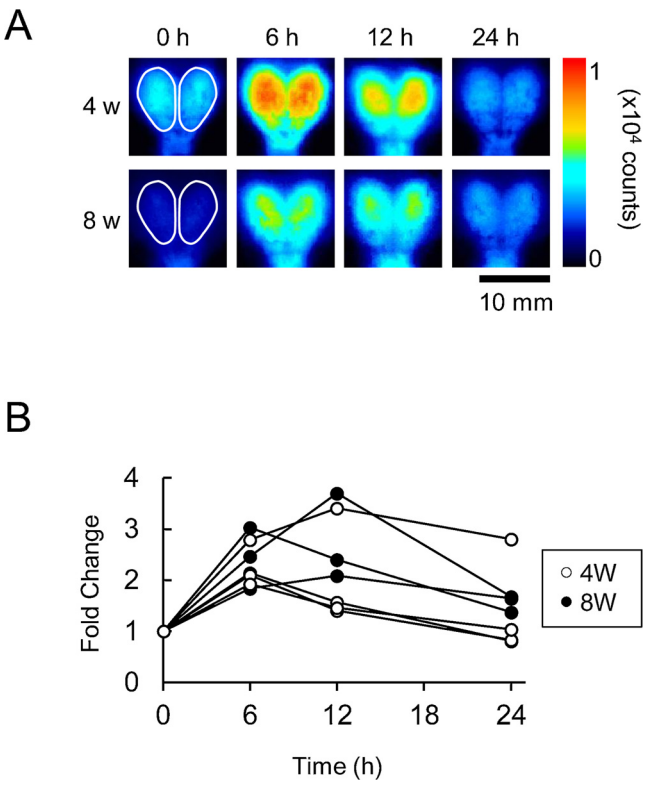


Fig. 2. Effect of acute DM treatment on *Arc-Luc* induction at 4 and 8 weeks of age.

A. Photon emission in the *Arc-Luc* Tg HL mice at 4 W and 8 W. Representative images before (at 0 h) and after (at 6, 12, and 24 h) acute DM treatment (3.5 mg/kg BW i.p.) are shown (pseudocolored, 0–10,000 counts). Locations of ROIs for forebrain (white circles) are indicated in images at 0 h. *Scale bar*, 10 mm.

B. Fold changes in bioluminescence signals induced by acute DM treatment. Results of each mouse are shown as the fold changes in signals relative to the basal level (signal intensity at 0 h) and compared between 4 W (n = 4, open circles) and 8 W (n = 3, closed circles) at the same BLI points (6, 12 and 24 h). Each line is a different animal. One of the mice at 4 W exhibited a higher increase in signals and choreoathetosis.

with NAC and IMI treatments showed no significant bioluminescence changes during 6 h.

In addition, we detected behavioral changes of the mice. For example, KA-treated mice showed convulsive responses to KA (head nodding, forelimb clonus, and rearing) within 1 h after the treatment, which continued for several hours. Moreover, treatment with DM induced lower activity of mice followed by forelimb and tail extension. GLA treatment induced frequent jumping activity as a distinguishing neurobehavioral effect observed 5 h after the treatment. Furthermore, with NAC treatment, the mice became motionless for several hours. However, they did not show any specific signs of twitches or seizures as seen in the KA-, DM-, and GLA-treated groups. A similar motionless state was observed in the group with IMI treatment. The saline-injected mice did not show any changes in behavior. Consequently, we concluded that the pesticides induced the increase in bioluminescence of *Arc-Luc* Tg mice in a manner correlated with the convulsive seizures (Supplemental Fig. 1).

3.2. Temporal dynamics of Arc-Luc induction by acute DM and GLA treatments during mouse development

As shown above, DM and GLA treatments induced aberrant neuronal activation at the adult stage. Next, we examined the effects of acute DM and GLA treatments on *Arc-Luc* expression during mouse development. For this purpose, we used newly established *Arc-Luc* Tg HL mice in subsequent experiments to avoid interference of the photon signal from the brain caused by black fur, skin pigmentation, and/or hair regrowth after depilation during development (Izumi et al., 2017).

The basal level (signal intensity at 0 h) of photon emission from *Arc-Luc* Tg HL mice at 4 weeks of age (4 W) was more than 1.5-fold higher than that of mice at 8 W (*$p < 0.05$; Student's t test, Fig. 2A). This result is consistent with the developmental changes of Arc and Luc protein expression as previously reported (Izumi et al., 2017). On the other hand, BLI of the *Arc-Luc* Tg HL mice showed similar temporal patterns of fold change in photon signal after DM treatment at each age The changes in the photon signal were up to about 2.2- (4 W) and 2.6-fold (8 W); however, the signal intensity had returned to the basal level by 24 h (Fig. 2A and B). The mice with DM treatment at 4 W showed more severe signs of salivation. Alternating limb movement with a wave-like movement of the abdomen (choreoathetosis) was observed in one of the mice in this group.

After acute GLA treatment, BLI was performed on the mice with s.c. implantation of an osmotic pump filled with luciferin solution in their back. Treatment with GLA produced a strong change in the bioluminescence signal in the mice at 8 W (Fig. 3A). The photon signal after GLA treatment began to increase in the entire forebrain and changed its pattern into a focal increase in the rostral area. The signal increase began at 6 h after GLA treatment and reached more than a 10-fold increase over 24 h (Fig. 3B). This temporal change in signal distribution with GLA treatment differed from that with DM treatment in the 8 W mice. Additionally, we examined the effect of an *N*-methyl-D-aspartate receptor antagonist, MK-801, which is also well known as an inhibitor of *Arc* induction. The mice at 8 W pretreated with MK-801 showed only an approximately twofold increase of the signal after GLA treatment (Supplemental Fig. 2) and did not exhibit characteristic jumping behavior induced by this treatment. In contrast to the large changes in the signal in the mice at 8 W, no significant changes in BLI or behaviors were detected in the 4 W group mice during 24 h after acute GLA treatment (Fig. 3B). Because it has been reported that the treatment with GLA causes tonic–clonic convulsions in rodents (Matsumura et al., 2001), we investigated the incidence rate of convulsions after acute GLA treatment at different ages (Fig. 3C). The treatment with GLA elicited convulsions in all of the mice at an age of over 8 W (15/15). In contrast, the incidence rate of convulsions of the mice at 4–5 W old was significantly lower frequency (4/19).

3.3. Effect of chronic treatment with low-dose DM and GA on neuronal activity

As shown in Fig. 4A, the control group showed a gradual decrease in bioluminescence signal from P28 to P42. The signal pattern and intensity at older age points (P42, P49, and P56) were similar among the different ages. The statistical analysis of BLI revealed that there was not a significant difference between DM and control groups (Fig. 4B). During DM treatments, no significant difference in BW was observed between the groups (Fig. 4C) and there were no obvious changes in the gross appearance of their skin, such as tumorigenesis or dermatitis.

The results of BLI in the chronically GLA-treated group and the control (saline) group are shown at each age (P28, 35, 42, 49, and 56) in Fig. 5A. Although no interaction was detected between treatment and age ($p = 0.33$), two-way ANOVA showed a statistical difference in two main factors ($p = 0.02$ for treatment, $p = 3.2 \times 10^{-7}$ for age). GLA-treated mice showed a slight but significant decrease in bioluminescence signal 3 weeks after the treatment (at P49) and the decrease of the signal continued until P56 compared with that in the control mice (Fig. 5B). The control group showed a gradual decrease in bioluminescence signal from P28 to P42, similar to that shown in Fig. 4B. During GLA treatments, no significant difference in BW was observed between the groups (Fig. 5C) and there were no obvious changes in the gross appearance of their skin.

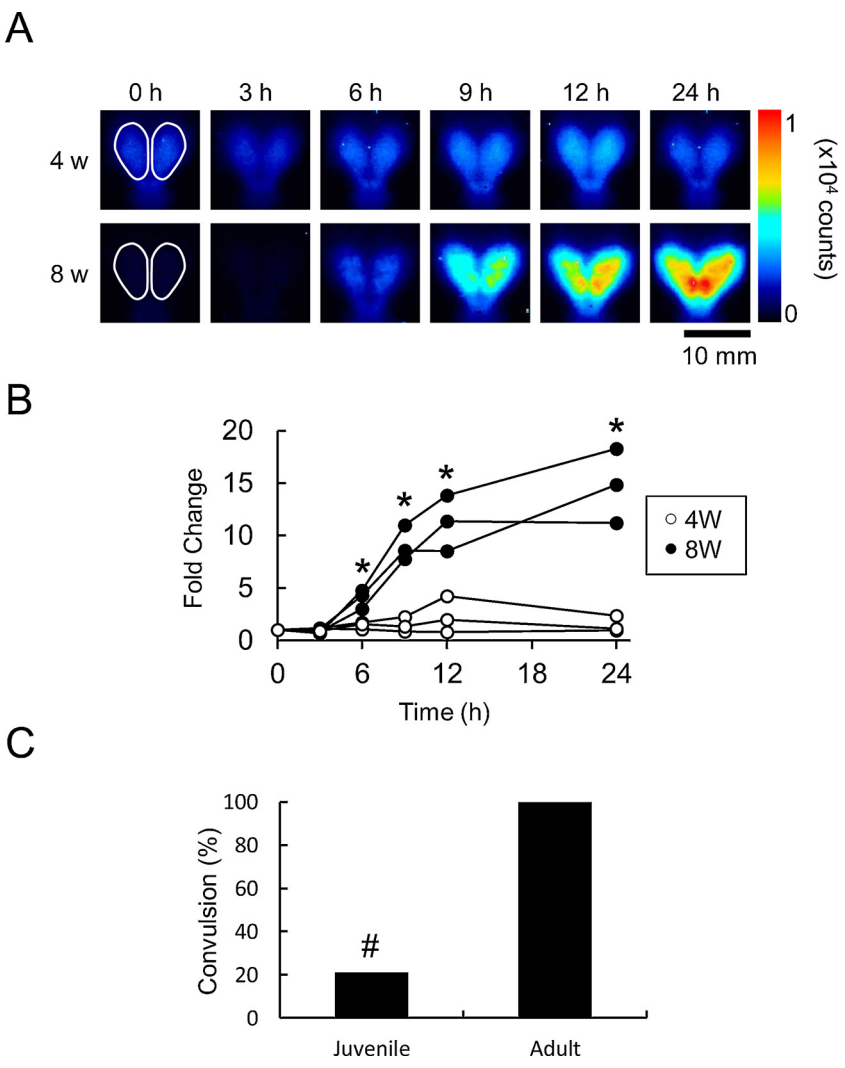


Fig. 3. Effect of acute GLA treatment on *Arc-Luc* induction at 4 and 8 weeks of age.

A. Representative images of photon emission in *Arc-Luc* Tg HL mice at 4 W and 8 W with acute GLA treatment (80 mg/kg BW i.p.). Mice were subcutaneously implanted with luciferin-filled osmotic pumps and bioluminescence signal images in the forebrain (pseudocolored, 0–10,000 counts) were examined before (at 0 h) and after (at 3, 6, 9, 12, and 24 h) acute GLA treatment. Location of ROIs for the forebrain (white circles) is indicated in images at 0 h. *Scale bar*, 10 mm.

B. Fold changes in bioluminescence signals induced by acute GLA treatment. Results of each mouse are shown as the fold changes in signals relative to the basal level (signal intensity at 0 h) and compared between 4 W (n = 3, open circles) and 8 W (n = 3, closed circles) at the same BLI points (3, 6, 9, 12, and 24 h). Each line is a different animal. $^{*}p < 0.05$; two-way ANOVA with Turkey-Kramer post hoc test.

C. Age-dependent incidence of convulsions after acute GLA treatment. Bar graph shows the incidence of convulsions until 24 h in juvenile (4–5 W old, n = 19) and adult mice (more than 8 W old, n = 15). The difference in the incidence of convulsions was highly significant. $^{\#}p < 0.01$; Fisher's exact test.

4. Discussion

The mechanisms underlying the association of exposure to pesticides with neurobehavioral and neurodevelopmental outcomes remain to be elucidated. To address this issue, it is essential to detect abnormal changes in neuronal activity in the CNS after exposure to pesticides. In this study, we examined the usefulness of BLI with *Arc-Luc* Tg mice to monitor the effects of pesticides on neuronal activity in the brain. Our data demonstrated abnormal neuronal-activity-dependent *Arc* expression after exposure to several different pesticides, which might be involved in neurological dysfunction or disease. Our study provides sensitive, quantitative, and real-time monitoring of abnormal neuronal activity associated with exposure to pesticides.

The pesticide concentrations used in our acute treatment study might be different from those of human exposures. However, some literatures reported the concentration of pesticide used for human suicide (Kaul et al., 2015; Mao et al., 2012; Mundhe et al., 2017), and these concentrations are much higher than our study. We found that exposure to a high dose of DM or GLA induced a gradual increase in bioluminescence signal in the brain of *Arc-Luc* Tg mice. This temporal pattern of changes is distinct from the case upon KA treatment (Fig. 1). These results imply that there are different mechanisms of neuronal overexcitation associated with each pesticide. In the case of KA treatment, which induces the activation of KA-type glutamate receptor channels, both rapid Arc expression and neurobehavioral change, that is, seizures, were significantly induced, as reported previously (Izumi et al., 2011, 2017; Mori et al., 2017). In the case of acute DM treatment, its primary target is considered to be the voltage-gated sodium channel, after which this channel is activated, followed by calcium flux through the activation of receptors including NMDA receptor and/or L-type Ca^{2+} channel (Cao et al., 2011). In addition, DM disrupts the regulation of genes related to neurite branching and neuronal morphogenesis (Harrill et al., 2008). Thus, the multistep actions of DM might result in Arc induction and a slow increase in bioluminescence signal (Figs. 1 and 2). In addition to a slow increase in bioluminescence similar to the effect with DM, acute GLA treatment produced a more sustained effect on *Arc-Luc* expression during 24 h, with temporal changes in the photon distribution pattern (Figs. 1 and 3). We used the NMDA receptor antagonist MK-801 and confirmed that the NMDA receptor is activated in the neuronal excitation process induced by GLA *in vivo* (Supplemental Fig. 2). On the other hand, the latency to detect a significant increase of bioluminescent signal upon GLA treatment (6 h) was longer than that upon KA treatment (3 h) (Fig. 1). Similar temporal differences in patterns were also observed regarding the time of onset of convulsions upon treatment with GLA (~6 h) and KA (< 1 h). Taking these findings together with a report describing that several metabolites of GLA in mammals are potentially associated with a risk of cumulative neurotoxicity (Lantz et al., 2014), the gradual and persistent effect of GLA on Arc-Luc expression might be explained.

This study also confirmed that an important factor determining the susceptibility to pesticides is age. The comparison of BLI between the mice at 4 W and 8 W indicated that the juvenile mice might be more

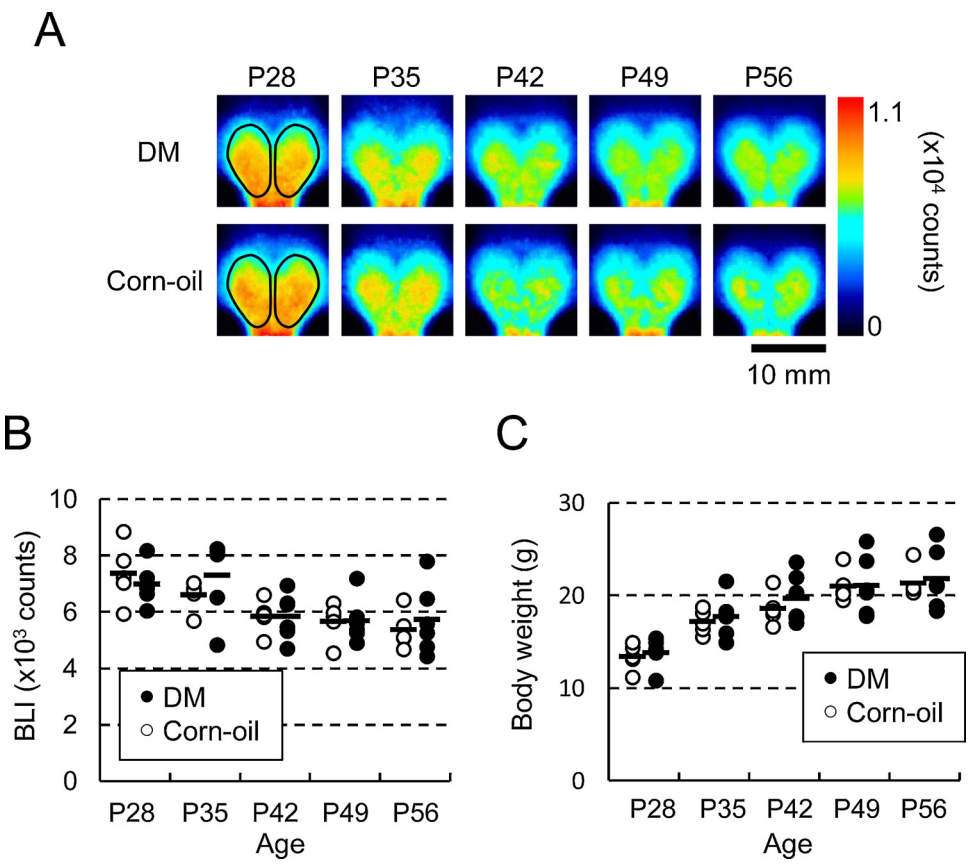


Fig. 4. Monitoring of long-term effect of low-dose DM treatment on neuronal activity.
A. Representative *in vivo* BLI of the *Arc-Luc* Tg HL mice under chronic treatment with low-dose DM. Representative images (pseudocolored, 0–11,000 counts) are shown from each time point at 1-week intervals (at P28, 35, 42, 49, and 56). Mice were orally administered DM (3 mg/kg BW) or corn-oil as a control every other day for 4 weeks from P28. Location of ROIs (black circles) is indicated in the images at P28. *Scale bar*, 10 mm.
B. Quantitative analysis of BLI for mice administered DM (n = 6, closed circles) and corn-oil (n = 5, open circles) during the 4-week treatment.
C. Changes of body weight of the DM-treated group (closed circles) and the corn-oil-treated group (open circles) during the 4-week treatment.
Means are marked by the thick horizontal lines in B and C.

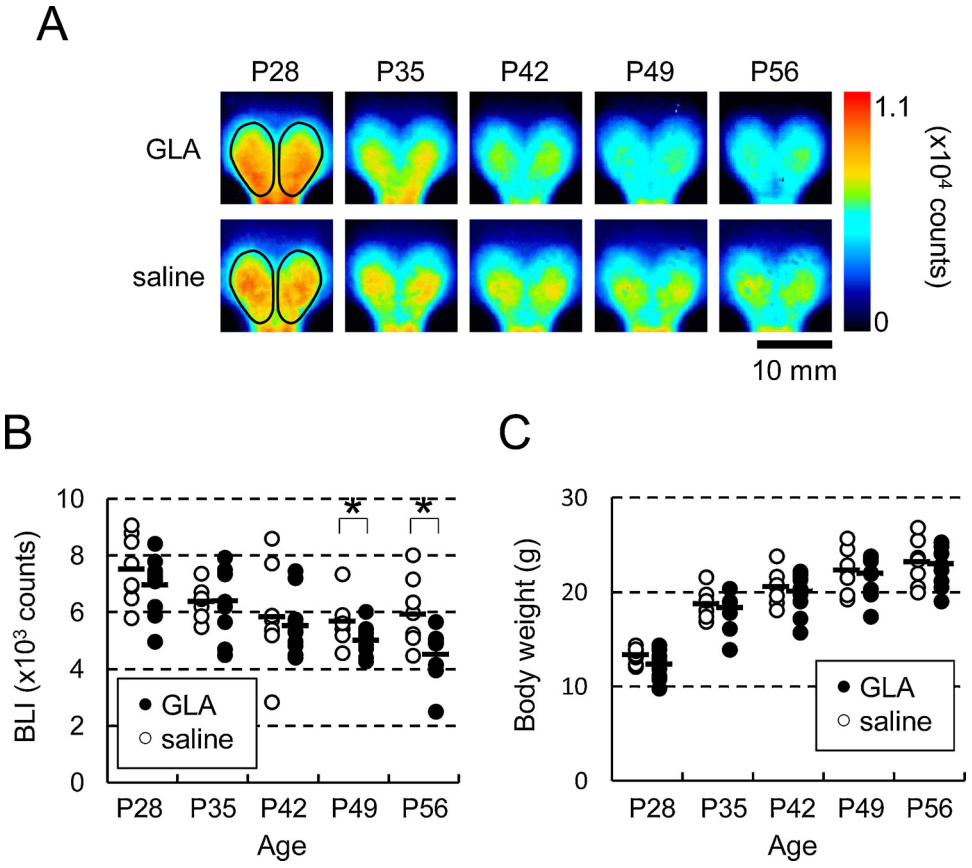


Fig. 5. Monitoring of long-term effect of low-dose GLA treatment on neuronal activity.
A. Representative *in vivo* BLI of *Arc-Luc* Tg HL mice under chronic treatment with low-dose GLA. Representative images (pseudocolored, 0–11,000 counts) are shown from each time point at 1-week intervals (at P28, 35, 42, 49, and 56). Mice were intraperitoneally injected with GLA (8 mg/kg BW i.p.) or saline as a control every day for 4 weeks from P28. The location of ROIs (black circles) is indicated in the images at P28. *Scale bar*, 10 mm.
B. Quantitative analysis of BLI for GLA-treated mice (n = 10, closed circles) and saline-treated mice (n = 8, open circles) during the 4-week treatment.
C. Changes of body weight of the GLA-treated group (closed circles) and control (open circles) during the 4-week treatment.
Means are marked by the thick horizontal lines in B and C. Data from each group were compared at the same time points. *$p <$ 0.05; two-way ANOVA with Turkey-Kramer post hoc test.

sensitive to DM (Figs. 2 and 4). While the adult mice exhibited obvious changes in bioluminescence signal and behavior, the juvenile mice showed only a slight increase in photon signal and a low rate of convulsions following acute GLA treatment (Fig. 3). As can be seen from the results in Figs. 4 and 5, Arc expression at the basal level decreased as the mice grew. This suggests that the structural remodeling of neural networks occurs widely in the developing brain, because of the involvement of Arc in synapse elimination to shape neural circuits (Mikuni et al., 2013). It is also recognized that the expression of metabolizing enzymes such as cytochrome P450, glutathione S-transferases, and microsomal epoxide hydrolase are altered in a manner depending on physiological (development, sex, pregnancy) and pathological conditions (diabetes, inflammation) (Kim and Novak, 2007). Therefore, the process of poisoning by a pesticide could vary depending on the developmental stage. Furthermore, developmental neurotoxic effects due to NAC were recently reported and it was suggested that a mechanism different from the inhibition of acetylcholinesterase (AChE) may underlie them (Lee et al., 2015). Several reports also described that IMI and its metabolites could have stimulatory effects as agonists of mammalian nicotinic acetylcholine receptor (nAChR) (Chao and Casida, 1997; Tomizawa et al., 2001). Kimura-Kuroda et al. (2016) reported the excitatory effect of IMI on the developing CNS using primary cultured cerebellar neurons from neonatal rats. Considering these reports, it remains possible that NAC and IMI cause aberrant neuronal activation during mouse development.

Although, in humans, epidemiological studies have suggested that deficits in cognitive and/or psychomotor functions follow cumulative exposure to pesticides, the neurotoxicity produced by repeated or prolonged exposures remains unclear. Thus, animal studies are important for our understanding of the effects (Moser, 2007). In this study, the chronic DM-treated group (3 mg/kg p.o., every other day) did not show significant differences in bioluminescence signal, although there was a tendency for the signal to increase at P35 (Fig. 4). In contrast to the lack of a significant effect of DM, we detected a decrease of bioluminescence signal in the brain area around the somatosensory cortex after GLA treatment (8 mg/kg i.p., every day) (Fig. 5). Considering these results with no obvious signs of symptoms, the doses used for chronic treatments are still well below the threshold for neuronal overexcitation as seen in the acute treatments (Figs. 1–3). Several reports have shown that chronic exposure to DM or GLA caused the impairment of spatial learning and memory in mice (Calas et al., 2008; Hossain et al., 2015). Although there are differences in the conditions of pesticide treatment and the age of the analyzed mice between those reports and our experiment, it is assumed that the adverse effects on neuronal activity shown in Fig. 5 lead to deficits in neurobehavioral function through interference with neural network integration after chronic exposure to low-dose pesticide as discussed below. We showed previously that the *Arc-Luc* Tg mice enable the detection of changes in neuronal activity associated with the object recognition task in home cages (Izumi et al., 2017). The development of photon counting technology makes it possible to measure bioluminescence signals in free-moving animals (Ono et al., 2015; Yamaguchi et al., 2016). In principle, it is possible to evaluate the association of abnormal changes in neuronal activity with neurological dysfunction by performing behavioral analysis and BLI on *Arc-Luc* Tg mice. Finally, from the perspective of the contribution of Arc to experience-dependent changes in neural connectivity (Evert and Greenberg, 2013; Jenks et al., 2017; Mandel-Brehm et al., 2015; Mikuni et al., 2013; Peebles et al., 2010; West and Greenberg, 2011; Zhang et al., 2015), the synapse must be carefully investigated after chronic exposure as a next step to elucidate the mechanistic basis for the effect of each pesticide on the CNS.

Thus, we evaluated the *Arc-Luc* Tg mice as an *in vivo* monitoring system for detecting the effects of pesticides on neuronal-activity-dependent Arc expression. These mice provided different spatiotemporal bioluminescence signal changes upon acute treatment with the four different pesticides (DM, GLA, NAC, and IMI). Furthermore, we detected a significant decrease of bioluminescence signal in this reporter mouse model after chronic treatment with low-dose GLA. Based on our results, we suggest that the *Arc-Luc* Tg mice are useful to assess the effects of pesticides on the CNS not only in the acute phase, but also across the lifespan. With pharmacological manipulations shown as Supplemental Fig. 2, we will also speculate some mechanisms of pesticide-induced neurotoxicity using *Arc-Luc* Tg mice and BLI.

Funding

This work was supported by a grant from the Food Safety Commission of Japan (No. 1001).

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

We thank Drs. Masaaki Tsuda (University of Toyama) and Mamoru Fukuchi (University of Toyama) for their constructive comments, Miki Shoji (University of Toyama) for technical advice regarding optical imaging and Hirofumi Nishizono (University of Toyama) for setting up the imaging experiments.

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