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Assessments of the effects of nicotine and ketamine using tyrosine hydroxylase-green fluorescent protein transgenic zebrafish as biosensors

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ABSTRACT

Transgenic zebrafish are a common vertebrate model system for the study of addictive behavior. In the present study, plasmid constructs containing green fluorescent protein (GFP) and the promoter of tyrosine hydroxylase (TH), a key synthetic enzyme for catecholamines, were produced. The TH-GFP constructs were microinjected into zebrafish embryonic cells. Three days post-fertilization, GFP began expressing in distinct catecholaminergic areas. The TH-GFP transgenic zebrafish were employed as live biosensors to test the effects of the commonly abused drugs nicotine and ketamine. First, locomotion assays were used to study the general excitatory effects of the drugs. Maximal locomotor activity was obtained after treatment with a high concentration of nicotine (10 μM), but with a much lower concentration of ketamine (0.1 μM). Second, TH protein levels in zebrafish brains were assessed by Western blot. TH protein levels were significantly increased, with maximal protein levels found after treatment with the same drug concentrations that gave maximal locomotor activity. Importantly, analysis of GFP in the zebrafish catecholaminergic areas revealed the same expression patterns as was obtained by Western blot. The present results indicate that increased locomotor activity can be correlated to TH protein expression, as indicated by Western blot and expression of TH-GFP. We have shown that TH-GFP expression is a reliable method to show the effects of drugs on TH expression that may be employed as a novel high-throughput live biosensor for screening drugs of abuse.

1. Introduction

The zebrafish, *Danio rerio*, is widely used in different areas of biological research (Zon, 1999; Panula et al., 2010), including neuroscience (Panula et al., 2006; Orger et al., 2008). Our previous report has strongly suggested that transgenic zebrafish are a good live biosensor for persistent organic pollutants (Hung et al., 2012). Zebrafish have a functional nervous system after 4–5 days of embryonic development, and thus may be an excellent model for studying vertebrate brain development. The basic structure of the central nervous system in zebrafish has all of the major domains that are found in the mammalian brain. Neurotransmitters such as dopamine and other catecholamines are also found in both interneuron systems and in long neuronal pathways (McLean and Fetcho, 2004; Panula et al., 2006; Filippi et al., 2010).

Tyrosine hydroxylase (TH) is necessary for catecholamine biosynthesis. It is commonly used as a marker for catecholaminergic neurons (Pickel et al., 1975; Arenzana et al., 2006). The distributions of catecholaminergic neurons in amniotes of mouse and rat have been extensively described (Schweitzer and Driever, 2009; Filippi et al., 2010; Panula et al., 2010). The largest difference in the zebrafish catecholaminergic system compared with the mammalian systems is the lack of midbrain dopaminergic neuron populations (Filippi et al., 2010; Panula et al., 2010). A projection of dopaminergic neurons in the posterior tuberculum (TPp) in the zebrafish is found to be equivalent to the mammalian ascending dopaminergic system (Reiner and Northcutt, 1992; Kaslin and Panula, 2001; Rink and Wullimann, 2001), which is the major focus of the present study.

Nicotine is highly addictive and has both stimulative and sedative effects to the brain. It can also decrease body temperature in animals, induce analgesic effects and enhance cognitive functions in mice (Biala and Kruk, 2009; Damaj et al., 1994). However, nicotine produces antidepressant-like effects in the rat forced swim test (Nowakowska et al., 2006; Popik et al., 2005), and acts as a typical anxiolytic drug (Ouagazzal et al., 1999). The stimulative effect was demonstrated by an increase in locomotor activity in rats (Clarke and Kumar, 1983; Vezina et al., 2007; Zaniewska et al., 2009). A biphasic effect of nicotine on activity has been shown, i.e., hyperactivity at low doses of nicotine, but hopomotility at high doses of nicotine (Clarke and Kumar, 1983;

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Picciotto, 2003; Vezina et al., 2007). Nicotine exposure also induces changes in dopaminergic activity in the zebrafish brain (Rice and Cragg, 2004; Stefano et al., 2007).

Ketamine is a noncompetitive antagonist of N-methyl-D-aspartate (NMDA) receptors (McDowell, 2004; Annetta et al., 2005; Ross, 2008; Chen et al., 2010). NMDA receptors are a subclass of ionotropic glutamate receptors that mediate excitatory transmission throughout the central nervous system (Dingledine et al., 1999). Several studies have demonstrated that ketamine can stimulate the activity of mesolimbic and mesostriatal dopaminergic neurons (French et al., 1993; Murase et al., 1993). In rats, activation of motor behavior was observed after a ketamine-induced increase in dopamine levels in the striatum (Wheeler et al., 1995; Vollenweider et al., 2000). After treatment with MK-801, another NMDA receptor antagonist (Javitt and Zukin, 1991), pronounced psychomotor disturbances like head twitching and head weaving were noted (Breese et al., 2002; Gilmour et al., 2009). Blocking NMDA receptors was also found to affect neurotransmitters and locomotion in zebrafish (Engeszer et al., 2007; Gerlai et al., 2000; Swain et al., 2004; Speedie and Gerlai, 2008).

Objectives of the present study were as follows: construct a TH-GFP plasmid, microinject the plasmids, obtain larval TH-GFP transgenic zebrafish, and employ these TH-GFP transgenic zebrafish as live biosensors. Detection of GFP expression was determined after treatment with nicotine and ketamine to determine TH protein levels. The intensities of the GFP fluorescence signals obtained were correlated with the locomotor activities of the larval fish and the levels of TH protein.

2. Methods

2.1. Animal care and maintenance

Standard fish care and maintenance protocols were carefully followed (Westerfield, 1995). Environmental variance was kept to a minimum for all behavioral experiments. Adult zebrafish were maintained in tap water, were kept on a photoperiod with 14 h of light and 10 h of dark and were fed twice daily with flake food. Embryos were sorted and cultured in purified water with methylene blue. Embryos and larval zebrafish were raised in a 28 °C humidified incubator.

2.2. Preparation of the TH-GFP plasmid

The zebrafish TH sequence was adopted from the Ensembl zebrafish database. A pair of primers was designed complimentary to the beginning of the promoter and to the end of the second exon sequence. The forward primer was 5′-TTT GAA ACA TTC AAA TGA CTC TTT-3′ and the reverse primer was 5′-CAC CTT GAG GTT TTC GAG-3′.

After cloning, the TH fragment was inserted into the pCR2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen). Digestion of pZsgreen1-1 plasmid (Clontech) and the pCR2.1-TOPO plasmid with the TH fragment (TOPO-TH) was performed with HindIII and ApaI (New England Biolabs) restriction enzymes. Then, the TH-GFP plasmid was produced by ligation of the TOPO-TH fragment and the pZsgreen1-1 plasmid with DNA ligase (New England Biolabs) at 16 °C overnight.

Finally, the ligated TH-GFP plasmid was confirmed by electrophoresis and sequencing.

2.3. Microinjection

Zebrafish embryos in the one cell stage were obtained by mating of adult male and female zebrafish. Adult male and female zebrafish were separated into two chambers overnight, and when the barrier between the two chambers was removed the next day, natural mating occurred and at least several dozen embryos were produced.

Fresh embryos were placed on an agar dish with troughs and microinjected with the aid of a stereomicroscope (Olympus) and a micromanipulator (Harvard Apparatus). Micropipettes were made on a needle puller using 1–2 mm fiber-filled glass capillary tubing. The plasmids were injected through a continuously flowing pipette; the flow rate was controlled by an application of pressurized air.

2.4. Six-hydroxydopamine (6-OHDA) treatment

Zebrafish larvae were treated 5 days post-fertilization (dpf) in the presence of 62.5 μM of 6-OHDA (Sigma) and 0.02% ascorbic acid for 24 h. After 6-OHDA treatment and a phosphate buffered saline (PBS) wash, zebrafish were fixed in 3% paraformaldehyde and mounted in DAKO Fluorescent mounting medium (Invitrogen) for further microscopic observation. Anti-TH immunostaining and analysis of GFP intensity was performed in TH-GFP transgenic zebrafish to evaluate the viability of dopaminergic neurons in the brain.

2.5. Nicotine and ketamine treatment

Nicotine- and ketamine-induced behavioral, physiological and neuroregulatory effects were examined in different assays. A dose range from 0.1 to 50 μM of (-)-nicotine hydrogen tartrate salt (Sigma) or ketamine hydrochloride (Jiang Su Heng Rui Medicine Company Limited, China) was employed on 5 dpf zebrafish. The nicotine solution was prepared by dissolving nicotine hydrogen tartrate salt powder, whereas the ketamine-containing media was adjusted by diluting the ketamine hydrochloride solution in aquarium water. After preparation, zebrafish were directly exposed to nicotine or ketamine in solution during treatment.

2.6. Locomotion assay

Swimming activities of zebrafish larvae were recorded in 90 mm × 15 mm Petri dishes. Because environmental changes could evoke transient changes in activity, larvae were placed in the treatment dish for at least 10 min prior to visualization and recording. The spontaneous locomotor activity of each group of zebrafish was recorded with a digital camera suspended above the treatment dish. The experimental setup was designed to capture displacements of larvae in a horizontal plane and the locomotor activity was measured as the total distance swam by the larvae. Changes in movement were obtained by overlaying the initial photograph on the fourth photograph in each series and measuring the distance between the images using Adobe Photoshop software.

2.7. Immunohistochemistry

Zebrafish were fixed with 3% paraformaldehyde for 4–6 h at 4 °C. After PBS and distilled water washes, an acetone shock was performed at −20 °C for 7 min. The fixed fish were then washed again with distilled water followed by PBS. Larvae were then blocked with 5% normal goat serum in 1% bovine serum albumin (BSA) and 1% DMSO in PBS (BSA/DMSO/PBS) for 30 min, and stained with rabbit anti-TH primary antibody (1:2000, Chemicon) or rat anti-DAT primary antibody (1:2000, Chemicon) overnight at 4 °C. After rinsing with BSA/DMSO/PBS, a secondary incubation was performed with fluorochrome-conjugated goat anti-rabbit secondary antibodies (Alexa 488, Molecular Probes) or with goat anti-rat secondary antibodies (Alexa 633, Molecular Probes) in

PBS/BSA/DMSO overnight at 4 °C. The immunoreactions were then washed 3 times with PBS/BSA/DMSO and mounted in DAKO Fluorescent mounting medium for microscopic observation.

2.8. Western blot analysis

Zebrafish larvae subjected to Western blot analyses were de-yolked manually with a razor blade and homogenized in lysis buffer using a tissue homogenizer. Lysates were then centrifuged at 14,000g for 10 min at 4 °C. The supernatant was stored at −80 °C.

After the protein was quantified, lysates were denatured at 100 °C for 5 min. An equal amount of protein (30 μg/well) was electrophoresed on an 8% SDS-PAGE gel. The proteins were then electroblotted to PVDF membranes (Bio-Rad) and blocked with 5% non-fat skim milk for an hour. Subsequently, the blot was incubated in rabbit anti-TH primary antibodies (1:3000, Chemicon) supplemented with 2% non-fat skim milk for 2 h at room temperature. After washing, the membrane was incubated in horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibodies (1:5000, Zymed Laboratories) in 2% non-fat skim milk for an hour at room temperature. Bands on the membranes were visualized using chemiluminescence detection reagents (Abfrontier WESTSAVE Up (Western Blotting Substrate)). Distinct immunoreaction products were revealed and images of the bands were developed on film (Biomax X-ray film, Kodak).

2.9. Microscopy and imaging

Confocal z-stack images of fluorescent proteins and brightfield images were obtained with a Laser Scan Confocal Microscope (FluoView^{TM} FV1000, Olympus). Optical slices 5 μm thick, were scanned sequentially to obtain Z-stack images of larval GFP expression in whole brain. Intensities of the images were measured with Metamorph software.

2.10. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with SPSS software. Data were determined to be statistically significant if the P value was <0.05. Each point represents the mean ± SEM.

3. Results and discussion

3.1. Production of transgenic TH-GFP zebrafish

The TH-GFP plasmid was constructed by inserting the TH fragment into the pZsgreen1-1 plasmid (Fig. 1). The plasmids were then microinjected into zebrafish embryos. TH and DAT immunostaining results are shown in Fig. 2. The expression of green fluorescent protein was identical to previous studies that showed that the distribution of TH throughout the olfactory bulb (OB), telencephalon (Tel), posterior tuberculum (TPp), pretectal area (PPv) and periventricular hypothalamus (PTN). Anti-DAT immunostaining was performed to confirm the localization of TH. However, the intensity of the fluorescence between the immunostaining and TH-GFP transgenic zebrafish were slightly different (Fig. 1).

Compared to the immunostaining, greater GFP expression was found in the TH-GFP transgenic zebrafish in PPv and TPp areas of the dorsal diencephalon, yet GFP expression in the TH-immunostained zebrafish was greater in the OB. It has previously been shown that TH distribution in zebrafish was greater in the diencephalic area, which was similar to the results obtained in the transgenic fish, rather than those obtained by immunostaining (Ryu et al., 2006; Chen et al., 2009; Filippi et al., 2010). The deviation of TH expression

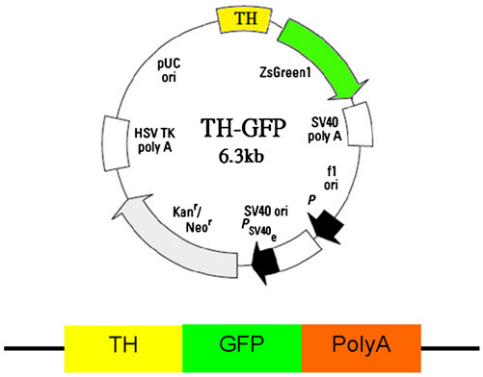

Fig. 1. TH-GFP plasmid construct. The TH sequence was inserted into pZsgreen1-1 plasmid. The linear DNA fragment showed the construction of fusion DNA.

intensity might be due to the antibody used for immunostaining. Because the antibodies used in the present study were produced in rabbits, the difference in homology between rabbit and zebrafish TH might have caused the different results obtained with the antibody staining compared with those obtained in the transgenic zebrafish.

Weak green fluorescence was observed in the control zebrafish. This autofluorescence expressed throughout the whole body of the zebrafish with no specific localization. The relative contribution and the cause of the autofluorescence are still unknown. Other studies also mentioned this problem; it was suggested that the background might due to weak laser reflection of the zebrafish scales. However, no solution to this problem has been suggested. This problem was improved by narrowing the absorption spectra to eliminate the autofluorescence. Therefore, the major fluorescent signal recorded from the TH-GFP fluorescence, minimizing the background autofluorescence.

The level of fluorescence was higher 5 dpf compared with the others. This may be due to different transfection efficiencies during different developmental stages of the zebrafish. Other studies support the data that reduced transfection efficiency was observed in older zebrafish (Hendricks and Jesuthasan, 2007). Because the greatest expression of TH was observed at 5 dpf, all of the experiments in the present study were performed with zebrafish that were 5 dpf.

3.2. 6-OHDA treatment confirmed successful transfection of the TH-GFP plasmid

Transfection of the TH-GFP plasmid was further confirmed by a 6-hydroxydopamine (6-OHDA) challenge in the transgenic zebrafish (Fig. 3). Anti-TH immunostaining was used to compare the viability of dopaminergic neurons in zebrafish receiving the same doses of 6-OHDA. After treatment with neurotoxic 6-OHDA, TH expression was dramatically reduced. Previous studies also demonstrated 6-OHDA toxicology; zebrafish exposed to 6-OHDA undergo a significant loss of dopaminergic neurons and reduced locomotion behavior (Anichtchik et al., 2004).

In the control zebrafish, TH fluorescence intensity was not changed, whereas changes were significantly observed in anti-TH immunostained as well as transfected zebrafish after 6-OHDA treatment. Nearly a 50% reduction was consistently observed in the TH-immunostained and transfected zebrafish, mainly in the ventral diencephalic area.

3.3. Drug treatment induced an aberrant swimming pattern

The normal larval zebrafish swimming pattern is a horizontal zig-zag pattern, the same pattern as an adult zebrafish (Giacomini et al., 2006). Although the swimming path varied among the

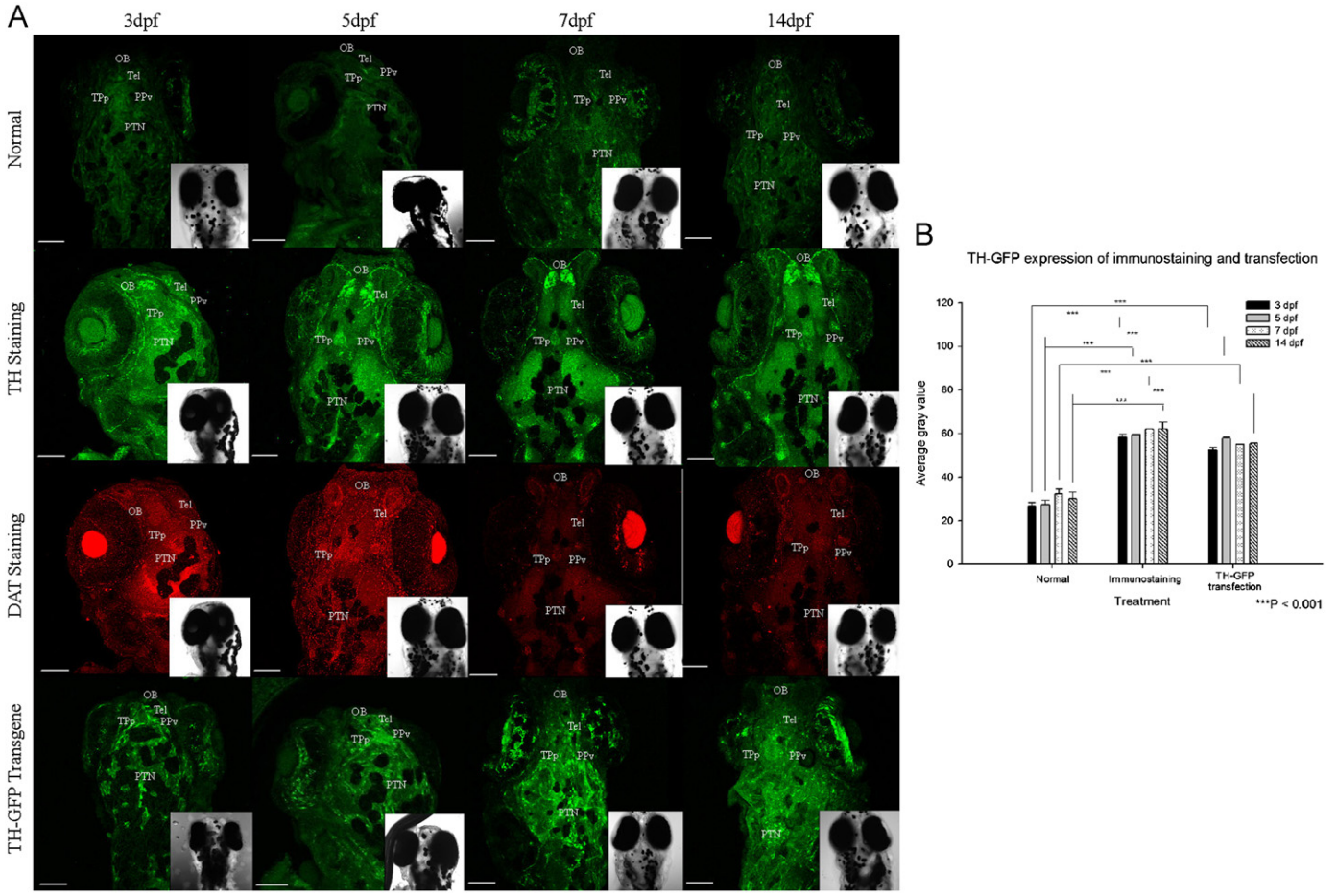

Fig. 2. (A) Confocal micrographs showing normal, anti-TH, anti-DAT immunostained and transgenic zebrafish on 3, 5, 7 and 14 dpf. Expression of fluorescence was found mainly in the telencephalon (OB, PPa,) and diencephalon (PPv, TPp, PTN) areas. The consistent fluorescent expression confirmed the transfection of the TH-GFP plasmid in the zebrafish. $N=5$ for each result. Scale bar: 100 μm (valid for all micrographs). (B) Histogram showing the intensity of green fluorescence in control, anti-TH, anti-DAT immunostained and TH-GFP plasmid transfected zebrafish. The fluorescence intensities were measured as average gray value ± standard error of the mean (SEM). The intensities were compared by one-way ANOVA using Turkey's multiple range test at a significance level of 95% ($P<0.05$) with SPSS. There was a significant increase in green fluorescence in zebrafish after anti-TH immunostaining or TH-GFP plasmid transfection. $N=5$ for each result.

individual fish, the characteristics of the swimming pattern were the same.

Aberrant swimming patterns were defined as rapid bouts of swimming, which was highly unpredictable, and interrupted by direction changes (Maximino et al., 2010). The normal zebrafish swimming pattern was altered by substances such as nicotine and ketamine. After treatment, the zebrafish swimming direction changed, and more loop-like swimming patterns were found (Fig. 4). There were also continual spontaneous turns, in which the zebrafish turned more than 90°. Previous studies have shown a relationship between anxiety and erratic swimming behavior in zebrafish (Clarke and Kumar, 1983; Picciotto, 2003; Vezina et al., 2007). Because the abnormal swimming behavior observed previously was similar to the observations in the present study, it was conceivable that exposure to high doses of nicotine and low doses of ketamine promoted anxiety in the zebrafish. Because the underlying mechanisms were unknown, the erratic swimming pattern was hypothesized to be due to impairment of the neuromuscular synapses in the zebrafish motor neurons that led to abnormal muscle contraction or exaggerated synaptic transmission.

3.4. Drug treatment enhanced locomotion activity dose dependently

Previous studies had demonstrated that rodent locomotor activity was induced by nicotine (Clarke and Kumar, 1983; Vezina et al., 2007; Zaniewska et al., 2009). More surprisingly, the effect was dose dependent and biphasic, as shown by hyperactivity after treatment with low nicotine doses, and hopomotility after treatment with extremely high doses (Clarke and Kumar, 1983; Picciotto, 2003; Vezina et al., 2007). The underlying mechanism of the stimulatory effect of acute nicotine exposure was direct; nicotine acted on nicotinic acetylcholine (nACh) receptors, which further induced the release of dopamine and excitatory synaptic transmission (Heinemann et al., 1990; McGehee and Role, 1995; Elgoyhen et al., 2001; Mansvelder and McGehee, 2002). This hypothesis was also tested in a study that used 6-OHDA-induced lesions to study locomotor activity in relation to dopamine in the rodent nucleus accumbens (Anichtchik et al., 2004). This study provided an interesting perspective by suggesting that dopamine was involved in the regulation of nicotine-induced locomotor activity.

These results demonstrated the dose-dependent relationship of nicotine to increase locomotor activity in zebrafish. From increasing nicotine exposure of 0.1–10 μM, the distances swam were increased in a dose-dependent manner, and peaked at the 10 μM dose. This result illustrated that after 10 μM nicotine treatment, the α7 nACh receptors were highly activated, whereas the non-α7 nACh receptors were mostly desensitized. A dose of 10 μM nicotine induced a large dopamine release and excitatory synaptic transmission compared with to treatment with 0.1 μM, 1 μM and 50 μM nicotine. However, the inverted U-shaped dose-dependent response shows a

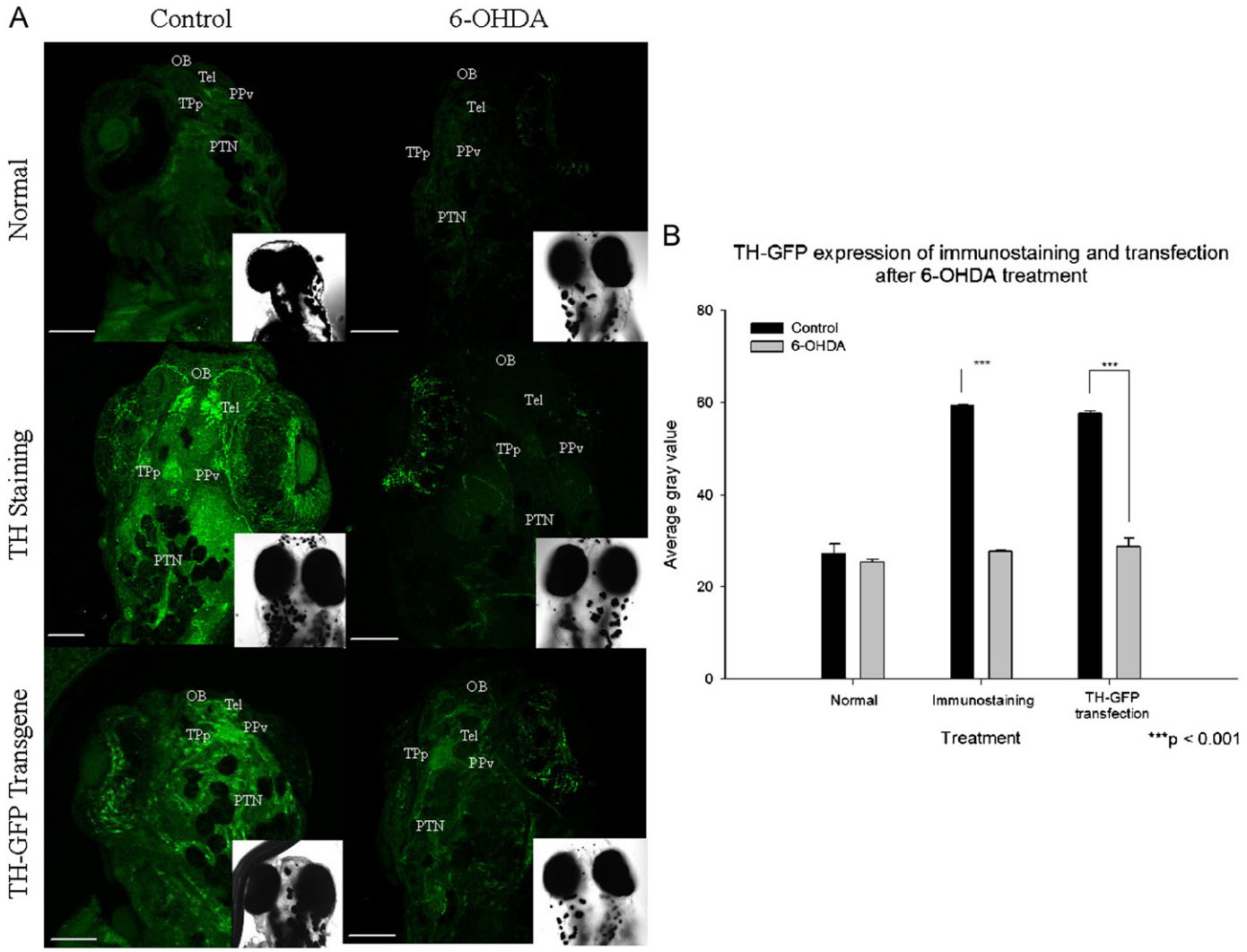

Fig. 3. (A) Confocal micrographs showing reduced TH expression in immunostained and transgenic zebrafish after 6-OHDA treatment. Fluorescence was sharply reduced after 6-OHDA treatment. The consistent fluorescent expression confirmed the transfection of the TH-GFP plasmid in the zebrafish. $N=5$ for each result. Scale bar: 100 μm (valid for all micrographs). (B) Histogram showing the intensity of green fluorescent protein in control, anti-TH immunostained and TH-GFP plasmid transfected zebrafishes after 6-OHDA treatment. The fluorescence intensities were measured as average gray value ± SEM. The intensities were compared by one-way ANOVA with Turkey's multiple range test at a significant level of 95% ($P<0.05$) with SPSS. There was significant decrease of green fluorescent protein expression in zebrafish after 6-OHDA treatment. $N=5$ for each result.

slight inhibitory effect of swimming activity after treatment with 50 μM nicotine, which indicated that the excitation of downstream transmission had reached its peak. This might be explained by desensitized nACh receptors or other inhibitory effects induced by high doses of nicotine.

However, zebrafish tended to swim faster during exposure to 0.1 μM and 1 μM ketamine. Previous studies similarly found that circling and swimming activities of zebrafish were dramatically increased after exposure to ketamine or MK-801, which is an NMDA receptor antagonist (Riehl et al., 2011). Ketamine, through NMDA receptor antagonism, also initiated behavioral alterations in other animal models (Ali et al., 1995; Wilson et al., 2005).

The increased activity was explained by an increase in dopamine release evoked by ketamine exposure. Ketamine triggered the NMDA receptor, which indirectly regulates the release and synthesis of dopamine. (Krebs et al., 1991; Johnson and Jeng, 1991; Desce et al., 1992; Wheeler et al., 1995) Because blocking NMDA receptors led to reduced NMDA receptor function, glutamatergic excitatory transmissions should have been suppressed. However, this study had opposite results, and suggested that ketamine treatment caused an increase in glutamatergic transmission (Gunduz-Bruce, 2009). Grunze suggested that interactions between excitatory and inhibitory neurons played an important role in the response to ketamine. Furthermore, NMDA receptor blockade was associated with approximately 10-fold greater effects on GABAergic cells than on glutamatergic cells (Grunze et al., 1996). Therefore, these results supported that the reduced function of the NMDA receptor leads to increased release of glutamate and dopamine to disinhibit major excitatory pathways.

Additionally, high doses of ketamine had anesthetic features that apparently inhibited swimming activity in the zebrafish. The zebrafish seemed inactive and did not swim, but drifted after exposure to 10 μM or 50 μM ketamine. The highest doses of ketamine might exceed the subanesthetic range of the larval zebrafish. Cachat also found that zebrafish treated with hallucinogenic or opioid drugs might exhibit trance-like passive swimming or epilepsy-like states (Cachat et al., 2011). Ketamine, like other anesthetics, has anesthetic effects in zebrafish because of NMDA receptor antagonism (Wagner et al., 2001). Altogether, the subanesthetic and anesthetic effects of ketamine were clearly exhibited by increased or and inhibited swimming activity in zebrafish at low and high doses of ketamine, respectively.

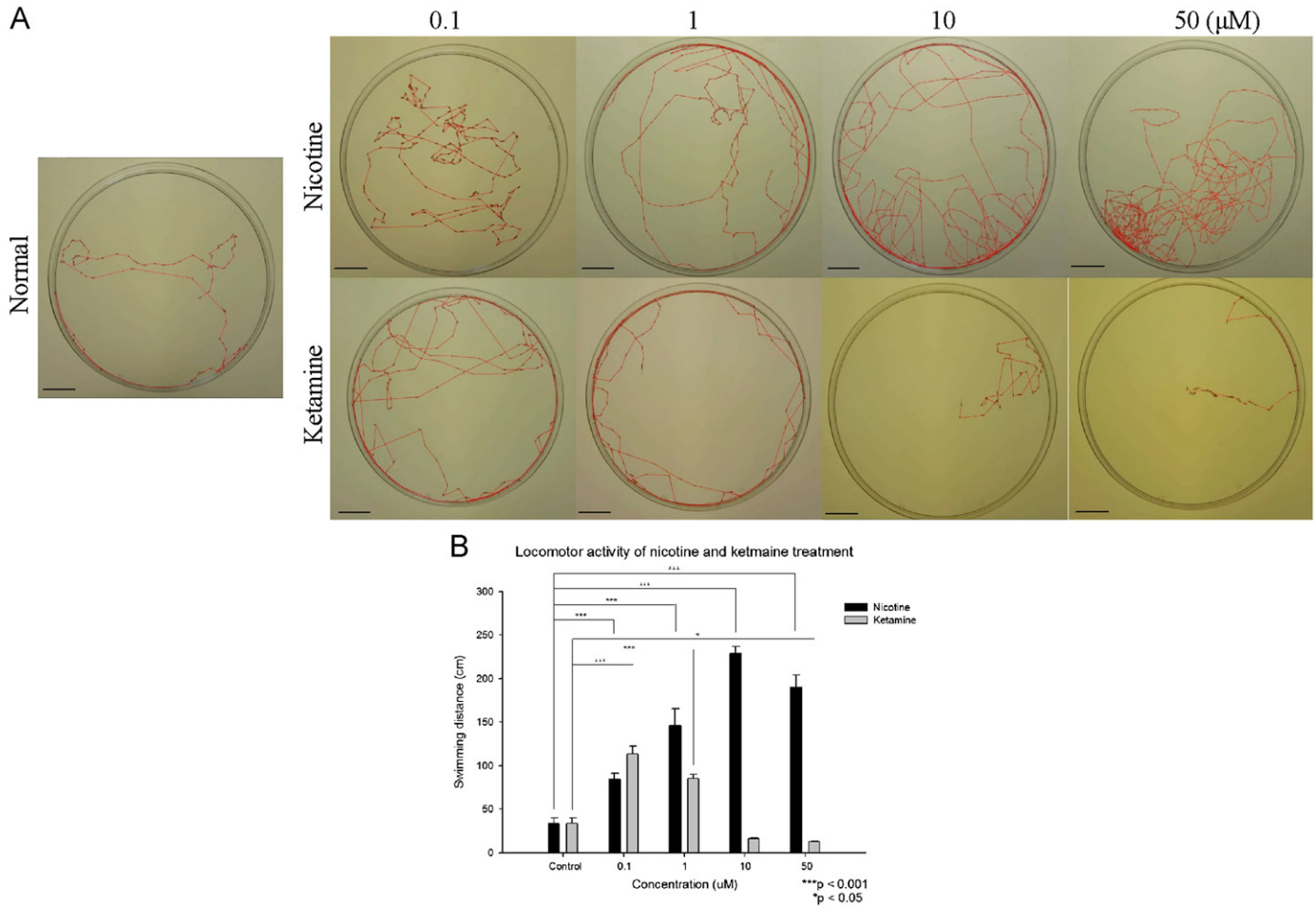

Fig. 4. (A) Swimming patterns of zebrafish during nicotine or ketamine treatment. Normal zebrafish swam smoothly and in a horizontal zig-zag pattern. After 0.1 μM, 1 μM, 10 μM or 50 μM nicotine or ketamine treatment, swimming patterns of zebrafishes were observed individually. During nicotine exposure, swimming distances and speeds were increased, and the aberrant swimming pattern was also observed by frequent changes in swimming direction and unpredictable trails. Aberrant swimming patterns were observed in low-dose and high-dose nicotine treatment; however, the distance swam and aberrant swimming pattern occurred more frequently after high-dose nicotine exposure. During ketamine exposure, swimming distances and speeds were increased at low-dose treatments, and the aberrant swimming pattern was also observed by frequent changes in swimming direction and unpredictable trails. However, suppressed swimming activities were observed after high-dose ketamine treatment. $N=5$ for each result. Scale bar: 1.4 cm (valid for all micrographs). (B) Histogram showing the locomotion activities of zebrafish during nicotine or ketamine treatment. The swimming distances were measured as average gray value ± SEM. The intensities were compared by one-way ANOVA with Turkey's multiple range test at a significant level of 95% ($P<0.05$) with SPSS. Locomotor activity was increased after treatment with increasing doses of nicotine. $N=5$ for each result.

3.5. Drug treatments increased TH protein level dose-dependently

Because increased locomotor activity was caused by dopamine release from the glutamatergic terminal, TH protein levels were further assessed by Western blot.

After serial doses of nicotine, an inverted U-shape dose-dependent response to nicotine is shown in Fig. 5. The increased TH levels found after treatment with nicotine concentrations of 0.1–50 μM were explained by activation of nACh receptors. As more nACh receptors were activated, more dopamine was released, which resulted in elevated TH protein levels. However, the increase in TH protein levels was slightly reduced after treatment with 50 μM nicotine. This may be explained by saturation and desensitization of activated nACh receptors, high doses of nicotine start to inhibit the increased synthesis and release of dopamine. The detailed mechanism was discussed above.

In the zebrafish that were treated with ketamine, a similar trend showed increases in TH levels after treatment with 0.1 and 1 μM ketamine. The increased levels of TH also accounted for the increased dopamine release. When NMDA receptors are blocked, dopamine is indirectly released because of disinhibition of GABAergic neurons (Zhang et al., 1993; Andersson et al., 1994; Svensson et al., 1995). Furthermore, TH levels after exposure to 10 and 50 μM ketamine did visibly change compared with control zebrafish. Because treatment with high doses of ketamine were expected to cause a narcotic and analgesic response (Nishizawa et al., 2002; Bencan and Levin, 2008), the unchanged TH levels indicate that there were no changes in the total amounts of dopamine in the anesthetic stage of zebrafish. Similar studies support this result that ketamine treatment did not change total dopamine levels in mice (Kelland et al., 1990; Irifune et al., 1997), and had no effect on striatal dopamine metabolism in rats (Koshikawa et al., 1988).

3.6. Drug treatments increased TH-GFP expression in transgenic zebrafish in a dose-dependent manner

In addition to Western blot analysis of TH protein levels, the intrinsic TH levels could also be assessed in the previously produced TH-GFP transgenic zebrafish.

Expression of TH in the transgenic zebrafish after the same treatment was consistent with former assessments. The similar inverted U-shape dose-dependent results showed increasing TH expression with increasing nicotine concentration from 0.1 to 10 μM. Strong green fluorescence intensity indicative of increased

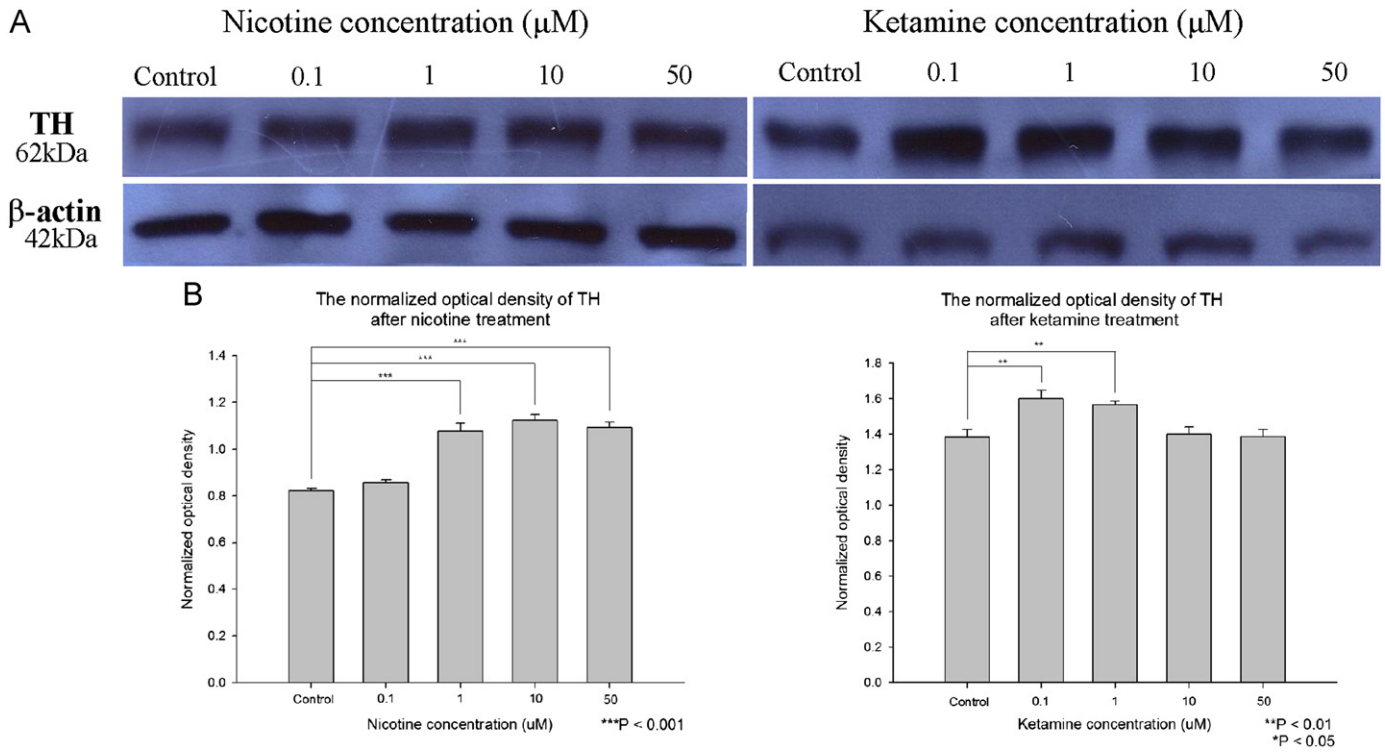

Fig. 5. (A) Western blot analysis of TH protein levels after nicotine or ketamine treatment in zebrafish. TH proteins (~62 kiloDalton (kDa)) were examined after treatment with 0.1 μM to 50 μM of nicotine or ketamine and normalized using the optical densities of β-actin proteins (~42 kDa). There was a marked increase in TH expression found after treatment increasing nicotine concentrations from 0.1 μM to 10 μM, while there was a slight decrease found after treatment with 50 μM nicotine. There was marked increase in TH expression found after treatment with increasing ketamine concentrations of 0.1 μM and 1 μM, whereas TH protein expression recovered to the control level after treatment with 10 μM and 50 μM ketamine. (B) Histogram showing the normalized optical density of TH after treatment with 0.1 μM to 50 μM nicotine or ketamine. The intensities were measured as average gray value ± SEM. The intensities were compared by one-way ANOVA with Turkey's multiple range test at a significant level of 95% ($P < 0.05$) with SPSS. $N = 5$ for each result.

TH expression illustrated greater release of dopamine and elevation of dopaminergic neurons in the diencephalic area of the zebrafish (Fig. 6). As mentioned above, the elevated TH expression was explained by the direct actions of nicotine on nACh receptors, which further induce the release of dopamine. As the response reaches its peak after 10 μM nicotine treatment, it was hypothesized that the dopamine levels were saturated and no further induction of dopamine could occur after treatment with higher doses of nicotine.

Nevertheless, in the high-dose 50 μM nicotine treatment, there was a slight drop in TH expression. It was also mentioned above that high doses of nicotine inhibited both locomotor activity and TH protein levels. Similar inverted U-shaped dose effects were also reported for many other drugs that inhibit activation of the target at high doses. The underlying mechanism was discussed, that saturation and desensitization of activated nACh receptors occurred after treatment with high doses of nicotine, thus inhibiting the increased synthesis and release of dopamine.

After a treatment with increasing doses of ketamine, TH expression increased, especially in the ventral tegmental area, as shown in the zebrafish after treatment with 0.1 and 1 μM ketamine. The increased TH expression was explained by dopamine release. Pharmacologically, ketamine blocked NMDA receptors, which further increased dopamine release, as was discussed above in detail.

However, after treatment with 10 and 50 μM ketamine, TH levels were reduced to the control level. Although high-dose ketamine treatment of zebrafish showed anesthetic characteristics, earlier studies proved that ketamine did not alter dopamine metabolism in the frontal cortex, nucleus accumbens, striatum or hippocampus of mice (Irifune et al., 1997). Furthermore, the results are consistent with the electrophysiological findings that high doses of ketamine did not change the basal firing rates of nigrostriatal dopamine neurons during anesthesia in rats (Kelland et al., 1990; Irifune et al., 1997). Because ketamine exerted its anesthetic effects by inhibiting the NMDA receptor, prior studies suggest that it might further block sodium channels with features similar to other local anesthetics (Wagner et al., 2001). The detailed complex mechanisms that regulate dopamine homeostasis in the zebrafish anesthetic stage remain unclear.

3.7. Sensitivities of the biosensor to ketamine and nicotine

Analysis of the western blotting and the GFP expression revealed that the nicotine- and ketamine-treated transgenic zebrafishes increased the TH expression and GFP expression respectively. In the examination of the GFP expression, for nicotine, a dose-response was found from 0.1 μM to 50 μM, while the transgenic zebrafishes responded to the ketamine from 0.1 μM to 1 μM. This zebrafish-based biosensor was found to have a relatively wider detection range for the nicotine and the lower limits of detection to both of the drugs were the same at 0.1 μM (Fig. 6).

4. Conclusion

Dose responses of drugs were clearly shown in the TH-GFP transgenic zebrafish, thus suggesting its use as a biosensor for drugs of abuse. Exposure to nicotine and ketamine markedly induced intrinsic TH levels in the TH-GFP transgenic zebrafish, thus suggesting that these fish could be used to detect the presence of drugs. The results from the present study strongly suggest that despite different physiology and habitat, the simple and easily assessed zebrafish appear to be an adequate model for studying intrinsic dopaminergic neuron regulation in the brain, especially in respect to behavioral and physiological responses.

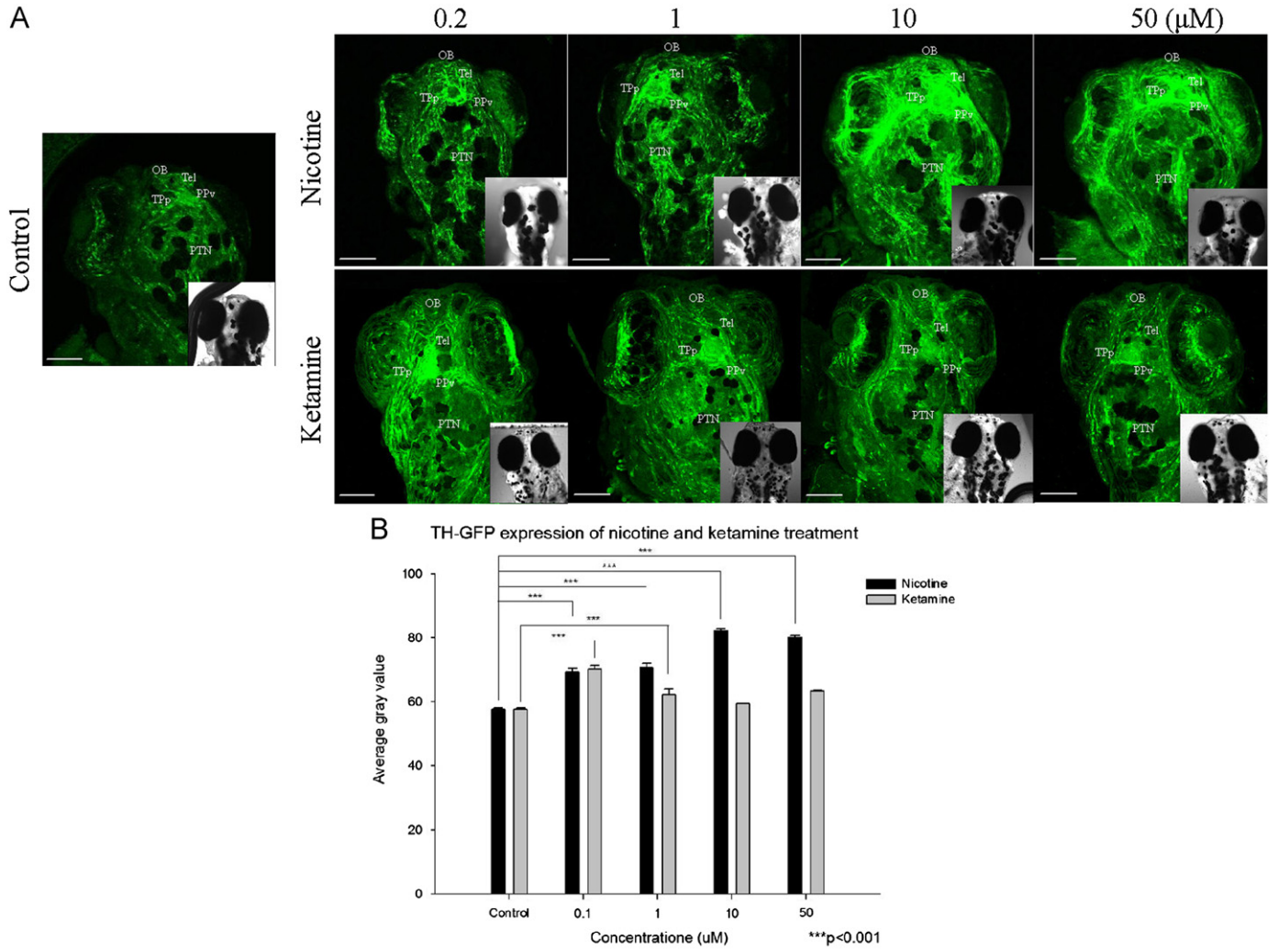

Fig. 6. (A) Confocal micrographs showing TH-GFP expression in zebrafish after nicotine or ketamine treatment. Zebrafish were treated with 0.1 μM, 1 μM, 10 μM and 50 μM nicotine or ketamine. Increased TH-GFP expression was shown after treatment with increasing nicotine concentrations from 0.1 μM to 10 μM, whereas there was a slight decrease of TH-GFP expression after treatment with 50 μM nicotine. Increased TH-GFP expression was shown after treatment with increasing ketamine concentrations from 0.1 μM to 1 μM, whereas the expression of TH-GFP returned to original levels in the fish that were treated with 10 μM and 50 μM ketamine. $N=5$ for each result. Scale bar: 100 μm (valid for all micrographs). (B) Histogram showing the intensity of TH-GFP expression of zebrafish after nicotine or ketamine treatment. The fluorescence intensities were measured as average gray value ± SEM. The intensities were compared by one-way ANOVA with Turkey's multiple range test at a significant level of 95% ($P<0.05$) with SPSS. $N=5$ for each result.

Taken together, parallel responses to drugs obtained from three assays suggested that the TH-GFP transgenic zebrafish model is an excellent vertebrate model system that reveals the direct effects of intrinsic TH levels after treatment with chemicals or metabolites in a rapid, time- and cost-effective manner. This novel, high-throughput biosensor is highly recommended to be used for drug testing.

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