

Benzo[a]pyrene represses synaptic vesicle exocytosis by inhibiting P/Q-type calcium channels in hippocampal neurons

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ABSTRACT

Humans are exposed to the common carcinogen benzo[a]pyrene (BaP) by ingesting contaminated foods and water or inhaling polluted air. Given the enriched lipids and reduced antioxidative properties in the brain and the accumulation of BaP in the brain due to its high lipophilicity, the brain is susceptible to BaP-induced toxicity. Exposure to BaP leads to impairments in learning and memory, increased anxiety behavior, and neuronal death. It induces protein dysfunctions in neuronal compartments that play essential roles in neuronal activity or physiology. However, the neurotoxicity of BaP on presynaptic terminals, which is crucial to neurotransmission by releasing synaptic vesicles that contain neurotransmitters, has not yet been investigated. In the present study, we investigated the toxicity of BaP at presynaptic terminals in living hippocampal neurons. These neurons were sourced from transgenic mice pups (postnatal 1-day, a total of 12 pups, equal numbers for each sex) that endogenously express synaptic vesicle-fused pHluorin, which is a green fluorescent protein that enables monitoring of synaptic vesicle dynamics. We observed that BaP suppressed synaptic vesicle exocytosis by inhibiting presynaptic Ca^{2+} entry via P/Q-type Ca^{2+} channels. Together with molecular docking simulation, we speculate that BaP and metabolites may bind to the P/Q Ca^{2+} channels. These results suggest the toxic mechanism of BaP exposure-induced abnormal behavior that provides a basis to evaluate the risk assessment of BaP-induced neurotoxicity.

1. Introduction

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) commonly found in cigarette smoke, broiled foods, grilled meat, industrial processes, traffic emissions, and other sources of air pollution (Ramesh et al., 2004). Due to its prevalence in our food and environment, exposure to BaP primarily occurs through the ingestion of contaminated food and water or the inhalation of polluted air. In fact, dietary BaP intake is the major source of BaP exposure in humans ranging from 2 to 500 ng/day (Lioy et al., 1988). The carcinogenicity of BaP is well-established, as BaP is classified as a carcinogen by the International Agency for Research on Cancer (IARC) and Environmental Protection Agency. BaP and its metabolites can induce DNA adducts and double-strand breaks, and they can activate aromatic hydrocarbon receptor (AhR), which plays an essential role in carcinogenesis. Additionally, BaP and its metabolites can cause genotoxicity, epigenotoxicity, and teratogenicity (Bukowska et al., 2022).

Many studies suggest that the brain is highly susceptible to BaP-induced toxicity. This is due to the brain's enriched lipid content,

diminished antioxidative properties, and rapid metabolic rate (Cobley et al., 2018; Saunders et al., 2002). As BaP is a highly lipophilic compound, it easily penetrates the blood–brain barrier (BBB) and accumulates in the brain (Grova et al., 2011). BaP exposure has been shown to induce brain toxicity in several species, leading to a range of effects such as behavioral impairment, cognitive dysfunction, anxiety behavior, neuronal death, glial cell-activated neuroinflammation, and increased oxidative stress (Bukowska et al., 2022). Also, BaP aggravates behavioral deficit and neuropathology in neurodegenerative disease by promoting plaque deposition, neuroinflammation, and neuronal loss (Liu et al., 2020). The need to evaluate BaP in neurotoxicity has become evident, as this aspect has been overlooked in the risk assessment of BaP (Chepelev et al., 2015). Given that BaP-induced neurotoxicity primarily occurs at lower doses and with less exposure time than BaP-induced carcinogenicity, the brain is particularly vulnerable to BaP-induced toxicity. However, compared to toxicity in other organs, the neurotoxicity of BaP is less well-understood. Therefore, investigating the neurotoxic effects of BaP and identifying the detailed mechanism of its action are essential for accurate risk assessment of BaP's impact on the brain.

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In neurons, BaP decreases the number and complexity of dendritic spines (Zhang et al., 2022), reduces N-methyl-D-aspartate receptor (NMDAR) expression (Schellenberger et al., 2013), and disrupts neurotransmission (Yang et al., 2017). Although the toxic effects of BaP in neurons have been investigated using behavioral and biochemical analyses, little is known about the toxic effect of BaP on presynaptic activity, which plays a critical role in neurotransmission by controlling synaptic vesicle (SV) release and recycling. In the present study, we examined the toxic effect of BaP on presynaptic activity and identified the mechanism in living hippocampal neurons using live cell imaging.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee of Chung-Ang University (approved protocol number: 2020-00049). B6;CBA-Tg(Thy1-spH) 21Vnmu/J (spH 21) transgenic mice were donated by Dr. Venkatesh N Murthy (Li et al., 2005) and purchased from Jackson's laboratory (Strain # 014651) for presynaptic imaging. After confirming the initial transgenic mice, they were maintained in breeding pairs of each sex. In breeder cages, the carrier and the noncarrier mice were distinguished by genotyping, and noncarrier mice were used as wild type (WT). Mice were maintained in a 12-h light/dark cycle at 15–25 °C with ad libitum access to food and water. C57BL/6J mouse pups were purchased from Nara Biotech (Seoul, Republic of Korea) for presynaptic Ca^{2+} imaging. Postnatal 1-day mouse pups (weighting 1.4–1.6 g) were used for hippocampal neuron culture. A total of 12 spH pups (6 males and 6 females) were used for measuring presynaptic activity. A total of 16 C57BL/6J pups (8 males and 8 females) were used for investigating voltage-gated Ca^{2+} channel (VGCC) subtypes, and 8 C57BL/6J pups (4 males and 4 females) were used for cell viability measurement. The culture number in each group was described in figure legends.

2.2. Genotyping

Newborn mice were genotyped within a day of birth by using tail tissue. Genomic DNA was eluted by incubation with lysis buffer (DirectPCR Lysis Reagent, CA) and 0.2 mg/mL proteinase K (Merck, NJ, USA) at 95 °C for 4 hr in a shaking heat block. Lysates were centrifuged for 3 min at 12,000 rpm, and supernatants were collected and stored at 4 °C until PCR analysis. A total of 2–5 µg of DNA was amplified by PCR using specific primers and polymerase (Promega, WI, USA). The PCR primer sequences were as follows: transgene forward 5'-CTG CAC CTC CTC CAA ACC TTA CTA-3', transgene reverse 5'-ATG ATG AGG ATG GCG CAG A-3', WT forward 5'-CAA ATG TTG CTT GTC TGG TG-3', and WT reverse 5'-GTC AGT CGA GTG CAC AGT TT-3'. PCR was performed according to the manufacturer's instructions. The PCR products were separated by electrophoresis on a 1% agarose gel containing Midori Green Advance (NIPPON Genetics Europe, Düren, Germany) and visualized using a UV light device (Corning Axygen Gel documentation systems, Merck). Transgenic pups were sacrificed for hippocampal neuron culture.

2.3. Hippocampal neuron culture and treatment

Hippocampal neuron cultures were performed in mouse pups as described in a previous report (Wu et al., 2016). Briefly, postnatal 1-day pups were sacrificed, and hippocampi were dissected under an optical microscope (C-PSN, Nikon, Japan). Hippocampal tissues were dissociated and seeded onto poly-D-lysine-coated coverslips or plates for further analysis. Neurons were grown in neurobasal medium (Thermo Fisher Scientific, MA, USA) supplemented with 2 % B27 (Thermo Fisher Scientific), 1% glutamax (Thermo Fisher Scientific), and 1 % antibiotics (penicillin and streptomycin, Thermo Fisher Scientific). Neurons were

maintained in a humidified incubator with 5 % CO_2 at 37 °C, and half of the medium was replaced with fresh medium every 2 or 3 days.

BaP was dissolved in dimethyl sulfoxide (DMSO). BaP was administered to neurons at the indicated concentrations before 48 h of live cell imaging. Vehicle (Veh) was treated with the same volume of DMSO without BaP. In spH 21 neurons, NH_4Cl was applied to the bath solution after the end of the electrical stimulation (40 APs 20 Hz) to obtain the resting pool. To calculate the P/Q-type Ca^{2+} channel contribution, electrical stimulation was initially applied to vGluT1-GCaMP5G-expressing neurons in the presence of ω -agatoxin IVA (agatoxin, 500 nM, Tocris, MN, USA) in the bath solution. Subsequently, electrical stimulation was applied in the presence of ω -conotoxin GVIA (conotoxin, 1 µM, Tocris) in bath solution to calculate the N-type Ca^{2+} channel contribution. An action potential was applied to neurons after 10 min of incubation with each inhibitor.

2.4. Cell viability

Cell viability was measured using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Neurons (21 DIV) were treated with BaP at the indicated concentrations. After the incubation of 48 h, neurons were further treated with 100 µM MTT and incubated for 1 hr at 37 °C. The medium was removed, and insoluble formazan crystals were eluted with DMSO. Absorbance was measured at an excitation wavelength of 540 nm with a microplate reader (BioTek Instruments, VT, USA), and the absorbance was normalized to the mean value of veh.

2.5. Transfection

The pCAG-vesicular glutamate transporter (vGluT1)-GCaMP5G (Addgene, #105008) was gifted by Franck Polleux. Neurons were transfected (DIV 5) with a plasmid containing vGluT1-GCaMP5G to measure presynaptic Ca^{2+} channel contribution using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's instructions. Fresh medium was added to neurons 4 h after transfection to avoid cellular toxicity.

2.6. Live cell imaging

As described in a previous report (Li et al., 2005), hippocampal neurons from transgenic mice were recorded at DIV 28 when the fluorescence expression was strong in neurons. Presynaptic Ca^{2+} imaging was performed at DIV 21–28. Images of living neurons were captured at 15–25 °C in a bath solution containing (in mM): 119 NaCl, 2 CaCl_2 , 2 MgCl_2 , 2.5 KCl, 25 HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffered pH 7.4, 30 glucose, with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM, Tocris, MN) and (±)-2-amino-5-phosphonopentanoic acid (APV, 50 µM, Tocris) to block glutamatergic activity. Live cell imaging was performed using an inverted microscope (Ti2, 60× objective NA 1.4, Nikon, Japan) with a metal halide lamp light source and fluorescein isothiocyanate filter set. Images were captured at 500 ms intervals with a high-sensitivity electron-multiplying charge-coupled device camera (iXON-L-888, Andor Technology, Belfast, UK) controlled by NIS-Elements software (Nikon). Action potential was evoked by passing a 1-ms pulse (20 mA) via platinum electrodes of a pulse stimulator (Model 2100, A-M Systems). Every experiment was monitored in the absence of electrical stimulation for at least 5 s and performed without photobleaching.

2.7. Image analysis

The region of interest (ROI) was a square 1.5 µm in height and width, considering presynaptic bouton size. The fluorescence intensity of each ROI was obtained by NIS-Elements software (Nikon). The resting value (F_0) was defined as the mean value of fluorescence intensity during the

initial 5 s recording before field stimulation. Changes in fluorescence (ΔF) were normalized by dividing by F_0 and multiplying by 100: $\Delta F = (F - F_0) \times 100/F_0$. The mean peak fluorescence upon application of action potential or NH_4Cl was obtained. In spH 21 neurons, the decay time constant of poststimulus endocytosis was obtained by fitting the fluorescence of synaptopHluorin from the peak to the baseline with a single exponential decay using Igor Pro 6 (WaveMetrics, OR). Presynaptic Ca^{2+} channel proportions were obtained by calculating the decreased value of peak amplitude by application of each inhibitor. The concentration of each blocker was determined by previous studies (Brockhaus et al., 2019), and the remaining Ca^{2+} responses by electrical stimulation in the presence of both channel blockers were referred to as "other" in accordance with a previous study (Ullman et al., 2018).

2.8. Molecular modeling study

The protein sequence of the P/Q-type Ca^{2+} channel (CAC1A.HUMAN; UniProt id: O00555) was used to detect remote protein homology by HHpred (MPI Bioinformatics Toolkit), which employs profile hidden Markov models (HMMs) for comparing sequence similarity (Gabler et al., 2020). The 3D structure of the P/Q-type Ca^{2+} channel (Cav2.1) was retrieved from the AlphaFold structural database (AF-O00555-F1-model_v4) (Varadi et al., 2022), and the unnecessary parts of the model structure (primarily disordered loops or terminal regions, i.e., residues 1–43, 410–462, 794–1230 and 1982–2506) were omitted. The final model structure was prepared using *Protein Preparation Wizard* in Schrödinger Maestro v.13.2 (Schrödinger, LLC, New York, NY) at a physiological pH 7.4. The potential binding sites in the channel were predicted using the *SiteMap* module (Halgren, 2009), and we suggest two major binding sites for the BaP molecule (Supplementary Fig. 1). The ligand structures of BaP and its known metabolites (BaP-7, 8-diol and BPDE) were prepared using the *LigPrep* module with *Epik* at pH 7.4. Then, by defining two binding sites, i.e., center pocket (grid center with $x = -6.891$, $y = -6.763$, $z = -1.225$, inner box size = 10 and outer box size = 46) and side pocket (grid center with $x = 9.144$, $y = 11.565$, $z = 15.501$), *Glide* docking was performed in standard precision (SP) mode. During the whole simulation, the optimized potential for a liquid simulation (OPLS) 2005 force field was used. The molecular graphic figures were generated by PyMOL software v.2.0 (Schrödinger, LLC).

2.9. Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM) for at least three independent experiments. Graphs were drawn using Igor Pro (WaveMetrics) or GraphPad Prism 5 (GraphPad Software, Inc., CA). Statistical significance was identified by Student's *t*-test when the data were normally distributed or one- or two-way analysis of variance followed by Tukey's *post-hoc* test for multiple comparisons. Differences were considered statistically significant when the *p*-value was less than 0.05. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software).

3. Results

3.1. Verification of spH 21 transgenic mice

To investigate the toxicity of BaP on presynaptic terminals, we employed spH 21 transgenic mice that expressed the genetically encoded fluorescent probe synaptopHluorin (spH) under the control of the mouse thymus cell antigen 1 (Thy1) promoter. spH is a fusion protein of the synaptic vesicle-associated membrane protein 2 (VAMP2) and super-ecliptic pHluorin, which is a pH-sensitive green fluorescence protein that quenches at acid circumstances and dequenches at neutral pH (Miesenböck et al., 1998). spH 21 transgenic mice enable monitoring of presynaptic activity by measuring SV exocytosis and endocytosis in

cultured neurons or slice cultures (Li et al., 2005). Transgenic and noncarrier (WT) mice were verified by genotyping (Fig. 1A). Primary cultured hippocampal neurons from transgenic pups endogenously expressed green fluorescence and spH fluorescence showed a rapid increase in response to electrical stimulation (40 APs at 20 Hz) and a slow decrease after the end of stimulation (Fig. 1B). An increase in the fluorescence intensity by electrical stimulation reflects SV exocytosis, and the decay rate of fluorescence indicates SV endocytosis (Li et al., 2005). Since SV endocytic mode was varied by the intensity of electrical stimulation (Wu et al., 2016), we examined the fluorescence decay rate in the application of electrical stimulation with 40 APs and 200 APs. Captured pseudocolor images of spH and traces showed a significantly higher increase in fluorescence by 200 APs at 20 Hz (Fig. 1C), and rapid decay was observed at 200 APs compared to 40 APs (Fig. 1D). Thus, spH 21 transgenic mice enabled the measurement of hippocampal presynaptic activity at various electrical stimulations by measuring fluorescence changes, which reflect SV exocytosis and endocytosis without genetic modification, consistent with a previous study (Li et al., 2005).

3.2. BaP suppressed SV exocytosis at the hippocampal presynaptic terminal

To investigate the effects of BaP on presynaptic terminals, we measured the spH fluorescence change in BaP-treated hippocampal neurons from transgenic mice in response to electrical stimulation. BaP concentrations were determined by MTT assay, which exhibited no significant cellular toxicity (Supplementary Fig. 2). Electrical stimulation of 40 APs at 20 Hz was used to verify the readily releasable pool (RRP), which is a small subset of SVs that are primarily released by electrical stimulation (Hoopmann et al., 2010). Traces showed that BaP repressed the increase in spH fluorescence intensity by electrical stimulation, and quantification of the peak amplitude was significantly decreased by BaP treatment (Fig. 2A and B). However, the decay rate of fluorescence and resting pool measured by NH_4Cl treatment were similar to the results observed in the BaP treatment.

We further confirmed the effect of BaP on SV exocytosis under stronger electrical stimulation. Traces and quantification of spH fluorescence intensity showed that the fluorescence increase was further enhanced by 200 APs compared to 40 APs, and the peak amplitude was significantly decreased by BaP treatment (Supplementary Fig. 3A and B). Decay rates were similar in the BaP treatment, consistent with previous results. These results indicate that BaP repressed SV exocytosis at the hippocampal presynaptic terminal.

3.3. BaP inhibited P/Q-type Ca^{2+} channels at the hippocampal presynaptic terminal

P/Q- and N-type Ca^{2+} channels are voltage-gated Ca^{2+} channels (VGCCs) that are predominantly expressed at presynaptic terminals in the central nervous system, and the contribution of channels is identified with sequential treatment with agatoxin and conotoxin, which are selective blockers of P/Q- and N-type Ca^{2+} channels, respectively (Catterall and Few, 2008). The activity of presynaptic VGCC subtypes was investigated using a sequential application of Ca^{2+} channel blockers in neurons expressed with vGlut1-GCaMP5G, a genetically encoded presynaptic Ca^{2+} sensor (Kwon et al., 2016). Agatoxin caused a significantly greater reduction in the increase in vGlut1-GCaMP5G fluorescence by electrical stimulation than conotoxin (Fig. 3A and B), indicating that P/Q-type Ca^{2+} channels contribute more to presynaptic Ca^{2+} entry than N-type channels in the hippocampal presynaptic terminal (Brockhaus et al., 2019). BaP-treated neurons showed a significant reduction in total presynaptic Ca^{2+} entry, and the decrease was derived by inhibition of P/Q- and other VGCC-mediated presynaptic Ca^{2+} entry, in contrast to N-type-dependent Ca^{2+} entry, which was not changed in BaP-exposed neurons. These results indicate that BaP significantly repressed SV exocytosis by inhibiting P/Q-type Ca^{2+}

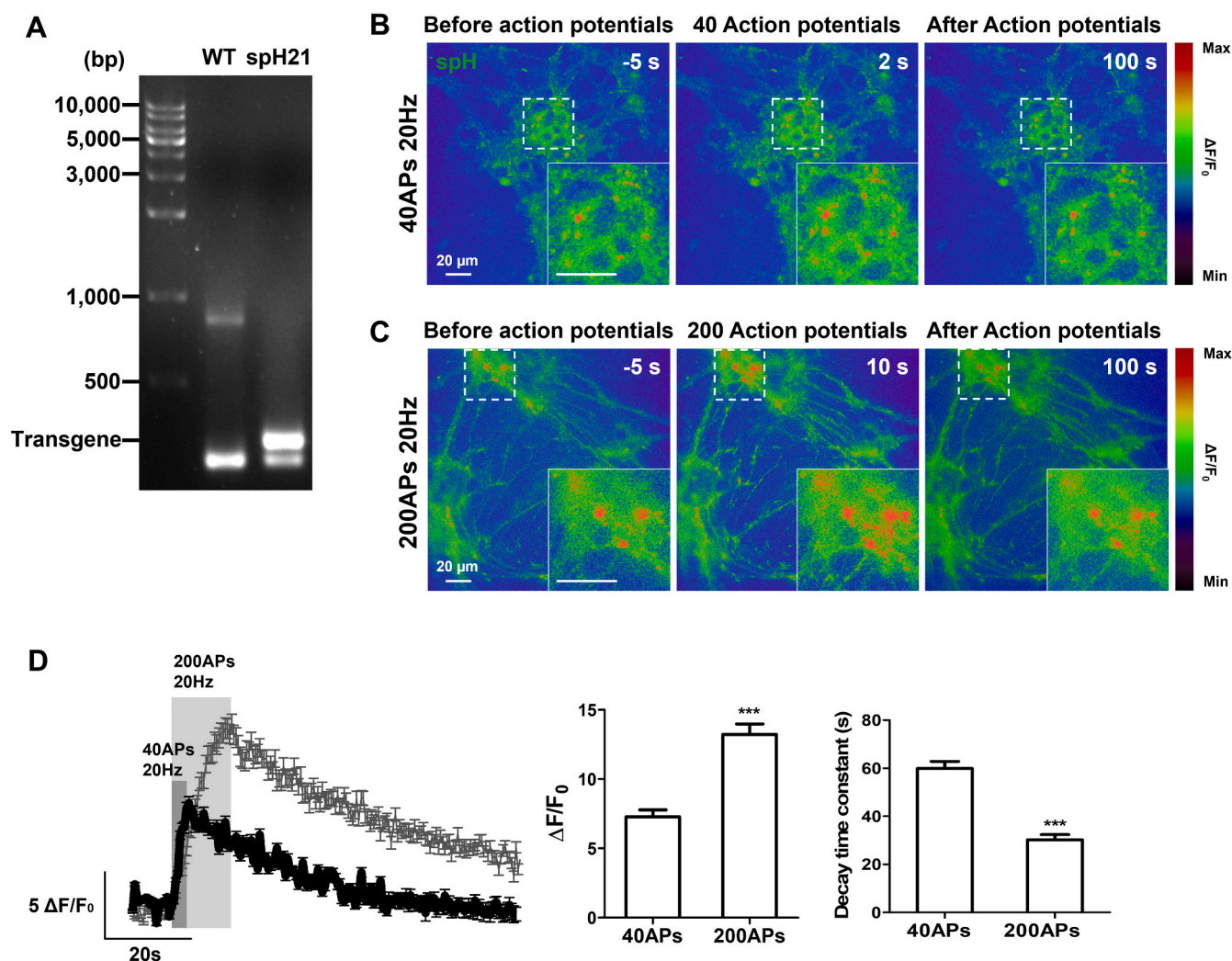


Fig. 1. Genotyping spH 21 transgenic mice and fluorescence change of spH by electrical stimulation in hippocampal neurons. (A) Genotyping tail tissue from spH 21 transgenic mice by PCR using transgene primers and PCR products. (B) Change in spH fluorescence in cultured hippocampal neurons from transgenic mice in response to electrical stimulation (40 APs at 20 Hz). Captured pseudocolor time-lapse images of cultured hippocampal neurons from spH 21 transgenic mice and magnification of the dashed box (inset). Scale bar = 20 μ m. (C) Similar to Panel B but with 200 APs at 20 Hz. (D) The average and merged normalized traces of ROIs from Panels B and C. Gray region indicates electrical stimulation. Quantification of the peak amplitude of fluorescence change in response to electrical stimulation and endocytic rate. Data are expressed as the mean $\pm$ SEM. $n = 4$ independent cultures for 40 APs at 20 Hz, $n = 5$ independent cultures for 200 APs at 20 Hz. *** $p < 0.001$ versus 40 APs. Student's t -test.

channels at the presynaptic terminal.

3.4. Binding mode prediction of BaP onto the P/Q-type Ca^{2+} channels

To explore the binding mechanism of BaP on the P/Q-type Ca^{2+} channel subunit $\alpha 1A$ (encoded by *CACNA1A*), we performed a molecular docking simulation. Since the experimental 3D structure of the P/Q-type Ca^{2+} channel is not yet available, the AlphaFold2 (AF) model was used. The AF model showed significant similarity to the structures of N-type (Cav2.2, PDB ID: 7MIY (Gao et al., 2021)) or R-type (Cav2.3, PDB ID: 8EPL (Yao et al., 2022)) Ca^{2+} channels, whose structures were also detected by HHpred search, a remote sequence homology detection tool (Gabler et al., 2020). Based on these structural similarities and pLDDT scores (confidence level) in the AF algorithm (Jumper et al., 2021), the AF model of the P/Q-type Ca^{2+} channels are considered quite reliable for further modeling studies.

By analyzing the ligand binding sites in the previously resolved Ca^{2+} channel structures, we speculated the approximate position of hydrophobic pockets where the compound BaP might bind. We first examined the 'center pocket' (Fig. 4A, yellow box), which is a binding pocket of

several antagonistic drugs for Ca^{2+} or sodium channels (e.g., PDB ID: 6JPA (Zhao et al., 2019), 6JPB (Zhao et al., 2019) and 6LQA (Li et al., 2021)). Additionally, since BaP is a very hydrophobic molecule, we considered a hydrophobic pocket located at the interface of the repeated domains (Fig. 4A, red box). The side pocket is a common site for cholesterol molecules to occupy in multiple structures (e.g., PDB ID: 7MIY (Gao et al., 2021), 7MIX (Gao et al., 2021), 8EPL (Yao et al., 2022), 6JPA (Zhao et al., 2019)), making it a likely location for hydrophobic molecules to bind. Therefore, the docking study of BaP was conducted considering both sites defined as 'center' and 'side', respectively.

BaP is known to be metabolically activated by enzymatic pathways, including cytochrome P450 and epoxide hydrolase (Murray, 2000; Wohak et al., 2016). The binding modes of BaP and its representative metabolites, i.e., BaP-7,8-diol and anti-BaP-7,8-diol-9,10-epoxide (BPDE) (Barnes et al., 2018), were predicted as shown in Fig. 4B and C. The flat ring of BaP occupies the center or hydrophobic side pockets well. Because BaP lacks polar atoms, it can interact with specific amino acid residues in the pocket via hydrophobic interactions. In particular, a stacking interaction with W335 has been identified as a possible binding mechanism for BaP. In the case of the metabolites, the hydroxyl group of

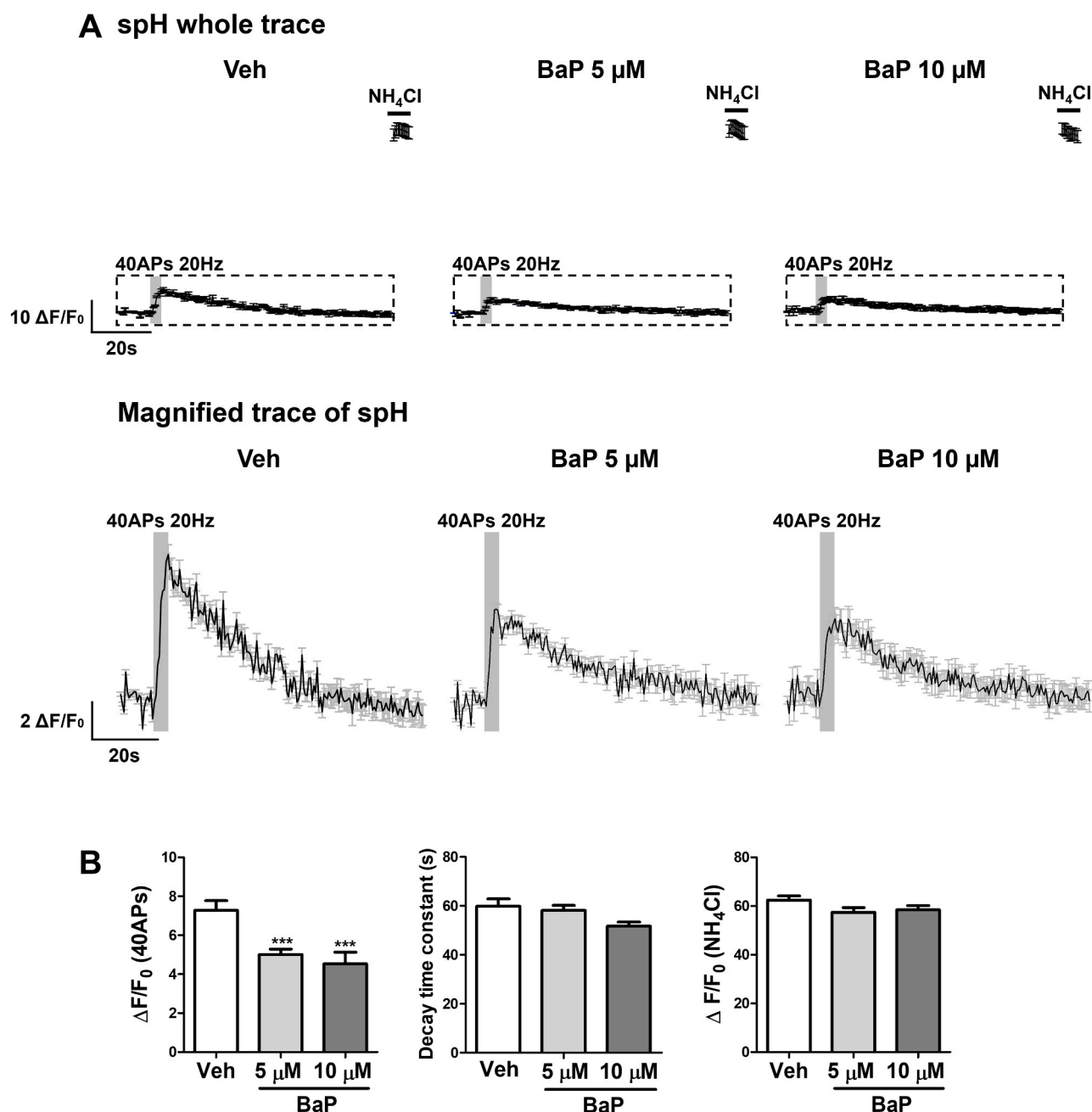


Fig. 2. Effects of BaP exposure on SV exo- and endocytosis at the hippocampal presynaptic terminal. (A) Cultured hippocampal neurons from spH 21 transgenic mice were treated with BaP. Electrical stimulation (40 APs at 20 Hz) was applied to cultured hippocampal neurons (gray region), and NH_4Cl was administered after the end of electrical stimulation to observe the resting pool. Averaged normalized traces of spH fluorescence intensity in ROIs. Whole traces (upper) and magnification of dashed box (below) are shown. (B) Quantification of peak amplitude in response to electrical stimulation, decay time constant after the end of the electrical stimulation, and peak amplitude by NH_4Cl treatment. Data are expressed as the mean $\pm$ SEM. $n = 3$ independent cultures for Veh, $n = 4$ independent cultures for BaP 5 μM , $n = 4$ independent cultures for BaP 10 μM . *** $p < 0.001$ versus Veh. Statistical differences were analyzed using one-way ANOVA followed by Tukey's post hoc test.

BaP-7,8-diol formed a hydrogen bond with the F247 backbone (Fig. 4B) or T316 backbone (Fig. 4C). BPDE also formed hydrogen bonds with N1655, S1657, and F1658 in the side pocket (Fig. 4B) and T316 and T665 in the center pocket (Fig. 4C). These additional polar interactions of the metabolites can help form a stable complex with the channel. Although confirming the BaP binding site with the currently available information may be challenging, we suggest that these two sites would be possible for BaP binding in the Ca^{2+} channel structures.

4. Discussion

Presynaptic activity is essential for synaptic efficacy and plasticity by controlling SV exocytosis and endocytosis. Regarding initial occurrence of presynaptic dysfunction or structural changes at the presynaptic terminal during development in neurodegenerative diseases (Kamat et al., 2016; Yasuda et al., 2013), measuring SV exocytosis and endocytosis is a key determinant for early neuropathology diagnosis. In this study, we explored the detrimental effects of BaP on presynaptic activity, focusing on SV exocytosis and endocytosis.

Many methodologies to investigate SV exocytosis and endocytosis

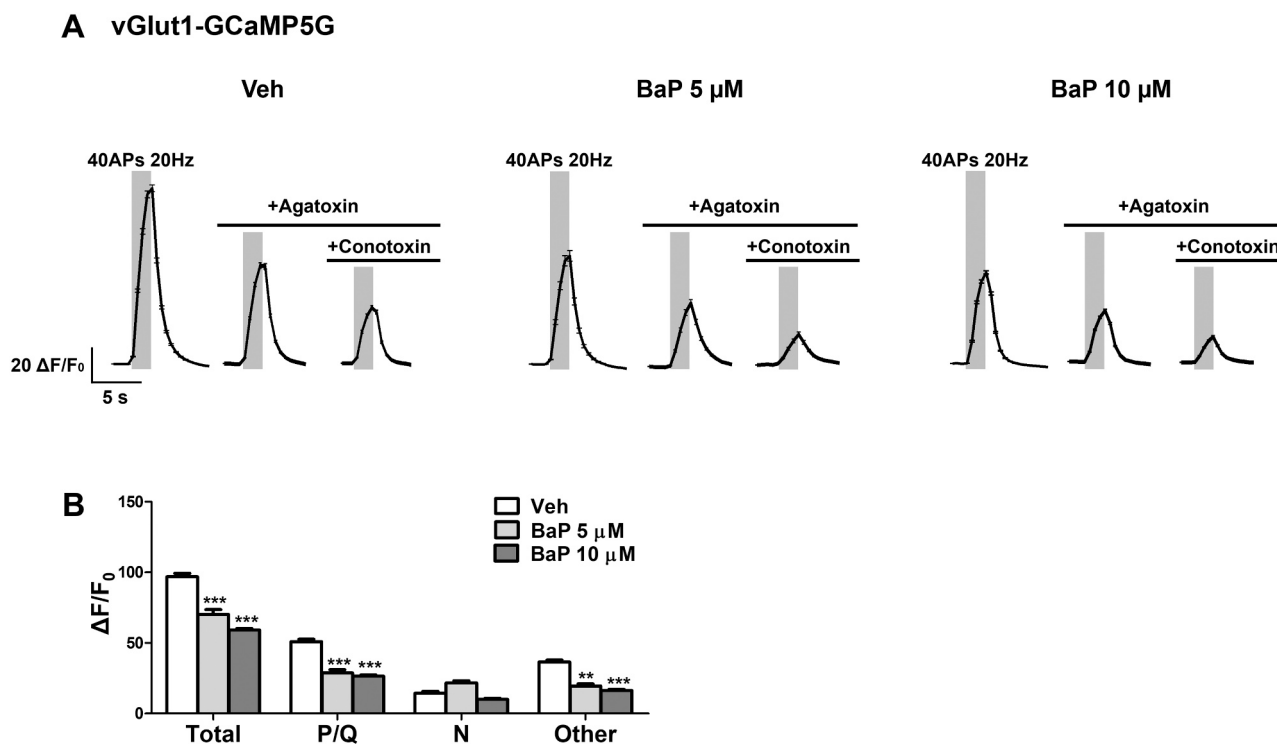


Fig. 3. Effects of BaP exposure on presynaptic VGCC subtypes. (A) Hippocampal neurons expressing vGlut1-GCaMP5G were treated with BaP. Electrical stimulation (40 APs at 20 Hz) was applied to neurons (gray region) in the sequential treatment of agatoxin and conotoxin. Averaged normalized traces of vGlut1-GCaMP5G fluorescence intensity in each ROI. (B) Quantification of the peak amplitude of vGlut1-GCaMP5G fluorescence change by electrical stimulation. Data are expressed as the mean $\pm$ SEM. $n = 4$ independent cultures for Veh, $n = 5$ independent cultures for BaP 5 μ M, $n = 5$ independent cultures for BaP 10 μ M. ** $p < 0.01$ and *** $p < 0.001$ versus Veh. Statistical differences were analyzed using two-way ANOVA followed by Tukey's post hoc test.

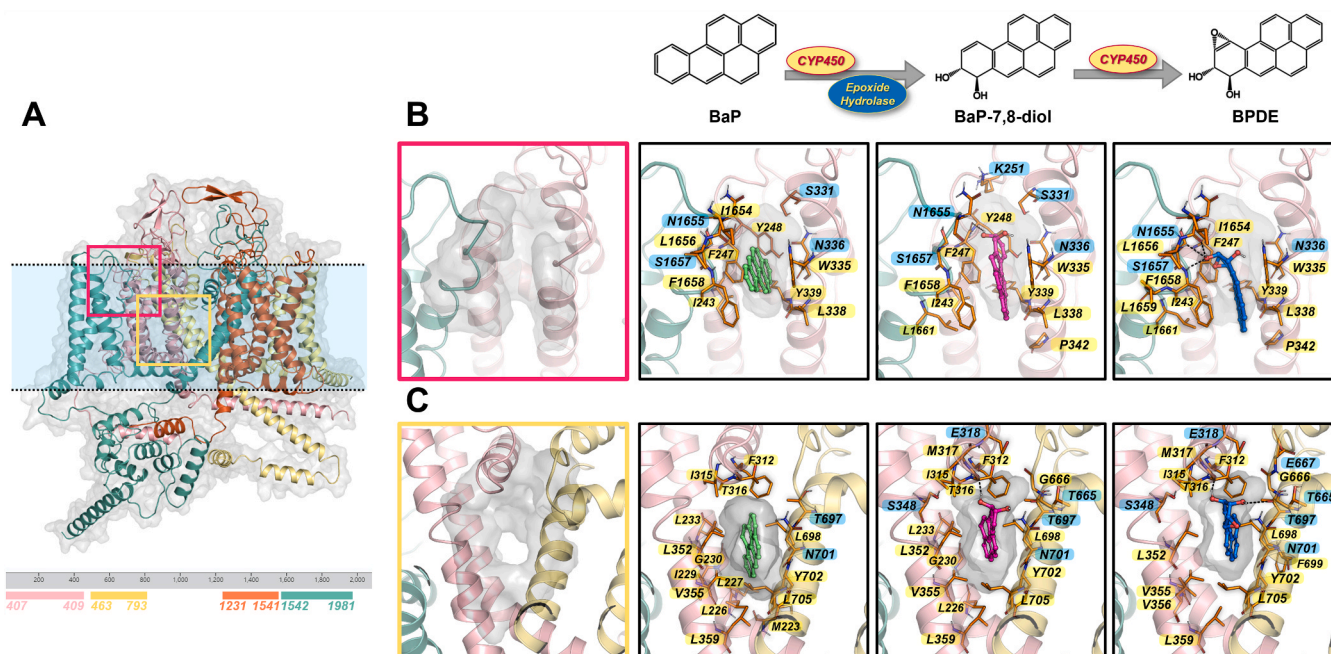


Fig. 4. Molecular docking analysis of BaP and metabolites in the P/Q-type Ca^{2+} channel model. (A) 3D structure of voltage-dependent P/Q-type Ca^{2+} channel subunit $\alpha 1A$ predicted by AlphaFold2 (AF-000555-F1-model_v4). Four homologous repeat domains are colored pink, yellow, orange, and green, respectively, with gray molecular surfaces. The two expected ligand binding sites are marked in magenta and yellow boxes. (B) Close-up view of the 'side pocket' and the docked conformations of the BaP metabolites. (C) Close-up view of the 'center pocket' and the docked conformations of the BaP metabolites. BaP (green), BaP-7,8-diol (magenta), and *anti*-BaP-7,8-diol-9,10-epoxide (BPDE, blue) are depicted as sticks, and the interacting residues are shown as orange sticks. The cyan-highlighted residues indicate polar residues, and the yellow-highlighted residues indicate nonpolar or hydrophobic residues. H-bonding interactions are shown as black dashed lines. The molecular surfaces of the binding pockets are colored gray.

have been reported, such as Styryl dyes for labeling SVs, electron microscopy, membrane capacitance measurement, and optical stimulation with high-pressure freezing (Gan and Watanabe, 2018; Jin et al., 2019). Live imaging methods with pHluorin-tagged SV proteins are commonly employed at mammalian hippocampal presynaptic terminals. However, this requires genetic manipulation to visualize SVs with fluorescence (Kononenko and Haucke, 2015), and the use of transfection reagents may perturb pHluorin activity by impairing mitochondrial function (Soykan et al., 2017). spH transgenic mice overcome these disadvantages by endogenously expressing pHluorin in the SV protein, negating the need for genetic modification. In a previous study, SV exocytosis and endocytosis have been observed at a single synapse both in slice and dissociated cultures from spH 21 transgenic mice (Li et al., 2005).

SV exocytosis is tightly regulated, controlling the release of neurotransmitters into the synaptic cleft, and plays a crucial role in synaptic transmission (Brunger et al., 2019). Studies using neurotoxins or genetic approaches have shown that impairment of SV exocytosis is highly associated with neurodevelopmental, neuromuscular, seizure, and neurodegenerative diseases (Melland et al., 2021). Presynaptic Ca^{2+} channels are essential for SV exocytosis as they modulate Ca^{2+} entry at the presynaptic terminal (Dolphin and Lee, 2020). Some neurotoxins, such as PhTx3–3, PhTx3–4, and kurtotoxin, are known to suppress SV exocytosis by inhibition of VGCCs (Dos Santos et al., 2002; Sidach and Mintz, 2002).

The expression of VGCC subtypes varies depending on neuronal or presynaptic terminal types. P/Q- (Cav2.1) and N-type (Cav2.2) Ca^{2+} channels are principal neuronal VGCCs that mainly mediate SV exocytosis at presynaptic terminals (Brockhaus et al., 2019). P/Q-type Ca^{2+} channels predominantly mediate SV release in both developing (Nakamura et al., 2015) and mature neurons (Nanou and Catterall, 2018). This is due to their enriched expression at presynaptic terminal and closer proximity to active zone compared to N- or R-type Ca^{2+} channels (Wu et al., 1999). P/Q-type Ca^{2+} channels enhance presynaptic activity by coupling neuronal excitation to the fusion of neurotransmitter-containing SVs with the presynaptic membrane. P/Q-type Ca^{2+} channels also facilitate the assembly of the SNARE proteins (Ishikawa et al., 2005), which provide the energy necessary for SV fusion with presynaptic membranes (Weber et al., 1998). Impaired presynaptic activity resulting from disruption of SV exocytosis and inhibition of presynaptic P/Q-type Ca^{2+} channels are highly linked to neurodevelopmental disorders, such as autism spectrum disorders, epilepsy, and intellectual disability (Bonnycastle et al., 2021; Ullman et al., 2018). Given that prenatal exposure to BaP impairs cognitive function in children (Perera et al., 2009), this study may shed light on the adverse effects of BaP on the brain by influencing presynaptic activity and the P/Q-type Ca^{2+} channels.

Selective inhibitors of P/Q- and N-type Ca^{2+} channels were used to identify the underlying mechanism of SV exocytosis by VGCC subtypes. Although sorting VGCC subtypes is facilitated by electrophysiological methods, this is not feasible at the hippocampal presynaptic terminal due to undetectable currents. Our study reveals that BaP inhibited P/Q-type Ca^{2+} channels, potentially causing conformational changes of the receptor. It leads to significantly reduced Ca^{2+} transients, thus impacting SV release at the presynaptic terminal. Since the proximity of the P/Q-type Ca^{2+} channels to the active zone is critical to presynaptic transmission (Dolphin and Lee, 2020), BaP might change the distribution of P/Q-type Ca^{2+} at the presynaptic terminal zone.

Oxidative stress and apoptotic mediators are known to cause neurotoxicity in the hippocampus (Ogut et al., 2019). Enhanced reactive oxygen species (ROS) levels and caspase activation are linked to presynaptic dysfunction in neurodegenerative diseases (Kamat et al., 2016). Although the exact mechanisms remain unclear, oxidative and apoptotic stresses impair presynaptic functions by altering presynaptic structure, damaging presynaptic proteins, or losing the mitochondrial function, which supplies energy for presynaptic transmission (Li and Sheng, 2022). Given that BaP induces apoptosis and oxidative stress by

producing ROS while suppressing the antioxidant enzyme activity (Bukowska et al., 2022; Lin et al., 2018), it is possible that BaP suppresses presynaptic activity in hippocampal neurons by elevating oxidative or apoptotic stress.

5. Conclusion

Our study has made important strides in understanding the neurotoxic impact of BaP, presenting concrete evidence that BaP is capable of inhibiting SV exocytosis at the hippocampal presynaptic terminal. This occurs via the suppression of P/Q-type Ca^{2+} channels, a critical player in synaptic neurotransmission, under conditions of mild to moderate electrical stimulation. In addition, we utilized molecular docking simulations, a powerful tool in computational biology, to uncover potential binding sites of BaP and its metabolites within the molecular structure of the P/Q-type Ca^{2+} channels. By integrating our experimental findings related to neuronal physiology with modeling studies, we can explore the intricate neurotoxic mechanisms of BaP more comprehensively. These findings provide information that can significantly enhance the accuracy of risk assessments related to BaP-induced neurotoxicity and highlight the urgent necessity of mitigating BaP exposure to prevent potential neurotoxicity in the brain.

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CRediT authorship contribution statement

YK: Methodology, Validation, Formal analysis. **YE:** Methodology, Investigation. **HY:** Investigation, Data curation. **YL:** Writing, Resources, Supervision, Writing – original draft, Writing – review & editing. **SHL:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors have no relevant financial or nonfinancial interests to disclose.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2023.115301](https://doi.org/10.1016/j.ecoenv.2023.115301).

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