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# Application of electrophysiological technique in toxicological study: From manual to automated patch-clamp recording

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ABSTRACT

Ion channels are common targets of a range of environmental substances, including pharmaceuticals, toxins, metals and many organic pollutants. Patch-clamp technique is gold standard in studying ion channel characteristics, contributing to the improved accuracy in active compound identification, the increasing studies of molecular mechanism behind diseases, and the better prediction of potential toxicants. Nevertheless, low throughput and high technical capability requirement limit the application of conventional manual patch-clamp recording and an emerging transformation towards automation and simplification is rising. The evolving automated patch-clamp technique shows obvious advances over manual technique in many aspects, including reproducibility, throughput, operability and standardization. The application of emerging automated patch-clamp platform has created valuable consequences in many research areas, especially in high throughput screening. In this study, we provided an overview on the progresses made by conventional manual and emerging automated patch-clamp techniques in toxicological studies.



## 1. Introduction

Ion channels represent a membrane-bound protein family that regulates and facilitates ion movement across membranes. As a result of their special structure, many ion channels have ion selectivity, mainly consisting of sodium, potassium, calcium, and chlorine channels. Moreover, each type of ion channel includes several subtypes, representing different proteins associated with various drug or toxin targets. The physiological function of ion channels involves participation in cell activities such as cell differentiation, neural transmission, and cell migration and apoptosis. Therefore, abnormal ion channel activity triggers human diseases such as skeletal muscle disorders, cardiac arrhythmias, and epilepsy [1–3]. Indeed, ion channels can act as both a target of drugs to cure disease and as a target of toxic substances, leading to adverse reactions.

Previous studies have reported on the regulation of ion channels by environmental substances leading to various malfunctions [4,5]. For example, tetrodotoxin (TTX), a natural toxin found in many marine animals, is a powerful sodium channel inhibitor that can selectively block voltage-gated sodium channels in nerves and muscles, leading to the disruption of neuronal and muscular activities [6]. Drugs in clinical use have been shown to block the cardiac $K_v11.1$ voltage-gated potassium channel, with dangerous side effects, resulting in the prohibition of various drugs affecting this ion channel [2]. Similarly, certain environmental pollutants have been shown to prevent normal neural transmission based on their interaction with ion channels. Polychlorinated biphenyls (PCBs), a family of persistent organic pollutants, can interact with ryanodine receptors in neurons and change the spatial and temporal properties of calcium signals [7]. Thus, the toxicity of many chemicals found in the environment is based on their effect on ion channels, suggesting the necessity of further research on this topic.

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At present, the patch-clamp technique has become the gold standard for studies on ion channel properties. Conventional manual patch-clamp records the channel current or voltage in real time and provides precise electrophysiological data with respect to ion channel function at a single-cell or single-channel level [8]. Recently emerging automated patch-clamp techniques are now able to provide a precise measurement of ion channel features with an improved throughput and a reduced skill requirement [9,10]. In many toxicological studies, automated patch-clamp has been used to provide adequate information on ion channel activities and greatly saves time and efforts. For instance, automated patch-clamp systems have been applied for the early safety assessment of a large number of drug candidates according to their potency to block the cardiac human ether-a-go-go-related gene (hERG) channel. These studies have led to valuable quantitative statistics, suggesting the potency of this evolving technique [2]. As the rapid and precise screening of toxicants and the identification of toxic effects are greatly needed in toxicology, an overview of automated patch-clamp systems and their potential to be applied in toxicological studies is of significance.

To the best of our knowledge, there are a limited number of reviews discussing the application of automated patch-clamp techniques in toxicological studies due to the techniques having just emerged in recent years. Herein, we first briefly describe the conventional manual and emerging automated patch-clamp techniques followed by a general overview of the application of manual patch-clamp technique in studying the effects of environmental substances on ion channels. Recent advances in toxicological studies resulting from the automation of the patch-clamp techniques are then discussed. Finally, we compare the manual and automated patch-clamp techniques on multiple dimensions, including cell preparation, throughput, operability, and data quality.

The literature was screened via Web of Science for articles published between January 1990 and March 2020, with key topic terms such as patch-clamp, high throughput electrophysiology, and ion channel, along with specific pollutants, metals, toxins, or drugs.

## 2. Electrophysiological methods

### 2.1. Manual patch-clamp technique

The conventional manual patch-clamp technique measures the ion current when the membrane voltage is clamped by a feedback amplifier and uses a microelectrode to record the membrane voltage [11]. During a patch-clamp measurement, a glass micropipette is pressed onto the surface of the cell membrane to conduct a single channel experiment. Through light suction by the pipette, the pipette tip and the cell membrane establish an intense contact, creating a giga-ohm resistance seal between the pipette and the cell, electrically isolating the area under the pipette tip [12]. By creating this special isolated membrane patch, patch-clamp can eliminate most of the current noise and increase the resolution of single-channel recordings. Additionally, the whole-cell patch mode, achieved by breaking the patch under the pipette tip to establish direct access to the cytoplasm, allows for recordings of the summated ion channel activities in a cell, and is commonly used to assess the neurotoxicity of compounds [13]. To date, conventional patch-clamp has been developed in many different configurations, including cell-attached, whole-cell, inside-out, and outside-out modes, to adapt to the different research needs [14]. The patch-clamp technique is able to offer real-time, high-resolution, and complete information on the ionic currents and membrane voltage for most ion channel types, indicating the changes in ion channel functions. In toxicological studies, the patch-clamp technique has generated many valuable consequences in a common experimental framework, as shown in Fig. 1.

In order to form a high resistance seal, direct contact between the pipette tip and the cell membrane is necessary, representing the largest obstacle in the application of this technique. Visually guided patch-clamp allows the researcher to observe the pipette and the targeted cell under microscopy [10]. The differential interference contrast microscopy provides an optimized imaging quality in patch-clamp processes and decreases the difficulty in giga-seal formation. This method is valuable for the effective characterization of specific types of neurons in the brain slice, allowing for studies of certain neuronal subsets.

Despite the considerable advances, conventional manual patch-clamp is a typical low throughput and labor-intensive methodology, with the operations being extremely time-consuming and difficult, requiring highly skilled and trained personnel and thus preventing it from wide application in drug testing, toxin screening, and toxicological studies [13,14]. Indeed, in the case of manual patch-clamp *in vivo*, only 20−30% of pipettes are able to form a stable giga-seal between cells, while the success rate of *in vitro* manual patch-clamp recording can be achieved at a range of 50−80% [15,16]. The combination of some high throughput screening technologies and patch-clamp has partially alleviated the throughput problem, generating more valuable studies. For example, Pillai et al. applied an osmotic lysis assay to screen active plasmodial surface anion channel inhibitors and further characterized their potency by patch-clamp [17].

### 2.2. Automated patch-clamp technique

In order to increase the throughput and the application of this technique, methods such as radioligand binding, fluorescence, and flux assays have been proposed as substitutions for or supplements to conventional patch-clamp [8]. However, these methods only provide a limited and indirect description of ion channel activities. In contrast, automated patch-clamp attempts to increase the throughput and does not compromise the data quality and temporal resolution [18]. Currently, many available automatic patch-clamp systems have been produced with comparative success at various levels in different directions. Fig. 2 shows some common configurations used by conventional manual and automated patch-clamp systems. One of the most popular attempts in automated patch-clamp aims to increase the throughput of this technique by recording more cell-attached structures simultaneously, leading to the advent of planar-patch-clamp systems [14,19]. Based on a bottom-top configuration, planar automated patch-clamp brings suspended cells to the multi-microelectrode plate, where many parallel wells act as micropipettes [20]. When separated and suspended cells approach the wells on the plate, light suction is applied to absorb these cells and automatically form a resistance seal. This method is suitable for the characterization of voltage-gated ionic channels and fast-desensitizing ligand-gated channels, providing medium throughput recording with a higher recording quality compared to other non-patch-clamp techniques. Many commercially available automated patch-clamp systems choose this method, including IonWorks, PatchXpress, and Qpatch [9]. Aside from planar patch-clamp, there also are many non-planar automated patch-clamp systems available. For instance, with innovation in the way of cells approaching pipettes, Flyion moves cells from internal space of pipette to the pipette tip, instead of absorbing suspended cells in the sample, and therefore allow for the application of the conventional glass pipettes in their automated patch-machine [9]. However, given the high costs, throughput, and technical requirements, the application of non-planar automated patch-clamp systems remains greatly limited.

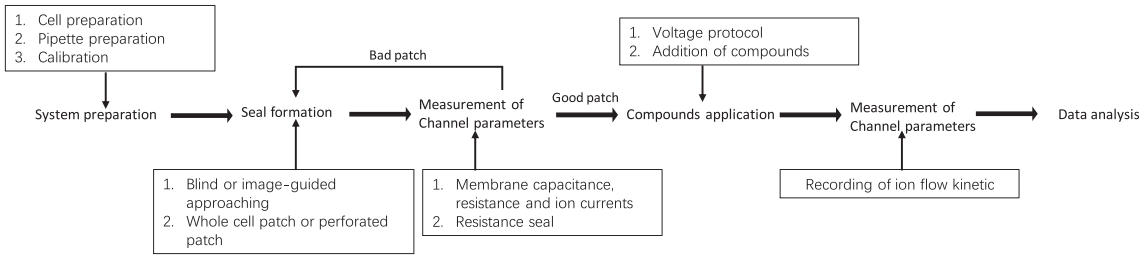


**Fig. 1.** The normal processes of patch-clamp recording for toxicological electrophysiological study.

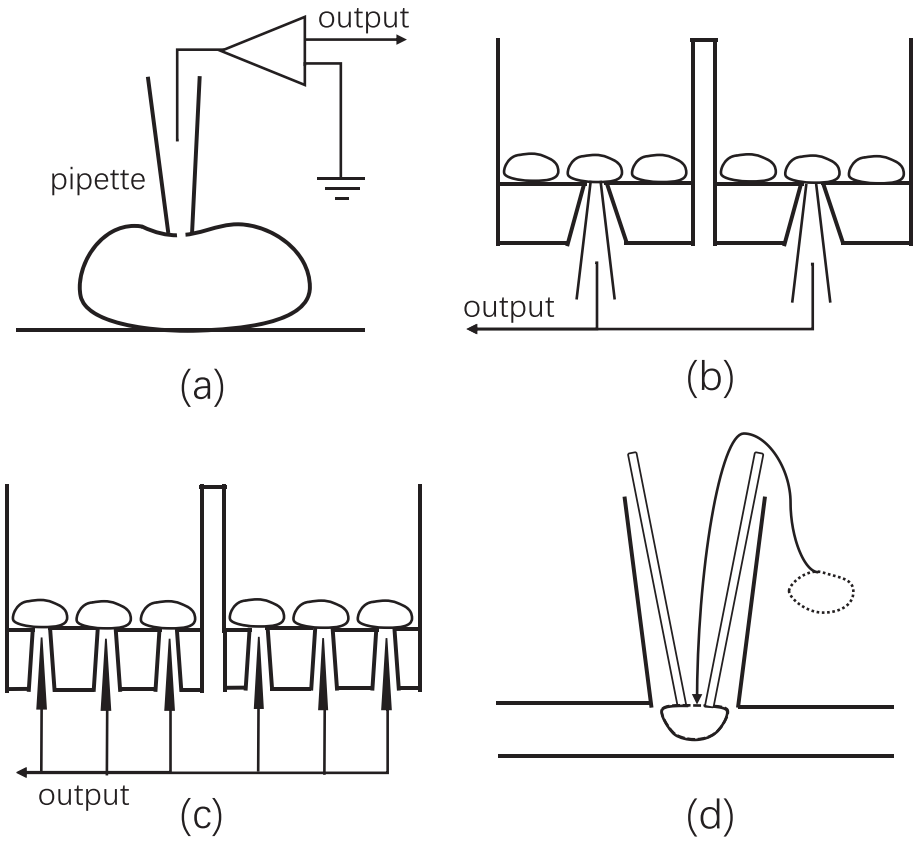


**Fig. 2.** Common configurations of patch-clamp instruments. (a) Manual patch-clamp; (b) planar patch-clamp; (c) population patch-clamp; (d) Automated patch-clamp of Flyscreen format.

Image-guided patch-clamp techniques propose a new potential mechanism for full automation of patch-clamp recording. In the early stage of development of the technique, a programmed computer system was used to replace the experimenter in manual patch-clamp recording, but it was proved to be nonsense to increase throughput because the computer works in a serial mode and spends as much time on the processes as conventional methods [12]. In 2017, an automatic and efficient patch-clamp system was proposed by Wu et al., which utilizes an optimized computer algorithm to automatically detect the fluorescence of a given cell and guide the pipette to form a high-resistance seal [10]. Moreover, a robotic "PatcherBot" system equips the machine with more functional components and replaces conventional manual exchanging pipettes by automatic cleaning, making the full automation of patch-clamp (without any human attending) practical [21]. This advanced technique reveals a powerful and standardized tool for functional studies of environmental pollutants.

## 3. Application of patch-clamp technique in toxicological studies

To date, increasing clinical evidence has linked many abnormal symptoms in the nervous and cardiorespiratory systems with exposure to common environmental contaminants. Generally, ion channels and receptors are common targets for environmental contaminants. Analytical methods originating from the application of patch-clamp have been widely used to study the characteristics of ion channels and search for active neurotoxicants. The application of patch-clamp technique has revealed the potential neurotoxicity and cardiac toxicity of a variety of categories of environmental substances. This technique examines the effects of these substances on different types of ion channels belonging to diverse cells, providing vital information for studies of toxicity and the molecular mechanism. The results are summarized in Table 1.

### 3.1. Environmental contaminants

#### 3.1.1. Pharmaceuticals and personal care products

Pharmaceuticals and personal care products may have unpredicted functional activity when interacting with ion channels. For example, the relationship between the transient receptor potential (TRP) channel in sensory neurons and the pain sensation caused by pharmaceutical use has been assessed. Clotrimazole, a widely used pharmaceutical with potential side effects of topical pain and an inflammatory reaction, is characterized as an effective TRPV1 and TRPA1 channel activator and possibly triggers the pain feedback [22]. Since the sodium channels, particularly TTX-resistant channels, play a vital role in the conduction of sensation, they are active targets of many pharmaceuticals. Indeed, lidocaine and bupivacaine reduce the sodium current amplitude when receiving high frequency stimulation at micromolar concentrations. In terms of a TTX-resistant channel, electrophysiological experiments recorded a progressive depression of current amplitude, supporting the finding that neural information transmission is gradually blocked during spinal anesthesia [23]. Another drug, dexmedetomidine, induces analgesic effects via blockade of the $Na_v1.8$ channel, shifting the steady-state inactivation curve towards a hyperpolarizing orientation and lifting the threshold of action potential, thereby decreasing the excitability of small sensory neurons [24]. The studies on the $Na_v1.8$ channel have proposed that pain sensation is associated with the activities of voltage-gated sodium channels in sensory neurons. Therefore, in order to explore the link between sensory information and channel activity, researchers designed and synthesized an engineered molecule to modulate sodium and calcium channels. This modified molecule has been used to reduce the excitability of peripheral nociceptor and exhibits significant antinociception effect in the experiment [25].

Many personal care products also alter ion channel function. Previous studies reported on the impact of triclosan (TCS), an antibacterial agent widely used in cosmetics and sanitary products, on calcium currents across the cell membrane. It has been suggested that micromolar concentrations of TCS (5 μM) increased the basal calcium levels in the cell by activating the TRPA1 channel [26]. Additionally, exposure to TCS and many other chemicals in personal care products, such as 3-(4-methyl benzylidene) camphor and α-zearalenol, can activate the sperm-specific calcium channel and affect the calcium homeostasis of sperm [27]. Parabens, a series of amphipathic compounds widely used in food preservatives and cosmetics, potentially block the voltage-gated sodium channels in mammalian cells. Treatment with over 100 μM of parabens obviously shifted the steady-state inactivation curve of the sodium channel toward the hyperpolarizing direction [28].

The $K_v11.1$ channel, coded by the *hERG* gene, is highly important in the ventricular repolarization of cardiomyocytes as a common target of many pharmaceuticals and personal care products. The abnormal change in ventricular repolarization caused by hERG blockade can result in severe cardiac diseases such as irregular atrial and ventricular beats [29]. Indeed, the ratio of total concentration of a drug in plasma over the hERG inhibiting $IC_{50}$ value is considered as a valuable parameter to predict the level of ventricular depolarization and repolarization duration prolongation, emphasizing the need to obtain the hERG blocking statistics of common drugs [30]. To date, many antipsychotic drugs block the hERG channel and produce QT interval prolongation, including disopyramide, astemizole, risperidone, and paliperidone [31–33]. For example, electrophysiological studies have shown that disopyramide is an effective hERG channel blocker at clinically relevant concentrations (7.5 μM), inducing obvious arrhythmia. The reduction in hERG current induced by disopyramide has been observed when the hERG channels are closed, suggesting a state-independent inhibitory mechanism [31]. Astemizole and its analogues also block the hERG channel and lead to long QT syndrome. The modified chemical structure of analogues resulted in a predictable inhibition, suggesting the possibility of designing new engineering molecules for medical uses [32]. Risperidone and its metabolite, paliperidone, potently reduce the hERG currents at a sub-micromolar concentration ($IC_{50}$ = 0.16 and 0.57 μM, respectively) [30]. The risperidone-induced hERG inhibition is mainly attributed to interactions between the drug and the activated or inactivated channel, rather than by disruption of channel protein trafficking [33]. However, the latter mechanism has been used to explain the inhibition caused by other compounds such as ketoconazole and fluconazole [34].

Inhibitory features derived from patch-clamp recording have led to the elucidation of molecular mechanisms and binding sites of active compounds. Chloroquine can block the L-type calcium current with no dependence on the G-protein related pathway [35]. Chloroquine also can interact with the cardiac ATP-sensitive inward rectifier potassium channel ($K_{ir}$ 6.2/SUR2A), reducing the current amplitude ($IC_{50}$ = 11.8 μM) through at least two different action mechanisms [36]. Recently, a modified fluorescent metabolite of astemizole, which is a potent hERG inhibitor, was utilized as a valuable visual probe for potassium channel structure detection [37]. Further, the relationship of hERG channel affinity with the molecular structure of drugs has been explored by patch-clamp. It is suggested that the binding kinetic parameters of compounds also are of importance in determining the potency of inhibition, while the $IC_{50}$ values of two tested compounds ($IC_{50}$ = 0.182 μM and 0.107 μM, respectively) were apparently linked to the binding duration times. Compounds that have a longer receptor-ligand residue time display a higher potency in inhibition [38].

#### 3.1.2. Halogenated organic compounds

Halogenated organic compounds include many persistent organic pollutants that possibly have a high affinity to ion channel proteins or receptors and alter the activity of the nervous system. For example, the potential effect of tetrachlorodibenzo-p-dioxin on cardiomyocytes was examined in recent studies, indicating that exposure to the chemical at nanomolar concentrations (10 nM) obviously prolonged the action potential duration, reduced the depolarization, and increased the total transient inward current. The intracellular calcium overload was explored, and was attributed to the increase in L-type calcium current [39]. PCBs are positively correlated with neurotoxicity, including impaired learning and memory capacity, specific attentional deficit, and slow cognitive development [5,40]. Recently, studies on acute neurodegeneration after PCB exposure found that PCBs increased intracellular $Ca^{2+}$ and generated reactive oxygen species, associated with ryanodine receptor activation [41–43]. A quantitative structure-activity relationship investigation confirmed that the ryanodine receptor can be a sensitive target of various congeners of PCBs [44]. Electrophysiological studies found that the L-type calcium channel and N-methyl-D-aspartic acid (NMDA) receptor had no influence on the PCB-induced increase of intracellular $Ca^{2+}$ [45], suggesting that the extracellular calcium influx had no effect on the increase in intracellular calcium level [42]. Moreover, some congeners of PCBs act as agonists of γ-aminobutyric acid (GABA) receptor and antagonists of the excitatory nicotinic acetylcholine receptor (nAChR) [46,47]. These additive effects perhaps represent an augmented shift of postsynaptic excitability, helping to explain the observed neurodevelopmental effect. Dichlorodiphenyltrichloroethane (DDT), a typical organochlorine pesticide, apparently prolongs the duration of action potential and prevents the inactivation of sodium channels. Recent studies revealed a new possible mechanism behind the neurotoxicity of DDT via modulation of

**Table 1**
Environmental contaminants affecting ion channels.

| Ingredient | Organism | Targeted receptor/channel | Dose | Response | Extra information | Ref. |
|---|---|---|---|---|---|---|
| **PPCPs** | | | | | | |
| Tamoxifen & Toremifene | Embryonic hypothalamic neuron | Sodium and potassium channel | 1–2 μM | Decrease current | | [1] |
| Lidocaine & Bupivacaine | Dorsal root ganglia neuron | Voltage-gated sodium channel | 13–210 μM | Block | More efficiently inhibit TTX-sensitive channels | [23] |
| Dexmedetomidine | Dorsal root ganglia neuron | $Na_v$ 1.8 | 1 μM | Decrease current and regulate activation and inactivation | Effects based on G(i/o)/AC/cAMP/PKA pathway | [22] |
| Nifedipine, Amiodarone, Quinidine, Amitriptyline & Flecainide | Embryonic heart | hERG | 10–50 μM | Block | | [24] |
| Disopyramide | Embryonic heart | hERG | 125 μM | Block | | [24] |
| Indapamide | Embryonic heart | hERG | 2.2 mM | Block | Low risk | [24] |
| Mibefradil | Embryonic heart | hERG | 2.5 μM | Block | | [24] |
| Disopyramide | Chinese hamster ovary cells | hERG | 7.5 μM | Block | Binding to both resting and open channels | [31] |
| Astemizole | Xenopus oocytes | hERG | 0.1 μM | Block | Structural variety decides inhibitory potency | [32] |
| Risperidone & Paliperidone | Modified HEK293 cell | hERG 3.1 | 0.1–1 μM | Decrease current and regulate activation and inactivation | No disruption of protein trafficking | [33] |
| Chloroquine | Modified HEK293 cell | ATP-sensitive potassium channel | 10 μM | Block | Enters the pore and induces direct block | [36] |
| Clotrimazole | Modified HEK293 cell | TRPV1 & TRPA1 | 5 μM | Opening channel | | [155] |
| **Halogenated organic compounds** | | | | | | |
| Dioxin | Rat ventricular myocyte | L-type calcium channel | 1–100 nM | Increase current and regulate activation | Prolonged repolarization | [39] |
| PCB 136 | Hippocampal neuron | Ryanodine receptor | 10 nM | Activate receptor | Increasing intracellular $Ca^{2+}$ | [40] |
| PCB 126 | Rat ventricular myocyte | Rapidly activating delayed rectifier potassium channel | 300 nM | Increase current | | [29] |
| PCB 95 | Hippocampal neuron | Ryanodine receptor | 2–200 nM | Activate receptor | Increasing intracellular $Ca^{2+}$ | [41] |
| 6-OH-PBDE-47 | Xenopus oocyte | GABA(A) & nAChR | 10–100 μM | Activate GABA(A) and inhibit nAChR | Additive inhibition of nAChR | [47] |
| DDT & DDE | Modified HEK293 cell | Ryanodine receptor | 0.1–10 μM | Activate receptor | | [49] |
| PBDE 209 | Hippocampal neuron | Voltage-gated sodium channel | 0.05–2 μM | Block and regulate activation and inactivation | Mechanism underlies peroxidation | [51] |
| Tetrabromobisphenol A | Rat cerebellar granule cell | Membrane potential | 25 μM | Depolarization | Mediated by ionotropic glutamate receptors and VGSCs | [53] |
| PFOA & PFOS | Hippocampal neuron | Potassium & sodium channel | 1–100 μM | Increase potassium current | | [59] |
| **Pesticides** | | | | | | |
| Cypermethrin & permethrin | Antennal lobe neuron | Voltage-gated sodium channel | 10–50 μM | Slow inactivation | Regulation in a state-dependent manner | [60] |
| Tetramethrin & permethrin | Olfactory receptor neuron | Voltage-gated sodium channel | 10 μM | Slow inactivation | | [61] |
| Bifenthrin | Cerebral cortical neuron | Voltage-gated sodium channel | 10 μM | Slow inactivation | | [62] |
| Tetramethrin | Smooth muscle | L-type calcium channel | 1 μM | Block | | [156] |
| α-cypermethrin | Hippocampal neuron | Transient outward potassium channel | 1–100 nM | Increase current | | [71] |
| Tefluthrin | Hypothalamic neuron | Sodium and L-type calcium channel | 1–10 μM | Increase sodium current and decrease calcium current | Effects are inhibited by TTX | [72] |
| Carbamate pesticide | Xenopus oocyte | nAChR | 1–10 μM | Block | Noncompetitive and sequential mechanism | [157] |
| Pyrazoline | Xenopus oocyte | Voltage-gated sodium channel | 1–5 μM | Block | | [158] |
| Indoxacarb | Front thoracic ganglia | Voltage-gated sodium channel | 1 μM | Block | Several subtypes are resistant to inhibition | [159] |

(*continued on next page*)

**Table 1** (*continued*)

| Ingredient | Organism | Targeted receptor/channel | Dose | Response | Extra information | Ref. |
|---|---|---|---|---|---|---|
| **Heavy metals** | | | | | | |
| $Zn^{2+}$ | Spinal dorsal horn neuron | glycine receptor | 1−100 μM | Increase current at lose dose (1 μM) and decrease at high dose (100 μM) | Activation of voltage-gated $Na^+$ and $Ca^{2+}$ channels | [88] |
| $Zn^{2+}$ | Carp retinal horizontal cell | Calcium-permeable AMPA Receptor | 300 μM | Decrease current | Regulate the receptor function | [160] |
| $Pb^{2+}$ | Outer hair cell | Rectifier $K^+$ channel | <1 μM | Decrease current | | [79,81] |
| $Pb^{2+}$ | Hippocampus (CA1) neuron | Voltage-gated sodium channel | <1 μM | Decrease current and regulate activation and inactivation | Participate in lipid peroxidation reaction | [78] |
| $Pb^{2+}$ | Brain neuron | Calcium channel | <10 μM | Block | Modulate ryanodine receptor | [82,83] |
| $Pb^{2+}$ | Pheochromocytoma (PC12) cell | Voltage-gated calcium channel | 25 μM | Increase current | | [80] |
| $Pb^{2+}$ | Xenopus oocyte | nAChR | <1 μM | Increase or decrease the current | Depend on specific subunit | [84] |
| Trimethyl lead | Dorsal root ganglion neuron | Voltage-gated calcium channel | 1 mM | Decrease current | | [161] |
| $Ni^{2+}$ | Atrioventricular node myocyte | Hyperpolarization-activated channel | 5 mM | Increase current | Na/Ca exchange block | [80,89] |
| $Ni^{2+}$ | Modified HEK293 cell | NMDA receptor | 50 μM | Increase current | Target GluN1 and GluN2B subunits | [90] |
| $Cd^{2+}$, $Hg^{2+}$ | Renal epithelial cell | Epithelial $Na^+$ channel | <2 mM | Decrease current | | [162] |
| $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$ | Renal epithelial cell | Epithelial $Na^+$ channel | <2 mM | Increase current | $Cu^{2+}$ reduces $Na^+$ self-inhibition | [162] |
| $Hg^{2+}$ | Cortical neuron | Whole-cell inward current | 0.1 μM | Increase current | Over-activate NMDA receptor | [85] |
| $Hg^{2+}$ | Dorsal root ganglia neuron | TRPV1 | <1 μM | Decrease current | Affect extracellular cysteine residue | [86] |
| $Hg^{2+}$ and MeHg | Modified HEK293 cell | TRPC channel | 1 μM | Opening channel | Affect extracellular cysteine residue | [87] |
| MeHg | Neonatal cerebellar granule cell | Calcium channel | 0.25 mM | Block | No subtype selectivity | [77] |
| **Air pollutants** | | | | | | |
| CO | Snail buccal neuron | Sodium channel | 50 μM | Decrease current | Inhibition of VGCCs | [99] |
| $SO_2$ | Human atrial cell | L-type calcium channel | 5 μM | Block | | [93] |
| $SO_2$ derivatives | Hippocampal neuron | Delayed rectifier potassium channel | 10 μM | Increase current | | [98] |
| Sodium metabisulfite | Hippocampal neuron | Transient outward potassium channel | 10 μM | Increase current and regulate activation and inactivation | | [96] |
| NO | Human cardiac fibroblast | Calcium-sensitive potassium channel | 100 μM | Increase current | Associated with PKG and PKA pathways | [102] |
| $H_2S$ | Rat pituitary tumor cell | Calcium-sensitive potassium channel | 300 μM | Increase current | | [103] |
| $NH_3$ | Midbrain dopamine neuron | Acid-sensitive ionic channel | 50 mM | Modulate gating kinetic | | [104] |
| $SO_2$ derivatives | Dorsal root ganglion neuron | Sodium channel | 1−10 μM | Modulate gating kinetic | Increase or decrease current in a voltage-dependent manner | [163] |
| **Toxin** | | | | | | |
| TTX | Mammalian cell | Sodium channel | 1 μM | Block | Several subtypes obtain resistance | [112] |
| TTX | Dorsal root ganglia neuron | TTX-s channel | 0.1 μM | Block | Decrease amplitude by 97% | [110] |
| ImKTx1 | Modified HEK293 cell | $K_V$ 1.1−1.3 | 1−10 μM | Block | No effect on sodium channels | [114] |
| *Echis coloratus* | Modified HEK293 cell | TRPV1 | | | | |
| KTx family | Human peripheral lymphocyte | Potassium channel | ~10 nM | Block | | [116] |
| Cobrotoxin | Dorsal root ganglia neuron | A-type and delayed rectifier potassium channel | 1 μM | Increase current | Associated with the activation of M3R | [117] |
| Crotoxin | cardiomyocyte | L-type calcium channel | 2 μM | Increase current | | [118] |
| α-Conotoxin | Xenopus oocyte | nAChR | <1 μM | Block | Associated with receptor subunit combination | [120] |
| Kappa-conotoxin | Xenopus oocyte | Voltage-gated potassium channel | 0.1 μM | Block | Binding to the protein and occluding the pore | [121] |

calcium channels [48]. DDT and its metabolites, for example, dichlorodiphenyldichloroethylene, can also engage with ryanodine receptor and increase the $Ca^{2+}$ transient amplitude, demonstrating potential damage to muscles [49].

Brominated flame retardants, including polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol-A (TBBPA), are emerging neurotoxic chemicals pervasive in the environment. PBDEs and their hydroxylated or methoxylated derivatives have attracted remarkable attention due to their acute and developmental neurotoxicity [50]. Evidence revealed that PBDEs and TBBPA have the capability of enhancing calcium current via interaction with the ryanodine receptor [7]. PBDEs also can efficiently inhibit voltage-dependent sodium channel activity and prevent recovery from inactivation [51]. Moreover, PBDEs and TBBPA potently modulate postsynaptic GABA receptor and nAChR, suggesting a different mechanism behind the regulation of calcium homeostasis except for ryanodine related signal pathways [47,52]. Additionally, the glutamate receptor was found to be a potential target of TBBPA, leading to the modulation of voltage-gated sodium channel and membrane hyperpolarization [53]. Bromophenol from marine animal secretion or industrial production can be used as a flame retardant or pesticide, with adverse ecological and toxicological functions. Calcium release from intracellular calcium stores induced by bromophenol is considered as main reason for the abnormal increase of intracellular calcium concentration [54].

Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are neurotoxic contaminants ubiquitously present in the environment [54,55]. Previous studies observed abnormal intracellular calcium overload in hippocampus neuron after PFOS exposure [56]. Further, the accumulation of PFOA and PFOS in neurons can induce the release of intracellular calcium stores and increase intracellular $Ca^{2+}$ levels, likely triggered by interaction with 1,4,5-trisphosphate receptor and ryanodine receptor [57]. PFOS also enhances the inward $Ca^{2+}$ current [58]. An electrophysiological study found that PFOS and PFOA could evoke a less negative membrane potential that cannot be completely explained by the alteration in calcium channel activities, indicating the effect of other potential targeted channels. Indeed, a delayed rectifier current as well as transient outward current is obviously enhanced by PFOS in hippocampal neurons [59]. Recent studies observed the activation of glutamate receptor and modulation of sodium channels after PFOS exposure, probably due to the alteration of membrane potential. These changes may further affect the function of NMDA receptors and influence synapse growth, resulting in chronic neurotoxicity [56,58].

### 3.1.3. Pyrethroid pesticides and insecticides

Ion channels are a sensitive target of many pesticides, underlying potent neurotoxicity to insects and, potentially, to human. Pyrethroids, originally extracted from pyrethrum, cause significant disruption in the nervous system of insects and mammals primarily via modification of voltage-gated sodium channels [60,61]. Many changes in channel kinetics have been observed by patch-clamp recording after pyrethroid exposure, including the prolongation of action potential, an obvious alteration in steady-state potential, and delayed inactivation [62]. Based on the observed associations between chemical structure and clinical syndromes of intoxication, a nomenclature was proposed to divide pyrethroids into two subgroups, type I and type II; these two subgroups exhibit distinct action on neural activities. Type II pyrethroids, which contain the α-cyano-3-phenoxybenzyl moiety in their chemical structure, more significantly modulate voltage-gated sodium channel [63,64]. For example, deltamethrin, a type II pyrethroid, produced a potent sodium tail current in mammalian neurons that lasted 9 times longer than that of S-bioallethrin (a type I pyrethroid) [65]. A toxicological study found that type II pyrethroid pesticides selectively block TTX-resistant sodium channels in dorsal root ganglion (DRG) neurons, mainly $Na_v1.8$ [66]. More recent studies further indicated that, among all sodium channel subtypes, $Na_v1.2$ exhibited a remarkably low sensitivity to type II pyrethroids while $Na_v1.3$ and $Na_v1.6$ were demonstrated to be highly sensitive to a group of pyrethroids [65]. Additionally, the prolonged sodium currents were found to be TTX-sensitive when they were induced by type I pyrethroids, including tetramethrin and permethrin [61]. Interestingly, pyrethroids were observed to modulate sodium channels in a state-dependent manner, indicating that they preferentially bind to the sodium channel in the open state [67]. It has been reported that the application of high-frequency depolarizing pulses enhances the type II pyrethroid-induced sodium channel modification, indicating that the transition of conformation is potentially associated with pyrethroid-mediated effects [68,69].

Aside from sodium channel modulation, studies also found that type I pyrethroids can partially block mammalian calcium channels at low micromolar concentrations (10 μM). T-type calcium channels in cardiac cells (completely blocked at 0.1 μM) exhibited a higher sensitivity to several pyrethroids than that in muscle cells (30 μM). However, the L-type calcium current is reduced by approximately 85% in muscle cells after 2 μM tetramethrin exposure, compared to a 50 μM active threshold in terms of heart cells [70]. Action on chloride channels was also identified as a consequence of pyrethroid exposure [63]. Several type II pyrethroid compounds, for instance, deltamethrin, significantly decreased the chloride current via inhibition of both voltage-dependent and GABA-gated chloride channels. Moreover, some pyrethroids, including both types, also comparatively slightly inhibit the potassium channel, particularly reducing the voltage-gated rectifying potassium current [71,72].

Organochlorine pesticides can evoke obvious alterations in sodium and calcium channel activities as discussed above. In contrast, organophosphorus pesticides are well known for their effects on nicotinic acetylcholinesterase. By binding to the active sites located on nicotinic acetylcholinesterase, organophosphorus pesticides prevent the normal degradation of acetylcholine and lead to a high excitability of postsynaptic ion channels [73]. Phosphinothricin, an active organophosphorus herbicide, was found to be responsible for the inhibition of rectifier potassium channels, obviously reducing the inward current ($IC_{50} = 34.5$ μM). This finding may help describe the contribution of potassium channel blockade to the neurotoxicity of organophosphorus compounds [74].

### 3.1.4. Heavy metals

Among the known environmental pollutants, heavy metals, such as mercury, cadmium, nickel, and lead, have been extensively studied since they are severe neurotoxic agents. These elements, including the cations and their derivatives, are quite active in disrupting neural activities.

Generally, because of their similarity with $Ca^{2+}$ in terms of ionic charge, hydrated ionic radius, and electron distribution, divalent cations such as $Pb^{2+}$, $Hg^{2+}$, $Cd^{2+}$, and $Ni^{2+}$ can easily flow across membranes via various types of calcium channels [75,76]. This mechanism not only decreases the effective calcium current but also contributes to the toxicity derived from intracellular metal ions [75]. Some of the organic derivatives of these cations, for example, methylmercury, target certain active sites in ion channels, probably causing irreversible inactivation [77]. Thus, due to the high need for direct detection of ion currents, patch-clamp is one of the most popular tools for such studies.

Lead, commonly released from the combustion of fossil fuels and the fabrication of metallic instruments, has shown potent affinity to diverse types of sodium, potassium, and calcium channels [78]. $Pb^{2+}$ potently inhibits voltage-gated sodium and potassium

channels, increasing both the peak current of calcium channels and the amplitude of sustained calcium current, collectively preventing the outflow of $Ca^{2+}$ [79–81]. $Pb^{2+}$ can depress the calcium current evoked by ryanodine receptor activation and possibly increase the concentration of intracellular $Ca^{2+}$, impairing the long-term potentiation of hippocampal neurons [82,83]. $Pb^{2+}$ is also able to affect the postsynaptic response in neurotransmission by binding with certain subunits of nAChR [84].

Mercury is able to degenerate the nervous system and disturb neuronal excitability. $Hg^{2+}$-induced over-activation of the NMDA receptor and the responding reaction in synaptic current has been observed in central neurons [85]. Because of the high affinity with sulfhydryl groups, mercury and its derivatives are effective in interacting with the extracellular cysteine residues of ion channels, leading to abnormal activation or inactivation [86,87]. Methylmercury has been reported as a wide range inhibitor of various types of calcium channels reducing the amplitude of calcium currents at low micromolar concentrations (0.25–1 μM). No preference of specific subtypes of calcium channels is found in such an effect and the current–voltage relationship is constant [77].

Zinc is a typical physiologically relevant metal released regularly by cells. Generally, $Zn^{2+}$ modulates the glycine-induced current in synaptic transmission, which mainly depends on the extracellular concentration of $Zn^{2+}$. This effect may lead to increasing or decreasing release of glycine and to an alteration in receptor activity [88]. It is comparatively harder for nickel to produce a clinical neurotoxicity due to the low environmental exposure, although it really induces an obvious ion channel modulation at a high concentration (5 mM) in the laboratory [89,90].

#### 3.1.5. Air pollutants

Air pollutants include a large group of compounds from natural or artificial sources. Limited evidence demonstrates a potential association between long-term exposure to polluted air and an increased risk of neural dysfunction. The mechanism underlying this neurotoxic effect may be linked to the modulation of ion channels.

Sulfur dioxide ($SO_2$) is a common air pollutant mainly released from the combustion of fossil fuels. There is evidence suggesting that $SO_2$ inhalation may lead to synaptic damage and alteration of synaptic plasticity [91]. $SO_2$ and its derivatives (such as bisulfite and sulfite) elicit negative inotropic effects in cardiovascular toxicity, involving L-type calcium and ATP-sensitive $K^+$ channels [92]. Electrophysiological studies indicate that 50 μM of $SO_2$ derivatives are able to attenuate L-type calcium currents in cardiomyocytes, obviously depressing the peak current but inducing no effect on the reversal potential and voltage threshold [93]. Moreover, a high concentration (1 mM) of $SO_2$ derivatives reduces intracellular calcium levels [94]. $SO_2$ derivatives are also effective in modulating voltage-gated potassium channels and in increasing extracellular $K^+$ [95]. They prominently augmented the rectifier potassium current, decreasing the time constant of activation and inactivation, and reducing the excitability of neurons [96–98]. Interestingly, some studies demonstrated that the steady-state inactivation curve of sodium channels in ganglion neurons was obviously shifted towards the depolarizing orientation by $SO_2$ derivatives, which may lead to greater activation of action potential, representing a positive effect on neural excitability [99].

Other gaseous air pollutants, including CO, $H_2S$, and $NH_3$, are potential ion channel modulators. Micromolar concentrations (50 μM) of CO decrease neuronal excitability via inhibition of sodium and calcium currents. CO blocks persistent sodium channels and depolarizes the resting membrane potential, which mediates the inhibition of voltage-gated sodium channels [99]. This effect is partially derived from the CO-induced increased production of intracellular NO, which may lead to S-nitrosylation of the $Na_v1.5$ channel [100,101]. NO can enhance the potassium current through a calcium-sensitive potassium channel, which probably performs as a potential mechanism behind the toxicity of CO [102]. $H_2S$ is a physiologically active gas in living cells. Recent studies have found that $H_2S$ can enhance the calcium-activated potassium channels and regulate electrical activity in nerves and muscle [103]. $NH_3$ plays a crucial role in gating of the acid-sensitive ion channel, which usually participates in pain sensation, suggesting the function of ion channels in $NH_3$-induced neuronal disorder [104].

Suspended particulate matter has increasingly become one of the most important air pollutants worldwide, with a complicated composition, including metallic particulate matter, inorganic mineral fragments, and organic adsorbates. Atmospheric fine particulate matter with a size of <2.5 μm ($PM_{2.5}$) has the capability to enter the cardiorespiratory system and elicit pulmonary and cardiovascular diseases [105]. $PM_{2.5}$ exposure apparently increases intracellular $Ca^{2+}$ concentrations, probably associated with the ryanodine receptor. It has been reported that the silencing of related genes, for example *Orai1*, effectively prevented a $PM_{2.5}$-induced intracellular $Ca^{2+}$ overload [106]. Probes for intrinsic and intracellular reactive oxidative species were used to detect an increased intrinsic free radical production after $PM_{2.5}$ exposure, indicating that oxidative stress probably accounts for the alteration in calcium homeostasis [107]. Thus, the application of antioxidants obviously alleviated the disruption of calcium homeostasis, further confirming the crucial role of oxidative stress in $PM_{2.5}$-induced toxicity [108,109].

### 3.2. Toxins

It is well known that several toxins released by animals, such as spiders, marine animals, and snakes, are natural ion channel modulators. Studies using patch-clamp platforms are useful for the characterization of the physiological activities of those toxins. For example, intoxication by TTX can lead to a deadly rapid muscle paralysis. Detailed kinetic studies based on TTX selectivity divided the sodium channels into two subgroups as TTX sensitive and TTX resistant, which helps to follow-up research identify the potential target of novel toxins [66]. TTX can bind to the exposed part of the sodium channel protein and efficiently block TTX-sensitive channels at nanomolar concentrations (100 nM) [110]. Sodium channels in skeletal muscle and heart can also be blocked by TTX, albeit at much lower sensitivity than that of nerves [111,112].

Similarly, toxins from cone snails, spiders, and scorpions show specific selectivity when interacting with various ion channels [113,114]. Viper venom contains many functional proteins with the capacity to activate the TRPV1 channel. A patch-clamp system was utilized to search for active compounds from the extract of viper venom and led to information about the TRPV1 activating mechanism [115]. Six novel peptides extracted from Mexican scorpion venom were shown to have high affinities to voltage-gated potassium channels, selectively inhibiting the $K_v1.2$ subtype at nanomolar concentrations (~10 nM) [116]. Cobrotoxin, a novel toxin isolated from venom of *Naja atra*, increases the transient potassium current in rat DRG neurons in a concentration-dependent manner [117]. Crotoxin, an active component of the venom from the South American rattlesnake, prolongs the duration of action potential and obviously increases the L-type calcium current, suggesting a potential cardiotoxicity [118]. Neuronal nAChR is a common target of several kinds of toxins [119]. The effect of toxins is determined, to a large extent, by their chemical structure and the combination of receptor subunits, including α2β2, α2β4, α3β4, etc. For example, an alpha-conotoxin AuIB

potently blocks the nAChR expressed in *Xenopus oocytes*, especially the α3β4 receptor [120].

The inhibition characteristics recorded by patch-clamp systems, including voltage dependence, channel kinetics, and inhibitory fraction, are associated with the molecular mechanism of certain toxins, providing the necessary information for structure−activity relationship studies [114]. The conotoxin kappa-PVIIA has a high affinity to bind voltage-gated potassium channels associated with the electrostatic surface of the receptor and ligand, providing a basis for investigation of binding orientation [121]. Crotamine is a typical toxin from rattlesnakes that induces hind-limb paralysis, which has long been attributed to the inhibition of sodium channels. However, recent studies found no obvious alteration in sodium channel characteristics although a reaction of fast muscle was observed [3]. An α-conotoxin isolated from cone snail showed high potency in inhibiting high-voltage-activated calcium channels in DRG neurons, which is abolished by the pertussis toxin, suggesting the possibility that the toxin blocks calcium channels via G-protein-coupled receptor [122].

### 3.3. Challenges in manual patch-clamp recording

Although the traditional manual patch-clamp technique is a valuable tool for identifying toxic chemicals in the environment and further characterizing their function in neural activities, the application of manual patch-clamp technique in toxicological studies is limited by its low throughput and requirements of proficient skill and experience.

The confirmed positive correlation between the ability to modulate ion channels and the observed dysfunction in nerve and muscle forms the basis for the need of early toxicological estimation of chemicals. However, the limitations of manual patch-clamp with regards to throughput, success rates, and time consumption, highly decrease the research progress in these areas. Generally, manual patch-clamp provides a low throughput, ranging from 20 to 40 effective data points per day, which falls short of the usual toxic compound library that contains millions of compounds [9]. Other high throughput screening methods have been applied to perform a primary screen followed by a precise manual patch-clamp recording to avoid the throughput drawbacks [123]. Nevertheless, although this strategy partially avoids the low throughput restrictions of manual patch-clamp, the throughput of tens of data points per day can hardly match the millions of compounds screened with true high-throughput screening methods such as fluorescence assays [9]. Furthermore, the low throughput of cell examination in manual patch-clamp results in random deviation in recording derived from the variable ion channel expression across different cells, reducing the comparability [124].

For manual patch-clamp, researchers must manipulate the pipette onto the cell surface. Although there may be devices allowing visual conduction, the steps required to form a high resistance seal, such as approaching orientation estimation and seal property judgement, have a high technical requirement. Thus, lowering the necessary technical capability is vital for increasing the recording throughput [125]. Factors like technical manipulation play an important role in conventional patch-clamp processes, which accounts for the observed discrepancies between documented data from different sources.

## 4. Development and application of the automated patch-clamp technique

The development of automated patch-clamp systems undoubtedly brought major innovation to toxicological experimental tools, truly improving the efficiency of studying ion channels and making the fast screening of dangerous compounds practical. Adequate data about compounds and their toxicity form the basis of studies of structure−activity relationships. Compared to conventional manual patch-clamp, automated patch-clamp has shown various degrees of success in diverse areas, including ion channel characterization, safety testing, and toxicant screening.

### 4.1. Ion channel characterization

To date, automated patch-clamp systems have been applied in the fast and effective identification of potential environmental toxicants, especially in the characterization of potential molecular targets. Polycyclic pyridines, a class of small organic compounds, are widely used in chemical and biological research and production. Previous studies had found some polycyclic pyridines to be structurally associated with some popular neural inhibitors, demonstrating their potential to inhibit ion channel activities [126]. An automated patch-clamp system, Qpatch, characterized the inhibitory potency of a polycyclic pyridine, 2,6-bis(2-benzimidazolyl) pyridine, on both calcium-gated and voltage-gated potassium channels. It was proven to be an effective inhibitor of calcium-gated potassium channels (~93% inhibition at 1 μM), especially for three subtypes of the small conductance calcium-gated potassium channels ($IC_{50}$: ~0.4 μM). This effect was probably associated with one specific amino acid, H491N, of the tested channel, while the H491N mutant channel was not affected by pyridines but still blocked by typical small conductance potassium channel blockers [127]. The bispyridinium compound, which is structurally similar to 2,6-bis(2-benzimidazolyl) pyridine, was a non-competitive antagonist of nAChR, and co-application of nicotine and bispyridinium compounds led to an enhanced maximum current amplitude and prolonged current decay. Based on the prediction of pharmacological effect using the chemical structure, several pyridinium compounds were developed to recover the desensitized nAChR receptor in intoxication of organophosphorus compounds [128].

A previous study used an automated patch-clamp system to distinguish active ingredients from extract of the herbal medicine, hangeshashinto, finding two major active gradients, 6-gingerol and 6-shogaol. Additionally, the study revealed that the relief of pain was attributed to the inhibition of voltage-gated sodium channel $Na_v1.8$, which is associated with the pain response activated in primary pain-related sensory neurons [129]. Phlotoxin-1 was an effective ion channel modulator and the inhibitory fraction of sodium channel $Na_v1.7$ at 1 μM concentration of exposure reached 90%, with an $IC_{50}$ value of 39 nM [130]. Because of the affinity and selectivity for the $Na_v1.7$ channel subtype, the adjustment of phlotoxin-1 to synthesize updated compounds with improved affinity and selectivity for $Na_v1.7$ channel has started to create new antinociceptive drugs [131]. A 35-amino acid peptide extracted from the venom of the Chinese bird spider, huwentoxin-IV, was popular for its high affinity with the Nav1.7 channel; its interaction with the Nav1.6 channel has also been recently investigated. The signal acquisition and data analysis with automated patch-clamp systems indicated a functional inhibition of the Nav1.6 subtype, which was approximately twice as strong as that for TTX [132]. Similarly, paralytic shellfish poisoning toxins target voltage-gated sodium channels. Electrophysiological experiments demonstrated that nine paralytic shellfish poisoning analogues greatly inhibited the Nav1.2 and Nav1.6 channels at nanomolar concentrations. In summary, the automated patch-clamp technique in combination with analytical methods provide a reliable high throughput screening platform for examination of the ion channel affinities of these molecules, especially promoting the characterization of specific targeted ion channel subtypes [133].

Azaspiracids are a family of marine toxins produced by algae and contained in many types of seafood. The appearance of abnormal arrhythmias in victims revealed their potent cardiotoxicity. Patch-clamp recording suggested that no apparent alteration in hERG currents occurred with the presence of azaspiracids but a remarkable change in hERG channel density on the cell surface was detected, which showed a fourfold increase in hERG protein after exposure. Therefore, these toxins were believed to be associated with the hERG channel trafficking to lead to more non-functional hERG channels [134]. Aconitine, a major ingredient of the famous herb aconitum, has shown a remarkable potency in inhibiting L-type calcium channels, decreasing both the duration and amplitude of the calcium current. Additionally, the possible inhibition of sodium channels was observed in patch-clamp tests on human induced pluripotent stem cell-derived cardiomyocytes. A modest suppression on the rapid delayed rectifier potassium channel was found but it could not account for the aconitine-induced proarrhythmic action [135].

### 4.2. Safety tests for drug discovery

The application of conventional patch-clamp systems in drug discovery has accomplished many achievements, as discussed above. However, the development of new drugs went far beyond the capability of safety testing with conventional low-throughput electrophysiological method, leading to the demand of a more convenient screening method. The recent advances in automated patch-clamp provide a reliable platform that permits the fast screening required in drug discovery.

Following reporting of the potential inhibition of hERG caused by many structurally and functionally unrelated pharmaceuticals, rapid tests for side effects on hERG channels became necessary for all drug candidates. An assessment model of potential cardiotoxicity based on high-throughput electrophysiological techniques has been proposed to rapidly provide a reliable evaluation of potential cardiotoxic chemicals. A novel parameter termed 'cardiac safety index' is now used as a metric of cardiotoxicity, derived from the normalization of several toxicity values recorded by automated patch-clamp [123]. The obvious prolongation of field potential discovered in electrophysiological recordings pointed out several active compounds that induce arrhythmia, including vandetanib, disopyramide, and quinidine [136]. Dofetilide, which led to a significant prolongation of hERG repolarization ($IC_{50}$ = 7 nM), was confirmed, by automatic patch-clamp, to inhibit the rapid delayed rectifier potassium current and calcium-activated potassium channels simultaneously [2,126]. The measurement of the $IC_{50}$ value of cisapride ($IC_{50}$ = 18 nM) targeting at hERG1 channel was duplicated on automated patch-clamp platform, suggesting that the admitted dose of cisapride applied to human potentially led to negative effects on cardiac activities [137]. Moreover, some intermediate risk compounds, such as loratadine, astemizole, and terfenadine, are probably potent hERG channel inhibitors at a high doses [138]. Except for those that were studied well using conventional patch-clamp, the wide range screening of marketed drugs discovered, by using automated patch-clamp, considerable amount of new potent blockers of hERG, such as trimethoprim, famotidine, and protopine. Generally, these new discovered compounds exhibit intermediate or low risk of inducing cardiac arrhythmia, and their $IC_{50}$ values are always larger than 1 μM [139,140]. Methadone, an opioid drug, is associated with cardiac arrhythmias attributed to the inhibition of the hERG channel. However, based on the assumption that opioids may lead to cardiotoxicity by inhibition of the sodium channel $Na_v1.5$, researchers found an obvious shift of the peak hyperpolarized potential and inactivation of sodium channels [141]. GABA is one of the inhibitory neurotransmitters in synaptic transmission. It is reported that some drug candidates act as potential positive GABA modulators in the environment and affect the neural signal transmission. Amplification of GABA current in the presence of MIDD0301, a novel drug candidate, was confirmed [142]. Other structurally and functionally unrelated drugs, such as diazepam and HZ166 (a benzodiazepine agonist), were also reported to be active GABA modulators in assay assessment [143].

### 4.3. Toxicant screening

Based on an automated patch-clamp platform, it is practical to establish a powerful high-throughput screening method for different categories of toxicants. In earlier studies, active screening of a large compound library applied non-electrophysiological high-throughput method to reduce the number of chemicals truly analyzed by patch-clamp technique. For example, a previous study used molecular docking to give a compound specific score according to their active binding sites, indicating the likelihood of being positive. After primary screening with molecular docking, a subset of 89 potentially toxic compounds were distinguished from a large category containing nearly 20,000 compound structures. Automated patch-clamp measurements identified 14 positive compounds from these 89 compounds exhibiting apparent inhibition of voltage-gated potassium channel at a micromolar concentration ($IC_{50}$ = 0.58–6 μM), accounting for 17% of the total compounds tested. As the virtual screening methods reduce the required throughput for electrophysiological experiments, they represent a practical strategy for decreasing the requirements of time, skills, and cost [144]. Based on the recent development of automated patch-clamp systems, it is possible to obtain nearly 10,000 data points per day by using automatic platforms, and the cost for each data point has been estimated as nearly 0.1 dollar [9]. In comparison, the manual patch-clamp system can only receive tens of data points per day, and the cost relies on the success rate and would be higher than that of automated system.

To date, the development of an automated patch-clamp technique improves the throughput and accuracy of compound identification, making the overall screening of compound libraries possible. A mushroom metabolite from the *Hypholoma lateritium* extract showed considerable ion channel affinity. Multiple components from the purified fungal extract was tested in an automated patch-clamp system. One of the compounds elicited a remarkable inhibitory effect on the G-protein gated inwardly rectifying K (GIRK) channel ($IC_{50}$ = 395.1 nM) and a weaker effect on the hERG channel (7.9% inhibited at 100 μM) [145]. The screening of novel selective inhibitors of the $Na_v1.7$ channel from 117 distinct toxins using automated patch-clamp recording has been conducted. A peptide from spider venom was found to effectively inhibit not only the $Na_v1.7$ channel but also all TTX-sensitive channels in a high nanomolar range ($IC_{50}$ = 72.0–129.5 nM) [146]. In order to study the activation of the neural voltage-gated potassium channel $K_v7.2$, a fluorescence-based high-throughput screening technique was utilized as a primary screening method to distinguish the active compounds from a collection of 80,000 compounds. Although this technique offers much less information that pertains to the kinetics of inhibited channels and the mass-dependent relationship, it has advantages in throughput, cost, and easy operation. The examination of 565 compounds distinguished from primary screening was performed by an automated patch-clamp system, identifying 38 compounds

(7% of the total number) as $K_v7.2$ channel activators [123]. Additionally, the high-throughput screening method based on automated measurement provides a fast and convenient tool for cardiotoxicity testing of drugs, especially for those derived from hERG inhibition. Twelve compounds from 1408 substances of a toxicological collection were validated as effective hERG inhibitors by automated whole-cell patch-clamp experiments. The collected statistics representing the inhibitory function of a large number of compounds in this study were applied to investigate the quantitative structure−activity relationship; the results showed that the inhibitory potency of quaternary ammonium compounds was associated with their aliphatic side chains [147]. Another study collectively tested the hERG inhibition of over 300,000 compounds from the Molecular Library Small Molecular Repository. The $IC_{50}$ value of each compound was calculated, obtaining the standard to divide the compound class according to their potency. Approximately 1.64% of compounds in the library were proven to possess a high inhibitory potency, meaning that their $IC_{50}$ values were lower than 1 μM. By analyzing such a large group of chemicals, it was found that positive compounds were more likely to be hydrophobic, high-molecular weight, flexible, and polarizable, which provided a novel perspective of hERG inhibition [148].

### 4.4. Advances and limitations

Since the automated patch-clamp technique was introduced, it has experienced a decade of development and several times of innovations. To date, automated patch-clamp has shown major advantages, bringing enormous changes to electrophysiology in various fields. However, some defects have also been identified in practical use as demonstrated below. A comparison between manual and automated patch-clamp techniques is provided in Table 2.

#### 4.4.1. Cell preparation

Compared with conventional manual patch-clamp, the automated patch-clamp systems set a higher request for the cell preparation processes, mainly due to the requirements of good cell quality. While an experienced electrophysiologist positively chooses the target cells under visual guide during conventional patch-clamp, automated systems randomly choose the recording cells, possibly leading to confusion in data quality if only a small set of cells are tested. Instead of using cells directly isolated from animal tissues, in automated patch-clamp systems, the use of stable cell lines that have been confirmed as suitable in high throughput electrophysiology is preferred [14]. This is because the cells applied to automatic patch-clamp need to be suspended for capture of pipettes or plates and have a stable expression of specific types of ion channel. At present, there is limited success in generating applicable cell lines for automatic patch-clamp, especially within the range of channel expression optimization in chosen cell line. Some questions remain in our comprehension of how ion channels or receptors work in organisms. For example, the α7 nicotinic receptor works only if there is stable expression of the related protein ric-3, which makes early attempts to express the α7 nicotinic receptor in subclones of other stable testing cell lines unsuccessful until the profiling of the chaperone protein [149]. Therefore, identification of a certain part that determines the function of ion channels when expressed in common cell line is necessary, but time-consuming. Alternatively, developing a specific cell line based on the research needs for application in automated systems is also a feasible option provided that requirements of time, cost, and effort can be covered.

To fulfill the requirements of appropriately cultured cells and to increase the experiment success rate, precise control of the environmental conditions in cell preparation is crucial. By designing a special system for suspension cell culture, a high cell density suspension with acceptable cell viability could be obtained [150].

#### 4.4.2. Throughput

Throughput is one of the greatest limitations of the conventional manual patch-clamp technique. Since an experienced electrophysiologist can only record less than 10 data points per day on a manual patch-clamp platform, it is impossible for the screening of large quantities of environmental contaminants or drugs to be performed [13]. To increase the throughput, various upgrades have been performed. One of these advances is the increase of recording sites. Early IonWorks HT and Quattro systems use a planar 384-well plate to automatically form resistance seals with attached cells and finish the recordings. The simultaneous collection of 384 data points in these systems provides considerable throughput for electrophysiological studies, although it compromises in the resistance of the membrane seal (~100 MΩ) compared with the conventional standard (>1 GΩ). More recently, the drawbacks of the seal quality are addressed in many new automated patch-clamp systems, truly combining high recording quality with high throughput [9]. Additionally, the successful patch-clamp rate determined by seal resistance and holding current of these automated systems in a single recording is acceptable, at >50%, which is higher than that obtained for manual experiments (35.3%) [12]. Moreover, the population measurement mode applied in IonWorks Quattro additionally provides an improved success patch-clamp rate (~95%) because several data points are recorded in a single well and averaged, further increasing the effective throughput when applied.

Because of the occurrence of a new generation of automated patch-clamp systems, the cost of obtaining a single data point has been greatly reduced (nearly 0.1 dollar), making the high throughput screening of compounds economically available. It is obvious that the reduction in cost for obtaining a single data point will have a positive effect on the toxicological studies associated

**Table 2**
Comparison of automated and manual patch-clamp systems.

| Configuration | Substrate | Preparation requirement | Automation | Throughput | Operability | Data quality | Application |
|---|---|---|---|---|---|---|---|
| Automated planar-array system | | | | | | | |
| Planar plate | Cell culture | Cells in suspension with high viability | High but not complete | High | Simplified, enhanced but less flexible | Lower resistance seal; frequent deviation; suspected | High throughput screening |
| Automated electrophysiological system with conventional pipette | | | | | | | |
| Conventional pipette | Brain slice | Conventional | Depending on the software | Low | Depending on the software | Similar to Manual patch-clamp system | Studies of high-order nervous system feedback |
| Manual patch-clamp system | | | | | | | |
| Conventional pipette | Cell cultures & Brain slice | Conventional | None | Low | Complicated and hard | Acknowledged | Chemical identification; molecular toxicological study |

with ion channels [9]. Considering all of the above, it is not difficult to conclude that the automated patch-clamp technique increases data acquisition by several orders of magnitude, allowing companies or laboratories to examine more compounds or save time.

#### 4.4.3. Operability

Compared with the manual patch-clamp platform, many automated systems allow easier and more precise control of the experimental conditions. The most obvious change brought by the automation of patch-clamp is the simplified manipulation. Although completely automating the processes is not practical in most automated systems, the assistance in key procedures is quite helpful for researchers to perform the recording, at least thus reducing the time needed for pipette movement, seal formation, and break-in Refs. [12]. The reduced requirement of skill and labor saves trained electrophysiologists from repeating experiments and attracts more research in the field. Moreover, the development of image-guided patch-clamp has effectively improved the operability of manual patch-clamp. The combination of image-guided patch-clamp and an automated pipette or plate control system has led to advances in recording of human brain slices. Some developed systems have even further optimized the automatic pipette cleaning and image-guided cell capture, allowing computers to finish the recording without any help of experimenters [10,21].

In most automated patch-clamp machines on the market, multiple dosing to a single well and changing the internal cell solution during recordings are permitted, which is possible but limited in manual patch-clamp systems. Several automated electrophysiological platforms currently allow for the perfusion of external and internal solutions, providing a valuable tool for studying the function of intracellular factors. For example, by utilizing the internal perfusion system, Sauter et al. conveniently changed the pH value of the intracellular solution and found that the two-pore domain potassium channel (TREK) is sensitive to intracellular pH [151]. Moreover, the automated systems can be integrated with a temperature control unit, although only half of the systems on the market allow for an automatic temperature control. Compared to manual patch-clamp systems, temperature control in automated system does not need extra unit and the usage can be easily programmed during experiment. For example, recordings of the cystic fibrosis transmembrane conductance regulator (CFTR) showed the efficiency of the automatic technique in assessing ion currents, especially with regards to precise temperature control and current–voltage relationship measurements. Recordings under distinct temperatures demonstrated that the mutation of ion channels altered the temperature sensitivity, leading to a discrepancy in results from laboratory or clinical measurements [18].

However, notably, the real-time feedback and adjustment to actual conditions by experienced experimenters are crucial, and the highly automated systems hardly permit this. Based on the presented experimental conditions, such as pipette pressure, location of target cell, and approaching direction, skilled researchers always make reasonable adjustments to the system state and protocol to obtain a better recording. In other words, the real-time interpretation of documented data and the following manipulation are vital for assessing physiological processes and properties, which is a characteristic of conventional manual patch-clamp. In contrast, the automated system can easily provide a large dataset from a standardized, specifically designed and high throughput electrophysiological trial, which is useful in toxicant screening, drug discovery, and quantitative structure–activity relationship analysis. Therefore, although the flexibility of manual patch-clamp has also caused many distinctions between laboratories and studies, automated patch-clamp systems cannot completely replace the manual one in some exploratory studies, while they are more suitable to be used in fast screening and repetitive recording.

#### 4.4.4. Data quality

For data quality and reliability, there remains controversy regarding both the manual and automated patch-clamp techniques. Since the conventional manual technique has been the gold standard in electrophysiological studies, the data collected through the automatic technique raises several doubts. In general, the reliability of automated patch-clamp is obtained by replicating results obtained with the manual machine, though there have been studies indicating that the planar substrate of the automated platform may have a more accurate recording compared to the glass pipette used by the manual platform [152]. Many studies have reported a positive correlation between the results from the two techniques in many aspects, including the capacitance, resistance, and holding current [12,18,123,147]. However, differences have also been found in various studies, partially due to the alteration in experimental conditions. A higher variation in $IC_{50}$ values obtained with automated patch-clamp compared with manual patch-clamp was previously observed [153]. In another study, an apparent consistent rightwards shift of $IC_{50}$ values is frequently detected in high throughput screening systems, which may represent a systematic deviation [14]. Meanwhile, it has been reported that assessment of sodium channel inhibitors generated apparent distinct statistics, which showed that, in terms of Nav1.6 subtype inhibition, the $IC_{50}$ estimation by the automatic technique was 2.5-fold greater than that by the conventional technique [131].

Many automated systems prefer to operate in a static solution rather than in continuous perfusion as performed in the manual system, thus leading to distinct results [13]. Additionally, the use of a planar chip instead of conventional glass pipette likely changes the optimized cell culture strategy and recording protocol. For example, when a similar voltage protocol is implemented, automated and manual patch-clamp generate distinct data in terms of some hydrophobic compounds, suggesting the necessity of protocol optimization [126]. Obviously, the introduction of a specific protocol based on the kinetics of ion channels and on features of high throughput screening system would be practical in reducing the uncertainty of measurements [154]. Due to the uncertainty of automated patch-clamp recordings, some studies use this technique as a primary screening tool and the manual technique is then applied to finally characterize the ion channel function [8].

## 5. Conclusions and future directions

Ion channels, as key molecules, play essential roles in many physiological activities. The conventional manual patch-clamp technique is the gold standard in studies of ion channel activities. This technique relies on the manual manipulation of micropipettes, can provide real-time data for physiological studies, and has been used in toxicological studies, where it provides accurate active compound identification, elucidates the underlying toxic mechanisms, and allows for screening of potential toxicants. Based on previous toxicological studies using this system, a wide acknowledged dataset containing a group of compounds with inhibiting potencies has been established. Molecular mechanisms that contribute to the toxicity of compounds are elucidated via the exploration of the correlation between certain signaling pathways and ion channel abilities. The selective actions of several organic pollutants, toxins, and pharmaceuticals on ion channels have attracted considerable attention and have been harnessed to explain the observed toxicity of many compounds as well as to

reveal the intoxication effects on other biosystems from a structural perspective. Through quantitative structure–activity relationship studies, researchers can obtain direct and abundant information from patch-clamp recordings.

Nevertheless, there are some intrinsic limitations in manual patch-clamp recordings, such as a low throughput and high technical researcher skill requirements. Thus, the automation of patch-clamp shows obvious advances over the manual technique in aspects such as standardization, reproducibility, and throughput. The transition from the utilization of conventional micropipettes to the application of planar chips changes the way that resistance seals form, simultaneously reducing the difficulty in manipulation and increasing the throughput of data. Ion channel characterization by using an automated patch-clamp platform becomes significantly easier due to the optimization and automation of processes. Moreover, the screening of a large number of compounds is practical on the automated patch-clamp platform, which is meaningful for toxicological studies with regard to complex environmental contaminants or rapidly increasing number of pharmaceuticals. Based on the progressive understanding of ion channel mechanisms, novel drugs can be produced starting from thoroughly characterized compounds. Finally, the automatic high throughput screening method offers a valuable tool for the early assessment of potential neurotoxicity and cardiotoxicity of synthetic compounds.

Nevertheless, there are certain limitations of the automated patch-clamp system. First, the cell preparation in automatic patch-clamp is more complicated than that in the manual technique. When recording adherent cells or tissue cells derived from brain slices, automated patch-clamp needs extra steps to isolate and suspend cells of interest and cannot target a specific cell type in a solution containing multiple types of cells. Second, the electrophysiological data from automated patch-clamp system is wished to mirror that from manual patch-clamp system. However, the cells and protocols suitable for the two distinct systems are different, questioning the data quality of the automated patch-clamp technique [126]. Third, although the application of the automated patch-clamp system could further standardize electrophysiological studies in the future, discrepancies in recording data from different laboratories currently remain. Therefore, a gold standard protocol to guide all technical aspects of the automated patch-clamp machines is urgently needed. Finally, within the range of toxicological studies, the application of automated patch-clamp is far from adequate. Although the automated patch-clamp technique has made the screening of compound libraries feasible, few results have been reported.

Actually, the throughput of early automated patch-clamp systems is still limited considering the millions of compounds in existence. Most of the currently available automated patch-clamp systems provide a medium throughput if no compromise on data quality is made, which is an advance compared to the conventional technique but not enough to test millions of compounds. Therefore, a combination of high throughput screening technology with conventional toxicity identification will be an attractive method for toxicological studies. Furthermore, increasing the throughput of automated patch-clamp systems can also be a valuable orientation for future studies. The second generation of automated patch-clamp systems has recently become commercially available at reduced cost per data point and increased throughput, with improvements in the throughput of screening compounds through an increase in recording sites and improvements in cell culturing [9]. This evolution provides a great opportunity for future toxicological studies. Moreover, further and fully automating *in vitro* patch-clamp technology can be an evolution for neural activity studies at the cellular level. The application of fluorescent labels in patch-clamp systems will allow software to search and contact targeted cells automatically. An emerging system named “Patcherbot” has further developed the approach to identify and track non-labeled cells in image-guided patch-clamp systems [20]. Automatic visual identification the algorithm can find and track cells that the experimenter initially selected under the microscope, proposing a feasible strategy for the development of a fully automated patch-clamp system.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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