

## Title page

# ***In vivo* patch-clamp recording from locus coeruleus neurones in the rat brainstem**

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## **Abstract**

Locus coeruleus (LC) neurones extend noradrenergic projections throughout the neuroaxis and are involved in homeostatic functions such as pain modulation, arousal and cardio-respiratory control. To address the cellular mechanisms underlying pain modulation we have developed a patch-clamp recording technique from LC neurones in anaesthetized rats. These recordings showed LC discharge *in vivo* to be driven by both spontaneous membrane potential oscillations and CNQX-sensitive EPSCs opposed by bicuculline-sensitive IPSCs. Hindlimb pinch evoked a biphasic action potential response underpinned by a slow monophasic excitatory current. This approach allows detailed characterisation of the synaptic and integrative mechanisms of LC responses to naturalistic stimulation.

## **Abbreviations**

LC – locus coeruleus, AP – action potential,

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione, TH– tyrosine hydroxylase

TH – tyrosine hydroxylase

## **Introduction**

The whole-cell patch-clamp recording technique (Neher & Sakmann, 1976) has become a ubiquitous method for studying the electrophysiological properties of cellular membranes and synaptic inputs. This technique has been mainly applied to *in vitro* preparations such as cultured cells and brain slices, contributing greatly to our understanding of the ionic mechanisms of neurone excitability, and synaptic transmission in neuronal circuits. However, the physiological significance of many neuronal and synaptic activities observed in such *in vitro* preparations remains to be determined, since the entire neuronal circuits are seldom preserved and it is typically not possible to apply natural or physiological stimulation.

To overcome these problems the whole-cell patch-clamp recording methodology has been extended to allow *in vivo* recordings from regions of the CNS in the intact animal including: spinal cord; thalamus; cerebellum; cortices and olfactory bulb. Such *in vivo* recordings these have provided compelling evidence of physiological circuit mechanisms (Sakmann, 2006; Furue *et al.*, 2007). However, to date there are no reports of patch-clamp recordings from brain stem neurones *in vivo* despite the crucial roles of brainstem circuits in vital physiology. This is likely to be due to technical difficulties in obtaining stable recordings, since the brain stem is rather inaccessible, is heavily myelinated making imaging challenging, and is relatively mobile showing pronounced respiratory and cardiac related pulsations.

The locus coeruleus (LC) is a dense cluster of noradrenergic neurones in the dorsal pons located either side of the floor of the fourth ventricle. These noradrenergic neurones send extensive projections to most regions of the CNS. The LC is implicated in the control of many homeostatic functions including pain control (Pertovaara, 2006; Howorth et al., 2009) and circadian regulation of arousal and performance. The activity of LC neurones is thought to be driven by the integration of fast excitatory and inhibitory synaptic inputs, and modulated by a range of peptidergic inputs to the LC and peri-LC area (Aston-Jones, 2004). Subthreshold synaptic and peptidergic mechanisms in LC neurones have been studied in detail using brain stem slices and dissociated preparations (Williams *et al.*, 1984; Ishibashi *et al.*, 2009) but these types of study offer a limited insight into the integrated function of the LC. In this study, we developed a novel method to obtain recordings from the brainstem using the *in vivo* “blind” patch-clamp technique (Furue *et al.*, 1999) to evaluate the physiological importance of those subthreshold responses.

## Methods

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences, and were performed in accordance with the institutional guidelines for animal experiments and were consistent

with the ethical guidelines of the International Association for the Study of Pain. Every effort was made to minimize the number of animals used.

### **Animal preparation**

Sprague-Dawley rats (62 males, aged 4-8 weeks) were anaesthetized with urethane (i.p., 1.2-1.5 g/kg). After tracheostomy, each animal was mechanically ventilated (to an end-tidal CO<sub>2</sub> of 3-4%) to reduce active respiratory movement and positioned in a stereotaxic frame (SG-3N, Narishige, Tokyo, Japan) (Fig. 1A). Body temperature was maintained at 37°C by an infrared heating lamp and heating pad. The preparations remained in good condition for recording for more than 6 h. At the end of the experiment, the animals were killed with supplemental administration of urethane (i.p., 2.0-4.0 g/kg).

### **Extracellular recordings from LC neurone**

Extracellular single-unit recordings of LC neurones were performed as described previously (Pineda *et al.*, 1993). A tungsten microelectrode (impedance, 10-12 MΩ) was stereotactically placed into the LC through a 3 mm occipital burr hole and neurones were identified by established criteria (Pineda *et al.*, 1993).

### ***In vivo* patch-clamp recordings from cells in the LC**

To target patch electrodes to the dorsal pons we exposed the floor of the fourth ventricle through a posterior occipital craniotomy using gentle suction aspiration to remove the central portion of the cerebellum. The surface of the exposed brainstem was irrigated with Krebs solution (mM: NaCl 117, KCl 3.6, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11, NaHCO<sub>3</sub> 25) equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> at 38 ± 0.5°C at a flow rate of 5-10 ml/min. Using this approach we were able to insert patch pipettes into the LC from the dorsal surface of the pons (Fig. 1B). The patch pipettes had a long shank with a resistance of 5-10 MΩ when filled with either of recording solutions of the following composition (mM): K-gluconate 135, KCl 5, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2, EGTA 5, ATP-Mg 5, HEPES-KOH 5; pH 7.2, or Cs<sub>2</sub>SO<sub>4</sub> 110, TEA-Cl 5, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2, EGTA 5, ATP-Mg 5, HEPES-CsOH 5; pH 7.2.

The electrodes were advanced into the vicinity of the LC (3.5 mm caudal to lambda, 0.85-1.0 mm lateral to the midline) at an angle of 45° from the horizontal using a micromanipulator according to coordinates derived from the atlas of Paxinos and Watson (Fig.1A, B). Cells in this region were shown to be within the LC using coronal brainstem slices of same age rats with immuno-staining for tyrosine hydroxylase (TH, Fig.1B). Gigaohm seals (4.2 ± 2.1 GΩ, n=37) were successfully formed using the “blind” patch technique with cells at a depth of 10-250 µm from the dorsal surface of the pons. In some cases cell-attached recordings were made from the LC neurones that were identifiable by their spontaneous firing pattern and their responses to sensory

stimuli as per the extracellular recordings. The whole-cell recording configuration was then obtained by applying further suction. The success rate for achieving whole-cell recording from neurones was ~20%, presumably because a gigaohm seal could be made on either an unfavourable location on a neurone or upon a glial cell. In some instances, the recorded neurone was filled with biocytin (0.4%) for post-hoc identification (as shown in Fig. 1C). For immunocytochemistry, the brainstem was perfusion fixed in 4% paraformaldehyde, sliced into 40 µm-thick sections and incubated overnight with rabbit anti-tyrosine hydroxylase (TH) antibody (Biomol International L.P., Exeter, UK), followed by AlexaFluor488-labeled donkey anti-rabbit secondary antibody (1:500, 2 hrs) and AlexaFluor555-labeled streptavidin (1:100) to identify the biocytin-filled neurone.

### **Cutaneous sensory stimulation**

Mechanical nociceptive stimulation was applied to the ipsilateral or contralateral hind limb by pinching the skin with toothed forceps by hand (using a force that was determined as noxious when applied to the experimenters own fingertip) as we have reported previously (Furue *et al.*, 2007). Innocuous touch stimulation was applied to the contralateral hind paw by brushing the skin.

### **Drug application**

Drugs were dissolved in Krebs solution and applied to the dorsal pontine surface via the perfusate. The drugs used were 6-cyano-7-nitroquinoxalone-2, 3-dione (CNQX) - a selective AMPA receptor antagonist (Sigma, St. Louis, MO, USA) and bicuculline - a selective GABA<sub>A</sub> receptor antagonist (Sigma).

### **Statistical analysis**

All data are expressed as means  $\pm$  SD. Statistical significance was determined as  $P < 0.05$  using one way analysis of variance or a two-tailed Student's *t* test. In all cases,  $n$  refers to the number of neurones studied.

## **Results**

### **Responses of the LC neurones to sensory stimuli recorded with extracellular and cell-attached recordings**

We first used the conventional extracellular technique to record spontaneous firing and stimulus evoked responses of the LC. These neurones showed a stable spontaneous discharge with an average frequency of  $7.4 \pm 3.3$  Hz ( $n=10$ ). When noxious pinch stimulation was applied to the contralateral hind limb, the firing frequency characteristically transiently increased and then decreased (Fig. 2A). Patch-clamp cell-attached recordings (at a holding potential of -70 mV) showed spontaneous action currents (Fig. 2B). The frequency of spontaneous firing was  $6.6 \pm 4.0$  Hz ( $n=16$ ) and the

neurones showed a biphasic excitation-inhibition response to pinch stimulation applied to the contralateral (but not ipsilateral) hindpaw (Fig. 2B,  $n=12$ ). The response profile and time course were similar to those recorded with extracellular recordings (Fig. 2C).

Innocuous touch stimulation applied to the contralateral hind limb also showed a similar biphasic response (the lowest trace in Fig. 2B). In 7 cells tested, the amplitude of maximum frequency for 1-2 s during pinch stimulation was higher than that during touch stimulation (pinch,  $172 \pm 48\%$  of control; touch,  $131 \pm 11\%$  of control;  $p < 0.05$ ) in the same neurones.

### **Whole-cell voltage and current recordings of LC activity and responses to sensory stimulation**

Stable whole-cell patch-clamp recordings were made from 63 LC neurones *in vivo*. All of these neurones exhibited spontaneous action potential (AP) discharge with an average frequency of  $6.0 \pm 2.7$  Hz ( $n = 23$ ). There was no significant difference in the spontaneous frequency of AP obtained from extracellular, cell-attached and whole-cell recordings (Fig. 2F), suggesting that tight seal formation and subsequent dialysis with pipette solution does not fundamentally alter their excitability. The resting membrane potential of the LC neurones was  $-55.4 \pm 8.3$  mV and the input resistance was  $215.9 \pm 56.4$  M $\Omega$ . The injection of hyperpolarising current showed that some of the LC neurones exhibited large spontaneous oscillations in membrane potential whose

frequency was altered by the degree of hyperpolarization (Fig. 2E). Pinch stimulation elicited a biphasic AP response without obvious prolonged depolarization or hyperpolarization (upper trace in Fig. 2D). We could also record the membrane currents under voltage-clamp conditions during pinch stimulation. Interestingly, the voltage clamp recordings of the response to pinch at a holding potential of -70 mV did not demonstrate the presence of fast EPSCs, but rather a slow, monophasic, inward current was observed. Outward currents were not detected (lower trace in Fig. 2D).

### **Recordings of excitatory and inhibitory synaptic currents**

When the Cs-containing pipette solution was used, EPSCs and IPSCs could be isolated under voltage-clamp conditions at holding potentials of -70 and 0 mV, respectively. Although recordings with K-gluconate solution also showed EPSCs, the recordings with the Cs-solution had a higher signal to noise ratio; this is due to the blockade of potassium channels. As shown in Fig. 3A, LC neurones exhibited spontaneous EPSCs with an amplitude of  $14.7 \pm 5.7$  pA and a frequency of  $3.7 \pm 2.8$  Hz ( $n=21$ ). Analysis of the EPSC time course (Fig. 3A) showed the presence of populations with fast (half width: 0-4 ms) and slow (half width: >4 ms) kinetics, perhaps consistent with a distinct cellular locations or differential receptor subunit compositions. Local application of CNQX (20  $\mu$ M) to the surface of the pons completely inhibited EPSCs within 1 min, followed by recovery on washout within 5 min (left traces in Fig. 3C & D). The

amplitude and frequency of spontaneous IPSCs were  $13.6 \pm 2.0$  pA and  $9.0 \pm 3.8$  Hz ( $n=7$ ) (Fig. 3B). Bicuculline (4  $\mu$ M) applied to the surface of the brainstem also completely and reversibly suppressed IPSCs (right traces in Fig. 3C & D). Even when Cs-solution was used, pinch stimulation applied to the contralateral but not ipsilateral hind limb elicited a similar slow current ( $23.6 \pm 4.7$  pA) to that recorded with K-gluconate solution without eliciting any fast EPSCs in most (5 out of 7) cells examined.

## Discussion

In the present study, we report an *in vivo* whole-cell patch-clamp recording technique from rat LC neurones which enables us to record at high fidelity the membrane potentials (including sub-threshold mechanisms), underlying synaptic currents (EPSCs and IPSCs) and gives good access for pharmacological investigation. This method is therefore useful to quantitatively elucidate which neurotransmitters are involved in the physiological responses of LC neurones; which are known to receive and respond to a variety of fast and slow transmitters (Aston-Jones, 2004). We have been able to show for the first time that adult LC neurones *in vivo* show spontaneous membrane potential oscillations like those attributed to gap junctional coupling from *in vitro* studies (Ishimatsu & Williams, 1996). We also show that LC neurones receive spontaneous small EPSCs (at a frequency lower than their spontaneous AP discharge rate) which are

opposed by a higher frequency of incoming IPSCs.

The LC is believed to play an important role for pain modulation. The LC receives projections from periaqueductal grey in the midbrain which integrate inputs from limbic forebrain and diencephalon including the anterior cingulate, insular cortex and amygdala with ascending nociceptive spinal inputs. Direct spinal projections are also found within the LC (Basbaum & Fields 1978). An illustration of the potential advantages inherent in our approach is the observation that the cutaneous pinch-evoked, biphasic firing of LC neurones was not accompanied by large depolarization or hyperpolarization. Voltage-clamp recordings from the same neurones demonstrated that a transient slow inward current was evoked by pinch and fast EPSCs were not evoked, suggesting that G-protein-coupled pathway may be involved in the excitatory phase of the response. It also suggests that the inhibitory phase is a consequence of the intrinsic properties of the LC neurones (which are known to become relatively refractory following a period of high frequency activation mediated both by an increased calcium activated potassium conductance and also feedback autoinhibition mediated by  $\alpha$ 2 receptors) rather than a fast inhibitory synaptic input. However, given that LC neurones are known from *in vitro* studies to be gap junction coupled into clusters (Ishimatsu & Williams, 1996); then it is conceivable that the LC neurons exhibiting the slow inward current response to pinch are coupled to neighbouring LC neurones that receive noxious afferent fast synaptic inputs. Further experiments are now possible

using perfusion of glutamatergic antagonists / gap junction blockers or intracellular application of G-protein signalling uncouplers to distinguish between these possibilities.

We anticipate that our recording approach to the brainstem will allow the function of the LC and other ponto-medullary structures to be probed in unprecedented detail and will facilitate mechanistic investigation of the key circuits involved in the regulation of sensory, autonomic and motor control.

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## **Author contributions**

This work was conducted in the laboratory of K.I. at the National Institute for Physiological Sciences, Japan. All authors contributed to the concept and design of the experiments as well as to the collection, analysis and interpretation of data. H. F., D. S. and A.E.P. developed the *in vivo* patch-recording technique from LC neurones. D.K. provided the histology. D.S., H.F and A.E.P. drafted the manuscript and all authors approve the final version of the manuscript.

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## Figures and legends

### **Figure 1. *In vivo* patch-clamp recording from LC neurones in the brainstem.**

A. Schematic diagrams of *in vivo* preparation and patch pipette insertion into the LC. The urethane-anaesthetised animal was fixed in ear bars and patch pipettes were inserted into the LC from the dorsal surface of the pons after cerebectomy (*left*). The topography of the LC (mauve) was mapped from Paxinos and Watson's - The Rat Brain (*right*). B. Location of cells recorded with *in vivo* whole-cell patch-clamp method (*left*). This recording area was identified as including the LC whose location was verified by TH-immunohistochemistry and Nissl staining of coronal sections in age matched rats. C. An example of a recording from a TH-positive LC neurone (double-stained for anti-TH (*green*) and neurobiotin (*red*)).

### **Figure 2. *In vivo* recordings of AP discharge in LC neurones.**

*A. Extracellular recording of AP discharge in response to cutaneous pinch stimulation applied to the contralateral hind limb showing a characteristic biphasic response with excitation followed by inhibition.* *B. Cell-attached patch recording - currents in response to voltage-step pulses (5 mV) were negligible after a giga-ohm seal formation (top trace before and middle trace after seal formation).* Spontaneous AP discharge was typically observed (indicated by arrow heads, shown on trace expanded below). These cell-attached LC recordings also showed a biphasic response to contralateral pinch and touch stimulation. These two sensory responses were obtained from the same neurone.

*C. Comparison of the time courses of AP discharge in response to pinch stimulation for the neurones in A (open circles) and B (closed circles).* *D. An example of *in vivo* whole-cell current clamp recording.* Pinch stimulation elicited APs followed by an inhibition. Note hyperpolarization was not detected during the inhibitory phase. An AP is shown on an expanded time base (*right trace*). In voltage clamp the pinch was seen to elicit a monophasic slow inward current ( $V_{hold}$  -90 mV). Noise in this trace was relatively higher than those in traces in Fig. 3. This is due to the presence of our hand in the Faraday cage for sensory stimulation. *E. Tonic activity in an LC neurone at rest was driven by underlying intrinsic membrane potential oscillations (right trace, revealed by the injection of hyperpolarising current) whose frequency was slowed by increasing hyperpolarisation.* *F. Summary graph showing the frequency of spontaneous APs in LC neurones recorded with *in vivo* extracellular, cell-attached and whole cell recording*

techniques. There were no significant differences in AP discharge between the three recording methods ( $p > 0.05$ , one way ANOVA).

**Figure 3. *In vivo* excitatory and inhibitory synaptic currents in LC neurones.**

A, Spontaneous EPSCs were clearly resolved ( $V_{\text{hold}} -70$  mV) and showed different kinetics manifesting as a wide range of half widths. The histogram shows one dominant population and a long tail of longer duration events. Insets in the histogram show the averaged traces of 10 spontaneous EPSCs having fast (half width  $< 2.5$  ms) and slow (half width  $> 4$  ms) kinetics. Dotted lines show the decay phase of the fast ( $\tau_{\text{fast}}$ , 2.4 ms) and slow ( $\tau_{\text{slow}}$ , 7.4 ms) EPSCs fitted with a single exponential. B, Spontaneous IPSCs ( $V_{\text{hold}} 0$  mV) resolved as outward currents (shown on an expanded timebase below). C, Effect of drugs applied to the dorsal surface of the pons. CNQX (20  $\mu\text{M}$ ) and bicuculline (20  $\mu\text{M}$ ) reversibly suppressed spontaneous EPSCs and IPSCs, respectively. D, Time courses of the averaged amplitude of spontaneous EPSCs (*open circle*) and IPSCs (*closed circle*) following application of and recovery from CNQX and bicuculline, respectively.

Fig.1 Sugiyama et al.

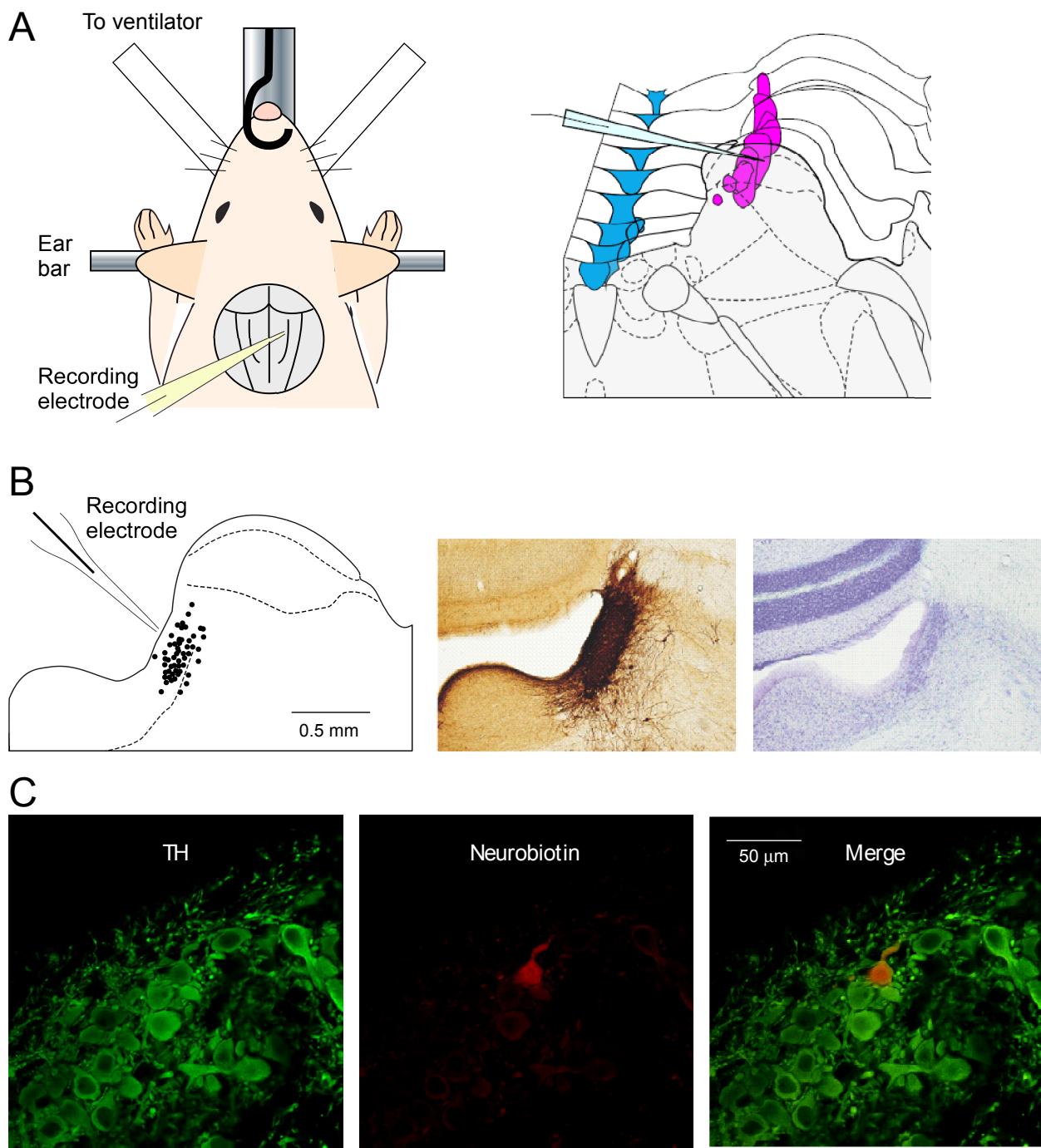


Fig.2 Sugiyama et al.

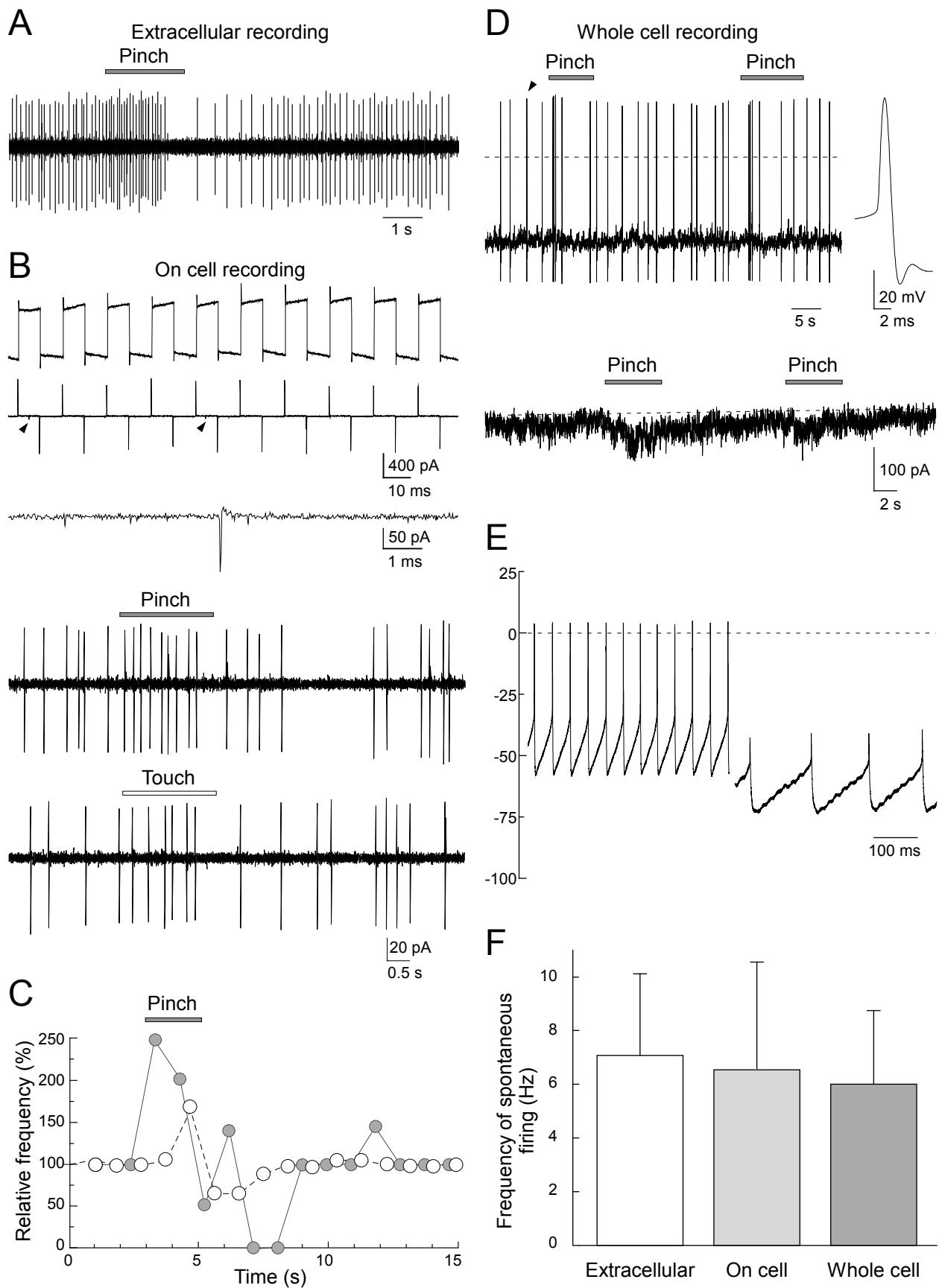


Fig.3 Sugiyama et al.

