



# Localization of nectin-2 $\alpha$ at the boundary between the adjacent somata of the clustered cholinergic neurons and its regulatory role in the subcellular localization of the voltage - ...

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**Localization of nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered cholinergic neurons and its regulatory role in the subcellular localization of the voltage-gated A-type K<sup>+</sup> channel Kv4.2 in the medial habenula**

Short running title: nectin-2 $\alpha$  in the medial habenula

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6

## 1 ABSTRACT

2  
3 The medial habenula (MHb), implicated in stress, depression, memory, and  
4 nicotine withdrawal syndromes, receives septal inputs and sends efferents to  
5 the interpeduncular nucleus. We previously showed that the  
6 immunoglobulin-like cell adhesion molecules nectin-2 $\alpha$  and nectin-2 $\delta$  are  
7 expressed in astrocytes in the brain, but their expression in neurons remains  
8 unknown. We showed here by immunofluorescence microscopy that  
9 nectin-2 $\alpha$ , but not nectin-2 $\delta$ , was prominently expressed in the cholinergic  
10 neurons in the developing and adult MHbs and localized at the boundary  
11 between the adjacent somata of the clustered cholinergic neurons where the  
12 voltage-gated A-type K<sup>+</sup> channel Kv4.2 was localized. Analysis by  
13 immunoelectron microscopy on this boundary revealed that Kv4.2 was  
14 localized at the membrane specializations with plasma membrane darkening  
15 in an asymmetrical manner, whereas nectin-2 $\alpha$  was localized on the apposed  
16 plasma membranes mostly at the outside of these membrane specializations,  
17 but occasionally localized at their edges and insides. Nectin-2 $\alpha$  at this  
18 boundary was not colocalized with the nectin-2 $\alpha$ -binding protein afadin,  
19 other cell adhesion molecules, or their interacting peripheral membrane  
20 proteins, suggesting that nectin-2 $\alpha$  forms a cell adhesion apparatus different  
21 from the Kv4.2-associated membrane specializations. Genetic ablation of  
22 *nectin-2* delayed the localization of Kv4.2 at the boundary between the  
23 adjacent somata of the clustered cholinergic neurons in the developing MHb.  
24 These results revealed the unique localization of nectin-2 $\alpha$  and its regulatory  
25 role in the localization of Kv4.2 at the membrane specializations in the MHb.

26  
27 Keywords: Nectin, Kv potassium channel, cell adhesion, habenula, cholinergic  
28 neuron  
29

## 1 | INTRODUCTION

The habenula is a bilateral structure which is located in the dorsal end of the diencephalon and faces to the third ventricle (Figure 1a,b). It consists of the medial habenula (MHb) and the lateral habenula (LHb). The MHb primarily receives afferents from the triangular septum and the medial septal complex (the medial septum and the nucleus of diagonal band) and projects efferents almost exclusively to the interpeduncular nucleus (IPN) in the midbrain through the fasciculus retroflexus (Carlson, Noguchi, & Ellison, 2001; Herkenham & Nauta, 1977, 1979). The MHb responds to glutamate, which is produced in the triangular septum, and  $\gamma$ -aminobutyric acid (GABA), which is produced in the medial septal complex (Qin & Luo, 2009), and releases three neurotransmitters in the IPN: acetylcholine, glutamate, and substance P, which are produced in the MHb (Carlson et al., 2001; Herkenham & Nauta, 1979). The IPN projects efferents to the ventral tegmental area and the raphe nuclei and regulates dopamine and serotonin levels, respectively (Balcita-Pedicino, Omelchenko, Bell, & Sesack, 2011; Cuello, Emson, Paxinos, & Jessell, 1978; Groenewegen, Ahlenius, Haber, Kowall, & Nauta, 1986). Thus, the MHb is a relay nucleus connecting the basal forebrain with the midbrain nuclei and regulating the activities of these monoaminergic neurons (Christoph, Leonzio, & Wilcox, 1986; Nishikawa, Fage, & Scatton, 1986; Nishikawa & Scatton, 1985; Wang & Aghajanian, 1977). The MHb is implicated in stress, depression, memory, and nicotine withdrawal syndromes (Kobayashi et al., 2013; Mathuru & Jesuthasan, 2013; Molas, DeGroot, Zhao-Shea, & Tapper, 2017; Shumake, Edwards, & Gonzalez-Lima, 2003).

The MHb is divided into five subnuclei depending on their topographic positions: the superior part (MHbS), the ventral region of the inferior part (MHbI), the lateral part (MHbL), the dorsal region of the central part (MHbCd), and the ventral region of the central part (MHbCv) (Figure 1b) (Aizawa, Kobayashi, Tanaka, Fukai, & Okamoto, 2012; Andres, von Düring, & Veh, 1999). The MHbS exclusively produces glutamate; the MHbCd produces both glutamate and substance P; and the MHbI, the MHbCv, and the MHbL produce both glutamate and acetylcholine (Aizawa et al., 2012). The MHb abundantly expresses the nicotinic acetylcholine receptors: the whole regions of the ventral MHb (MHbI,

1 MHbCv, MHbL) express the  $\alpha 3$  and  $\beta 4$  subunits of the nicotinic acetylcholine  
2 receptor, while the some, but not all, regions of the ventral MHb express the  $\alpha 6$ ,  
3  $\beta 2$ ,  $\beta 3$ , and  $\alpha 4$  subunits (Shih et al., 2014). The MHb also expresses high  
4 concentrations of the GABA receptors (Bischoff et al., 1999; Charles et al., 2001;  
5 Durkin, Gunwaldsen, Borowsky, Jones, & Branchek, 1999; Wang, Gong, Luo, &  
6 Xu, 2006). The MHb expresses the glutamate receptors, but their distribution in  
7 each subnucleus remains unknown (Gall, Sumikawa, & Lynch, 1990; Khan et al.,  
8 2000).

9       The immunoglobulin-like cell adhesion molecules nectins play roles in cell  
10 adhesion, proliferation, differentiation, survival, and migration (Takai, Ikeda,  
11 Ogita, & Rikitake, 2008a; Takai, Miyoshi, Ikeda, & Ogita, 2008b). They are  
12 further involved in cellular patterning, such as checkerboard patterning of hair and  
13 supporting cells of the auditory epithelium in the organ of Corti in the inner ear,  
14 and the olfactory neurons and supporting cells in the olfactory epithelium of the  
15 nasal cavity (Katsunuma et al., 2016; Togashi et al., 2011). Nectins comprise a  
16 family with four members (nectin-1, nectin-2, nectin-3, and nectin-4) and further  
17 comprise a superfamily with the nectin-like molecule (Necl) family with five  
18 members (Necl-1, Necl-2, Necl-3, Necl-4, and Necl-5) (Takai et al., 2008a; Takai  
19 et al., 2008b). Necls also show a variety of cell functions, including cell adhesion,  
20 migration, proliferation, differentiation, and survival. All members of the nectin  
21 superfamily have three extracellular immunoglobulin-like domains, a single  
22 transmembrane region, and a cytoplasmic region. The extracellular region of  
23 nectins or Necls on a cell interacts in *trans* with the same or different members of  
24 the nectin superfamily on the adjacent cell to form cell-cell adhesion. The  
25 cytoplasmic region of nectins binds the actin filament-binding protein afadin and  
26 is associated with the actin cytoskeleton, whereas that of Necls does not bind  
27 afadin.

28       In the brain, nectin-1 and nectin-3 are localized at the puncta adherentia  
29 junctions of hippocampal mossy fiber synapses, which are formed between the  
30 axons of the dentate granule cells and the dendrites of the pyramidal cells in the  
31 CA3 region of the hippocampus (Mizoguchi et al., 2002). Nectin-1 and nectin-3  
32 are asymmetrically localized at the pre-synaptic and post-synaptic sides,  
33 respectively, whereas afadin and N-cadherin are symmetrically localized at both  
34 the pre-synaptic and post-synaptic sides (Mizoguchi et al., 2002). This

1 asymmetric localization of nectin-1 and nectin-3 is involved in the selective  
2 interaction of axons and dendrites cooperatively with afadin and N-cadherin  
3 (Togashi et al., 2006). Nectin-1 is implicated in contextual fear memory  
4 consolidation in the hippocampus (Fantin, van der Kooij, Grosse, Krummenacher,  
5 & Sandi, 2013), whereas nectin-3 is implicated in cognitive impairment induced  
6 by stress *via* corticotropin-releasing hormone receptor 1 on the hippocampal CA3  
7 pyramidal cells (Wang et al., 2013). Nectin-1 and nectin-3 are expressed in  
8 Cajal-Retzius cells and neocortical projection neurons, respectively, in the  
9 developing brain, and the nectin-1- and nectin-3-mediated interaction between  
10 Cajal-Retzius cells and migrating neurons is critical for radial migration of  
11 neurons cooperatively with afadin and N-cadherin (Gil-Sanz et al., 2013). In  
12 addition, nectin-1 is localized independently of afadin or cadherins at the contacts  
13 between the lateral dendrites of the mitral cells in the developing olfactory bulb  
14 and homophilically *trans*-interacts with each other to form a novel type of  
15 cell-cell adhesion apparatus, named “nectin-1 spot”, which is distinct from the  
16 thus far identified cell-cell adhesion apparatuses, such as puncta adherentia  
17 junctions, adherens junctions, tight junctions, desmosomes, and gap junctions  
18 (Inoue et al., 2015). The nectin-1 spot regulates branching of the lateral dendrites  
19 of the mitral cells (Fujiwara et al., 2015). Mutations in the *NECTIN1* gene are  
20 responsible for an autosomal recessive cleft lip/palate ectodermal dysplasia  
21 syndrome, also called Zlotogora–Ogur syndrome and Margarita Island ectodermal  
22 dysplasia (Suzuki, Bustos, & Spritz, 1998; Suzuki et al., 2000). The clinical  
23 characteristics include cleft lip/palate, hidrotic ectodermal dysplasia,  
24 developmental defects in the hands, and intellectual disability in some cases  
25 (Suzuki et al., 2000).

26 In contrast to nectin-1 and nectin-3, nectin-2 has not been well characterized  
27 in the brain. Nectin-2, also termed as poliovirus receptor related 2 or CD112, has  
28 two splice variants, nectin-2 $\alpha$  (a shorter variant) and nectin-2 $\delta$  (a longer variant),  
29 and both transcripts are ubiquitously expressed in the tissues and organs of the  
30 mouse and the human (Aoki et al., 1997; Eberlé, Dubreuil, Mattei, Devilard, &  
31 Lopez, 1995). The amino acid sequences of the extracellular regions excluding the  
32 juxtamembrane region consisting of 12 amino acids are the same between these  
33 two mouse variants, whereas those of the other regions, including the  
34 juxtamembrane regions, the transmembrane regions, and the intracellular regions,

1 are different between them (Eberlé et al., 1995). The *nectin-2* gene was originally  
2 cloned as a murine homolog of the poliovirus receptor gene (Morrison &  
3 Racaniello, 1992), but its gene product was later shown to serve as an entry  
4 receptor for herpes simplex virus (Warner et al., 1998). In the preceding study, we  
5 showed that nectin-2 $\alpha$  is expressed in both cultured mouse neurons and astrocytes  
6 whereas nectin-2 $\delta$  is selectively expressed in cultured astrocytes (Miyata et al.,  
7 2016). In immunofluorescence microscopy on the mouse brain, nectin-2 $\alpha$  and/or  
8 nectin-2 $\delta$  (nectin-2 $\alpha/\delta$ ) is observed intensively in the pia mater, walls of the lateral  
9 ventricles, the choroid plexus, and the habenula in the forebrain. Nectin-2 $\alpha/\delta$  is  
10 also observed around the blood vessels to a lesser extent. Nectin-2 $\delta$ , but not  
11 nectin-2 $\alpha$ , is localized at the adhesion sites between adjacent cultured astrocytes,  
12 but it is localized on the plasma membrane of the perivascular astrocytic endfoot  
13 processes facing the basement membrane of blood vessels in the brain. Genetic  
14 ablation of *nectin-2* causes degeneration of perivascular astrocytic endfoot  
15 processes and neurons in the cerebral cortex during adulthood (Miyata et al.,  
16 2016). These results uncovered for the first time the localization and functions of  
17 nectin-2 in the brain.

18       Extending this earlier study, we investigated here the subcellular  
19 localization of nectin-2 in the mouse habenula and showed that nectin-2 $\alpha$ , but not  
20 nectin-2 $\delta$ , was prominently expressed in the developing and adult MHbs and that  
21 nectin-2 $\alpha$  was localized at the boundary between the adjacent somata of the  
22 clustered cholinergic neurons and at the synaptic regions (Figure 1c–e). It was  
23 previously shown that the membrane specializations (MSs) are observed at the  
24 boundary between the adjacent somata of the unidentified neurons in the MHb  
25 and that the voltage-gated A-type K<sup>+</sup> channels, Kv4.2 and Kv4.3, are localized at  
26 the MSs of these clustered neurons (Kollo, Holderith, & Nusser, 2006). Kv4.2 and  
27 Kv4.3 belong to the family of the voltage-gated A-type K<sup>+</sup> channel with rapid  
28 activation and inactivation, resulting in transient A-type K<sup>+</sup> current (Birnbaum et  
29 al., 2004). The physiological function of the MSs or these voltage-gated A-type  
30 K<sup>+</sup> channels in the MHb remains unknown. We investigated here nectin-2 $\alpha$  at the  
31 boundary between the adjacent somata of the clustered cholinergic neurons, but  
32 not nectin-2 $\alpha$  at the synaptic regions, in the MHb, and showed that the  
33 Kv4.2-associated MSs are localized at the adjacent somata of the clustered  
34 cholinergic neurons in the adult MHb and that nectin-2 $\alpha$  was localized at the



apposed plasma membranes mostly at the outside of these MSs, but occasionally localized at their edges and insides. In addition, we showed here that nectin-2 $\alpha$  and Kv4.2 were colocalized at the boundary between the somata of the clustered cholinergic neurons in the developing MHb as well as in the adult MHb and that nectin-2 $\alpha$  regulated the localization of Kv4.2 at the MSs in the developing MHb.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). *Nectin-2*-deficient (aka, *nectin2*<sup>tm1Smu/tm1Smu</sup>) and *nectin-2*-heterozygous (aka, *nectin2*<sup>+ /tm1Smu</sup>) mice (Mueller, Rosenquist, Takai, Bronson, & Wimmer, 2003; Ozaki-Kuroda et al., 2002) were maintained on a C57BL/6J background. In the *nectin2*<sup>tm1Smu</sup> allele, the  $\beta$ -galactosidase gene was knocked-in. Therefore the  $\beta$ -galactosidase protein was expressed in the *nectin-2*-heterozygous mice and in the *nectin-2*-deficient mice. The day of birth was defined as postnatal day 0 (P0). All animal experiments were performed in accordance with the guidelines of the institution and approved by the administrative panel on laboratory animal care of Kobe University. This study was approved by the president of Kobe University after being reviewed by the Kobe University Animal Care and Use Committee (approval number: 24-03-04), and animal experiments were conducted in accordance with the regulations for animal experimentation of Kobe University.

### 2.2 | Antibody characterization

The primary antibodies (Abs) used in this study are listed in Table 1. They were characterized as follows. Rat anti-nectin-2 $\alpha$ / $\delta$  monoclonal Ab (mAb) (RRID: AB\_590848) recognized 56-kDa and 68-kDa bands in Western blotting of mouse heart and brain (Aoki et al., 1997; Miyata et al., 2016; Shiotani et al., 2017). Specificity was verified by Western blotting of the wild-type and *nectin-2*-deficient mouse hearts and cultured astrocytes and by

1 immunohistochemistry on the wild-type and *nectin-2*-deficient mouse hearts and  
2 brains (Miyata et al., 2016; Satomi-Kobayashi et al., 2009). Rat anti-nectin-3  
3 mAb (RRID: AB\_592587) recognized a 90-kDa band in Western blotting of  
4 mouse brain (Miyata et al., 2016; Satoh-Horikawa et al., 2000; Shiotani et al.,  
5 2017). Specificity was verified by Western blotting of the wild-type and  
6 *nectin-3*-deficient mouse brains and by immunohistochemistry on the wild-type  
7 and *nectin-3*-deficient mouse brains (Honda et al., 2006). Rabbit anti-Necl-1  
8 polyclonal Ab (pAb) recognized a 45-kDa band in Western blotting of mouse  
9 brain (Kakunaga et al., 2005; Shiotani et al., 2017). Specificity was verified by  
10 Western blotting of the cultured wild-type L cells and the cultured L cells stably  
11 expressing mouse Necl-1 (Kakunaga et al., 2005). Rat anti-Necl-5 mAb  
12 recognized a 100-kDa band in Western blotting of mouse brain (Shiotani et al.,  
13 2017). Specificity was verified by Western blotting of the cultured wild-type L  
14 cells and the cultured L cells stably expressing mouse Necl-5 and by  
15 immunocytochemistry of the cultured wild-type and Necl-5-knockdown NIH3T3  
16 cells (Ikeda et al., 2003; Minami et al., 2010). Rabbit anti-l-afadin pAb (RRID:  
17 AB\_257881) recognized a 205-kDa band in Western blotting of rat brain (Mandai  
18 et al., 1997). Specificity was verified by Western blotting of the wild-type and  
19 *afadin*-knockout embryonic stem cells (Ikeda et al., 1999) and the wild-type and  
20 *afadin* conditional knockout mouse brains and by immunohistochemistry on the  
21 wild-type and *afadin* conditional knockout mouse brains (Toyoshima et al., 2014;  
22 Yamamoto et al., 2013). Rabbit anti-Necl-3 pAb recognized a 60-kDa band in  
23 Western blotting of mouse brain (Shiotani et al., 2017). Specificity was verified  
24 by Western blotting of the cultured mouse endothelial cells and the cultured  
25 mouse Necl-3-transfected HEK293 cells (Yamana et al., 2015). The Abs listed  
26 below were purchased from commercial sources. Mouse anti-NeuN mAb (EMD  
27 Millipore, Billerica, MA; Cat# MAB377X, RRID: AB\_2149209) recognized  
28 48-kDa and 46-kDa bands in Western blotting of mouse brain. Specificity was  
29 verified by Western blotting of various tissues (Mullen, Buck, & Smith, 1992).  
30 Rabbit anti-nectin-2 $\delta$  mAb (abcam, Cambridge, UK; Cat# ab135246) recognized  
31 a 58-kDa protein in Western blotting of mouse brain. Specificity was verified by  
32 Western blotting of the wild-type and *nectin-2*-deficient mouse hearts and  
33 cultured astrocytes and by immunohistochemistry on the wild-type and  
34 *nectin-2*-deficient mouse brains (Miyata et al., 2016). Goat anti-choline

1 acetyltransferase (ChAT) pAb (EMD Millipore, Cat# AB144P, RRID:  
2 AB\_11214092) recognized a 68-kDa band in Western blotting of postmortem  
3 human brain (Grosman, Lorenzi, Trinidad, & Strauss, 1995). Specificity was  
4 verified by Western blotting of the wild-type and mouse ChAT-transfected  
5 cultured COS-1 cells (Rathenberg, Gartner, Koenen, & Witzemann, 2002). Mouse  
6 anti- $\beta$ -galactosidase mAb (Promega, Fitchburg, WI; Cat# Z3781, RRID:  
7 AB\_430877) recognized a 116-kDa band in Western blotting of the wild-type and  
8  $\beta$ -galactosidase-expressing transgenic mouse brains. Specificity was verified by  
9 Western blotting of the wild-type and  $\beta$ -galactosidase-expressing transgenic  
10 mouse brains (Jurado-Arjona, Llorens-Martín, Ávila, & Hernández, 2016). Mouse  
11 anti-bassoon mAb (Enzo Life Sciences, East Farmingdale, NY; Cat#  
12 ADI-VAM-PS003, RRID: AB\_10618753) recognized a 400-kDa band in Western  
13 blotting of the cultured SH-SY5Y cells (Mandemakers et al., 2013). Specificity  
14 was verified by immunohistochemistry on the wild-type and *Bsn*-deficient mouse  
15 retinas (Dick et al., 2003). Rabbit anti-Kv4.2 pAb (Alomone Labs, Jerusalem,  
16 Israel; Cat# APC-023, RRID: AB\_2040176) recognized a 73-kDa band in  
17 Western blotting of mouse brain (Arroyo, Kim, Biehl, Yeh, & Bett, 2011).  
18 Specificity was verified by immunohistochemistry on the human myometrium  
19 (Novaković et al., 2015). Chicken anti-Necl-2 mAb (MBL, Nagoya, Japan; Cat#  
20 CM004-3, RRID: AB\_592783) recognized 90-kDa and 80-kDa bands in Western  
21 blotting of mouse brain. Specificity was verified by Western blotting of the  
22 wild-type and Necl-2-knockdown cultured hippocampal neurons (Yamada et al.,  
23 2013). Rat anti-nectin-1 mAb (MBL, Cat# D146-3, RRID: AB\_590847)  
24 recognized a 90-kDa band in Western blotting of mouse brain (Miyata et al.,  
25 2016; Shiotani et al., 2017). Specificity was verified by Western blotting of the  
26 wild-type and *nectin-1*-deficient mouse brains and by immunohistochemistry on  
27 the wild-type and *nectin-1*-deficient mouse brains (Honda et al., 2006). Goat  
28 anti-nectin-4 pAb (R&D Systems, Minneapolis, MN; Cat# AF2659, RRID:  
29 AB\_416659) recognized a 66-kDa band in Western blotting of the of pregnant  
30 mouse mammary epithelium (Kitayama et al., 2016). Specificity was verified by  
31 Western blotting of the wild-type and mouse nectin-4-transfected cultured COS-1  
32 cells (Noyce et al., 2011). Mouse anti-Necl-4 mAb (NeuroMab, Davis, CA; Cat#  
33 75-247, RRID: AB\_10676101) recognized a 55-kDa band in Western blotting of  
34 mouse brain (Shiotani et al., 2017). Specificity was verified by Western blotting

1 of the wild-type and *Necl-4*-knockdown cultured Caco-2 cells (Sugiyama et al.,  
2 2013). Mouse anti-N-cadherin mAb (BD Biosciences, San Jose, CA; Cat# 610921,  
3 RRID: AB\_398236) recognized a 130-kDa band in Western blotting of mouse  
4 brain (Shiotani et al., 2017). Specificity was verified by immunohistochemistry on  
5 the mammary glands of the control and *Cdh2*-knockin mice (Kotb, Hierholzer, &  
6 Kemler, 2011). Rat anti-P-cadherin mAb (Takara Bio, Shiga, Japan; Cat# M109)  
7 recognized a 118-kDa band in Western blotting of the cultured PSA5-E cells.  
8 Specificity was verified by Western blotting of the cultured trypsin-treated  
9 PSA5-E cells in the presence of  $\text{Ca}^{2+}$  or EGTA (Nose & Takeichi, 1986). Mouse  
10 anti-cadherin-13 mAb (Santa Cruz Biotechnology Inc., Cat# sc-166875, RRID:  
11 AB\_10612090) recognized a broad band of 130-kDa in Western blotting of the  
12 human cadherin-13-transfected cultured cells. Specificity was verified by Western  
13 blotting of the wild-type and human cadherin-13-transfected cultured cells. Rabbit  
14 anti- $\beta$ -catenin pAb (Sigma-Aldrich, St. Louis, MO; Cat# C2206, RRID:  
15 AB\_476831) recognized a 90-kDa band in Western blotting of mouse brain  
16 (Shiotani et al., 2017). Specificity was verified by Western blotting of the  
17 wild-type and  $\beta$ -catenin-knockdown cultured glial progenitor cells (He & Shen,  
18 2009). Mouse anti-p120-catenin mAb (BD Biosciences, Cat# 610134, RRID:  
19 AB\_397537) recognized a 110-kDa band in Western blotting of mouse brain  
20 (Shiotani et al., 2017). Specificity was verified by Western blotting of the  
21 wild-type and p120-catenin-knockdown cultured A431 cells (Davis, Ireton, &  
22 Reynolds, 2003). Rat anti-E-cadherin mAb (ECCD2) was kindly provided by Dr.  
23 Masatoshi Takeichi (RIKEN CDB, Kobe, Japan). The mAb recognized a 124-kDa  
24 band in Western blotting of the cultured F9 cells (Shirayoshi, Nose, Iwasaki, &  
25 Takeichi, 1986). Specificity was verified by Western blotting of the cultured  
26 E-cadherin-positive cell line MCF-7 and the cultured E-cadherin-negative human  
27 breast cancer cell line MDA-MB-231 (Mbalaviele et al., 1996). The  
28 anti-cadherin-8 mAb (CAD8-1, RRID: AB\_2078272) developed by Dr.  
29 Masatoshi Takeichi (RIKEN CDB) was obtained from the Developmental Studies  
30 Hybridoma Bank (Iowa City, IA). The mAb recognized a 135-kDa band in  
31 Western blotting of mouse prefrontal cortex and striatum (Friedman et al., 2015).  
32 Specificity was verified by Western blotting of the wild-type and *cdh8*-deficient  
33 mouse spinal cords (Suzuki et al., 2007). For immunofluorescence microscopy,  
34 primary Abs were visualized using goat or donkey fluorochrome-conjugated

secondary Abs. The fluorochromes used were Alexa Fluor 488, 555, 568, and 647 (Thermo Fisher Scientific, Waltham, MA). For nuclear counter staining, 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Nacalai Tesque, Kyoto, Japan) or 1 µM DRAQ5 (Biostatus, Leicestershire, United Kingdom, Cat# DR50050, RRID: AB\_2314341) was used.

### 2.3 | Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Toyoshima et al., 2014). In brief, mice were deeply anesthetized and transcardially perfused at room temperature with 1 × Hanks' Balanced Salt Solution with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS; Thermo Fisher Scientific) containing 10 mM HEPES, 1 mM sodium pyruvate, 4% sucrose, heparin, and protease inhibitor cocktail (cOmplete Mini; Roche Diagnostics, Mannheim, Germany), followed by perfusion of 2% paraformaldehyde (PFA) in the abovementioned HBSS-based buffer. After dehydration with 30% sucrose in phosphate buffered saline, whole brains were embedded in OCT compounds (Sakura Finetek, Tokyo, Japan). Cryostat sections were incubated at 62°C for 20 min in HistoVT One antigen retrieval solution (Nacalai Tesque) and then incubated with 1% bovine serum albumin, 10% normal goat serum, and 0.25% Triton X-100 in phosphate buffered saline at room temperature for 30 min. The sections were stained with the indicated Abs, and then with appropriate fluorophore-conjugated secondary Abs (1:600). Confocal image acquisition was performed on a C2 confocal laser-scanning microscope (Nikon, Tokyo, Japan) using a 20×/0.75 or 40×/0.95 objective lens (Plan Apo numerical aperture water immersion objective lens (Nikon)). Images captured on the C2 confocal laser-scanning microscope were analyzed using NIS Elements acquisition software (Nikon).

### 2.4 | Immunoelectron microscopy

Immunoelectron microscopy was performed using the silver-enhanced immunogold method as previously described (Mizoguchi et al., 2002; Omiya et al., 2015). Deeply anesthetized mice were transcardially perfused with 1 × HBSS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  containing 10 mM HEPES, 1 mM sodium pyruvate, 4%

sucrose, and 4% or 2% PFA. After postfixation, the brains were soaked in the cacodylate-buffered solution containing 2 mM CaCl<sub>2</sub>, 30% sucrose, and cOmplete Mini protease inhibitor cocktail tablets (Roche Diagnostics) at 4°C overnight. The samples were viewed by the JEOL 1011 or the JEM 1400 electron microscopy (JEOL, Tokyo, Japan). For double staining immunoelectron microscopy, the rat anti-nectin-2 $\alpha$ / $\delta$  mAb, which recognizes the extracellular regions of nectin-2 $\alpha$  and nectin-2 $\delta$ , and the rabbit anti-Kv4.2 pAb, which recognizes the cytoplasmic region of Kv4.2, were mixed and used as a primary Ab. This mixed primary Ab was visualized by a mixture of anti-rat and anti-rabbit immunogold particles. Because it was confirmed that the single staining immunoelectron microscopy for the anti-nectin-2 $\alpha$ / $\delta$  mAb or the anti-Kv4.2 pAb detected almost exclusive localization of immunogold particles at the intercellular or intracellular spaces, respectively, at the apposed plasma membranes, the immunogold signals located at the extracellular or intracellular spaces were regarded as those labeled by the anti-nectin-2 $\alpha$ / $\delta$  mAb or anti-Kv4.2 pAb, respectively, with the mixed primary Ab.

## **2.5 | High-resolution immunofluorescence microscopy**

High-resolution immunofluorescence microscopy was performed with the FV3000 confocal laser-scanning microscope (Olympus, Tokyo, Japan) using a 40 $\times$ /0.90 or 60 $\times$ /1.35 objective lens. For quantitative analysis of the signal for Kv4.2 at the boundary between the adjacent somata of the clustered cholinergic neurons, the z-stack image was obtained from the five images taken at every 0.5  $\mu$ m. Then, the intensity of the linear signal for Kv4.2 at the boundary between the clustered neurons was analyzed by the cellSens software (Olympus) and the ImageJ software. Neurons were defined as cells of which nuclei were larger than 5  $\mu$ m. When the intensity of the signal for Kv4.2 at the boundary showed more than twice the cytoplasmic background intensity, the boundary was considered as a Kv4.2-positive boundary. Then, the percentage of the neurons with Kv4.2-positive boundaries was calculated.

## **2.6 | Statistical analysis**

Statistical analysis of the difference between two mean values was performed with the two-tailed, unpaired Student's *t*-test. The criterion for statistical significance was set at  $p < 0.05$ . All values are reported as the mean  $\pm$  s.e.m.

### 3 | RESULTS

#### 3.1 | Expression and localization of nectin-2 $\alpha$ in the adult MHb

We first performed immunofluorescence microscopy on the brain sections from adult mouse at P56 using the mAb against the extracellular regions of nectin-2 $\alpha$  and nectin-2 $\delta$ , which recognizes both variants (anti-nectin-2 $\alpha/\delta$  mAb). The immunofluorescence signal with the anti-nectin-2 $\alpha/\delta$  mAb was prominently observed in the habenula (Figure 2a,b, arrows). In addition, the signal with the anti-nectin-2 $\alpha/\delta$  mAb was observed in the choroid plexus in the third, fourth, and the lateral ventricles (Figure 2a,b, arrowheads), and the inferior olivary nucleus (Figure 2a, double arrowhead). These results were consistent with the previous observations (Miyata et al., 2016). The signal with the anti-nectin-2 $\alpha/\delta$  mAb was observed in the MHb, but not the LHb (Figure 2c–e). This signal was observed in the cells showing the signal for NeuN (Figure 2e). This signal was undetectable in the MHb of the *nectin-2*-deficient mouse (Figure 2f), indicating that the signal with the anti-nectin-2 $\alpha/\delta$  mAb in the MHb of the wild-type mouse was specific. The Ab which specifically recognizes the cytoplasmic region of nectin-2 $\alpha$  by immunofluorescence microscopy was not available, whereas the Ab which specifically recognizes the cytoplasmic region of nectin-2 $\delta$  (anti-nectin-2 $\delta$  mAb) was available. Therefore, we next performed immunofluorescence microscopy with the anti-nectin-2 $\delta$  mAb. The signal with the anti-nectin-2 $\delta$  mAb was not significantly observed in the MHb or the LHb, although the intense signal was observed in the choroid plexus and the ependyma (Figure 2g, arrow and arrowheads). These results indicate that nectin-2 $\alpha$ , but not nectin-2 $\delta$ , is prominently expressed in the adult MHb, but not the adult LHb.

#### 3.2 | Localization of nectin-2 $\alpha$ at the boundary between the adjacent somata

## of the clustered cholinergic neurons in the adult MHb

To determine the precise localization of nectin-2 $\alpha$  in the adult MHb, we performed immunofluorescence microscopy with a higher magnification on the MHb using the anti-nectin-2 $\alpha$ / $\delta$  mAb (Figure 3a–e). The immunofluorescence signal for nectin-2 $\alpha$  showed two different staining patterns: one is linear and the other is punctate (Figure 3b–e, arrowheads and arrows). The linear pattern was observed mainly in the MHbI and the MHbCv, whereas the punctate pattern was observed mainly in the MHbS and randomly in other whole regions of the MHb. The signal for the linear pattern was observed near the signal for DAPI (Figure 3f–i, arrows), suggesting that the signal for nectin-2 $\alpha$  with this linear pattern was localized near the neuronal somata. It is noted that the signal for DAPI was clustered, indicating that the neuronal somata expressing nectin-2 $\alpha$  were clustered. When the cholinergic neurons were identified with the anti-ChAT pAb, the cholinergic neurons were clustered and the signal for nectin-2 $\alpha$  with the linear pattern was observed at the adjacent somata of these clustered cholinergic neurons (Figure 3f–i, arrows). To identify the origin of the signal for nectin-2 $\alpha$  with the linear pattern, we utilized the *nectin-2*-heterozygous mouse in which  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele. The signal for nectin-2 $\alpha$  with the linear pattern was observed at the boundary between the adjacent somata of the clustered cholinergic neurons in both of which the signal for  $\beta$ -galactosidase was observed (Figure 3j–l, arrows). In contrast, the signal for nectin-2 $\alpha$  with the punctate pattern in the MHbS and other regions of the MHb was partly colocalized with the synaptic protein bassoon (Figure 3m–o, arrows). These results suggest that nectin-2 $\alpha$  is localized at the boundary between the adjacent somata of the clustered cholinergic neurons in the adult MHb, mainly in the MHbI and the MHbCv, and at the synaptic regions. We focused here on nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered cholinergic neurons, but not nectin-2 $\alpha$  at the synaptic regions.

### 3.3 | Immunofluorescence microscopic colocalization of nectin-2 $\alpha$ with Kv4.2 at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI and the MHbCv of the adult MHb



1 It was previously shown that Kv4.2 and Kv4.3 are localized at the boundary  
2 between the adjacent somata of the neurons in the rat MHb, although these  
3 neurons were not identified (Kollo et al., 2006). We therefore compared the  
4 distribution of nectin-2 $\alpha$  with that of Kv4.2 in the adult mouse MHb using the  
5 anti-nectin-2 $\alpha/\delta$  mAb and the anti-Kv4.2 pAb, the former of which recognizes the  
6 extracellular regions of nectin-2 $\alpha$  and nectin-2 $\delta$  whereas the latter of which  
7 recognizes the cytoplasmic region of Kv4.2. Consistent with the previous  
8 observations (Kollo et al., 2006), the immunofluorescence signal for Kv4.2 was  
9 observed as a linear pattern at the boundary between the adjacent somata of the  
10 clustered cholinergic neurons (Figure 4b,c). The signal for nectin-2 $\alpha$  was also  
11 observed as a linear pattern at this boundary (Figure 4a,c). Both the signals were  
12 frequently colocalized with each other (Figure 4a–c, arrows), although the signal  
13 for Kv4.2 with the linear pattern was less frequently observed at this boundary  
14 where the signal for nectin-2 $\alpha$  was not observed (Figure 4a–c, arrowheads) and  
15 the signal for nectin-2 $\alpha$  with the linear pattern was less frequently observed at this  
16 boundary where the signal for Kv4.2 was not observed (Figure 4a–c, double  
17 arrowheads). The average ratios of these three patterns of these signals in three  
18 independent experiments were  $44.6 \pm 6.7\%$  :  $29.3 \pm 5.8\%$  :  $26.1 \pm 2.8\%$ ,  
19 respectively, in the MHbI. Essentially the same results were obtained in the  
20 MHbCv (data not shown). These results indicate that nectin-2 $\alpha$  is frequently  
21 colocalized with Kv4.2 at the boundary between the adjacent somata of the  
22 clustered cholinergic neurons in the MHbI and the MHbCv of the adult MHb  
23 although either nectin-2 $\alpha$  alone or Kv4.2 alone is less frequently observed at this  
24 boundary. The signal for Kv4.2 in the *nectin-2*-deficient mouse in which  
25  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele was not different  
26 from that in the control wild-type mouse (Figure 4d–f, arrows). Essentially the  
27 same results were obtained in the MHbCv (data not shown). These results indicate  
28 that the localization of Kv4.2 at the boundary between the adjacent somata of the  
29 clustered cholinergic neurons in the MHbI and the MHbCv of the adult MHb is  
30 independent of nectin-2 $\alpha$ .

31

32 **3.4 | Immunoelectron microscopically different localization of nectin-2 $\alpha$  with**  
33 **Kv4.2 at the boundary between the adjacent somata of the clustered**  
34 **cholinergic neurons in the MHbI of the adult MHb**

1  
2 We then determined by immunoelectron microscopy the precise localization of  
3 nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered  
4 cholinergic neurons in the MHbI of the adult MHb, where Kv4.2 was localized.  
5 For this purpose, the anti-nectin-2 $\alpha/\delta$  mAb which recognizes the extracellular  
6 regions of nectin-2 $\alpha$  and nectin-2 $\delta$  and the anti-Kv4.2 pAb which recognizes its  
7 cytoplasmic region were used. In a low magnification of immunoelectron  
8 microscopy in the MHbI, the immunogold particles for nectin-2 $\alpha$  were observed  
9 at the boundary between the adjacent somata of the cholinergic neurons (Figure 5a,  
10 arrows). The somata (Figure 5a, S) were identified by the localization of the  
11 nucleus (Figure 5a, N). This localization of the immunogold particles for  
12 nectin-2 $\alpha$  was confirmed by immunoelectron microscopy with a higher  
13 magnification (Figure 5b, arrows). In these micrographs, the plasma membrane  
14 was not well preserved, but in another micrograph, the plasma membrane was  
15 preserved and the immunogold particles for nectin-2 $\alpha$  were observed at the  
16 intercellular spaces of the boundary between the adjacent somata of the  
17 cholinergic neurons (Figure 5c, arrows). These results indicate that nectin-2 $\alpha$  is  
18 localized on the apposed plasma membranes at the boundary between the adjacent  
19 somata of the clustered cholinergic neurons in the MHbI of the adult MHb. On the  
20 other hand, the immunogold particles for Kv4.2 were always localized at the MSs  
21 at the boundary between the adjacent somata of the cholinergic neurons in the  
22 MHbI in an asymmetrical manner, but occasionally in a symmetrical manner  
23 (Figure 5d, arrowheads). These results were consistent with the previous  
24 observations (Kollo et al., 2006). It is noted that both sides of the apposed plasma  
25 membranes of the MSs was darkened, compared with those of the regions other  
26 than the MSs, raising the possibility that the MSs serve as a cell adhesion  
27 apparatus.

28 We then performed double immunoelectron microscopy using the mixture  
29 of the anti-nectin-2 $\alpha/\delta$  mAb and the anti-Kv4.2 pAb. Because the anti-nectin-2 $\alpha/\delta$   
30 mAb recognizes the extracellular regions of nectin-2 $\alpha$  and the anti-Kv4.2 pAb  
31 recognizes the cytoplasmic region of Kv4.2, the immunogold particles in the  
32 intracellular regions represent the localization of Kv4.2, whereas those in the  
33 extracellular regions represent the localization of nectin-2 $\alpha$ . The immunogold  
34 particles for nectin-2 $\alpha$  were observed at the intercellular spaces of the apposed

plasma membranes (Figure 5e, arrows). They were mostly observed at the outside of the MSs where the immunogold particles for Kv4.2 were observed, but occasionally at the edges and insides of the MSs (Figure 5e, arrows and arrowheads). In addition, a cluster of nectin-2 $\alpha$  with 2-6 immunogold particles and a cluster of Kv4.2 with 2-6 immunogold particles were localized in an alternating manner. These results indicate that nectin-2 $\alpha$  and Kv4.2 are differently localized on the apposed plasma membranes at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI of the adult MHb. These results also indicate that the apparent colocalization of the immunofluorescence signals for nectin-2 $\alpha$  and Kv4.2 at this boundary shown in Figure 4a–c is caused by the less spatial resolution of the regular confocal microscopy used here than that of the immunoelectron microscopy.

### **3.5| No colocalization of nectin-2 $\alpha$ with other cell adhesion molecules and their interacting proteins except Necl-2 in the adult MHb**

Each member of the nectin superfamily homophilically and heterophilically *trans*-interacts with each other (Rikitake, Mandai, & Takai, 2012; Takai et al., 2008a). Nectin-2 homophilically *trans*-interacts with each other and further heterophilically *trans*-interacts with nectin-3. We examined the localization of other members of the nectin superfamily to determine whether nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI and the MHbCv of the adult MHb homophilically *trans*-interacts with each other or heterophilically *trans*-interacts with other members of the nectin superfamily and whether cell adhesion molecules (CAMs) other than the nectin superfamily are colocalized with nectin-2 $\alpha$ . The signal for Necl-2 was observed as a linear pattern in the MHbI, the MHbCv (Figure 6a–c, arrows and arrowheads), and the MHbL (data not shown) of the adult MHb. The signal for Necl-2 with the linear pattern was colocalized with that for nectin-2 $\alpha$  at  $41.7 \pm 0.9\%$  ( $n=3$ ) of the nectin-2 $\alpha$ -positive boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI, the MHbCv, and the MHbL. Thus, the signal for nectin-2 $\alpha$  with the linear pattern was partly colocalized with that for Necl-2 in these subnuclei, although nectin-2 $\alpha$  is known not to *trans*-interact with Necl-2 (Shingai et al., 2003). The signal for Necl-3 was observed as a linear pattern in the

1 MHbCv (Figure 6d–f, arrow) and the medial part of the MHbL (data not shown)  
2 of the adult MHb. The signal for Necl-3 with the linear pattern was observed at  
3  $15.3 \pm 2.3\%$  (n=3) of the nectin-2 $\alpha$ -positive boundary between the adjacent  
4 somata of the clustered cholinergic neurons in the MHbI, the MHbCv, and the  
5 MHbL. Thus, the signal for nectin-2 $\alpha$  with the linear pattern was rarely  
6 colocalized with that for Necl-3 in these subnuclei (Figure 6d–f). The signals for  
7 Necl-1 and Necl-4 were faintly observed near the boundary between the adjacent  
8 somata of the clustered cholinergic neurons in the MHbI of the adult MHb, but  
9 was not colocalized with that for nectin-2 $\alpha$  (Figure 6g–i, arrows). The signals for  
10 Necl-1, Necl-2, Necl-3, and Necl-4 were observed in the adult MHb of the  
11 *nectin-2*-deficient mice were similar to those observed in the adult MHb of the  
12 wild-type mice (data not shown). The signals for other members of the nectin  
13 superfamily, including nectin-1, nectin-3, nectin-4, and Necl-5, were not  
14 concentrated at the boundary between the adjacent somata of the clustered  
15 cholinergic neurons, where the signal for nectin-2 $\alpha$  was concentrated, in the MHbI  
16 and the MHbCv of the adult MHb (data not shown). These results indicate that  
17 nectin-2 $\alpha$  is not colocalized with other members of the nectin superfamily except  
18 Necl-2 at the boundary between the adjacent somata of the clustered cholinergic  
19 neurons in the most regions of the MHbI and the MHbCv of the adult MHb,  
20 although nectin-2 $\alpha$  is only partly colocalized with Necl-2.

21 The cytoplasmic tails of nectins bind two splice variants of afadin, l-afadin  
22 and s-afadin (Rikitake et al., 2012; Takai et al., 2008a). l-Afadin has an actin  
23 filament-binding region at the C-terminus whereas s-afadin lacks it. The pAb  
24 which recognizes both l-afadin and s-afadin (anti-l/s-afadin pAb) and the pAb  
25 which recognizes only l-afadin, but not s-afadin, (anti-l-afadin pAb) are available,  
26 whereas the Ab which recognizes only s-afadin, but not l-afadin, is not available  
27 (Mandai et al., 1997). The signal with the anti-l-afadin pAb was not concentrated  
28 in the MHbI and the MHbCv of the adult MHb (data not shown). The signal with  
29 the anti-l/s-afadin pAb was not concentrated there, either (data not shown). These  
30 results indicate that l-afadin or s-afadin is not colocalized with nectin-2 $\alpha$  at the  
31 boundary between the adjacent somata of the clustered cholinergic neurons in the  
32 MHbI and the MHbCv of the adult MHb.

33 None of the signals for other CAMs, such as N-cadherin, E-cadherin,  
34 P-cadherin, cadherin-13, cadherin-8, and their binding proteins  $\beta$ -catenin and

p120-catenin were not concentrated at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI and the MHbCv (data not shown). Thus, no CAMs or their binding proteins were not identified to be colocalized with nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHbI and the MHbCv of the adult MHb.

### 3.6| Different expression of nectin-2 $\alpha$ and ChAT in the developing MHb

We next examined whether nectin-2 $\alpha$  is required for the localization of Kv4.2 at the boundary between the adjacent somata of the clustered cholinergic neurons in the MHb during the developmental stages. We first examined the expression of nectin-2 $\alpha$  and ChAT in the developing MHb, because it has not been investigated how newly born neurons are differentiated into cholinergic neurons or how the differentiated neurons migrate and finally attach to each other to cluster in the developing MHb. For this purpose, we used the *nectin-2*-heterozygous mouse in which  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele. In the control *nectin-2*-heterozygous mouse at P1, the immunofluorescence signal for nectin-2 $\alpha$  was observed weakly in the ventral half of the MHb, although the signal for  $\beta$ -galactosidase was not observed in this area as well as other areas in the MHb (Figure 7a–d). The reason why the signal for  $\beta$ -galactosidase was not observed in the whole area of the MHb might be due to the lower expression level of  $\beta$ -galactosidase than that of nectin-2 $\alpha$  and/or due to the lower sensitivity of the Ab against  $\beta$ -galactosidase than that against nectin-2 $\alpha$  used here. The signal for ChAT was weakly observed in the more ventral area of the MHb, but not the dorsal area of the MHb. In the *nectin-2*-heterozygous mouse at P7, the signals for nectin-2 $\alpha$ , ChAT, and  $\beta$ -galactosidase were all clearly observed in the ventral half of the MHb where the signal only for nectin-2 $\alpha$  was clearly observed at P1 (Figure 7e–h). However, the signal for ChAT was not observed at some areas where those for both nectin-2 $\alpha$  and  $\beta$ -galactosidase were observed at P7 (Figure 7e–h, arrows). In the *nectin-2*-heterozygous mice at P14 and P28, the signal for ChAT was observed in most areas in which the signals for both nectin-2 $\alpha$  and  $\beta$ -galactosidase were observed (Figure 7i–p). The signal for each of ChAT and  $\beta$ -galactosidase in the *nectin-2*-deficient mice was not different from that in the control *nectin-2*-heterozygous mice at the respective developing stages, P1, P7, P14, and

P28 (data not shown). These results suggest that some newly born neurons in the more dorsal area in the ventral half of the MHb, which are differentiating into cholinergic neurons, express nectin-2 $\alpha$ , before they express ChAT, although it is unknown whether this is the case in the more ventral area in the ventral half of the MHb where the signals for both nectin-2 $\alpha$  and ChAT were observed at the developmental stage of P1. In addition, these results also suggest that nectin-2 $\alpha$  is not required for the development of the cholinergic neurons in the MHb.

### **3.7| Reduced localization of Kv4.2 at the boundary between the adjacent somata of the cholinergic neurons in the developing *nectin-2*-deficient MHb**

We then compared the localization of nectin-2 $\alpha$  and Kv4.2 in the developing MHb of the *nectin-2*-deficient mice with that in the developing MHb of the *nectin-2*-heterozygous mice at P1, P7, P14, and P28. In the *nectin-2*-heterozygous mouse at P1, the immunofluorescence signal for nectin-2 $\alpha$  was occasionally observed at the boundary between the adjacent somata of most ChAT-positive neurons (Figure 7a–d), but the signal for Kv4.2 was not observed at any areas (data not shown). In the *nectin-2*-heterozygous mouse at P7, the signal for nectin-2 $\alpha$  was more frequently observed at the boundary between the adjacent somata of most ChAT-positive neurons (Figure 7e–h) and the signal for Kv4.2 was occasionally observed at the boundary between the adjacent somata of the  $\beta$ -galactosidase-positive cholinergic neurons (data not shown). In the *nectin-2*-heterozygous mouse at P14, the signal for Kv4.2 as well as nectin-2 $\alpha$  was observed at the boundary between the adjacent somata of the neighboring  $\beta$ -galactosidase-positive cholinergic neurons after P14 (Figure 8a–d, arrowheads). Essentially the same results were obtained in the *nectin-2*-heterozygous mouse at P28 (Figure 8i–l). These results indicate that nectin-2 $\alpha$  is localized at the boundary between the adjacent somata of the clustered cholinergic neurons at the earlier developmental stages than Kv4.2 in the MHb.

In the *nectin-2*-deficient mouse, the signal for Kv4.2 was reduced markedly at P14 and slightly at P28 in the developing MHbs, compared with that for the *nectin-2*-heterozygous mice (Figure 8a–p). The reduction of the signal for Kv4.2 was not markedly observed in the *nectin-2*-deficient mice at P1 and P7, compared with that for the *nectin-2*-heterozygous mice (data not shown). This might be due

1 to the poor accumulation of Kv4.2 at the boundary between the adjacent somata of  
2 the clustered cholinergic neurons. It was noted that the signal for Kv4.2 was not  
3 significantly different between the *nectin-2*-heterozygous and *nectin-2*-deficient  
4 mice at P56 (Figure 4b,c,e,f) and between the wild-type and *nectin-2*-deficient  
5 mice at P56 (data not shown). To confirm the reduction in the signal for Kv4.2 in  
6 the *nectin-2*-deficient mouse at P14, we performed high-resolution  
7 immunofluorescence microscopy analysis on the wild-type and *nectin-2*-deficient  
8 mice at P14 (Figure 9a–h). The signal for Kv4.2 at the boundary between the  
9 adjacent neuronal somata in the primordial region for the MHbCv, the MHbL, and  
10 the lateral part of the MHbI of the wild-type mouse was observed in  $85.0 \pm 5.8\%$   
11 cells, whereas that of the *nectin-2*-deficient mouse was observed in about  $50.0 \pm$   
12  $4.4\%$  cells (Figure 9b,d,f,h,i). These results, together with the results obtained for  
13 the wild-type and *nectin-2*-deficient mice at P56 (Figure 4b,c,e,f), indicate that  
14 *nectin-2 $\alpha$*  is not essential for, but enhances the localization of Kv4.2 at the  
15 boundary between the adjacent somata of the clustered cholinergic neurons in the  
16 developing MHb.

17

18

## 19 **4 | DISCUSSION**

20

21 In the preceding paper, we showed that *nectin-2 $\delta$*  is localized at the astrocytic  
22 endfoot processes attaching to the vascular basement membrane in adult mouse  
23 brain and that the genetic ablation of *nectin-2* displays age- and region-dependent  
24 degeneration including neuron death in the brain (Miyata et al., 2016). We further  
25 showed that both *nectin-2 $\alpha$*  and *nectin-2 $\delta$*  are expressed in cultured astrocytes  
26 from the mouse cerebral cortex and hippocampus whereas *nectin-2 $\alpha$*  is selectively  
27 expressed in cultured neurons from the mouse hippocampus (Miyata et al., 2016).  
28 However, the localization of *nectin-2 $\alpha$*  in neurons in the mouse brain remained  
29 unknown. We showed here that *nectin-2 $\alpha$* , but not *nectin-2 $\delta$* , was prominently  
30 expressed in the MHb, particularly in the MHbI and the MHbCv, but not in the  
31 LHb, in the adult mouse at P56, where it was localized at the boundary between  
32 the adjacent somata of the clustered cholinergic neurons (Figure 1c–e). We also  
33 showed here that *nectin-2 $\alpha$*  was localized at the synaptic regions, but we focused

1 here on nectin-2 $\alpha$  at the boundary between the adjacent somata of the clustered  
2 cholinergic neurons and did not further study nectin-2 $\alpha$  at the synaptic regions. It  
3 is noted that nectin-2 $\alpha$  at the boundary between the adjacent somata of the  
4 clustered cholinergic neurons in the MHbI and the MHbCv was not colocalized  
5 with other CAMs except Necl-2, including nectin-1, nectin-3, nectin-4, Necl-1,  
6 Necl-3, Necl-4, Necl-5, N-cadherin, P-cadherin, E-cadherin, cadherin-13, and  
7 cadherin-8, indicating that nectin-2 $\alpha$  has thus far been identified as an only CAM  
8 localized at this boundary. Necl-2 was partly colocalized with nectin-2 $\alpha$  in the  
9 MHbI and the MHbCv, although nectin-2 $\alpha$  does not heterophilically  
10 *trans*-interacts with Necl-2 (Shingai et al., 2003). Any of these CAM-interacting  
11 proteins, including 1-afadin,  $\beta$ -catenin, and p120-catenin, were not colocalized  
12 with nectin-2 $\alpha$  at this boundary. In addition, nectin-2 $\alpha$  was concentrated at the  
13 boundary between the adjacent somata of the  $\beta$ -galactosidase-positive cholinergic  
14 neurons of the *nectin-2*-heterozygous mouse. These results suggest that nectin-2 $\alpha$   
15 at the boundary between the adjacent somata of the clustered cholinergic neurons  
16 in the MHbI and the MHbCv homophilically *trans*-interacts with each other to  
17 form a cell adhesion apparatus. Three types of nectin-based cell adhesion  
18 apparatuses have thus far been identified: (1) afadin- and cadherin-dependent; (2)  
19 afadin-dependent but cadherin-independent; and (3) afadin- and  
20 cadherin-independent (Mizutani & Takai, 2016). The third type is named “nectin  
21 spot”: the nectin-1-3 spot between the commissural axons and the floor cell plate  
22 cells in the developing neural tube (Okabe et al., 2004); the nectin-1 spot between  
23 the lateral dendrites of the mitral cells in the developing olfactory bulb (Inoue et  
24 al., 2015); and the nectin-1-4 spot between the luminal and basal cells in the  
25 mammary gland (Kitayama et al., 2016). The properties of the nectin-2 $\alpha$ -mediated  
26 cell adhesion apparatus shown here by immunofluorescence microscopy were  
27 similar to those of the nectin spots. Therefore, according to the classification  
28 criteria of the nectin-based cell adhesion apparatuses, this apparatus in the MHb  
29 may be classified into the third type and named tentatively here “nectin-2 $\alpha$  spot”,  
30 although the result similar to that obtained for the nectin-1 spot between the  
31 lateral dendrites of the mitral cells in the developing olfactory bulb (Inoue et al.,  
32 2015) has not been obtained by immunoelectron microscopy, because the Ab  
33 which recognizes the intracellular region of nectin-2 $\alpha$  was not available.



1           In general, the somata of neurons in the brain do not attach to each other,  
2 although axons of one neuron interact with the dendrites, the somata, and the  
3 axons of other neurons to form axodendritic, axosomatic, and axoaxonic synapses,  
4 respectively, and the dendrites of one neuron interact with the dendrites of other  
5 neurons to form dendrodendritic synapses (Kandel, Schwartz, Jessell, Siegelbaum,  
6 & Hudspeth, 2013). However, it was previously shown that the unidentified  
7 neurons are clustered and that their somata are exceptionally attached to each  
8 other in the MHb (Kollo et al., 2006). In addition, it was previously shown that  
9 the Kv4.2-associated MSs are localized at the boundary between the adjacent  
10 somata of these clustered neurons in the MHb (Kollo et al., 2006). We confirmed  
11 here these early observations and further showed here (1) that these clustered  
12 neurons were cholinergic neurons; (2) that the MSs were localized at the boundary  
13 between the adjacent somata of the clustered cholinergic neurons; and (3) that the  
14 plasma membrane of the MSs was darkened, compared with that of the regions  
15 other than the MSs. These structures of the MSs suggest that the MSs serve as a  
16 cell adhesion apparatus, but it was previously shown that Kv4.2 is mostly  
17 distributed on the apposed plasma membranes in an asymmetrical manner (Kollo  
18 et al., 2006) and we confirmed here this previous observation. These results  
19 suggest that Kv4.2 is not likely to serve as a CAM at the MSs.

20           We showed here that nectin-2 $\alpha$  was localized mostly at the outside of the  
21 MSs, but occasionally at their edges and insides, and that the genetic ablation of  
22 *nectin-2* did not affect the immunofluorescence signal for Kv4.2 at the boundary  
23 between the adjacent somata of the clustered cholinergic neurons in the adult  
24 MHb. These results indicate that nectin-2 $\alpha$  is not a CAM for the MSs and does not  
25 regulate the localization of Kv4.2 at this boundary in the adult MHb. It is noted  
26 that Necl-2 is partly colocalized with nectin-2 $\alpha$  at this boundary. It remains  
27 unknown whether Necl-2 is the CAM for the MSs, but this possibility is unlikely,  
28 because Necl-2 is not necessarily localized at all the boundary where nectin-2 $\alpha$   
29 and Kv4.2 are localized and Necl-2 does not *trans*-interact with nectin-2 $\alpha$   
30 (Shingai et al., 2003). Thus, the definitive CAM(s) for the MSs remains  
31 unidentified and it remains unknown how the MSs are formed.

32           It has not been investigated how newly born neurons are differentiated into  
33 cholinergic neurons or how the differentiated neurons migrate and finally attach to  
34 each other to cluster in the developing MHb. We showed here using the

1 *nectin-2*-heterozygous mice at various developmental stages in which  
2  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele that at least some  
3 newly born neurons in the more dorsal area of the ventral half of the MHb, which  
4 are differentiating into cholinergic neurons, express *nectin-2 $\alpha$* , before they express  
5 ChAT. We previously showed that *nectin-1* is expressed in the glutamatergic  
6 granule cells of the dentate gyrus whereas *nectin-3* is expressed in the  
7 glutamatergic pyramidal cells at the CA3 area in the hippocampus (Mizoguchi et  
8 al., 2002). It is noted that the cholinergic neurons in the MHb specifically express  
9 *nectin-2*, but not *nectin-1* or *nectin-3*, whereas these hippocampal glutamatergic  
10 neurons selectively express *nectin-1* and *nectin-3*, but not *nectin-2* (Miyata et al.,  
11 2016; Mizoguchi et al., 2002).

12 We further showed here that the signals for *nectin-2 $\alpha$*  and Kv4.2, which  
13 were observed at the boundary between the adjacent somata of the clustered  
14 cholinergic neurons in the adult MHb, were similarly observed in the developing  
15 MHb. In addition, the localization of Kv4.2 at this boundary in the developing  
16 MHb of the *nectin-2*-deficient mouse was reduced markedly at P14 and slightly at  
17 P28. Because this reduction was not observed in the *nectin-2*-deficient mouse at  
18 P56, these results indicate that *nectin-2 $\alpha$*  is not essential for, but enhances the  
19 localization of Kv4.2 at the boundary between the adjacent somata of the  
20 clustered cholinergic neurons in the developing MHb. We found here that Kv4.2  
21 and the MSs were always associated with each other at the boundary between the  
22 adjacent somata of the clustered cholinergic neurons of the adult MHb. Therefore,  
23 the results that the immunofluorescence signal for Kv4.2 at this boundary was  
24 reduced in the developing MHbs of the *nectin-2*-deficient mice at P14 and P28,  
25 suggest that the number and/or the length of the MSs are reduced in these  
26 developing MHbs. We previously showed that *nectin* first initiates cell-cell  
27 adhesion and then recruits cadherin to the *nectin*-based cell-cell adhesion sites  
28 through the *nectin*-binding protein afadin to adherens junctions in many types of  
29 cells including epithelial cells and fibroblasts (Takai et al., 2008a). In addition,  
30 *nectin* *cis*-interacts on the same plasma membrane with many growth factor  
31 receptors or integrins through their extracellular regions: *nectin-1* *cis*-interacts  
32 with the FGF receptor (Bojesen et al., 2012) and integrin  $\alpha_v\beta_3$  (Sakamoto et al.,  
33 2006); *nectin-3* *cis*-interacts with the PDGF receptor (Kanzaki et al., 2008) and  
34 integrin  $\alpha_v\beta_3$  (Sakamoto et al., 2006; Sakamoto, Ogita, Komura, & Takai, 2008);

1 and nectin-4 *cis*-interacts with the prolactin receptor (Kitayama et al., 2016). In  
2 the *cis*-interaction of nectin-4 with the prolactin receptor, nectin-4 forms a spot  
3 with *trans*-interacting nectin-1 at the boundary between the luminal and basal  
4 cells in the mammary gland (Kitayama et al., 2016). Because the  
5 nectin-2 $\alpha$ -mediated cell adhesion apparatus is similar to this nectin-1-4 spot,  
6 nectin-2 $\alpha$  may directly or indirectly *cis*-interact with Kv4.2 and/or the unidentified  
7 CAM(s) for the MSs through their extracellular regions, eventually leading to the  
8 formation of the MSs which subsequently attaches the adjacent somata of the  
9 cholinergic neurons in the developing MHb, although it remains unknown  
10 whether nectin-2 $\alpha$  *cis*-interacts with Kv4.2 and/or the unidentified CAM(s) for the  
11 MSs before or after the formation of the MSs. After the formation of the MSs and  
12 the attachment of the adjacent somata of the cholinergic neurons, Kv4.2 and  
13 nectin-2 $\alpha$  may be dissociated from each other to be localized at the MSs and  
14 mostly at the outside of the MSs, respectively. The identification of the CAM(s)  
15 for the MSs would be critical to elucidate the mechanisms for the formation of the  
16 MSs, the localization of Kv4.2, and the attachment of the adjacent somata of the  
17 cholinergic neurons in the MHb. Although these mechanisms have not fully been  
18 elucidated here, the present studies revealed the unique localization of nectin-2 $\alpha$   
19 in the MHb and its regulatory role in the localization of Kv4.2 at the boundary  
20 between the adjacent somata of the clustered cholinergic neurons in the  
21 developing MHb.

22 The *NECTIN2* gene was shown to be genetically associated with  
23 Alzheimer's disease (Harold et al., 2009; Logue et al., 2011; Takei et al., 2009).  
24 The MHb is implicated in stress, depression, memory, and nicotine withdrawal  
25 syndromes (Kobayashi et al., 2013; Mathuru & Jesuthasan, 2013; Molas et al.,  
26 2017; Shumake et al., 2003). Therefore, some MHb-mediated diseases may be  
27 caused by a combination of genetic or epigenetic alternation of the *NECTIN2* gene  
28 with some stresses at the developmental stages of the MHb. Further studies are  
29 needed to elucidate the physiological and pathological roles of nectin-2 $\alpha$  in the  
30 cholinergic neurons in the MHb.

31

1   **CONFLICT OF INTEREST**

2

3   The authors have no conflicts of interest.

4

5   **AUTHOR CONTRIBUTIONS**

6

7   All authors had full access to all the data in the study and take responsibility for  
8   the integrity of the data and the accuracy of the data analysis. Study concept and  
9   design: H.S., M.M, A.M., K.M., H.M., M.W., and Y.T. Acquisition of data: H.S.,  
10   M.M, Y.I., S.W., A.K., A.M., and M.Y. Analysis and interpretation of data: H.S.,  
11   M.M, A.M., M.Y., and K.M. Drafting and critical revision of the manuscript: H.S.,  
12   M.M, A.M., K.M., and Y.T. Obtained funding: A.M., K.M, and Y.T. Study  
13   supervision: Y.T.

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 32 [E1%3E3.0.CO;2-D](https://doi.org/10.1002/(SICI)1096-9861(19990913)412:1%3C1::AID-CNE1%3E3.0.CO;2-D)
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## FIGURE LEGENDS

**Figure 1.** Schematic drawings of the habenula in the adult mouse brain and the localization of nectin-2 $\alpha$ . (a) A sagittal view. MHb, the medial habenula; MS, the medial septum; NDB, the nucleus of diagonal band; TS, the triangular septum; FR, the fasciculus retroflexus; and IPN, the interpeduncular nucleus. (b) The habenula. A coronal view at the indicated rostrocaudal level in (a). The encircled area in the left panel is highlighted in the right panel. The habenula consists of the MHb and the lateral habenula (LHb). The MHb is divided into five subregions: the MHbS, the MHbI, the MHbCd, the MHbCv, and the MHbL. VL, the third ventricle. (c–e) Localization of nectin-2 $\alpha$  in the MHb. (c) A high magnification image of the encircled area in (b). (d) A high magnification image of the encircled area in (c). The black punctates in (c, d) and bold lines in (d) indicate neuronal expression of nectin-2 $\alpha$  with a punctate pattern and a liner pattern, respectively. (e) A high magnification image of the encircled area in (d). S, somata of the cholinergic neurons; and MSs, the membrane specializations.

**Figure 2.** Expression of nectin-2 $\alpha$  in the adult MHb. Immunofluorescence microscopy was performed using the wild-type and *nectin-2*-deficient mice at P56. (a, b) Nectin-2 $\alpha$  and nectin-2 $\delta$  in the wild-type whole brain. The anti-nectin-2 $\alpha/\delta$  mAb (the mAb against the extracellular region of nectin-2 which recognizes both nectin-2 $\alpha$  and nectin-2 $\delta$ ) was used. (a) A sagittal section. (b) A coronal section. Arrows, the MHb; arrowheads, the choroid plexus; and double arrowhead, the inferior olivary nucleus. (c–e) Nectin-2 $\alpha/\delta$  and NeuN in the habenula of the wild-type mouse. The anti-nectin-2 $\alpha/\delta$  mAb and the anti-NeuN mAb were used. The area encircled by the curve, the MHb. Inset, a magnified image for the area encircled by the square. (f) Nectin-2 $\alpha/\delta$  in the habenula of the *nectin-2*-deficient mouse. The anti-nectin-2 $\alpha/\delta$  mAb was used. Inset, a magnified image for the area encircled by the square. (g) Nectin-2 $\delta$  in the habenula of the wild-type mouse. The anti-nectin-2 $\delta$  mAb (the mAb against the cytoplasmic region of nectin-2 which recognizes specifically nectin-2 $\delta$ ) was used. Inset, a magnified image for the area encircled by the square. Arrow, the choroid plexus; and arrowheads, the ependyma. The results shown in (a–g) are representative of three independent experiments.

1  
2 **Figure 3.** Localization of nectin-2 $\alpha$  in the cholinergic neurons in the adult MHb.  
3 Immunofluorescence microscopy was performed using the wild-type and  
4 *nectin-2*-heterozygous mice at P56. (a–e) Nectin-2 $\alpha$  in the MHb of the wild-type  
5 mouse. The anti-nectin-2 $\alpha$ / $\delta$  mAb was used. (b, d) A magnified image for the  
6 encircled area in (a). (c, e) A magnified image for the encircled area in (b, d).  
7 Arrows, the signal for nectin-2 $\alpha$  with the punctate pattern; and arrowheads, the  
8 signal for nectin-2 $\alpha$  with the linear pattern. (f–i) Nectin-2 $\alpha$  and ChAT in the MHb  
9 of the wild-type mouse. The anti-nectin-2 $\alpha$ / $\delta$  mAb and the anti-ChAT pAb were  
10 used. DAPI was used for nuclear counter staining. Arrows, areas where the signal  
11 for nectin-2 $\alpha$  with the linear pattern was observed. (j–l) Nectin-2 $\alpha$  and  
12  $\beta$ -galactosidase in the MHb of the *nectin-2*-heterozygous mouse, in which  
13  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele. The  
14 anti-nectin-2 $\alpha$ / $\delta$  mAb and the anti- $\beta$ -galactosidase mAb were used.  $\beta$ -Gal,  
15  $\beta$ -galactosidase. Arrows, areas where the signal for nectin-2 $\alpha$  with the linear  
16 pattern was observed at the boundary between the adjacent somata of the  
17 cholinergic neurons in which the signal for  $\beta$ -galactosidase was observed. (m–o)  
18 Nectin-2 $\alpha$  and bassoon in the MHbS of the wild-type mouse. The anti-nectin-2 $\alpha$ / $\delta$   
19 mAb and the anti-bassoon mAb were used. Arrows, areas where the signal for  
20 nectin-2 $\alpha$  with the punctate pattern was observed in the MHbS. The results shown  
21 in (a–o) are representative of three independent experiments.

22  
23 **Figure 4.** Immunofluorescence microscopical colocalization of nectin-2 $\alpha$  with  
24 Kv4.2 at the boundary between the adjacent somata of the clustered cholinergic  
25 neurons in the MHbI of the adult MHb. Immunofluorescence microscopy was  
26 performed using the wild-type and *nectin-2*-deficient mice at P56. (a–c) Nectin-2 $\alpha$   
27 and Kv4.2 in the MHbI of the wild-type mouse. The anti-nectin-2 $\alpha$ / $\delta$  mAb and the  
28 anti-Kv4.2 pAb were used. Arrows, areas where the signals for both nectin-2 $\alpha$  and  
29 Kv4.2 with the linear pattern were observed; arrowhead, area where the signal for  
30 Kv4.2, but not nectin-2 $\alpha$ , with the linear pattern was observed; and double  
31 arrowheads, areas where the signal for nectin-2 $\alpha$ , but not Kv4.2, with the linear  
32 pattern was observed. (d–f)  $\beta$ -Galactosidase and Kv4.2 in the MHbI of the  
33 *nectin-2*-deficient mouse, in which  $\beta$ -galactosidase was expressed from the  
34 *nectin-2* knockin allele. The anti- $\beta$ -galactosidase mAb and the anti-Kv4.2 pAb

1 were used.  $\beta$ -Gal,  $\beta$ -galactosidase. Arrows, areas where the signal for Kv4.2 was  
2 observed at the boundary between the adjacent somata of the  
3  $\beta$ -galactosidase-positive cholinergic neurons of the *nectin-2*-deficient mouse. The  
4 results shown in (a–f) are representative of three independent experiments.

5  
6 **Figure 5.** Immunoelectron microscopically different localization of nectin-2 $\alpha$   
7 with Kv4.2 at the boundary between the adjacent somata of the clustered  
8 cholinergic neurons in the MHbI of the adult MHb. Immunoelectron microscopy  
9 was performed using the wild-type mouse at P56. The anti-nectin-2 $\alpha/\delta$  mAb and  
10 the anti-Kv4.2 pAb were used. The anti-nectin-2 $\alpha/\delta$  mAb recognizes the  
11 extracellular region of nectin-2 $\alpha/\delta$ , while the anti-Kv4.2 pAb recognizes the  
12 intracellular region of Kv4.2. (a–c) Nectin-2 $\alpha$ . (d) Kv4.2. (e) Nectin-2 $\alpha$  and Kv4.2.  
13 (a, b, d, e) Immunoelectron microscopy performed using the brain fixed with 4%  
14 PFA. (c) Immunoelectron microscopy performed using the brain fixed with 2%  
15 PFA. Arrows, the immunogold particles for nectin-2 $\alpha$ ; and arrowheads, the  
16 immunogold particles for Kv4.2. N, nucleus of a neuron; S, somata of neurons;  
17 MS, the membrane specialization; and M, mitochondrion. The results shown in  
18 (a–e) are representative of three independent experiments.

19  
20 **Figure 6.** Colocalization of nectin-2 $\alpha$  with Necl-2, but not with Necl-1, Necl-3,  
21 and Necl-4, in the adult MHb. Immunofluorescence microscopy was performed  
22 using the wild-type mouse at P56. (a–c) Nectin-2 $\alpha$  and Necl-2 in the MHbI and  
23 the MHbCv. The anti-nectin-2 $\alpha/\delta$  mAb and the anti-Necl-2 mAb were used.  
24 Arrows, areas where the signal for Necl-2, but not nectin-2 $\alpha$ , with the linear  
25 pattern was observed; and arrowheads, areas where the signals for both Necl-2  
26 and nectin-2 $\alpha$  with the linear pattern were observed. (d–f) Nectin-2 $\alpha$  and Necl-3  
27 in the MHbCv. The anti-nectin-2 $\alpha/\delta$  mAb and the anti-Necl-3 pAb were used.  
28 Arrow, an area where the signal for Necl-3, but not nectin-2 $\alpha$ , with the linear  
29 pattern was observed; and arrowheads, areas where the signal for nectin-2 $\alpha$ , but  
30 not Necl-3, with the linear pattern was observed. (g–i) Nectin-2 $\alpha$  and Necl-1 in  
31 the MHbI. The anti-nectin-2 $\alpha/\delta$  mAb and the anti-Necl-1 pAb were used. Arrows,  
32 areas where the signal for nectin-2 $\alpha$ , but not Necl-1, with the linear pattern was  
33 observed. (j–l) Nectin-2 $\alpha$  and Necl-4 in the MHbI. The anti-nectin-2 $\alpha/\delta$  mAb and  
34 the anti-Necl-4 pAb were used. Arrows, areas where the signal for nectin-2 $\alpha$ , but

not Necl-4, with the linear pattern was observed. The results shown in (a–l) are representative of three independent experiments.

**Figure 7.** Different expression of nectin-2 $\alpha$  and ChAT in the MHb of the developing mouse. Immunofluorescence microscopy was performed using the *nectin-2*-heterozygous mice, in which  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele, at P1, P7, P14, and P28. (a–p) Nectin-2 $\alpha$ , ChAT, and  $\beta$ -galactosidase in the ventral half of the MHb. The anti-nectin-2 $\alpha/\delta$  mAb, the anti-ChAT pAb, and the anti- $\beta$ -galactosidase mAb were used. The signal for  $\beta$ -galactosidase is depicted in blue in the merged images. (a–d) P1. (e–h) P7. (i–l) P14. (m–p) P28.  $\beta$ -Gal,  $\beta$ -galactosidase. Arrows, areas where the signals for nectin-2 $\alpha$  and  $\beta$ -galactosidase, but not ChAT, were observed. The results shown in (a–p) are representative of three independent experiments.

**Figure 8.** Reduced localization of Kv4.2 at the boundary between the adjacent somata of the cholinergic neurons in the MHb of the developing *nectin-2*-deficient mouse. Immunofluorescence microscopy was performed using the *nectin-2*-heterozygous and *nectin-2*-deficient mice, in which  $\beta$ -galactosidase was expressed from the *nectin-2* knockin allele, at P14 and P28. (a–p) Nectin-2 $\alpha$ , Kv4.2, and  $\beta$ -galactosidase in the primordial regions for the MHbI and the MHbCv. The anti-nectin-2 $\alpha/\delta$  mAb, the anti-Kv4.2 pAb, and the anti- $\beta$ -galactosidase mAb were used. The signal for Kv4.2 is depicted in blue in the merged images. (a–h) P14. (i–p) P28.  $\beta$ -Gal,  $\beta$ -galactosidase. Arrowheads, areas where the signal for Kv4.2 was observed at the boundary between the adjacent somata of the  $\beta$ -galactosidase-positive cholinergic neurons. (a–d, i–l) The MHb of the *nectin-2*-heterozygous mice. (e–h, m–p) The MHb of the *nectin-2*-deficient mice. The results shown in (a–p) are representative of three independent experiments.

**Figure 9.** Confirmation by high-resolution immunofluorescence microscopy of the reduced localization of Kv4.2 at the boundary between the adjacent somata of the cholinergic neurons in the MHb of the developing *nectin-2*-deficient mouse. (a–h) Localization of Kv4.2 in the primordial regions for the MHbCv, the MHbL, and the lateral part of the MHbI. High-resolution immunofluorescence



1 microscopy was performed using the wild-type and *nectin-2*-deficient mice at P14.  
2 The anti-nectin-2 $\alpha/\delta$  mAb and the anti-Kv4.2 pAb were used. DRAQ5 was used  
3 for nuclear counter staining. The signal for DRAQ5 is depicted in blue in the  
4 merged images. (a–d) The MHb of the wild-type mouse. (e–h) The MHb of the  
5 *nectin-2*-deficient mouse. (i) The average percentage of cells with the signal for  
6 Kv4.2 at the boundary between the adjacent neuronal somata in the primordial  
7 regions for the MHbCv, the MHbL, and the lateral part of the MHbI (n=6, three  
8 different specimens of each two MHbs of the wild-type mice or two MHbs of the  
9 *nectin-2*-deficient mice).