**In Situ** Hybridization Analysis of the Expression of *Futsch*, *Tau*, and *MESK2* Homologues in the Brain of the European Honeybee (*Apis mellifera* L.)

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Abstract

**Background:** The importance of visual sense in Hymenopteran social behavior is suggested by the existence of a Hymenopteran insect-specific neural circuit related to visual processing and the fact that worker honeybee brain changes morphologically according to its foraging experience. To analyze molecular and neural bases that underlie the visual abilities of the honeybees, we used a cDNA microarray to search for gene(s) expressed in a neural cell-type preferential manner in a visual center of the honeybee brain, the optic lobes (OLs).

**Methodology/Principal Findings:** Expression analysis of candidate genes using in situ hybridization revealed two genes expressed in a neural cell-type preferential manner in the OLs. One is a homologue of *Drosophila futsch*, which encodes a microtubule-associated protein and is preferentially expressed in the monopolar cells in the lamina of the OLs. The gene for another microtubule-associated protein, *tau*, which functionally overlaps with *futsch*, was also preferentially expressed in the monopolar cells, strongly suggesting the functional importance of these two microtubule-associated proteins in monopolar cells. The other gene encoded a homologue of Misexpression Suppressor of Dominant-negative Kinase Suppressor of Ras 2 (*MESK2*), which might activate Ras/MAPK-signaling in *Drosophila*. *MESK2* was expressed preferentially in a subclass of neurons located in the ventral region between the lamina and medulla neuropil in the OLs, suggesting that this subclass is a novel OL neuron type characterized by *MESK2*-expression. These three genes exhibited similar expression patterns in the worker, drone, and queen brains, suggesting that they function similarly irrespective of the honeybee sex or caste.

**Conclusions:** Here we identified genes that are expressed in a monopolar cell (*Amfutsch* and *Amtau*) or ventral medulla-preferential manner (*AmMESK2*) in insect OLs. These genes may aid in visualizing neurites of monopolar cells and ventral medulla cells, as well as in analyzing the function of these neurons.

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Competing Interests: Ms. Akiko Wakamoto, a co-author of this article, is employed by a company, DNA Chip Research Incorporation (JAPAN). The other authors collaborated with Ms. Wakamoto to construct cDNA microarray and perform hybridization. Actually, she printed cDNA subclones that we sent to her on the chips, for analyzing the function of these neurons. There is no consultancy, patents, products in development, or marketed products etc, that the company has related to our article. The authors hereby confirm that the Ms. Wakamoto’s employment does not alter at all our adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the ‘guide for authors’.

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Introduction

Some insect species possess a highly developed visual sense that is essential for adaptation to the environment [1,2]. The European honeybee (*Apis mellifera* L.) is a social insect, and its colony consists of three types of adults: queens (female reproductive caste), workers (female labor caste), and drones (reproductive males) [3]. In addition, the workers shift their labors from nursing their brood (nurse bees) to foraging (foragers), according to their age after adult emergence [3]. Highly developed visual ability is especially important for social Hymenopteran insects, because they must be able to return to their hive [1,2]. For example, queens and drones mate in the air several tens of meters above the ground (rendezvous flight), which might require visual memory to return to the hive [3]. Workers use various visual cues to memorize the locations of food sources and return to the hive from several kilometers away [3]. In particular, they use optic flow to gauge the distance from the hive to the food source and inform their nestmates of the location of the food source (distance and direction from the hive) using the well-known ‘dance communication’ [3–6]. In the honeybee, visual information perceived at the compound eyes is first projected to the optic lobes (OLs), a visual center of the...
insect brain, and is then projected to other brain areas, such as the mushroom bodies (MBs), a higher-order integration center of the insect brain [7–11]. The OLs are composed of three layers of neuropil: the lamina, which directly contacts the retina; the medulla; and the lobula [7]. Electrophysiological studies suggest that the proportion of neurons involved in the detection of light wavelength, location, and motion direction differs in each of the three neuropil layers [12–19]. Anatomically, neurons in the OLs comprise groups termed ‘cartridges’ in the lamina, and groups termed ‘columns’ in the medulla and lobula. Neurons in these cartridges and columns are classified into subtypes based on their morphology or projection patterns [7,20–23]. The ability of honeybees to discriminate various colors, shapes, patterns, and motion direction have been studied in both free-flying [4,24] and harnesses bees [25–27]. Due to the small and rather simple morphology or projection patterns [7,20–23]. The ability of honeybees to discriminate various colors, shapes, patterns, and motion direction have been studied in both free-flying [4,24] and harnesses bees [25–27]. Due to the small and rather simple morphology or projection patterns [7,20–23]. The ability of honeybees to discriminate various colors, shapes, patterns, and motion direction have been studied in both free-flying [4,24] and harnesses bees [25–27]. Due to the small and rather simple morphology or projection patterns [7,20–23].
with large somata located beneath the calyces of the MBs (black arrowheads in Fig. 2H). In addition, intermediate signals were sometimes detected in two other brain regions: 1) a few cells with large somata located in the lateral area of the subesophageal ganglion (SOG; arrows in Fig. 2I); and 2) some cells located between the OLs and MBs (arrows in Figs. S2G and S3F, for queen and drone brains, respectively). Frequent, but not constant, detection of these two signals may depend on the individual experiment or the depths of the sections used. In contrast, there were no intense signals in the other brain regions, including the inside of the calyces of the MBs (white arrowheads in Fig. 2G, H), cells between the lamina and medulla (white arrowheads in Fig. 2D, E), cells between the medulla and lobula (white arrowhead in Fig. 2F), or cells located around SOG (white arrowheads in Fig. 2I). These signals were not detected in sections hybridized with sense probe (Fig. 2C), indicating that the signals were due to the expression of Amfutsch. Although Amfutsch was expressed weakly in the whole brain cortex, it was expressed preferentially in restricted neural cell types, suggesting the importance of this gene product in these cells (Fig. 2I). Essentially the same expression profile was observed in both nurse bee (Fig. S4) and forager brains (Fig. 4). Furthermore, Amfutsch-expression in the honeybee brain resembled that of Drosophila Tau. Although weak signals were detected in the whole brain cortex (Fig. 4A, B), stronger signals were detected in two restricted brain regions in the forager brain: 1) in a part of the lamina cells (black arrowheads in Fig. 4D, E); and 2) a few cells with large somata and are located beneath the MBs (black arrowheads in Fig. 4H). In addition, intermediate signals were sometimes detected in two other brain regions: 1) a few cells with large somata located in the lateral area of the SOG (arrows in Fig. 4J); and 2) some cells located between the OLs and MBs (data not shown). In contrast, there were no intense signals in other brain regions (white arrowheads in Fig. 4D–J). These signals were not detected in sections hybridized with sense probe (Fig. 4C), indicating that the signals represented Amtau-expression. These results indicated that Amfutsch and Amtau were expressed in the similar brain regions. Similar to Amfutsch, although Amtau is expressed weakly in the whole brain cortex, it is expressed preferentially in restricted neural cell types, suggesting the importance of this gene product in these cells (Fig. 4B, C).

Next, to identify the cell types that express Amfutsch and Amtau as well as to further confirm the co-expression of Amfutsch and Amtau in the lamina, we performed double fluorescent in situ hybridization using Amfutsch- and Amtau-specific antisense RNA probes and horizontal sections of the forager brain, followed by nuclear staining with DAPI (Fig. 5). Expression of both Amfutsch and Amtau was detected preferentially in a subclass of lamina cells located between the retina and lamina (blue arrowheads in Fig. 5D–G). The neurons and glial cells exhibit unique distribution patterns in the lamina of the OLs [20]. Comparison of the Amfutsch/Amtau-expressing cells by staining the nuclei with DAPI suggested that these cells were monopolar cells (blue arrowheads in Fig. 5H–K) and not glial cells (white arrowheads in Fig. 5I–K), whose cell bodies are located at the inner and outer parts of the cortex between the retina and lamina, respectively [20], although we could not definitely conclude that they are neurons but not glial cells, without staining with glial markers. The signals for both Amfutsch- and Amtau-expression were detected in monopolar cells, and they overlapped at least in some monopolar cells (Fig. 5H–K), indicating that lamina monopolar cells preferentially expressed both Amfutsch and Amtau in the OLs.
Figure 2. *In situ* hybridization of *Amfutsch* in the nurse bee brain. *In situ* hybridization using DIG-labeled RNA antisense (B, D–I) and sense (C) *Amfutsch* probes with nurse bee brain sections. (A) Schematic representation of the signals detected in the left-brain hemisphere of the nurse bee brain. Black circles and a black check mark indicate stronger or intermediate signals, respectively. (D–I) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected in the lamina (D, E) and in the other region (H) are indicated by black arrowheads. White arrowheads indicate the regions with no significant signals (D–I). Black arrows indicated intermediate signals near the SOG (I). Scale bars = 100 μm. Asterisks indicate non-specific staining. D, dorsal; L, lateral; la, lamina; Ica, lateral calyx; lo, lobula; M, medial; me, medulla; mCa, medial calyx; Re, retina; SOG, subesophageal ganglion; V, ventral. Note that each panel (panels D–I) shows repeated views of the same section rather than multiple sections from multiple brains.

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that these genes are expressed in the differentiating neurons in the developing pupal brains. As expected, both Amtau and Anfutsch were expressed in the developing pupal brains (Supporting Information S1, Fig. S5 and S6). Unexpectedly, however, these genes were differentially expressed in the pupal brains: Anfutsch was expressed in the whole brain cortex except for the mushroom bodies (MBs), whereas Amtau was expressed around the proliferating MB cells, suggesting that these genes function in a brain-region dependent manner in developing pupal brains (Supporting Information S1, Fig. S5 and S6). We could not identify differentiating monopolar cells and thus examine the expression of these genes in these cells.

Gene Structure of Honeybee MESK2

Clone #2 corresponded to the putative intron region of a deduced gene, GB18470, which is located at Linkage group 6 (Fig. 6A). NCBI database search revealed that GB18470 encodes a protein that has the highest sequence similarity with Drosophila Misexpression Suppressor of dominant-negative KSR (Kinase Suppressor of Ras) 2 (MESK2). The Drosophila MESK2 isoform 1 (GenBank accession No. AAS64904.1) consists of 485 amino acid residues and contains an Ndr domain, which is the domain conserved among proteins encoded by the X-ray downstream regulated gene family (Fig. 6B) [48] (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The predicted protein encoded by GB18470 consists of 383 amino acid residues and contains an Ndr domain that also has sequence identity (74%) with that of DmMESK2 (Fig. 6B). Because there were no genes other than GB18470 similar to DmMESK2 in the honeybee genome, and the protein encoded by GB18470 had the highest sequence similarity with DmMESK2 in the honeybee genome, we concluded that GB18470 is the DmMESK2 homologue (AmMESK2).

To confirm that Clone #2 actually corresponded to the intron region of AmMESK2, we intended to examine whether Clone #2 is connected with the predicted 6th exon on a precursor AmMESK2 mRNA, by amplifying partial cDNA that contained both Clone #2 and the predicted 6th exon region of AmMESK2 by reverse transcription-polymerase chain reaction (RT-PCR) (Supporting Information S1, Fig. S7). A cDNA fragment of approximately 700 bp, which is consistent with the predicted size (678 bp), was obtained, supporting our notion that Clone #2 corresponds to an intron of AmMESK2. We can’t exclude the possibility, however, that there are some alternative splice variants and that Clone #2 partly includes sequences for these splice variants.

Expression Analysis of AmMESK2 in the Honeybee Brain

DmMESK2 was originally identified by screening genes with the potential to modulate RAS-signaling when misexpressed [49], and its actual functions and expression profile have not been analyzed. Therefore, we performed in situ hybridization of AmMESK2 using the forager brain sections and an ‘intron probe’ that corresponded to the sequence (Clone #2) obtained by the cDNA microarray analysis. Significant signals were detected in only a single brain region: a few dozen neurons whose somata were located at the ventral part of the cortex between the lamina and medulla of the OLs (Fig. 7A, B, E). No significant signals were detected in other brain regions including the OL (Fig. 7D, G), the inside of the calyces of the MBs (Fig. 7F), and cells around the SOG (Fig. 7H). These signals were not detected in sections hybridized with sense probe (Fig. 7C), indicating that the signals represented AmMESK2 expression. Interestingly, AmMESK2 was not expressed in the dorsal or middle part of the OLs (Fig. 7D), indicating that AmMESK 2 was expressed preferentially in the ventral part of the brain.

Figure 3. Gene structure of the predicted gene Amtau. Genomic organization of the predicted gene Amtau (A), comparison of the domain structures of Taup (B) and alignment of amino acid sequences of MTBD repeats (C). (A) Exon (closed boxes) and intron (lines) structures of the deduced gene and the location of the probe used for in situ hybridization are indicated below the corresponding linkage group (upper line). Closed boxes in (B) indicate microtubule-binding regions of Amtau and Dmtau. The numbers above these regions of Amtau indicate sequence identities with corresponding regions in Dmtau. (C) The amino acid sequences of MTBD Repeat 1–5 of Dmtau (upper amino acid sequence) and Amtau (lower amino acid sequence) are aligned, where the gray background indicate the identical amino acid residues. The numbers on the left and right of each sequence indicate amino acid positions in each protein.

To obtain evidence that Anfutsch and Amtau also function as ‘microtubule-associated proteins’ in the honeybee brain, we used in situ hybridization to examine the expression of Anfutsch and Amtau in the developing pupal brains, based on the assumption

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Figure 4. In situ hybridization of Amtau in the forager brain. In situ hybridization using DIG-labeled RNA antisense (B, D–I) sense (C) Amtau probes and the forager brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the forager brain. Black circles indicate stronger signals. (D–I) Magnified views of parts of (B) corresponding to the boxes shown in (A). (J) Magnified view of the same part as (I) of another section, which includes intermediate signals. The stronger signals detected in the lamina (D and E) and the other region (H) are indicated by black arrowheads. White arrowheads indicate regions with no significant signals (D–J). Black arrows indicated intermediate signals near the SOG (J). Scale bars = 100 µm. Asterisks indicate non-specific staining. D, dorsal; L, lateral; la, lamina; ICa, lateral calyx; lo, lobula; M, medial; me, medulla; mCa, medial calyx; Re, retina; SOG, subesophageal ganglion; V, ventral.

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Figure 5. Double in situ hybridization of Amfutsch and Amtau in the OLs of the forager brain. Fluorescent double in situ hybridization using DIG-labeled RNA antisense Amfutsch and Amtau probe and horizontal sections of forager brains, followed by DAPI staining. (A) Schematic representation of the structure in the vertical brain sections. White regions correspond to neuropil, whereas gray regions indicate cortex. (B) Schematic representation of the structure in the horizontal brain section corresponding to the dotted line in (A). (C) Schematic representation of the structure in the horizontal brain section corresponding to the box shown in (B). Black circles indicate monopolar cells with signals, and white circles indicate glial cells with no signals. (D–G) In situ hybridization of the box shown in (B): (D) with antisense Amfutsch probe, (E) with antisense Amtau probe, (F) nuclear staining with DAPI, and (G) merged images in (D–F). Red, green, and blue signals in (G) indicate Amfutsch- and Amtau-expression and nuclear staining with DAPI, respectively. (H–K) Magnified views corresponding to the boxes shown in (D–G), respectively. The stronger signals detected in the lamina monopolar cells are indicated by blue arrowheads, and regions with no significant signals are indicated by white arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. D, dorsal; Ich, inner chiasma; L, lateral; la, lamina; lo, lobula; M, medial; me, medulla; MB, mushroom body; Och, outer chiasma; Re, retina; V, ventral.
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AmMESK2 is expressed differentially between the anterior and proximal parts of the OLs by *in situ* hybridization with the horizontal drone brain sections. The AmMESK2-expressing cells were located ubiquitously along the anterior-posterior axis and around the outer chasm (Fig. 9I–L). The signals were not detected between the medulla and lobula, confirming that AmMESK2 was expressed preferentially in neurons located between the lamina and medulla in the honeybee brain.

Finally, we performed real-time RT-PCR to compare the amount of *Amfutsch*, *Amtau* and *AmMESK2* transcripts between the brains of nurse bees and foragers, in which the MB structure changes depending on the division of labor of the workers and correlates with the foraging experience of the foragers [28, 29]. The amount of *Amfutsch* and *Amtau*-transcripts did not differ significantly between the brains of nurse bees and foragers, whereas the amount of *AmMESK2*-transcript was 1.5-fold higher in the forager brain than in the nurse bee brain (Supporting Information S1, Fig. S11).

**Discussion**

The present study is the first to identify genes whose expression was more enriched in the OLs than in the other regions in the honeybee brain. The findings indicated three genes with neural cell type-preferential gene expression profiles in the OLs. To our knowledge, this is the first identification of genes that are expressed in a monopolar cell- (Amfutsch and Amtau) or ventral medulla-preferential manner (AmMESK2) in insect OLs.

One of these genes was a homologue of a gene for microtubule-associated protein, *futsch*/*map1*. Both mammalian MAP1 and *Drosophila* Futsch function to stabilize axon structures by binding to microtubules at axons or axon terminals [45, 50]. Although the MAP1 family contains several genes in mammals, in *Drosophila*, MAP1 function appears to be fulfilled by a single gene, *futsch* [44]. Similarly, *Amfutsch* was a single copy gene in the honeybee genome. In *Drosophila*, *DmFutsch* encodes the 22C10 antigen, which has been widely used as a neuronal marker [51]. In adult brain, 22C10-immunoreactivity is detected in some central nervous systems, such as chiasmas or antennal nerves, as well as in most peripheral neurons [52], whereas *Amfutsch* was expressed preferentially in the monopolar cells in the lamina of the adult honeybee brain. In addition, double *in situ* hybridization showed that *futsch* and *tau*, which functionally overlap in mice and flies, are co-expressed in the monopolar cells in the lamina. Similar to the situation for *Amfutsch*, although the Tau family contains several genes in mammals, *tau* represents a single copy gene in both *Drosophila* and the honeybee. Furthermore, although *Dmtau* was expressed in photoreceptors in *Drosophila* adults and as well as in brain and most peripheral neurons in *Drosophila* larvae [46], *Amtau* was expressed preferentially in the monopolar cells in the lamina of the adult honeybee brain. These results suggested that *Amfutsch* and *Amtau* are involved in the monopolar cell-specific cell characteristics or structures. Furthermore, the expression profiles of *DmFutsch* and *Dmtau* in the adult brain seem distinct from those of *Amfutsch* and *Amtau*, suggesting that both *futsch* and *tau*-expression might be differently regulated among these insect species. Considering that monopolar cells may be involved in detection of contrast of visual objects, and project axons into the medulla as well as into the lobula, *AmFutsch* and *AmTau* might play important roles in stabilizing axon structures, which is essential for neural functions of monopolar cells.

In *in situ* hybridization of *Amfutsch* and *Amtau* using developing pupal brain sections indicated that, although both genes were expressed in the pupal brains, they were differentially expressed in

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**Figure 6. Gene structure of the predicted gene AmMESK2.**

Genomic organization of the predicted gene AmMESK2 (A) and comparison of the domain structures of AmMESK2 and DmMESK2 (B). (A) Exon (closed boxes) and intron (lines) structures of the predicted gene and the location of the Clone #2 and the probe for *in situ* hybridization are indicated below the corresponding linkage group (upperline). (B) Closed boxes in AmMESK2 and DmMESK2 isoform I indicate Ndr domains. The number below the Ndr domain of AmMESK2 indicates sequence identities with that of DmMESK2.

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the pupal brains, suggesting that these genes function in a brain-region dependent manner during pupal stages (Supporting Information S1, Fig. S5 and S6). Considering that expression of both *AmFutsch* and *AmTau* is enriched in monopolar cells, it might be that the axons of monopolar cells are solid or undergo remodeling under certain circumstances, which requires more abundant *AmFutsch* and *AmTau* than the other brain regions. Because essentially the same *AmFutsch-* and *AmTau*-expression profiles were

**Figure 7. In situ hybridization with the intron probe of AmMESK2 in the forager brain.*** In situ hybridization using DIG-labeled RNA antisense (B, D–H) and sense (C) AmMESK2 probes with forager brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the forager brain. Black circles indicate the stronger signals. (D–H) Magnified views of parts of (B) corresponding to the boxes shown in (A). The signals detected in the cortex between the lamina and medulla are indicated by black arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. D, dorsal; L, lateral; la, lamina; lCa, lateral calyx; lo, lobula; M, medial; me, medulla; mCa, medial calyx; Re, retina; SOG, subesophageal ganglion; V, ventral. Note that stronger signals detected in magnified view (E) are scarcely detected in low magnification micrograph (B), and so the signals need to be examined closely with magnified views. This is also the case for Figs. 8, 9, S5, S6 and S7.

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detected in all forager, nurse bee, queen, and drone brains, it is likely that Amfutsch and Amtau function similarly in monopolar cells irrespective of the sex, caste, or division of labor of workers.

The function of Amfutsch and Amtau in the cells with large somata that are located beneath the MB calyces remains unknown. Based on the location of these cells, they might be neurosecretory cells. Axonal stabilization might also be required in these cell types. In the honeybee, immunoreactivity for pigment dispersing hormone, which is involved in insect circadian clock, is detected in neurons located at the medial margin of medulla as well as in large neurons.

Figure 8. *in situ* hybridization with the exon probe of AmMESK2 in the forager brain. *in situ* hybridization using DIG-labeled RNA antisense (B, D–H) and sense (C) AmMESK2 probes with forager brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the forager brain. Black circles indicate the stronger signals. (D–H) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected between the lamina and medulla are indicated by black arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. D, dorsal; L, lateral; la, lamina; Ica, lateral calyx; lo, lobula; M, medial; me, medulla; mCa, medial calyx; Re, retina; SOG, subesophageal ganglion; V, ventral.

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**Figure 9. In situ hybridization with the exon probe of AmMESK2 in the drone brain.** In situ hybridization using DIG-labeled RNA antisense (vertical sections; B, D–H, horizontal sections; J–M) and sense (vertical sections; C) AmMESK2 probes with drone brain sections. (A, I) Schematic representation of signals detected in the vertical (A) and horizontal section (I) of the left-brain hemisphere of drones, respectively. Black circles indicate stronger signals. The dotted line in (A) indicates the position of the horizontal sections for (I–J). (D–H) Magnified views of parts of (B) corresponding to the boxes shown in (A). (K–M) Magnified views of parts of (J) corresponding to the boxes shown in (I). The stronger signals detected in the cortex between the lamina and medulla are indicated by black arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. A, anterior; AL, antennal lobe; D, dorsal; L, lateral; la, lamina; ICa, lateral calyx; lo, lobula; M, medial; me, medulla; mCa, medial calyx; Och, outer chiasma; P, posterior; Re, retina; V, ventral.

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located beneath the MBs [53]. Although the latter cells resemble to
those expressing *Ampfutsch* and *Amtau*, the former cells differ from
the monopolar cells. Thus, the relationship between the circadian
clock and the *Ampfutsch*- and *Amtau*-expressing cells is unclear, at
present. Finally, *Amtau* was not identified in our cDNA microarray
screening as a gene whose expression was more enriched in the
OLs than in the other brain regions, possibly because our cDNA
microarray contained only 5000 cDNA subclones corresponding
to the differential display-positive bands, and thus the variety of
genome printed on the cDNA microarray was restricted.

The second gene was a homologue of *MESK2*. *DmMESK2* was
originally identified while screening for genes that have potentials
to modulate RAS-signaling [44]: this gene enhances KSR-
signaling, which functions downstream of RAS1, when mis-
expressed in the Drosophila retina. Its actual function and
expression profile in the adult brain has not been analyzed in
Drosophila. Because there was only a single gene for *MESK2*
in both honeybee and Drosophila, we concluded that *AmMESK2* is
a homologue of *DmMESK2*. The conserved Ndr domain of
*AmMESK2* suggests that *AmMESK2* has molecular functions
similar to those of *DmMESK2*. Interestingly, *AmMESK2* was
expressed strongly in only a few dozen cells located in the ventral
region between the lamina and medulla in the OLs. Analysis of
*AmMESK2* expression revealed that the *AmMESK2*-expressing
cells form a zone at the ventral part that spans from the anterior
to the posterior regions of the OLs, close to the outer chiasm. It
might be that these cells are important for detecting visual cues,
e.g., optic flow, present on the ground rather than in the air. Although
the function of neurons expressing *AmMESK2* is currently
unknown, *AmMESK2* may be involved in some kind of
neural plasticity by modulating RAS-signaling. These findings
indicate that the medulla contains a new subclass of cells
characterized by *AmMESK2*-expression, which has not been
identified by previous anatomic or electrophysiologic studies.

Neurons in the lamina and medulla form ‘cartridges’ and
‘columns’, each of which comprise several types of neurons
[7,20–23]. Thus, it is possible that the *AmMESK2*-expressing
cells comprise a part of the ‘cartridges’ or ‘columns’, and that these
‘cartridges’ or ‘columns’ represent a considerable part of the
ventral region between the lamina and medulla in the OLs.

Whether monopolar cell-preferential *Ampfutsch/Amtau*-expression
and ventral medulla-preferential *AmMESK2*-expression are
restricted to honeybees, are conserved among a part of social
Hymenopteran insects, or are conserved among various Hyme-
noptera insects, requires further investigation. From the view of
‘molecular dissection’ of the honeybee brain, *Ampfutsch/Amtau* and
*AmMESK2* may be useful tools for detecting axons of the cells that
express these genes: these genes aid in visualizing axons of
monopolar cells and ventral medulla cells that express *AmMESK2*,
for example, by immunochemical staining or by using reporter
genes in future experiments. Considering that the MB structure
changes depending on the division of labor of the workers and
correlates with the foraging experience of the foragers [51,52], it is
plausible that visual experience affects patterns or densities of
axonal projections of OL neurons to the MBs. In fact, the amount of
*AmMESK2*-transcript was 1.5-fold higher in the forager brains
than in the nurse bee brains, raising the possibility that the
function of *AmMESK2* is more necessary in the forager brain than
in the nurse bee brain [Supporting Information S1, Fig. S11].
Further studies to examine whether projection patterns of the
monopolar cells and ventral medulla neurons expressing *Ampfutsch/
Amtau* and *AmMESK2* depend on the foraging flight of workers can be
performed using *Ampfutsch/Amtau* and *AmMESK2* as cell type-
preferential markers.

### Materials and Methods

#### Animals

European honeybees (*Apis mellifera* L.) were purchased from a
local dealer and maintained at the University of Tokyo. Foragers
with pollen loads were captured at the hive entrance. Nurse bees
were collected when they were feeding their brood in honeycombs
[26,54]. Drones were collected in the hive. Queens were
purchased from the same local dealer.

#### cDNA Microarray Analysis

A cDNA microarray was performed as described previously
[39] with some modifications. We previously prepared a cDNA
microarray with over 5000 cDNA subclones representing various
genomes expressed in the adult honeybee brains: the subclones were
cloned from gel portions corresponding to the positive bands in the
differential display method used to identify genes expressed in
honeybee brain in a brain region- or role-preferential manner
[35]. In the present study, we used this cDNA microarray to
compare gene expression profiles between the OLs and the other
brain regions. Total RNA was extracted from the OLs and the
remaining brain regions dissected from the heads of 79 foragers
using TRIzol (Invitrogen). Total RNA (500 ng) from the OLs and
the remaining brain regions were amplified using an Amino Allyl
MessageAmp aRNA Amplification kit (Ambion). Total RNA from
the OLs and the other brain regions was divided into 4 groups and
two groups were labeled with fluorescent dye Cy5, while the other
two groups were labeled with Cy3 (Amersham Bioscience), to
prepare two sets of Cy5- or Cy3-labeled RNA from the OLs and
the other two sets of Cy5- or Cy3-labeled RNA from the other brain regions. Hybridization was performed twice using a pair of ‘Cy5-labeled
OL RNA and Cy3-labeled the other brain region RNA’, and a
pair of ‘Cy3-labeled OL RNA and Cy5-labeled the other brain
region RNA’. Data analyses were performed using Genespring
software (Silicon Genetics). Hybridization was performed twice by
exchanging the dyes, Cy5 or Cy3, that were used to label the
RNAs, and this hybridization process was repeated to confirm the
results.

We calculated the ratio of the expression level of each clone in the
OLs relative to that in the remaining brain regions and looked
for clones whose ratios were greater than 1.4-fold. Sequencing the
positive clones revealed many redundant clones, and 45
independent clones were identified as candidate genes whose
expression was more enriched in the OLs than in the other brain
regions. An expression analysis of 19 clones selected arbitrarily
from the 45 identified candidate clones performed using *in situ*
hybridization with a DIG-labeled RNA probe led to the
identification of two clones, Clones #1 and #2 (GenBank
accession Nos. BP538943 and BP539264), that were strongly
expressed in the OLs compared with the other brain regions.
Expression of 15 of the remaining 17 candidates was not clearly
detected in any brain region by *in situ* hybridization, possibly
because their expression levels were below the detection threshold,
whereas the expression of the remaining 2 candidates was detected
in both the OLs and other brain regions in the honeybee brain,
suggesting that these genes represented ‘false positive clones’.

#### In Situ Hybridization Analysis

*In situ* hybridization was performed as described previously with
some modifications [34,54,55]. Frozen vertical brain sections
(10 μm thick) were fixed in 4% paraformaldehyde in phosphate
buffer, pretreated, and hybridized with digoxigenin (DIG)-labeled
riboprobes. The DIG-labeled riboprobes were synthesized by T7 or
SP6 polymerase with a DIG labeling mix (Roche) from
template cDNAs containing the fragment cDNA for Clone #1 (BP530943, which corresponds to +9039 to +1003 of GB1510/Amfutsch), Antau (+2609 to +3103 of hmlJ4906), Clone #2 (BP539264, which corresponds to +213115 to +221462 of LG6/putative intron of JmMEX2), and 'exon probe' of AmMEX2 (44 to +1052 of GB15070), respectively. After stringent washes, DIG-labeled riboprobes were detected immunocytochemically with alkaline phosphatase-conjugated anti-DIG antibody using a DIG Nucleic Acid Detection Kit (Roche). To examine the monoclonal cell-preferential expression or co-expression of Amfutsch with Antau in monopolar cells, the Amfutsch riboprobe was labeled with DIG and the Antau riboprobe was labeled with biotin. DIG-labeled Amfutsch riboprobes were detected with the HNPP Fluorescent Detection Set (Roche), and biotin-labeled Antau riboprobes were detected with the TSA plus System (Perkin Elmer). As a negative control, sections were hybridized with sense probes and the antisense probe-specific signals were confirmed in every experiment. Micrographs of fluorescent in situ hybridization were taken using a fluorescent microscope (Axio Imager Z1, Carl Zeiss). 4',6-Diamino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) was used to stain the nuclear DNA [55,56]. Intensity and brightness of the micrographs were processed with Photoshop software (Adobe Systems).

Supporting Information

Supporting Information S1
Found at: doi:10.1371/journal.pone.0009213.s001 (0.06 MB DOC)

Figure S1 In situ hybridization of Amfutsch in the forager brains. In situ hybridization using DIG-labeled RNA antisense (B, D–I) and sense (C) Amfutsch probes with forager brain sections. (A) Schematic representation of the signals detected in the left-brain hemisphere of the forager brain. Black circles indicate the stronger signals. (D–I) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected in the lamina (D, E) and in another region (H) are indicated by black arrowheads. White arrowheads indicate regions with no signals (E–F). Black arrows indicated intermediate signals near the SOG (I). Scale bars = 100 μm. Asterisks indicate non-specific staining. D: dorsal, L: lateral, M: medial, Lo: lobula; M: medial, Me: medulla; mCa, medial calyx; Re: retina; SOG, subesophageal ganglion; V: ventral.

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Figure S2 In situ hybridization of Amfutsch in the forager brains. In situ hybridization using DIG-labeled RNA antisense (B, D–I) and sense (C) Amfutsch probes with forager brain sections. (A) Schematic representation of the signals detected in the left-brain hemisphere of the queen brain. Black circles and black check-marks indicate the stronger and intermediate signals, respectively. (D–I) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected in the lamina (D, E) and in another region (H) are indicated by black arrowheads. White arrowheads indicated the regions with no signals (D–I). Black arrows indicate intermediate signals near the MBs (G) and the SOG (I). Scale bars = 100 μm. Asterisks indicate non-specific staining. D: dorsal, L: lateral, Lo: lamina; ICa, lateral calyx; Lo: lobula; M: medial; Me: medulla; mCa, medial calyx; Re: retina; SOG, subesophageal ganglion; V: ventral.

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Figure S3 In situ hybridization of Amfutsch in the drone brains. In situ hybridization using DIG-labeled RNA antisense (B, D–I) and sense (C) Amfutsch probes with drone brain sections. (A) Schematic representation of the signals detected in the left-brain hemisphere of the drone brain. Black circles and black check-marks indicate the stronger and intermediate signals, respectively. (D–G) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected in the lamina (D, E) and in another region (F) are indicated by black arrowheads. White arrowheads indicate the regions with no signals (D–F). Black arrows indicate intermediate signals in regions near the MBs (F) and SOG (G). Scale bars = 100 μm. Asterisks indicate non-specific staining. AL: antennal lobe; D: dorsal; L: lateral; La: lamina; Ica, lateral calyx; Lo: lobula; M: medial; Me: medulla; mCa, medial calyx; Re: retina; V: ventral.

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Figure S4 In situ hybridization of Antau in the nurse bee brains. In situ hybridization using DIG-labeled RNA antisense (B, D–I) and sense (C) Antau probes with nurse bee brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the forager brain. Black circles indicate stronger signals. (D–I) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected in the lamina (D, E) and in another region (H) are indicated by black arrowheads. White arrowheads indicate the regions with no signals (E–F). Black arrows indicated intermediate signals near the SOG (I). Scale bars = 100 μm. Asterisks indicate non-specific staining. D: dorsal; L: lateral; La: lamina; IcA, lateral calyx; Lo: lobula; M: medial; Me: medulla; mCa, medial calyx; Re: retina; SOG, subesophageal ganglion; V: ventral.

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Figure S5 Expression analysis of Amfutsch in the developing pupal brain. In situ hybridization using DIG-labeled RNA Amfutsch antisense probes with developing pupal brain sections (Stage P2, P4, and P5). (A) Schematic representation of signals detected in the left hemisphere of the developing pupal brain. Grey regions indicate the part of the brain cortex with stronger signals. (B–D) Results of in situ hybridization using developing pupal brain sections at the P2, P4, and P5 stages [S1], respectively (for staging, also see legend for Fig. S6). Note that relatively strong signals were detected in almost the whole brain cortex, whereas only weak signals were detected in the developing MB regions surrounded by dotted lines [S1, 3, 4]. We could not identify the monoclonal cells undergoing differentiation in these developing pupal brain sections. Scale bars = 100 μm. D: dorsal; L: lateral; M: medial; MB: mushroom body; OL: optic lobe; V: ventral.

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Figure S6 Expression analysis of Antau in the developing pupal brain. In situ hybridization using DIG-labeled RNA antisense Antau probes with developing worker brain sections. (A) Results of the in situ hybridization using a section from the right hemisphere of the developing pupal brain. (B) A magnified view of the right pupal MB, indicated by the box in panel (A). (C, D) Schematic representation of signals detected in the right hemisphere of the developing pupal brain, which correspond to panels (A) and (B), respectively. Black circles indicate stronger signals. Grey regions indicate brain cortex with medium signals. Proliferating MB cells are represented by open circles in the inner core of the inside of developing calyces, and are indicated by arrows. (E: upper panel) Time-course of the developmental stages, including the larva, prepupa, pupa (P1–9), and adult. (E: lower panels) Magnified views of the in situ hybridization of the developing pupal MBs at stages P1, P2, P4, and P5 [S1]. Stronger signals were detected around the proliferative MB cells, indicated by arrows. Scale bars = 100 μm.
RNA antisense (B, D–H) and sense (C) *AmMESK2* probes with nurse bee brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the nurse bee brain. Black circles indicate stronger signals. (D–H) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected between the lamina and medulla are indicated by black arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. AL, antennal lobe; D, dorsal; L, lateral; la, lamina; ICa, lateral calyx; M, medial; me, medulla; mCa, medial calyx; OL, optic lobe; Re, retina; V, ventral.

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**Figure S10** *In situ* hybridization with the exon probe of *AmMESK2* in the queen brains. *In situ* hybridization using DIG-labeled RNA antisense (B, D–H) and sense (C) *AmMESK2* probes with queen brain sections. (A) Schematic representation of signals detected in the left-brain hemisphere of the queen brain. Black circles indicate stronger signals. (D–H) Magnified views of parts of (B) corresponding to the boxes shown in (A). The stronger signals detected between the lamina and medulla are indicated by black arrowheads. Scale bars = 100 μm. Asterisks indicate non-specific staining. AL, antennal lobe; D, dorsal; L, lateral; la, lamina; ICa, lateral calyx; M, medial; me, medulla; mCa, medial calyx; OL, optic lobe; Re, retina; V, ventral.

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**Author Contributions**

Conceived and designed the experiments: KK SH MMM. Performed the experiments: KK SH MMM TN AW ST. Analyzed the data: KK SH MMM TN ST. Contributed reagents/materials/analysis tools: KK SH MMM TN RKP TF KS AW ST. Wrote the paper: KK HT TK.

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