INTRODUCTION

In the brain, generation of new neurons throughout life, the adult neurogenesis, has important functional aspects. Adult neurogenesis is a multistep process covering proliferation of neuronal stem/precursor cells (NPCs), migration of neuroblasts, differentiation into mature neurons, and integration into pre-existing neuronal networks. Moreover, neurogenesis is heterogeneous among mammals depending on species and age.1 Under normal physiological conditions, adult neurogenesis takes place in specific regions of the adult mouse brain, known as “neurogenic niches,” such as spatial working memory and olfaction using mice with a targeted deletion of the P2Y2 receptor (P2Y2−/−). Proliferation, migration, differentiation, and survival of neuronal precursor cells (NPCs) were analyzed by BrdU assay and immunohistochemistry; signal transduction pathway components were analyzed by immunoblot. In P2Y2−/− mice, proliferation of NPCs in the SGZ and the SVZ was reduced. However, migration, neuronal fate decision, and survival were not affected. Moreover, p-Akt expression was decreased in P2Y2−/− mice. P2Y2−/− mice showed an impaired performance in the Y-maze and a higher latency in the hidden food test. These data indicate that the P2Y2 receptor plays an important role in NPC proliferation as well as in hippocampus-dependent working memory and olfactory function.
comprising mainly the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle. In the SGZ, the radial-glia cell like NPCs proliferate and give rise to neuroblasts which start to migrate for a short distance within the DG. In the DG, only a fraction survives and differentiates into granule cells that extend their dendrites within the molecular layer and their axons through the mossy fiber tract to the CA3 region. The adult-born granule cells become integrated into the hippocampal neuronal network and play a crucial role in learning and cognition. In the SVZ, the NPCs give rise to neuroblasts which migrate in chains for a longer distance along the rostral migratory stream (RMS) into the olfactory bulb (OB). Here they detach, and differentiate into functional interneurons of the granule cell layer (GCL) and the glomerular layer (GL). In the OB, this continuous supply of newly born interneurons plays a crucial role in olfactory function.

In both neurogenic niches, the highly dynamic process of adult neurogenesis is influenced by several extrinsic and intrinsic factors such as enriched environment, aging, circadian clock, and several neuro- and gliotransmitters including purinergic signaling. The purinergic signaling pathway is mediated by the extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP, UDP-glucose, and UTP). While adenosine binds to P1 receptors that involves four subtypes (A1, A2A, A2B, and A3), the other purines and pyrimidines bind to P2X ligand-gated ion channel receptors that possess two transmembrane domains and a large extracellular loop and/or to P2Y G protein-coupled receptors that feature an extracellular N-terminus, an intracellular C-terminus, and seven transmembrane domains. P2X receptors include seven subtypes P2X1-7; while P2Y receptors includes eight subtypes P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. ATP activates P2X receptors and P2Y2 receptors bind to P2Y2 while UDP activates P2Y6 and ADP stimulates P2Y1,12,13,14. In the central nervous system, purinergic receptors show a distinct spatial and even temporal distribution. The activation of P2 receptors is involved in ion transport, neuro-/gliotransmission, and reorganization of the cytoskeleton and implemented in a wide range of physiological and pathophysiological processes.

Purinergic receptors are involved in a variety of CNS pathological conditions including Alzheimer disease, dementia, sleep, and psychiatric disorders and, therefore, P2 receptors are considered as important potential therapeutic targets for neurological diseases. In adult neurogenesis, purines and pyrimidines acting on purinergic receptors modulate proliferation, migration, survival, and differentiation of NPCs (reviewed by). ATP released predominantly by astrocytes has been identified as a proliferative factor for NPCs. Interestingly, also the NPCs themselves are a source for ATP, exerting an autocrine effect on P2Y receptors to mediate the intracellular Ca\(^{2+}\) mobilization and hence trigger their own proliferation. Application of ATP and other purines as well as pyrimidines in vitro and in vivo promotes the proliferation of NPCs which can be blocked by P2Y receptor antagonists. The extracellular nucleotide breakdown enzyme ectonucleotidase (NTPDase2) and the P2Y receptors are highly expressed in the major neurogenic niches in the DG and SVZ. The deletion of NTPDase2 leads to enhanced progenitor proliferation in both niches, however, with no effect on neuron formation. Especially, the P2Y1 and P2Y2 receptors which utilize the second messenger (Ca\(^{2+}\)) and the mitogen-activated protein kinase (MAPK) cascades with extracellular signal regulated kinase-1 and 2 (ERK1/2) as well as the phosphoinositide 3-kinase (PI3K)/Akt pathway have been identified to be most effective in NPCs. In addition to their own proliferative potential, activation of P2Y1,2 receptors exerts a synergistic growth factors-mediated effect and hence, augments the NPC proliferation. P2Y2 receptors (receptor structure and function are reviewed in) are expressed by neuronal cells, astrocytes, oligodendrocytes progenitors, and also in neural progenitors.

While the majority of studies on purinergic signaling in adult neurogenesis were performed in vitro, few experiments were performed in vivo and most of them were done using purinergic receptor agonists or antagonists.

Interestingly, P2Y1-knockout mice showed reduced expression of rapidly dividing and transit-amplifying cells in the SVZ as well as a decreased proliferative capacity in neurospheres. P2Y13-knockout mice also displayed microglial-mediated enhanced progenitor proliferation and new neuron formation in the adult hippocampus. However, P2Y2-knockout mice have not been used yet to investigate the adult neurogenesis. Moreover, the availability for subtype specific agonists and antagonists is limited. Although selective antagonists have been recently described, the most commonly used P2Y2 antagonist is suramin, which is competitive but non-selective. Thus, the role of the P2Y2 receptors on adult neurogenesis and brain function is yet poorly understood. In the present study, we analyzed adult neurogenesis in the SGZ and the SVZ as well as certain brain functions such as spontaneous locomotor activity, spatial working memory, and olfaction in mice with a targeted deletion of the P2Y2 receptor.

2 | MATERIALS AND METHODS

2.1 | Experimental Animals

All animal experiments were approved by the local government, North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Germany (case number: 84-02.04.2015.A273) and conform to international guidelines for the ethical use of experimental animals.
efforts were exerted to minimize the number of animals used and their suffering.

Homozygous P2Y2-deficient mice (P2Y2−/−) and wild-type controls (P2Y2+/+) mice were generously provided by Dr Potthoff (Department of Urology, University Hospital Düsseldorf, Germany) and were kept for breeding at the local animal facility at the University of Düsseldorf. Adult male mice 12-16 weeks old, were used. In each experiment, age-matched P2Y2+/+ and P2Y2−/− mice were used. Mice were housed in standard cages in a temperature controlled environment under 12 hours light and 12 hours darkness, with lights on at 6:00 AM and off at 6:00 PM Mice had free access to food and water. Mice were viable, fertile, and did not manifest any physical or behavioral deficits in agreement with previous observations.47-49

2.2 | BrdU administration

Mice were injected intraperitoneally with the proliferation marker BrdU (Roche, Basel, Switzerland) at a dose of 100 mg/kg dissolved in 0.9% NaCl, twice daily for three consecutive days. One group of mice was perfused on the next day after the final BrdU administration to analyze the NPCs proliferation. A second group was sacrificed 4 weeks after the last BrdU injection to study the survival of NPCs and neuronal differentiation.

2.3 | Tissue processing

The mice were deeply anesthetized using Ketamine/Xylazine mixture (100/10mg, respectively, /kg). Mice were perfused transcardially using Ministar Peristaltic Pump (World Precision Instruments) with 0.9% NaCl followed by 4% paraformaldehyde. The brains were dissected from the skull, post-fixed in 4% paraformaldehyde for 24 hours followed by cryoprotection in 20% then 30% sucrose, each for 24 hours. The brains were divided into two hemispheres. One hemisphere from each brain was sectioned coronally in six series through the whole rostro-caudal extent of the hippocampus using a cryostat (Leica CM) into 40µm free floating sections. The other hemisphere was embedded in Optimal Cutting Temperature (OCT) compound (VWR) and cut into six series of 20 µm sagittal sections through the whole extent of the RMS and the OB. Sections were kept at −20°C until further processing.

2.4 | Histochemistry

One series, each of coronal and sagittal sections, was used for standard histological staining for the cell nuclei using a 0.1% cresyl violet (Sigma-Aldrich) staining solution. One series, each of coronal and sagittal sections, was stained against BrdU or DCX, respectively. Sections were rinsed with phosphate-buffered saline (PBS), and incubated with 0.6% H2O2 for 30 minutes at room temperature (RT). For BrdU staining, DNA denaturation was performed using 2N HCl for 30 minutes at 37°C followed by incubation with 0.1 M boric acid for 10 minutes at RT. Sections were rinsed in PBS, then incubated in 10% normal goat serum in PBS-T 0.2% for 1 hour at RT to block the nonspecific binding of secondary antibody, followed by incubation with rat monoclonal anti-BrdU antibody (1:800, AbD Serote) or rabbit polyclonal anti-DCX antibody (1:1000, Abcam) overnight at 4°C. Sections were incubated with biotinylated goat anti-rat IgG (1:500, Vector Laboratories) or biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories) for 1 hour at RT then incubated with VECTASTAIN® Elite® ABC solution (Vector Laboratories, Burlingame, CA, USA) for 1 hour at RT. Then, sections were rinsed and incubated with 0.05% 3, 3′-diaminobenzidine (Sigma-Aldrich) for 5 minutes. Finally, sections stained for BrdU were counter-stained with Cresyl violet and coverslipped using Depex (SERVA Electrophoresis).

One series, each of coronal and sagittal sections, was used for double immunofluorescence labeling with BrdU/DCX, BrdU/GFAP, or BrdU/NeuN, respectively. After DNA denaturation (as mentioned above), sections were incubated with the primary antibodies: rat monoclonal anti-BrdU (1:500, AbD Serotec) and rabbit polyclonal anti-DCX (1:1000); rabbit anti-GFAP (1:2000, DAKO) or polyclonal rabbit anti-NeuN (1:1000, Millipore-Chemicon) overnight at 4°C, followed by incubation with the secondary antibodies: Alexa Fluor 488 goat anti-rat IgG (1:500, Molecular Probes) and Alexa Fluor 568 goat anti-rabbit IgG (1:500, Molecular Probes) for 1 hour at RT. Sections were rinsed and nuclei were counterstained with NucBlue Fixed Cell Stain (Molecular Probes). Finally, slides were coverslipped using Vectashield Hard Set anti-fade reagent (Vector Laboratories) and stored in darkness at 4°C.

2.5 | Image acquisition and analysis

For image acquisition and analysis, the animal genotype was obscured to the investigator. For volume measurement, the entire DG and OB were outlined in one series of cresyl violet-stained coronal and sagittal sections, respectively. The total area of the outlined DG and OB in each mouse was then multiplied by six and by the section thickness (40 and 20 µm, respectively), to obtain the total volume according to Cavalieri method. The cytoarchitecture of DG and OB was also analyzed using cresyl violet-stained sections. The width of the respective cellular layers of OB was measured as described previously.50,51

BrdU+ and DCX+ cells were counted manually using the bright field mode with 40X objective on a KEYENCE BZ
900E microscope (Keyence). All settings were kept identical during image acquisition and processing. Fluorescent signals were recognized using respective filters and a 40X objective of KEYENCE BZ 900E fluorescent microscope. Image processing and co-localization were performed by BZ-II analyzer software (Keyence).

In the hippocampal DG, BrdU+ cells in the SGZ and the GCL of the DG were counted while the hilus was not taken into account. The SGZ was defined as a two-cell thick layer along the inner border of the GCL toward the hilus and the GCL was subdivided into inner, middle, and outer thirds (Figure S1A). The BrdU+ cell count was multiplied by six to obtain the absolute number per hippocampus. BrdU+ cells were ranked according to their spatial distribution in the SGZ or the GCL subdivisions. The percentage of BrdU+ cells from the whole count was analyzed in each subregion. DCX+ cells were counted in a delineated area within the DG and the mean cell density in each mouse was expressed as number of cells/mm².

Coronal sections were used for counting the BrdU+ cells in the SVZ, while the sagittal sections were used for counting the BrdU+ cells in the RMS (subregions: vertical limb “VL” and horizontal limb “HL”) and in the OB (layers: granular cell layer “GCL” and glomerular layer “GL”). Cells were counted in a delineated area and the mean cell density in each mouse was displayed as number of cells/mm³.

2.6 | Immunoblot

The mice were sacrificed using lethal dose of isoflurane. The hippocampus was carefully dissected out. Tissue was homogenized and lysed in RIPA buffer (Thermo Fisher Scientific) with 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) using PRECELLYS® Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux). Protein concentration was determined using BCA kit (Thermo Fisher Scientific). Polyacrylamide gel electrophoresis and immunoblot were performed using Novex XCell Sure Lock Electrophoresis and Blot module (Thermo Fisher Scientific) according to the manufacturer’s instructions and InvitrolonTM PVDF membranes (20 µm pores, Life Technologies). The membranes were washed in 0.1%TBS-Tween (TBST) and incubated with blocking solution (TBST containing 5% biotin-free BSA) for 1 hour. Membranes were incubated with rabbit polyclonal anti-phospho-AKT (1:2500, CST), rabbit polyclonal anti-phospho-ERK (1:2500, CST), mouse anti-b-Actin (1:5000, Santa Cruz, Paso Robles), or Rabbit polyclonal anti-ß-Tubulin (1:10 000, Abcam) over night at 4°C. After washing, membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG (1:40 000, Dianova) and HRP-conjugated goat anti-mouse IgG (1:5000, Carl Roth) for 1h at the room temperature. After washing, immunoreactive bands were visualized using Immobilon ECL (Millipore) by Chemi Only Gel Documentation System (VWR). Intensity of the respective immunoreactive bands was normalized against b-Actin (for p-Akt) or ß-Tubulin (for p-ERK) using densitometric analysis ImageJ tool.

2.7 | Behavioral assays

2.7.1 | Assessment of spontaneous locomotor activity

Spontaneous locomotor activity was continuously recorded for individually housed P2Y2+/+ and P2Y2−/− mice using on-cage infrared movement detectors linked to a monitoring system (Mouse-E-Motion, Germany). For experimental jet lag, mice were kept for 3 weeks in 12 hour light :12 hour darkness (lights on at 6:00 AM, LD12.1), then the lights/dark phases were delayed by 6 hours (+6, lights on at 12:00), after 3 weeks the light cycle was advanced by 6 hours back to the regular cycle (−6, lights on at 6:00 AM). After confirming the complete re-entrainment, mice were kept for additional 3 weeks in constant darkness (DD), followed by 3 weeks in constant light (LL), then finally for another 3 weeks in 12 hours light:12 hours darkness (lights on at 6 AM, LD12.2). Actograms, activity onset, activity profiles, chi-square periodograms, and amplitude of circadian rhythmicity were analyzed by Clocklab software (Actimetrics).

2.7.2 | Assessment of spatial working memory

To assess hippocampus-dependent spatial working memory, P2Y2+/+ and P2Y2−/− mice were subjected to a spontaneous spatial alternation behavior task using the Y-maze. This test utilizes the tendency of mice to explore novelty. The mice frequently visit the relatively novel places that have not been visited quite recently when freely allowed to choose among respective alternatives in the Y-maze. This spontaneous spatial alternation behavior is provoked by spatial novelty, thus requires the integrity of basic spatial working memory. The Y-maze (TSE Systems, Bad Homburg, Germany) was made of black plexiglas with three equally spaced arms, labeled A, B, and C (Figure 3E), each 4.5 cm wide, 30 cm long, with walls of 15 cm height, radiating from a triangle-shaped central platform. The Y-maze was placed in a sound-silenced room and was illuminated by diffuse white light with an intensity of 50 lux at the center of the apparatus. Mice were habituated to the investigator’s handling before testing. Each mouse...
was placed on the central platform and allowed to explore the maze for a total of 7 minutes trial duration. An arm entry was scored when the animal entered an arm with all four paws. The sequence of arm entries was manually recorded (ie, BCACB) using a video camera mounted 60 cm above the maze which was linked to a computer-based tracking system (Ethovision XT 8, Noldus, Wageningen, Netherlands). Experiments were conducted and scored by an experimenter blind to the genotype. Between trials, the apparatus was cleaned with 70% ethanol. The following parameters were calculated from the manually recorded arm entries sequence. Spontaneous alternation percentage (%), as a measure of spatial working memory, was computed by dividing the number of triads containing consecutive entries into all three arms without re-entries into an arm entered during the last two entries (ie, CAB, ABC, ACB, etc) by the number of possible alternation opportunities (total arm entries minus 2) x 100.

Apart from the manually scored sequence of arm entries, extra parameters using the Ethovision tracking system were conducted included: total distance traveled and animal speed inside the maze, total time spent within each arm and in the central platform. Novelty was guided by distinct spatial cues. Care was taken to ensure that the mouse was stress-free during testing. Experiment was conducted between 9:00 AM and 05:00 PM.

### 2.7.3 Assessment of mouse olfaction

For assessment of olfactory function in P2Y2+/+ and P2Y2−/− mice, a buried food test was performed. Briefly, the mice received one piece of food (Froot Loops, Kellogg’s, Battle Creek, MI, USA) daily for 2 weeks. Before the test, the mice were food-deprived for 18 hours. The mice were tested individually. The mouse was allowed to acclimate for 5 min in the test cage which contained fresh bedding 3 cm depth, then transferred temporarily into a holding cage. The mouse was transferred back to the test cage after a single Froot Loop was completely hidden in one corner of the cage 1 cm beneath the surface. The latency to find the Froot Loop was estimated as soon as the mouse touched the test cage bedding until it held the Froot Loop with its paws or when the mouse started to eat it. Mice that could not find the food within 300 seconds were excluded from the experiment.

### 2.8 Statistical analysis

Statistical analysis was performed using Graph Pad Prism software. After testing for normal distribution, student’s t test was used to determine the differences between two groups. \( P < .05 \) was considered statistically significant. Values were presented as mean ± SEM.

### 3 RESULTS

#### 3.1 The number of proliferating cells and neuroblasts in the hippocampal neurogenic niche is reduced in P2Y2−/− deficient mice

The hippocampal cytoarchitecture and DG size were comparable in both genotypes (Figure S1B) indicating comparable total granule cell number in both genotypes. Proliferation of NPCs was analyzed by the number of BrdU+ cells in the DG of both genotypes 1 day after the last BrdU injection. The BrdU+ cells were arranged in clusters or distributed sporadically (Figure 1A). The number of BrdU+ cells in P2Y2−/− (1436 ± 141, n = 4) was significantly lower than in P2Y2+/+ controls (2975 ± 348, n = 4) \( (P = .006) \) (Figure 1B). This is a reduction of more than 50% and suggests that NPCs proliferation is strongly affected by P2Y2 deficiency. Consistently, the number of DCX+ neuroblasts was significantly smaller in DG of P2Y2−/− mice (269.2 ± 21.1 cell/mm², n = 5) as compared to P2Y2+/+ mice (492.5 ± 80.6 cell/mm², n = 5) \( (P = .03) \) (Figure 1C,D).

The spatial distribution of the NPCs within the SGZ and the GCL subdivisions was not different between both genotypes (Table 1). This suggests that migration, at least at this short distance and during this short time period, is not affected by P2Y2 deficiency.

For characterization of the proportion of proliferating progenitor cells, we performed co-labeling of BrdU with GFAP (type-1 radial glia-like stem cells) or with DCX (neural precursors subtypes type-2b and type-3). There was no significant difference in the percentage of GFAP+/BrdU+ type-1 radial glia-like progenitor cells between P2Y2+/+ (3.2 ± 0.6%, n = 4) and P2Y2−/− mice (3.3 ± 0.8%, n = 4) \( (P = .9) \) (Figure S1C,D). Also the percentage of DCX/BrdU co-labeled neuroblasts was comparable between P2Y2−/− (31.3 ± 2.1%, n = 4) and P2Y2+/+ mice (41.77 ± 8.129%, n = 4) \( (P = .3) \) (Figure S1E,F). This suggests that P2Y2 deficiency does not affect the proportion of proliferating progenitor subtypes.

#### 3.2 Survival, neuronal differentiation, and migration of NPCs in the hippocampus are not affected in P2Y2−/− deficient mice

For the analysis of NPC survival, the number of BrdU+ cells in DG was analyzed 4 weeks after the last BrdU administration (Figure 2A). The number of BrdU+ cells was significantly lower in P2Y2−/− mice (290 ± 31, n = 4) as compared to P2Y2+/+ mice (518 ± 62, n = 4) \( (P = .02) \) (Figure 2B). This is a reduction of about 45%, consistent with the reduction of BrdU+ cells 1 day after the last injection (Figure 1). The percentage of BrdU+ cells 4 weeks after the last BrdU injection relative to the number of BrdU+ cells 1 day after the last BrdU injection was comparable between P2Y2+/+...
(17.39%) and P2Y2−/− (20.16%) mice. This suggests that survival of adult-born cells is not affected in the hippocampus of P2Y2-deficient mice.

For further characterization of the survived adult-born cells, the percentage of BrdU+ cells that differentiated into neurons and, therefore, co-expressing the neuronal marker NeuN was calculated. In both genotypes, around 70% of the BrdU+ cells co-expressed NeuN. The percentage of NeuN/BrdU+ cells was not significantly different between P2Y2−/− (76.6 ± 5.6%, n = 4) and P2Y2+/+ mice (64.3 ± 5.2%, n = 4) (P = .2) (Figure 2C,D). Thus, we conclude that the deletion of P2Y2 does not affect the differentiation of NPCs into neurons and the survival of adult-born neurons in the hippocampus.

To analyze the long-term migration of NPCs within the DG, the location of BrdU+ cells in SGZ, inner, middle, and outer thirds of DG was determined 4 weeks after the last BrdU injection. The BrdU+ cell fractions in the hippocampal subregions were comparable in both genotypes, suggesting that there was no difference in the migration of adult-born neurons to their final position within the neuronal network of the DG (Table 2).

**TABLE 1** Spatial distribution of NPCs in hippocampal subregions 1 day after the last BrdU injection

| Subregions     | P2Y2+/+ | P2Y2−/− | P value |
|---------------|---------|---------|---------|
| SGZ           | 57.4% ± 0.6 | 59.3% ± 1.9 | .4      |
| Inner third    | 39.0% ± 2.1 | 36.9% ± 0.99 | .4      |
| Middle third   | 1.9% ± 0.3  | 1.4% ± 0.55  | .3      |
| Outer third    | 3.8% ± 0.5  | 2.4% ± 0.7  | .1      |

(17.39%) and P2Y2−/− (20.16%) mice. This suggests that survival of adult-born cells is not affected in the hippocampus of P2Y2-deficient mice.

**3.3 | p-Akt expression in the hippocampus is reduced in P2Y2-deficient mice**

Next, we analyzed the two signal transduction pathway components of P2Y receptor activation which are involved in the regulation of NPC proliferation, p-AKT, and p-ERK.12 The expression level of p-Akt in the hippocampus was significantly reduced in P2Y2−/− as compared to P2Y2+/+ mice (n = 6 mice per genotype) (P = .008). However, the level of p-ERK was comparable in both genotypes (P = .5) (Figure 3A-D). This suggests that the p-Akt signal transduction pathway is affected by P2Y2 deficiency.

**3.4 | P2Y2 deficiency does not affect the spontaneous locomotor activity**

As adult neurogenesis is affected by intrinsic factors such as the circadian system,7 we analyzed spontaneous locomotor activity...
activity as a readout for circadian system integrity. Both genotypes showed a significantly higher activity during the dark phase as compared to the light phase (Figure S2) demonstrating an intact entrainment of the locomotor activity rhythm to the light/dark phase. Both genotypes had a comparable level of total activity under the different light conditions (Figure S2), suggesting a comparable balance of energy homeostasis.

3.5 | P2Y2 deficiency affects the hippocampus-dependent working memory

In the hippocampus-dependent spatial working memory test, the P2Y2-deficient mice showed a significantly reduced alternation percentage in Y-maze (P2Y2+/+: 44.7 ± 5.3%; P2Y2−/−: 31.3 ± 2.9%; n = 8 per group; P = .04) (Figure 3E,F). Whereas, the total arm entries, the number of triplets, and total time spent within each arm and in the central platform were comparable between both genotypes (P > .05) (Figure 3G, Table S1). This proves that the mice do not have an orientation preference for a certain arm. In P2Y2+/+ mice, the spontaneous alternation percentage was at a level expected by chance (50%). In contrast, in P2Y2−/− mice the alternation percentage was significantly below the chance level (P = .0004). This suggests that the spontaneous spatial alternation behavior, induced by the spatial novelty, is reduced in P2Y2−/− mice. This reflects the deficits in working memory. The speed and the total distance moved inside the maze were statistically comparable between both genotypes (Table S1). This is consistent with the comparable spontaneous locomotor activity levels in both genotypes (see above, Figure S2). Thus the impaired behavioral performance of P2Y2−/− mice in Y-maze is not confounded by motor deficits.

3.6 | P2Y2 deficiency affects the NPC proliferation in the SVZ

One day after the last BrdU injection, the number of BrdU+ cells in P2Y2−/− mice (353 041 ± 26 547 cell/mm³, n = 4) was
significantly lower as in P2Y2+/+ mice (471 933 ± 19 219 cell/mm³, n = 5) (P = .007) (Figure 4A,B). This is consistent with reduced NPC proliferation in the SGZ (see above). Nevertheless, in both regions of the RMS; vertical limb (VL) and horizontal limb (HL), the number BrdU+ cells was not different between P2Y2+/+ and P2Y2−/− mice (VL: P2Y2+/+: 301 613 ± 76 361 cell/mm³, P2Y2−/−: 253 080 ± 38 488 cell/mm³; n = 5; P = .6) (HL: P2Y2+/+: 230 819 ± 80 725 cell/mm³, n = 4; P2Y2−/−: 196 805 ± 19 541 cell/mm³, n = 4; P = .7) (Figure 4C-F). This indicates that the P2Y2 deletion does not affect the proliferation of NPCs and progenitor cells residing in the RMS.

### 3.7 Migration of neuroblasts to the OB is not affected by P2Y2 deficiency

One day after the last BrdU injection, the number of BrdU+ cells in the OB was not significantly different between both genotypes either in the GCL (P2Y2+/+: 631.2 ± 156.6 cell/mm³; P2Y2−/−: 784.9 ± 241 cell/mm³; n = 5; P = .6) or in the GL (P2Y2+/+: 1949 ± 728.1 cell/mm³; P2Y2−/−: 2171 ± 444.1 cell/mm³; n = 5; P = .8) (Figure 5A-D). This suggests that the early migration of progenitor cells toward the OB was not affected in P2Y2-deficient mice.

In line with this finding, the number of BrdU+ cells that survived and reached the OB 4 weeks after the last

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**FIGURE 3** Impairment of p-Akt expression and cognitive function in P2Y2−/− mice. A, Immunoblot and (B) quantification of p-Akt in the hippocampus of P2Y2+/+ and P2Y2−/− mice (n = 6 mice per genotype) (C) Immunoblot and (D) quantification of p-ERK in the hippocampus of P2Y2+/+ and P2Y2−/− mice (n = 6 mice per genotype). E, Experimental paradigm (Y-maze) for hippocampus-dependent spatial working memory. F, Spontaneous alternations in (%) of total arm entries and (G) total number of arm entries of P2Y2+/+ and P2Y2−/− mice in Y-maze, n = 8 mice per genotype. Values are shown as mean ± SEM. *P < .05. **P < .01
BrdU injection was also comparable between both genotypes in both the GCL (P2Y2+/+: 7480 ± 2525 cell/mm³; P2Y2−/−: 8936 ± 2428 cell/mm³; n = 4, P = .7) and the GL (P2Y2+/+: 1361 ± 288 cell/mm³; P2Y2−/−: 1013 ± 440 cell/mm³; n = 4, P = .5) of the OB (Figure 5E-H). This indicates that migration of neuroblasts to the OB was not affected by P2Y2 deficiency.

3.8 | OB cytoarchitecture is affected by P2Y2 deficiency

The OB of both genotypes (n = 4 per genotype) showed a comparable total volume and all layers were present (Figure 6A,C). However, the mitral cell layer was significantly thinner in P2Y2−/− mice (Figure 6B, Table 3).

3.9 | P2Y2 deletion affects the olfactory function

To test the olfactory function, the buried food test was applied. P2Y2+/+ mice showed a significantly lower latency to find the hidden food (64.5 ± 10.5 s; n = 8) as compared to P2Y2−/− mice (112.2 ± 16.58 s, n = 6) (P = .03) (Figure 6D). This indicates that olfactory function is affected by P2Y2 deficiency.

4 | DISCUSSION

There is increasing evidences that purinergic signaling, especially via the G protein-coupled P2Y receptors, modulates adult neurogenesis. However, few in vivo studies have
demonstrated the role of P2Y signaling on NPC proliferation and migration as well as on forebrain function. Here, we investigated adult neurogenesis in the two major neurogenic niches, the SGZ and the SVZ, in mice with targeted deficiency of the P2Y2 receptor; and analyzed the expression of two key signal transduction pathway components of P2Y receptor signaling as well as spontaneous locomotor activity, working memory, and olfaction as readout for forebrain function.

**Figure 5** Short- and long-term NPCs migration to olfactory bulb (OB) are not affected in P2Y2−/− mice. A-D, Short-term NPC migration to the OB analyzed by the number of BrdU+ cells (arrows) in P2Y2+/+ and P2Y2−/− mice 1 day after the last BrdU administration (n = 5 mice per group). (A) Representative photomicrographs of granule cell layer (GCL) and (B) respective quantification. C, Representative photomicrographs of the glomerular layer (GL) and (D) respective quantification. E-H, Long-term NPC migration to the OB analyzed by the number of BrdU+ cells (arrows) in P2Y2+/+ and P2Y2−/− mice 28 days after the last BrdU administration (n = 5 mice per group). E, Representative photomicrographs of GCL and (F) respective quantification. G, Representative photomicrographs of the GL and (H) respective quantification. Scale bars = 50 µm. Values are shown as mean ± SEM.
**FIGURE 6** Cytoarchitecture of the OB and olfactory function is affected in P2Y2−/− mice. A, Representative photomicrographs of OB of P2Y2+/+ and P2Y2−/− mice. Glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), and granule cell layer (GCL). Scale bar = 100 µm. B, Laminar width of the MCL in the OB of P2Y2+/+ and P2Y2−/− mice (n = 4 mice per group). C, OB volume of P2Y2+/+ and P2Y2−/− mice. D, Assessment of olfactory function in P2Y2+/+ (n = 8) and P2Y2−/− (n = 6) mice by latency to find a hidden food pellet. Values are shown as mean ± SEM. *P < .05

**TABLE 3** Volume and laminar width of the olfactory bulb

| Genotypes   | Glomerular layer (µm) | External plexiform layer (µm) | Mitral cell layer (µm) | Internal plexiform layer (µm) | Total volume (µm³) |
|-------------|-----------------------|-------------------------------|------------------------|-------------------------------|-------------------|
| P2Y2+/+     | 113.4 ± 8.6           | 198.7 ± 11.4                  | 33.4 ± 1.3             | 46.4 ± 5.2                    | 1.95 ± 0.09       |
| P2Y2−/−     | 121.2 ± 4.8           | 191.1 ± 14.1                  | 28.3 ± 0.7             | 36.5 ± 3.8                    | 1.99 ± 0.06       |
| P value     | .5                    | .7                            | .01                    | .2                            | .7                |

**4.1 | P2Y2 deficiency affects NPC proliferation in the SGZ**

In P2Y2-deficient mice, proliferation of NPCs is reduced compared to wild-type controls. This is in agreement with previous findings demonstrating a promoting effect of P2Y receptor activation on NPCs proliferation in the SVZ and in neurospheres and gives novel evidence of a crucial role of P2Y2 receptor in mediating this proliferative effect. However, the current study cannot exclude the possibility that in our constitutive P2Y2-knockout mouse, the number of progenitors was decreased during development. In the
SGZ and SVZ, the reduction of NPC proliferation in P2Y2-deficient mice is about 50%, indicating a stronger effect as in P2Y1-deficient mice where the number of BrdU+ and Mash+ cells was reduced by about 20%. Interestingly, in vitro, the effect of P2Y1 deletion on NPC proliferation was about 50%, thus higher as in vivo, suggesting a mechanism compensating for the loss of P2Y1 signaling which is effective mainly in the living animal. Given the synergistic activation of NPC proliferation by growth factors and nucleotides, this potential mechanism might include P2Y1 independent growth factor expressing cells which are missing in the in vitro system. Importantly, in P2Y1-deficient neurospheres, the P2Y2 agonists ATP and UTP are still effective in inducing intracellular Ca2+ signals and the P2Y2 agonist UTP is effective in increasing NPC proliferation, suggesting that these effects are mediated by the P2Y2 receptor.

In adult hippocampus, there are different types/stages of proliferating cells, the GFAP+ type-1 stem cells give rise to type-2a precursors, which generate type-2b and type-3 neuroblasts that migrate to GCL where they exit the cell cycle and mature into neurons of the hippocampal neuronal network. During aging and pathological conditions, especially type-2b and -3 neuroblasts decline, which can be detected by the DCX/BrdU colocalization. We found that P2Y2 deficiency does not affect the proportion of the type-1 and type-2b/type-3 cells, suggesting that P2Y2 is not involved in the neuronal fate decision of NPCs. Consistently, the percentage of new-born neurons co-expressing BrdU and NeuN, is comparable between both genotypes. This is in agreement with the finding that the capability of NPCs to differentiate into neurons is not affected upon treatment with suramin (P2Y2 receptor nonselective antagonist). Thus, it is likely that adult-born neurons in P2Y2-deficient mice are functionally integrated into the hippocampal neuronal network. Moreover, P2Y2 deficiency does not affect the survival of new-born neurons. Consistently, Suyama et al. showed that infusion of suramin does not lead to an increase in cell death or to change in long-term survival of BrdU+ cells in the SVZ. The reduction in the total number of DCX+ cells in the DG of P2Y2-deficient mice by about 50% relative to wild-type controls reflects the level of reduction in NPC proliferation.

4.2 | P2Y2 deficiency affects the PI3K/Akt pathway in the hippocampus

We further elucidated the expression of key components in P2Y2 receptor signal transduction pathways which are involved in direct and synergistic growth factor-mediated NPC proliferation. The PI3K/Akt pathway plays a crucial role in cell proliferation. Once PI3K is activated, it induces Akt activation via phosphorylation, leading to phosphorylation of downstream signaling proteins that are implicated in proliferation and self-renewal of NPCs. P2Y2 receptor activation induces Akt phosphorylation in a PI3K-dependent manner and subsequently stimulates the proliferation of human astrocytes as well as osteoblastic cells and mouse embryonic stem cells. Similarly, ATP promotes corneal endothelial proliferation via P2Y2-PI3K/Akt signaling. Consistently, we found that the reduced proliferation of NPCs in P2Y2-deficient mice is associated with a reduced p-Akt expression in the hippocampus. In contrast, expression of p-ERK, which is involved in the P2Y2 receptor-mediated enhancement of epidermal growth factor-dependent proliferation of astrocyte and NPCs is not affected in P2Y2-deficient mice. This suggests a possible compensatory response by other P2Y receptors, in particular P2Y1, that principally utilize Gq/G11 to activate the PLCβ/IP3 pathway to activate ERK1/2. Thus, P2Y2 deficiency unlikely affects the NPC proliferation via the synergistic epidermal growth factor-mediated NPC proliferation.

4.3 | P2Y2 deficiency affects spatial working memory

Adult neurogenesis in the hippocampus is interlaced with learning and memory formation and is correlated with the performance in spatial working memory tests. Thus, hippocampus-dependent spatial working memory was analyzed in P2Y2-deficient mice using the spontaneous alternation task in Y-maze. P2Y2-deficient mice displayed impaired memory formation while locomotion was not affected. This is the first evidence that the P2Y2 receptor has a functional role in the mammalian brain. In chicken, suramin affects memory retention by a yet unknown mechanism. P2Y2-deficient mice display deficits in memory which are associated with increased anxiety-like behavior and reduced locomotion, presumably, as a consequence of impaired brain noradrenergic signaling. In contrast, the impaired working memory in P2Y2-deficient mice is likely a consequence of reduced hippocampal neurogenesis as, at least in vitro, the expression of P2Y2 receptors is downregulated during neuronal maturation of NPCs. However, p-Akt is downregulated in the entire hippocampus of P2Y2-deficient mice, and it has been shown that p-Akt is involved in memory processing by modulating long-term potentiation in cortical neurons. Thus, we cannot exclude that P2Y2 signaling modulates synaptic plasticity in the hippocampal neuronal network.

4.4 | P2Y2 deficiency does not affect the circadian system

We and others showed earlier that adult neurogenesis is affected by intrinsic factors such as the circadian system.
Moreover, the P2Y\textsubscript{2} receptor is expressed in the circadian rhythm generator, the suprachiasmatic nucleus of the hypothalamus, in a time-of-day-dependent manner.\textsuperscript{16} Therefore, we analyzed the rhythmic spontaneous locomotor activity under different light conditions as a readout for the integrity of the circadian system. The P2Y\textsubscript{2} deficiency has no effect on the entrainment of rhythmic locomotor activity to the light/dark cycle, re-entrainment to changes in light-dark cycle or rhythmic locomotor activity under constant darkness demonstrating the integrity of the circadian system. Thus, the observed effects of P2Y\textsubscript{2} deficiency on NPC proliferation are not an indirect effect of chronodisruption.

4.5 | P2Y\textsubscript{2} deficiency affects NSC proliferation in the SVZ

The SVZ and RMS harbors a large population of different types/stages of proliferating cells. The type B1 stem cells residing in the SVZ give rise to transit-amplifying precursors (type C cells), which generate neuroblasts (type A cells) that divide one or two times while migrating tangentially along the RMS to the OB (reviewed by\textsuperscript{82}). In P2Y\textsubscript{2}-deficient mice, the NPC proliferation was significantly reduced in the SVZ, in agreement with the reduced proliferation of NPCs in the SVZ upon infusion of suramin.\textsuperscript{25} The RMS has been shown to be a distinct neurogenic niche that not only harbors neuroblasts but also multipotent precursors with different characteristics than the ones in the SVZ.\textsuperscript{83} Interestingly, P2Y\textsubscript{2} deficiency did not affect the proliferation in vertical limb or horizontal limb of RMS. This suggests a role of P2Y\textsubscript{2} receptor signaling on the proliferative capacity specifically for the type B1 stem cells in SVZ. In contrast to type C and type A cells, and presumably also the NPCs in the RMS, the apical surface of the type B1 cells is in contact with the cerebrospinal fluid which provides proliferative cues such as insulin-like growth factor (IGF).\textsuperscript{84} Interestingly, signal transduction of IGF includes the PI3K/Akt pathway (reviewed by\textsuperscript{85}). Thus, IGF and P2Y\textsubscript{2} receptor signaling converging on the same signal transduction pathway might be a possible mechanism for the specific effect of P2Y\textsubscript{2} deficiency on type B1 proliferation in SVZ. Proliferation of type C cells is modulated by P2Y\textsubscript{1} receptor and its agonist ATP.\textsuperscript{25} Thus, the two P2Y receptors seem to play a complementary role in modulating the proliferation of NPC subtypes.

4.6 | P2Y\textsubscript{2} deficiency does not affect the neuroblast migration

P2Y\textsubscript{2} deficiency had no effect on neuroblast migration in both main neurogenic niches. While in the hippocampal neurogenic niche, the neuroblasts migrate a short distance radially into the GCL of the DG, the neuroblast in the RMS migrate tangentially several millimeters to the OB at relatively high speed.\textsuperscript{86} Guidance of neuroblast migration along the RMS is multifactorial and presumably includes endogenous electrical currents.\textsuperscript{87}

Importantly, the directional migration of cultured SVZ neuroblasts in an electric field is mediated by P2Y receptor signaling as is can be inhibited with suramin or the specific P2Y\textsubscript{1} receptor antagonist MSR2179 as well as knockdown of P2Y\textsubscript{1}.\textsuperscript{87}

However, the number of BrdU\textsuperscript{+} cells reaching the OB was not affected by P2Y\textsubscript{2} deficiency. It is noteworthy that a gene knockout might lead to a compensatory upregulation of other genes,\textsuperscript{88} for example, inhibition of P2Y\textsubscript{1} in Cx43-deficient astrocytes results in an upregulation of P2Y\textsubscript{4} receptor expression.\textsuperscript{89} Therefore, a compensatory effect of other purinergic receptors could not be excluded, however, this needs to be tested experimentally.

4.7 | P2Y\textsubscript{2} deficiency affects OB cytoarchitecture and olfactory function

The OB is the first relay station in the olfactory system. The olfactory sensory neurons project their axons to the glomeruli harboring the dendrites of the excitatory principal neurons, the mitral and tufted cells.\textsuperscript{90} The mitral cells project their axons to the olfactory cortex to propagate odor information.\textsuperscript{91}

The inhibitory interneurons, the granule cells (GC) and the periglomerular cells (PGCs) greatly outnumber the principal neurons, suggesting that odor procession is predominantly shaped by local inhibitory circuits.\textsuperscript{92,93} Most of the adult-born neurons that migrate to the OB become mature interneurons replacing the pre-existing GCs and PGCs within the distinct OB circuits.\textsuperscript{91,94} The number of BrdU\textsuperscript{+} cells (new born interneurons) in both, the GCL and the GL was not different between P2Y\textsubscript{2}\textsuperscript{+}/+ and P2Y\textsubscript{2}\textsuperscript{−/−} mice suggesting a compensatory mechanism. Although the OB showed a comparable size and intact morphological layering in both genotypes, the P2Y\textsubscript{2}-deficient mice showed a significantly thinner MCL. This is unlikely a result of impaired adult neurogenesis, as the mitral cells are exclusively generated during embryonic life,\textsuperscript{95} but raises the assumption that P2Y\textsubscript{2} signaling plays a role in OB development. Consistent with the important role of the mitral cells in propagating odor information, the P2Y\textsubscript{2}-deficient mice showed a longer latency to find the hidden food and thus impaired the olfactory function. However, based on our data we cannot rule out, that this not only a result of reduced mitral cell number but also, at least partially, a result of impaired mitral cell function as purinergic signaling can modulate mitral cell electrical activity.\textsuperscript{96,97}
This study provides the first in vivo evidence that the P2Y2 receptor contributes to proliferation of NPCs in major adult neurogenic niches, the SGZ and the SVZ. Moreover, P2Y2 deficiency interferes with hippocampus-dependent working memory and olfactory function. In parallel, p-Akt, which plays an important role in cell proliferation and function is reduced in P2Y2−/− mice (Figure 7).

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CONFLICT OF INTEREST
The authors declare no conflict of interest in connection with this article.

AUTHOR CONTRIBUTION
A. A. H. Ali and C. Gall designed the research; A. A. H. Ali, L. Abdel-Hafiz, F. Tundo-Lavalle, and S. A. Hassan performed the research and analyzed the data; A. A. H. Ali, L. Abdel-Hafiz, and C. Gall wrote the paper.

ORCID
Amira A. H. Ali https://orcid.org/0000-0003-0058-0300

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.