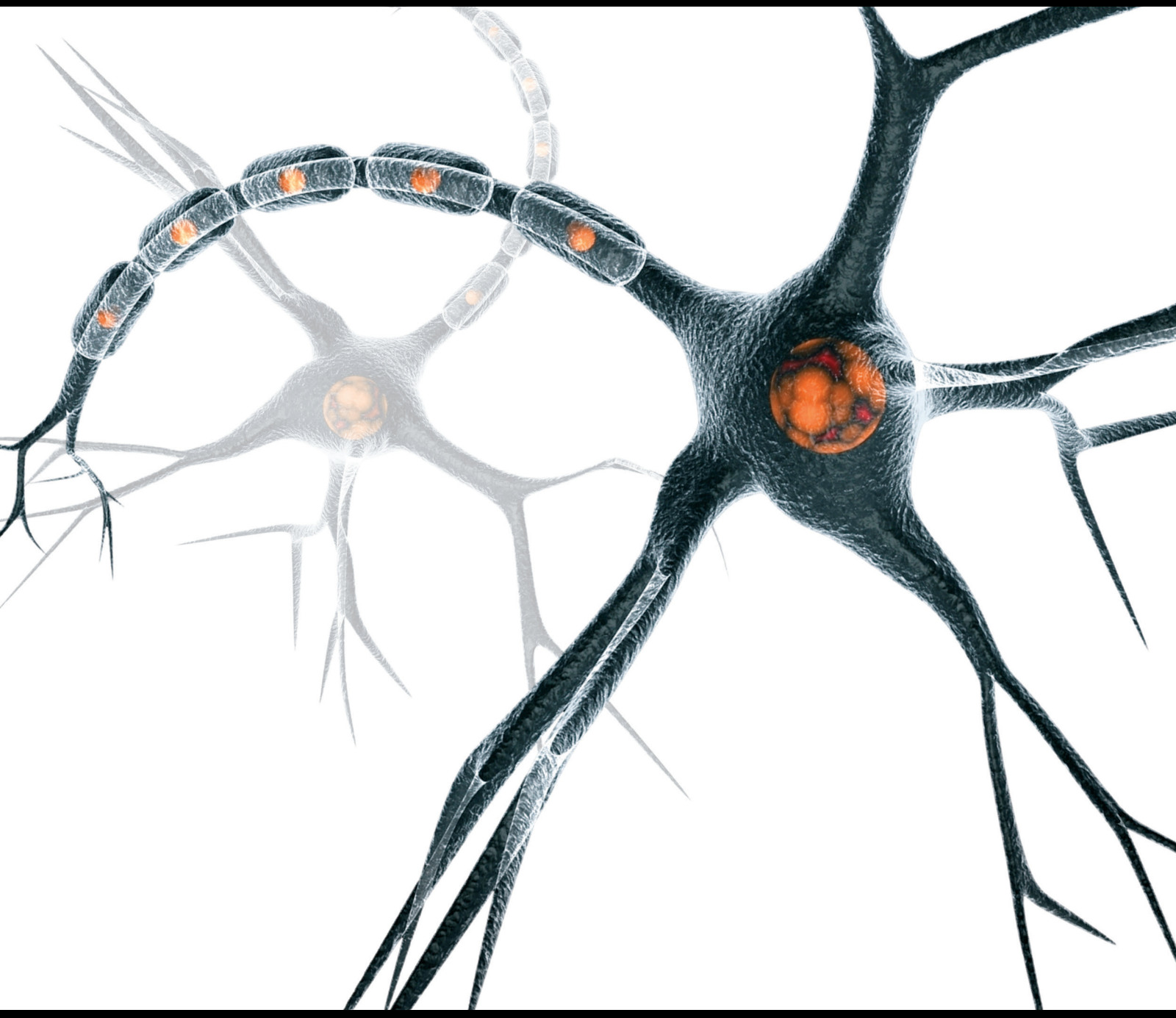
Neural Plasticity

Neurogenesis in Physiological and Pathological Conditions

Lead Guest Editor: Silvia Middei
Guest Editors: Stefano Farioli Vecchioli and Susanna Pietropaolo



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Hindawi
Neural Plasticity
Volume 2022, Article ID 9959044, 18 pages
https://doi.org/10.1155/2022/9959044

Review Article

Adult Hippocampal Neurogenesis in Alzheimer's Disease: An Overview of Human and Animal Studies with Implications for Therapeutic Perspectives Aimed at Memory Recovery

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Received 19 July 2021; Revised 21 October 2021; Accepted 15 December 2021; Published 15 January 2022

Academic Editor: Jiu Chen

The mammalian hippocampal dentate gyrus is a niche for adult neurogenesis from neural stem cells. Newborn neurons integrate into existing neuronal networks, where they play a key role in hippocampal functions, including learning and memory. In the ageing brain, neurogenic capability progressively declines while in parallel increases the risk for developing Alzheimer's disease (AD), the main neurodegenerative disorder associated with memory loss. Numerous studies have investigated whether impaired adult neurogenesis contributes to memory decline in AD. Here, we review the literature on adult hippocampal neurogenesis (AHN) and AD by focusing on both human and mouse model studies. First, we describe key steps of AHN, report recent evidence of this phenomenon in humans, and describe the specific contribution of newborn neurons to memory, as evinced by animal studies. Next, we review articles investigating AHN in AD patients and critically examine the discrepancies among different studies over the last two decades. Also, we summarize researches investigating AHN in AD mouse models, and from these studies, we extrapolate the contribution of molecular factors linking AD-related changes to impaired neurogenesis. Lastly, we examine animal studies that link impaired neurogenesis to specific memory dysfunctions in AD and review treatments that have the potential to rescue memory capacities in AD by stimulating AHN.

1. Introduction

Human adult neurogenesis, the generation of new neurons from neural stem cells in specific areas of the adult brain, has been at the center of an intense scientific research over the past years. New neurons are continuously generated in the human hippocampal dentate gyrus (DG), a brain region involved in learning and memory. This ongoing generation peaks at a young age but declines in adulthood and drops in old age, when memory decline also commonly occurs. The temporal correlation between reduced adult hippocampal neurogenesis (AHN) and impaired memory has been the rationale for animal studies investigating whether and how hippocampal newborn neurons contribute to memory. The general view that emerged from these studies is that new neurons are involved in distinct mechanisms of memory [1, 2].

The impact of ageing on altered AHN and the associated cognitive decline has encouraged researchers to investigate the possibility that deficits in AHN are a complicating factor in Alzheimer's disease (AD), the most frequent form of dementia and memory loss in ageing individuals. This possibility has been intensively debated, due to the lack of a clear and homogeneous methodology for the identification of new neurons in human hippocampal tissues. However, recent studies [3–5] provided convincing evidence for a massive decay in AHN in AD brains and shifted the focus of the debate towards new scientific questions concerning

therapeutic approaches that can reinforce neurogenesis in AD patients.

Thus, the present paper is aimed at providing a state-of-the-art review on studies that link AHN to memory in AD, as well as at delineating the questions that in our opinion should be addressed by scientific and clinical research in the near future. We review both relevant experimental investigations about adult neurogenesis in AD patients from the last two decades and studies on mouse models of AD. Although direct comparisons between humans and rodents cannot be made because of the huge species-specific variability [6], we will extrapolate from animal studies key information to understand neurogenesis in AD patients. We then focus on factors and therapeutic approaches that have the potential to trigger neurogenesis to contrast AD. Given the relevance of AHN for memory, we will limit our discussion to the hippocampus only.

2. Neurogenesis

2.1. Key Steps of Adult Hippocampal Neurogenesis. Since the discovery of adult neurogenesis, intensive research in rodent studies has been investigating the steps through which quiescent adult neural stem cells (qNSCs) become new mature neurons functionally integrated in the hippocampal trisynaptic circuit (Figure 1). Radial glia-like (RGL) NSCs (also known as type 1 cells) are located in the subgranular zone (SGZ), a restricted region of the hippocampal dentate gyrus (DG) on the border between the granule cell layer (GCL) and the hilus. This narrow area ensures an essential environmental niche where complex signaling pathways and support cells (astrocytes, microglia, and endothelial cells) allow the RGLs to maintain their quiescent state (qNSCs). Following appropriate intrinsic and/or extrinsic stimuli, the neurogenic niche plays a fundamental role in modulating the recruitment of qNSCs in the cell cycle, and in promoting the necessary differentiation/maturation steps and overseeing the functional integration of newly generated neurons.

The use of markers expressed specifically in the different subpopulations originating from the qNSCs and neural progenitors allows the defining of the cell lineage in hippocampal neurogenesis. Through this approach, studies have established that type 1 NSCs (expressing the specific markers GFAP, Nestin, SOX2, and BLBP) give rise to type-2 amplifying progenitors, which manifest their neural commitment, as evidenced by the coexisting expression of transcription factors NeuroD1 and Prox-1, as well as of the structural protein Doublecortin (DCX).

Proliferative type-2 cells differentiate into type-3 neuroblasts (characterized by the expression of DCX, NeuroD1, and PSA-NCAM), which exit from the cell cycle and start their migration towards the inner layer of the GCL, where they mature into granule cells by extending long axonal projections along the mossy fiber path. In the final maturation phase, the newborn neurons, which are specifically recognized by the expression of NeuN, Calbindin, and Prox1, send their axonal projections toward the CA3 layer of pyramidal neurons, providing an essential cue for the integration in the hippocampal circuitry [7, 8].

Under physiological conditions, adult hippocampal neurogenesis generates only one type of neuron, the granule cells, which represent the main glutamatergic excitatory neurons of the DG. Recent studies demonstrate that progenitor cells initially receive excitatory GABAergic synaptic inputs, which facilitate their maturation. About three weeks after birth, the response to GABA changes from depolarization to hyperpolarization, which corresponds to the beginning of glutamatergic excitatory signaling [9, 10].

2.2. Evidence of Adult Hippocampal Neurogenesis in Humans. The presence of newly generated neurons in the human hippocampus has raised widely contrasting, sometimes antithetic, results from different research groups. The first report of human hippocampal neurogenesis has been documented in 1998 by Eriksson et al. In this study, BrdU (a dye that intercalates DNA during cell division) was administered to 5 terminal cancer patients and 1 subject control, and the presence of positive BrdU cells ($BrdU^+$) was found in the postmortem autoptic DG samples [11]. However, the small sample size and the unethical implications of the study raised both doubts and contradictions concerning these results. A couple of decades later, Spalding et al. [12] employed carbon-14 dating to estimate the age of neurons in postmortem tissues of 55 people aged 15-92. Based on this indirect procedure, the study assessed the production of about 700 new neurons every day in the hippocampus of middle-aged men. It was then suggested that about 35% of the hippocampal cells renew during a lifespan, with an estimated turnover of around 2% every year [12, 13].

Over the last few years, three relevant publications reopened the debate about adult hippocampal neurogenesis. One of these studies [14], based on immunofluorescence examination of autoptic hippocampal samples, stated that there was no evidence of hippocampal neurogenesis from an adolescent stage onward. This "denial" study has raised a number of animated methodological criticisms concerning tissue selection and preservation, including (a) the scarcity of information about perimortem causes, which might affect tissue preservation; (b) the 48 hours postmortem delay (PMD), i.e., the time elapsed between death and brain fixation, which could be associated with protein rupture and the consequent disappearance of the antigenicity of several markers including DCX [15] the main neurogenic marker used in human studies; and (c) the morphometric analysis, which was run on a small number of samples randomly chosen and not taking into consideration that neurogenesis differs considerably between the dorsal and ventral region of the hippocampus.

On the ground of these and other remarks, many researchers rejected the idea that hippocampal neurogenesis in humans is interrupted during adolescence, as previously observed in the subventricular zone [6].

Two other studies [3, 16] then clearly demonstrated that adult neurogenesis in the human hippocampus is a robust process, which ensures a continuous supply of new neurons during adult life. However, while Boldrini et al. [16] showed that neurogenesis persists at high levels in the elderly, the work by Moreno-Jimenez et al. [3] stated that the generation

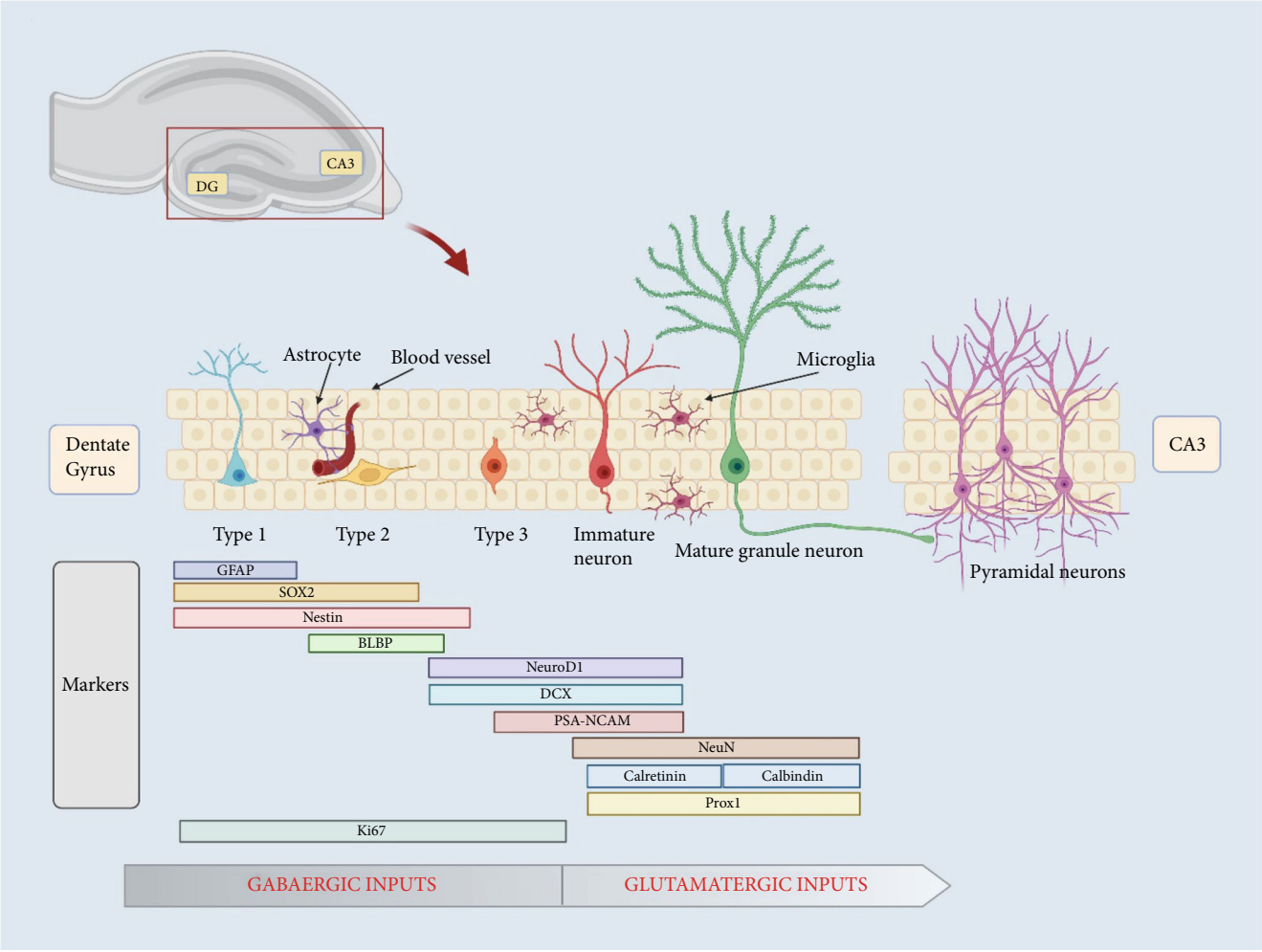

Figure 1: Main steps of AHN. Top left: image representing the position of the dentate gyrus (DG) and its main output target, the CA3 subregion, within the hippocampal circuit. Middle panel: morphological characterization and main markers related to the different cell types that identify the transition of a newborn neuron from a neural stem cell to a mature neuron integrated into a pre-existing circuit. As indicated by the scheme, the different stages undertaken by a newly generated neuron within the hippocampal dentate gyrus are characterized by a peculiar morphological identity and by the expression of specific cell markers (see boxes) that make it possible to study the proliferative and differentiative dynamics finely orchestrating the maturation of newborn neuron. Bottom diagram: neurotransmitters involved in the differentiation and maturation processes of newborn neurons. A first phase characterized by an excitatory GABAergic signaling that enables the maturation of the neural progenitors is followed by an excitatory glutamatergic signaling, which will permanently distinguish the electrophysiological properties of the new-generated granule neurons in the hippocampal dentate gyrus circuit. GFAP: glial fibrillary acidic protein; Sox2: SRY- (sex determining region Y-) box; BLBP: brain lipid binding protein; Neuro D1: neuronal differentiation 1; DCX: doublecortin; PSA-NCAM: polysialylated-neural cell adhesion molecule; NeuN: neuronal nuclear protein; Prox 1: prospero homeobox 1; Ki67: proliferative marker.

of new neurons declines with age, with a greater extent in the brains of Alzheimer's patients. The latter study reported the coexistence of numerous neural progenitor-associated markers (including DCX, PS-NCAM, and Prox-1) in DG single cells of 13 healthy individuals and received an almost unanimous scientific recognition for the optimization of some steps in the utilized protocol. For instance, brain samples were selected after a very short PMD and were maintained in fixation for a long time. Furthermore, autofluorescent quenching was significantly reduced while epitope retrieval and antibody selection were optimized. These methodological improvements allowed the authors to observe a higher number of DCX-positive (DCX^+) progenitors in the middle-aged DG as compared to similar studies [3].

Finally, Tobin et al. [5] confirmed the existence of hippocampal neurogenesis in 18 postmortem brains of elderly people ranging from 79 to 99 years of age. The relevance of this study lies in establishing with extreme clarity the presence of proliferation ($PCNA^+$ and $Ki67^+$ cells) both in NSCs ($Nestin^+$ and SOX^+ cells) and in DCX^+ neural progenitors. Furthermore, this study pointed to a regional cell distribution with proliferating and progenitor cells localized along the dorsal-ventral axis, while the NSCs ($Nestin^+$) were distributed preferentially in the dorsal portions of the hippocampus. In conclusion, at the time of writing this review, a

fairly supported agreement has been reached about the persistence of adult neurogenesis in the human brain, although with some discrepancies concerning the decline rate occurring with ageing.

2.3. The Contribution of Newborn Neurons to Memory. Computational models hypothesized the involvement of AHN in three key memory processes known as pattern integration, pattern separation, and memory erasure. Here, we describe these phenomena and provide experimental data from behavioral studies in rodents.

Associative memories are generated by the co-occurrence of two or more events within a limited time window. The neurobiological mechanism sustaining this process, known as pattern integration, consists in the simultaneous activation of distinct DG cells in response to coincident events. Given their peculiar hyperexcitability, newborn neurons are extremely efficient in detecting temporally related events [17, 18], which makes these cells pivotal for pattern integration. Indeed, reduction of DG neurogenesis has been shown to compromise mice's ability to pair both object-place associations in the object location task [19] and auditory (or visual) stimulus with an unconditioned stimulus in the eyeblink conditioning task [20]. Furthermore, loss of DG neurogenesis leads to low performance in contextual fear conditioning, a task in which rodents learn to associate an aversive event with the context in which it takes place [21, 22]. During memory encoding, the disambiguation of similar contexts through a process known as pattern separation allows the formation of a precise and accurate memory. Pattern separation depends on sparse activity of DG neurons leading to low network activity, which is implemented by highly excitable newborn neurons [23, 24]. In fact, reducing neurogenesis decreases overall DG inhibition while increasing it leads to interneurons activation and bigger DG inhibition [25, 26]. Hence, highly excitable newborn inhibitory neurons may act to modulate mature DG cells leading to a sparse activation necessary for pattern separation.

Consistently, deletion of newborn neurons in rodents compromises their pattern separation ability. Studies have shown that ablating AHN in mice resulted in impaired radial arm maze performance if surrounding contextual cues were presented in a complex spatial configuration with little spatial separation [27] and in reduced discrimination between two distinct contexts in fear conditioning [28]. Suppression of AHN also impaired performance in the Morris water maze (MWM), a task in which mice must learn the position of a submerged platform in order to escape from the water. As a further support to the idea that newborn neurons are necessary for pattern separation, increased discrimination between similar contexts was reported after enhancing AHN through genetic tools [29] or voluntary running [30].

Proactive interference is the process by which one encoded information overlaps with a new one limiting the possibility for both new memory encoding and old memory retrieval. The above-mentioned process of pattern separation acts to reduce proactive interference, but neurogenesis-associated clearance of previously existing memories can also play a role. In fact, one relevant study in rodents has demonstrated that enhancing AHN by wheel running reduces old contextual fear memory [31]. Avoiding the interference between old and new memories is also necessary for cognitive flexibility, which is the ability to adapt a new behavioral strategy to face environmental changes. This form of reversal learning, which has been examined in the MWM by moving the platform from a familiar to a novel position, was facilitated in mice with enhanced AHN [32].

3. Neurogenesis in AD

3.1. Evidence of Altered Neurogenesis in AD Patients. Alzheimer's disease (AD) is the most common neurodegenerative disease associated with ageing. The hippocampus and entorhinal cortex, two key regions for memory, are particularly vulnerable to AD neurodegeneration. Clinically, AD patients manifest a severe impairment in cognition that principally affects memory functions. Several studies have examined whether altered AHN plays a role in AD progression. However, contrasting results were obtained, likely due to both the scarcity and heterogeneity of the brain samples analyzed, and to the difficulty of preservation and immune-detection analysis of the tissues of postmortem brains.

Some studies in the first decade of the 2000s reported intact adult neurogenesis in patients with presenile AD [33], or even a sharp increase of proliferation in neuroblasts expressing the specific markers DCX, PS-NCAM, and Tuc-4 [34].

However, a reduced hippocampal neurogenesis was observed in most studies in AD patients [3, 5, 35–38]. In particular, Crews et al. [37] reported a sharp decrease in hippocampal neurogenesis, which was closely related to a significant increase in the expression levels of the growth factor BMP6, especially in proximity of the Aβ plaques [37]. Another study found that adult neurogenesis abnormalities in AD patients vary considerably between neurogenic stages and disease progression with a net decrease of stem cell number compensated by an enhanced proliferation. However, this enhanced proliferation did not lead to an increased number of new differentiated neurons [38]. More recently, two papers have provided additional information about this topic, comparing a fairly large number of brain samples of elderly individuals and AD patients. In both studies, what emerged quite unequivocally was a sharp drop of hippocampal neurogenesis in AD patients with respect to control individuals [3, 5]. In detail, Tobin et al. [5] observed a reduced number of neuroblasts at early stages of cognitive decline. Interestingly, these authors revealed a direct correlation between the number of proliferating neuroblasts (DCX^+, $PCNA^+$) and clinical diagnosis, observing a significantly reduced number of neuroblasts in the early stage of mild cognitive impairment (MCI). Based on this evidence, the authors speculated that depletion of hippocampal neurogenesis might represent an early neuropathological aspect that promotes or exacerbates cognitive deficits in AD [5].

Hence, a correlation between decreased adult neurogenesis and AD seems now established with a good degree of certainty, although it remains to be ascertained whether the neurogenic decline has a causal role or is a consequence of the AD-dependent neurodegenerative events.

3.2. Mouse Models to Understand the Mechanisms of Impaired Neurogenesis in AD. The hallmarks of AD brains include plaques made of fragments derived from amyloid precursor protein (APP) and intracellular aggregates of hyperphosphorylated TAU protein, respectively, known as amyloid plaques and neurofibrillary tangles. Most of the altered genes in the familiar forms of AD (FAD) relate to the expression of proteins involved in APP cleavage or clearance of its proteolysis products. Below, we review studies linking pathological neurogenesis processes to the expression of APP or to FAD-related genes involved in altered APP proteolysis. Furthermore, we discuss the relationship between AHN and hyperphosphorylated TAU or apolipoprotein E (ApoE), a key risk factor for AD.

3.2.1. Amyloid Precursor Protein (APP). APP is a membrane-bound protein involved in several physiological functions. Its role in neurogenesis has been assessed in studies exploring the effects of either overexpression or deletion of wild type APP in rodents.

In mice overexpressing APP, the number of BrdU-labeled ($BrdU^+$) cells was reduced as compared to controls but it increased after exposure to environmental enrichment, a condition that strongly potentiates cells differentiation [39]. On the contrary, mice with APP deletion displayed more $BrdU^+/DCX^+$ cells relative to controls, suggesting that APP restricts NPG cell proliferation. This modulation is likely acting through GABA regulation of stem cell quiescence [40] since selective deletion of APP from GABAergic neurons was sufficient to increase progenitor proliferation [41]. The number of proliferating cells that differentiate into neurons ($BrdU^+/NeuN^+$ cells) was comparable between mice overexpressing APP and wild-type ones, but in the former genotype, this number was reduced with ageing [41, 42]. Furthermore, dendritic length and branching were found reduced in differentiated neurons of APP KO mice [41, 42]. Overall, this set of evidence indicates that APP exerts a control function on proliferation, differentiation, and maturation of newborn cells.

3.2.2. APP Proteolytic Products. Age and genetic factors, including Presenilin1 (PS1) mutations, are at the origin of APP processing into proteolytic products that gradually accumulate in amyloid plaques. One study directly addressing whether the presence of amyloid plaques alters hippocampal neurogenesis was conducted in the plaque-bearing Tg2576 mouse model. This study reported that a reduced number of $BrdU^+$ cells was already evident in the DG of mutant mice before plaque appearance, while pharmacological rescue of neurogenesis did not alter plaque load [43]. This evidence, which indicates that the production of new neurons is already impaired before plaque manifestation, has been confirmed by other studies on mouse models of AD-like progression (see Table 1). In apparent contrast, one study using the APP/PS1ΔE9 mouse model [44] found that treating mutant mice with metformin resulted in both reduced plaque load and enhanced neurogenesis. However, it is likely that these events are unrelated and are linked to a common third factor, i.e., the inhibition of reactive astrogliosis and microgliosis induced by metformin.

Plaques are mainly composed of Aβ, a product of APP proteolysis that gradually aggregates into oligomers and fibrils. Aβ has been considered as potentially harmful for neurogenesis in AD by several studies documenting impaired neurogenesis in transgenic mice expressing the PS1 mutation, which dramatically increases Aβ levels long before plaque appearance (Table 1). More direct evidence of this hypothesis is that $A\beta_{1\text{-}42}$ delivery in brain ventricles of wild type mice resulted in reduced $BrdU^+/NeuN^+$ cell numbers as compared to controls [45]. Furthermore, treatments that drop Aβ levels, including β2-adrenergic agonist [46] or a drug that blocks amyloidogenic APP cleavage [47], are effective in restoring neurogenesis in AD mice.

Although the above evidence implies that the presence of Aβ interferes with neurogenesis, other studies lead to opposite conclusions. Wang et al. [48] reported a reduced number of $BrdU^+$ DG cells in 3xTgAD mice as compared to controls at 3 months of age, which is before the occurrence of Aβ signs in these mice. One other study [49] compared neurogenesis between hAPP-J20 and hAPP-I5 mice, which display different Aβ amounts. Although Aβ levels were higher in hAPP-J20 mice, survival of newborn cells was more compromised in hAPP-I5. The authors provided further support to the idea that impaired neurogenesis was not associated with Aβ levels by demonstrating that deletion of cystatin C, which is known to reduce Aβ, did not alter the number of DCX^+ neurons in hAPP-J20 mice.

Valero et al. [50] exposed Aβ overexpressing mice to the highly neurogenic condition of an enriched environment. Although Aβ levels remained unvaried upon enriched environment, this condition increased the number and complexity of newborn neuron projections to CA3, meaning that newborn neurons were integrated in the hippocampal circuit.

Several other studies have investigated neurogenesis in mice expressing mutations that contribute to amyloidogenic processing of APP (Table 1), leading to mixed results. The disparity among these correlative studies is likely due to differences in transgene expression, mouse line, animal age, or other experimental conditions. Despite discrepancies, some of these studies are noteworthy as they highlight several mechanisms that are triggered by Aβ and may affect neurogenesis. Among these, Aβ alters the expression neurotrophins BDNF, NGF, and NT3, as well as the expression and phosphorylation of receptors TrkA and TrkB in mice with reduced neurogenesis [51, 52]. Given the importance of neurotrophins in neurogenesis [53, 54], this evidence suggests that Aβ may act as a toxic agent that interferes with neurotrophic mechanisms. In fact, AHN reduction in AD mice could be rescued by treatment with L-NPB [52] or Osthole [51], two molecules that increased the levels of neurotrophins involved in neurogenesis. One other study [55] confirmed these results in APP751 mice, by showing that administration of Cerebrolysin, a peptidergic mixture that increases BDNF levels, improved survival of grafted NSCs in the DG. One possible mechanism involved in impaired neurogenesis lies in the amyloid-associated microgliosis

Table 1: Studies investigating AHN on AD mouse models.

Mouse model	Age	BrdU treatment	BrdU	DCX	BrdU/DCX	OTHER markers	Ref
APP/PS1	8 months	4 days - analysis 6 days later	↓	↓		↓ dendritic branches, ↓ dendritic spines, ↓synaptic markers	46
	10 months	-		↓		↓ dendritic branches, ↓synaptic markers	47
	8-9 months	7 days - analysis 18 hours later	↓			= Nestin, = Ki67/Nestin	51
	6 months	4 days (twice per day) - analysis 24 hours later	↓				52
	2, 4, 6 months	3 days - analysis 3 days later		↓ at 4 and 6 months	↓ at 6 months	↓ BLBP+ stem cells at 6 months	56
	12 months	3 days (twice per day) - analysis 2 weeks later	↓	↓		↓ Nestin, ↓ synaptic proteins	75
	12 months	7 days - analysis 1 day later	↓				78
	8 months	-				↓ Sox2	83
	2 months	EdU 7 days - analysis one month later				↓ EdU, ↓ EdU/NeuN	87
	3 months	3 days (twice per day) - analysis 5 weeks later	↓BrdU/NeuN			↓dendritic ramification ↓Spine density	109
	8 months	3 weeks - analysis at the end	↓	↓		↓BrdU/NeuN	113
	7 months	12 days - analysis 1 day (a) or 30 (b) days later	= (a), ↓ (b)		↓ (b)	= Ki67+ cells, ↓ Brdu+/NeuN+/S100b-, ↓ Brdu+/DCX+/NeuN-, ↓ Brdu+/DCX-/NeuN+	127
APP/PS1ΔE9	28 weeks	7 days (twice per day) - analysis 7 days later	↓	↓		↓ NeuN	44
	3 months	3 days - analysis 2 hours (a) or 4 weeks (b) later	=	↓		↓ NeuN	57
	2 months	3 days (twice per day)	↓		↓		67
	3,9,18 months	1 day - analysis 18 hours later	= (all ages)	↓ at 18 months		↓ total DG cells at 18 months	72
	3 months	3 days - analysis 1 day later	↓	↓		↓ NeuN	73
	4, 10 months	2 days (a), 3 days (b) - analysis 1 day later (a), (b)	↓ at 4 and 10 months	↑ at 3 months, = at 10 months			80
	9 months	-				↓Ki67, ↓synaptic proteins	82
	8-9 months	-				↓ newborn (GFP+), ↓ spine density	85
	2 months	1 injection - analysis 1 day and 14 days later	↓ in enriched mice		↓ in enriched mice	↓ DG cells and ↓ BrdU/NeuN in enriched mice	86
	3 months	3 days - analysis 3 hours (a) and 4 weeks later (b)	↓(a), = (b)		↓(a), = (b)	= (b) BrdU/NeuN	88
	3,5,10,15months	1 injection - analysis 1 day later	↓ at 3, 5, 10 months	↓			126

Table 1: Continued.

Mouse model	Age	BrdU treatment	BrdU	DCX	BrdU/DCX	OTHER markers	Ref
	3,10,13 months	5 days - analysis 4 weeks later	↓	=	=	↑ PCNA, ↑ PCNA/DCX at 3 months, ↓BrdU/NeuN	128
	9,13 months	4 days (twice per day) - analysis at day 4	↓			↓ GFAP/Sox	129
APP/PS1/ Nestin-GFP	7 days; 1, 3 7 months	1 day - analysis 2 (a) or 3(b) hours later	↓			↓ Nestin, ↑ DCX/Nestin at 3 and 7 months, ↓ Spine density at 3 and 7 months	84
PS1M146L	2 months	1 injection - analysis 1 day and 14 days later	↓ in enriched mice		↓ in enriched mice	↓ BrdU/Tub and BrdU/NeuN in enriched mice	86
PS1/PS2-KO	7-9, 18-20 months	1 injection - analysis 1 day later	↑	↑		↑Brdu/NeuN, ↑ BrdU/GFAP	130
APP/PS1 KI	2, 6 months	-		↓ at 6 months			71
	6 months	-		↓			79
aAPP751	1,3,6,9 months	1 day before grafting - analysis 1,3,6,9 months later	↓	↓		↓ Synaptic markers	55
APPSw,Ind	4 months (plus 7 weeks EE)	5 days- analysis 7 weeks later	↓	↓		↓BrdU/NeuN, ↓ dendritic ramification, ↓ DG volume	50
Tg2576	3,6, 9 months	3 days - analysis 1 day later	↓				43
	3,5 months	-		=		↑ PCNA, ↑ PCNA/DCX at 3 months	128
	3, 5, 12, months	1 day - analysis 1 day (a) or 30 days (b) later	↓ at 3 months			↓ Brdu/NeuN, ↓ dendritic ramification, ↓ spine density at 3 months BrdU+/DCX+/NeuN+ at 3 months, Brdu+/DCX-/NeuN- at 12 months	131
Tg19959	5 weeks; 10, 15 months	-		↑ at 5 weeks		↑ Ki67	132
Arg-61	3-12 months	-		↑ at 3 months, ↓ at 12 months			133
3xTgAD	3 months	1 day - analysis 1 day later	↓				48
PDAPP	2, 12 months	1 injection - analysis 2 hours (a) and 4 weeks (b) later	↓(a) and (b)	↓(a)		= Brdu/NeuN and =BrdU/GFAP (b)	134
PDGF-APP	3,12 months	3 days - analysis 7 days later	↑	↑			34
CRND8	3, 7 months	3 days - analysis 1 day (a) and 5 weeks (b) later	↓(a) and (b)	↓(a)		↓BrdU/NeuN (b)	74
	6, 7, 9, 11, 13 weeks	5 days - analysis 1 (a), 2 (b), 4 (c), 6 (d), 8 (e) weeks later	↑			= BrdU/NeuN at 1 and 8 weeks; ↓ Brdu/GFAP (a), = (e)	135

Table 1: Continued.

Mouse model	Age	BrdU treatment	BrdU	DCX	BrdU/DCX	OTHER markers	Ref
ApoE3, ApoE4	10 weeks	3 days (twice per day) - analysis 4 weeks later	=			= BrdU/Prox1, = Ki67/GFP/Nestin , ↓ dendritic ramification and ↓ spine density in ApoE4 vs ApoE3	62
ApoE3, ApoE4	6-7 months	1 day - analysis 1 day (a), 3 days (b), 4 weeks (c) and 10 weeks (d) later	↑ ApoE4 (a,c)		↑ ApoE4 (c)	↓ Brdu/Neun (c,d), ↑ ki67 (a), ↓ dendritic branching and ↓ GAD67 in ApoE4	63
ApoE2, ApoE3, ApoE4	10-12 weeks; 1 year	3 days - analysis 1 day later	↓ ApoE4, ↑ ApoE2/3, age- and sex-dependent				64
ApoE4	9-15 months	-	=				136
ApoE ko	10 weeks	3 days (twice per day) - analysis 4 weeks later	=			= BrdU/Prox1, ↓Ki67/GFP/Nestin, ↓ dendritic ramification , ↓ spine density	62
	6-7 months	1 day - analysis 1 day (a), 3 days (b), 4 weeks (c) and 10 weeks (d) later	↓ (b)		↓(c,d)	↓ Brdu/Neun (c,d) , ↑ BrdU/S100b (b,c,d)	63
	9-15 months	-		↑ in female, = in male			136

Table summarizes the studies investigating neurogenesis in distinct AD mouse models (column 1) at different age points (column 2). Column 3 reports details of BrdU treatment (days/number of injections and time between last BrdU injection and animal sacrifice). Columns 4, 5, and 6 refer, respectively, to quantifications of $BrdU^+$, DCX^+, and $BrdU^+/DCX^+$ cells in the dentate gyrus (DG) region. Column 7 refers to other neurogenic/synaptic markers found in the DG. Symbols indicate increase (↑), decrease (↓) or no variation (=) of specific markers as compared to wild type controls. Details including different time points (a, b) between BrdU and sacrifice of the animal are reported where necessary. Unless otherwise indicated, male mice were used in the studies. BrdU: bromodeoxyuridine; DCX: doublecortin; Ki67: proliferative marker; BLBP: brain lipid-binding protein; Nestin: nestin protein; Sox2: SRY- (sex determining region Y-) box; EdU: ethynyldeoxyuridine (BrdU analogue); S100B = S100 calcium-binding protein B; PCNA: proliferating cell nuclear antigen; GFAP: glial fibrillary acidic protein; Tub: tubuline; GAD67: glutamic acid decarboxylase 67 (GABA-synthesizing enzyme).

and the induction of proinflammatory cytokines as documented in studies reporting Aβ-induced microglial proliferation and impaired neurogenesis in mutant AD mice [56, 57]. Furthermore, altered signaling of GABA, which is essential for neuronal development, has been shown to depend on Aβ levels and to impact on differentiation and development of newborn neurons in the hippocampus of J20 mice [58]. Hence, on the basis of the above evidence, we suggest that Aβ accumulation is a key factor that triggers pathological events including altered neurotrophin levels, release of proinflammatory cytokines, and impaired GABA signaling, which in turn impact on AHN in AD brain.

3.2.3. Apolipoprotein E (ApoE). Apolipoprotein E (ApoE) is a molecule primarily secreted by astrocytes and involved in the regulation of lipid transport, synaptogenesis, and amyloid clearance [59, 60]. ApoE is also expressed by adult NSCs where it regulates their proliferative rate [61].

Among the allelic variants of ApoE gene, ApoE2 and ApoE3 are protective against the risk to develop AD, whereas ApoE4 associates with increased risk. Few studies investigated the contribution of ApoE variants to adult hippocampal neurogenesis, leading to mixed results.

As compared to relative controls, ApoE ko and ApoE4 mice display decreased dendritic complexity and spine density in adult-born neurons [62], indicating a reduced newborn neurons maturation.

Two studies [63, 64] reported that the expression of ApoE4 decreases adult neurogenesis in mutant mice as compared to wild type controls, while the expression of ApoE2 drives enhanced proliferation of DG progenitor cells and increased neurogenesis. Ageing in ApoE-expressing mice severely compromised neurogenesis, but the ApoE3 genotype was protective against this effect in female mice [64]. One other study [65] reported that the presence of ApoE4 was associated with enhanced neurogenesis in mice under standard conditions, whereas maintaining mice in environmentally enriched cages was associated with a drop of neurogenesis in ApoE4-expressing mice. Overall, these studies indicate that APOE polymorphisms play distinct roles in regulating the balance between neuronal birth and death.

3.2.4. TAU. In physiological conditions, TAU phosphorylation facilitates migration of DCX^+ cells [66], but levels of hyperphosphorylated TAU have been shown to be extremely high in neurogenic microenvironments of APP/PS1ΔE9 mice [67]. In particular, TAU immunoreactivity colocalized with $BrdU^+$, $GFAP^+$, and DCX^+, meaning that alterations of TAU phosphorylation may be detrimental to NSCs, NPCs, and neuroblasts. One mechanism through which phosphorylated TAU can impact on AHN has been recently described in an important paper [68]. This study reported that phosphorylated TAU accumulates in GABAergic interneurons of the DG in both AD patients and 3xTg mouse model. The consequent GABA reduction, local circuit disinhibition and astrogliosis in turn impair AHN. The evidence that these alterations are reported in mice mutant for APP suggests a role for APP products in triggering TAU phosphorylation that alters AHN. Consistent with this view, we recently reported that, at least in the subventricular zone of an AD mouse model, NSCs fail to terminally differentiate due to TAU-mediated microtubule alteration, while blocking the generation of Aβ oligomers rescues this effect [69]. Hence, the induction of TAU phosphorylation can be one other mechanism through which APP products alter AHN in AD brains.

4. Rescue Neurogenesis in AD: Implications for Cognitive Recovery?

4.1. Specific Contribution of Altered Neurogenesis to Memory Decline in AD: Behavioral Studies in Animal Models. Among AD symptoms, proactive interference and cognitive rigidity are two key features that are in common with the effects of AHN alteration. In particular, persistence of old memories at the expense of new ones, a process known as anterograde amnesia, is a typical clinical sign of AD that also manifests in conditions of impaired neurogenesis. Despite anterograde amnesia in AD can be explained by neuronal damage that prevents the formation of new memories [70], no studies so far directly investigated AD-related anterograde amnesia in relation to AHN, leaving the door open to new explanations.

Instead, numerous studies in AD mouse models (see Table 2) have reported that altered neurogenesis associates with deficits in pattern integration, pattern separation, and cognitive flexibility. Pattern integration deficit in AD mouse models has been evidenced by impaired spontaneous alternation in the Y-maze task [57, 71–73], reduced step-down latency in the inhibitory avoidance test [74, 75], increased frequency to enter dark compartments of the passive avoidance apparatus [51], and reduced freezing in the fear conditioning task [43, 76]. In particular, impaired contextual but not cue fear conditioning [43] is indicative of a selective pattern integration deficit. Pattern separation deficit has been evidenced by low MWM performance in numerous studies [44, 46, 50–52, 57, 74, 75, 77–84] although most of these do not detail the experimental context which is essential to understand if the test is suitable for the evaluation of pattern separation effect. Other studies evidenced impaired pattern separation in AD mice by demonstrating that their reduced neurogenesis was associated with compromised performance in the object location task, which requires spatial discrimination [85], and in the object recognition task [73, 77]. Cognitive flexibility, the third function affected by impaired AHN, has been evidenced by one study reporting impaired reversal learning in the MWM task [80].

The overall picture emerging from the above studies is that impaired neurogenesis in AD mouse models is associated with deficits in one or more of the above-mentioned memory functions. Below, we review the most relevant studies that probed whether restoring neurogenesis correlates with memory recovery in these mice.

4.2. Factors That Rescue Neurogenesis and Recover AD Symptoms

4.2.1. Enriched Environment and Physical Activity. Since enriched environment and physical activity are potent

Table 2: Studies investigating memory in relation to AHN in AD mouse models.

Mouse model	Age	Test	Behavioural outcome	Increased neurogenesis by	Ref
APP/PS1	8 months	MWM	↑ escape latency, ↓ time in target quadrant, ↓ platform crossings	b2AR activation (clenbuterol)	46
	8-9 months	PA (a)	↓ latency and ↑ frequency to enter in dark compartment	osthole (a), (b)	51
		MWM (b)	↑ escape latency, ↑ distance from platform, ↓ platform crossings		
	6 months	MWM	↑ escape latency	L-3-n-butylphthalide (L-NBP)	52
	12 months	MWM (a)	↑ escape latency, ↓platform area crossings, ↓ time spent in target quadrant	NSC translpant (a), (b)	75
		SD (b)	↓ latencies; ↑ error time		
	1,3,6,9,12 months	FC	↓ freezing from 6 months of age	social interaction	76
	2 months	EPM (a)	= time and number of entries in open arms	enriched environment (a)	87
		FC (b)	= time in freezing	-	
	6 months	NOR (a)	↓ recognition index	PDE7 inhibitor (S14) (a), (b)	77
		MWM (b)	↑ escape latency		
	12 months	MWM	↑ escape latency, ↓ time in target quadrant, ↓ platform area crossings	running	78
	9 months	MWM	↑ escape latency, ↓ platform area crossings, ↓ time in target quadrant	osthole	81
	8 months	MWM	↑ escape latency, ↓ time in target quadrant	MDA7 (CB2 receptors agonist)	83
	4, 7-8 months	MWM (radial version)	↑ error rate	FGF2	107
	18 weeks	NOR (a)	↓ recognition index	minocycline (a)	109
		YM (b)	= alternation rate		
	7 months	MWM (radial version)	↑ error rate	IL-10	113
APP/PS1ΔE9	28 weeks (female)	MWM	↑ escape latency, ↓ time in platform area, ↓ platform crossings	metformin	44
	3 months	nest building behavior (a)	↓ nesting score	yonkenafil (a, dose-dependent), (b), (c, dose-dependent)	57
		SA (b)	↓ alternation and arm entries		
		MWM (c)	↓ escape latency, ↓ platform crossings		
	9-18 months	SA	↓ alternation rate at 18 months age	Paroxetine	72
	3 months	OF (a)	= locomotor activity	xanthoceraside (b, dose-dependent), (c, dose-dependent)	73
		YM (b)	↓ spontaneous alternation		
		NOR (c)	↓ discrimination index		
	3, 9 months	NOR	= exploration for novel object at 3 months	-	80
		Locomotor activity	↑ activity at 3 months	-	
		YM	= time in open arms at 3 months	-	
		MWM	↑ escape latency at 9 months	-	
	9 months	MWM	↑ escape latency, ↓ platform area crossings and time in target quadrant	osthole	82
	8-9 months (female)	OL	↓ exploratory preference for displaced object	enhancing functional integration of new neurons	85
APP/PS1 KI	6 months	EPM (a)	↑ time in open arms	-	71
		YM (b)	↓ alternation rate	-	
	2-6 months	neurological evaluation (a)	↓ of vertical activity (age-related), = other parameters, hyperactivity	-	79
	6 months	EPM (b)	↑ time in open arms	-	

Table 2: Continued.

Mouse model	Age	Test	Behavioural outcome	Increased neurogenesis by	Ref
	2, 4, 6 months	MWM (c)	↓ in goal target (age-related), ↓ accuracy (age-related)	-	
APPSw,Ind	4 months (plus 7 weeks EE)	MWM	↑ escape latency, ↓ time in platform area, ↓platform crossings	Environmental Enrichment	50
Tg2576	3,6, 9 months	FC (contextual and cue)	↓ freezing to context after stress (isolation) at 6 and 9 months	Fluoxetine	43
3xTgAD	6 months	OL	↓ exploratory preference for displaced object	THIP (Gaboxadol)	68
		FC (pattern separation)	↑ trials for discrimination		
	3 months	TEC	↓ conditioned responses	Allopregnanolone	48
CRND8	3, 7 months	SD (a)	↓step-down latency	Lithium salts (a) 3 months only, (b) 3 months only	74
		MWM (b)	↑ escape latency, ↓ time in target quadrant		
ApoE3-Ki and ApoE-4 KI	10-17 months	MWM (a)	↓ ApoE4-KI preference for target quadrant (respect to Apoe3-KI)	Transplant of embryonic interneuron progenitor (a), (b), (c)	119
		OF (b)	↓ ApoE4-KI time in central area (respect to Apoe3-KI)		
		EPM (c)	↓ ApoE4-KI time in open arms (respect to Apoe3-KI)		

Table summarizes studies investigating neurogenesis and memory in distinct AD mouse models (column 1) at different age points (column 2). Column 3 reports the memory test(s) used in each study. Column 4 refers to results from each memory test with symbols indicating increase (↑), decrease (↓), or no variation (=) of specific behavioral outcomes as compared to wild type controls (unless otherwise indicated). Please note (↑) latency corresponds to lower memory. When available, treatments that were associated with a rescue in neurogenesis and also ameliorated memory are reported in column 5. MWM: Morris water maze (standard or radial); PA: passive avoidance; SD: step down; FC: fear conditioning (contextual or cue); EPM: elevated plus maze; NOR: novel object recognition; OF: open field; YM: Y maze; SA: spontaneous alternation; OL: object location; TEC: trace eye-blink conditioning.

neurogenic stimuli (Figure 2), numerous studies have investigated their potential therapeutic effect on AD mice. Although some of these studies found no evidence of a relation between environmental stimulation and AHN or improved memory [71, 86], other studies reported positive effects of environmental enrichment [87, 88] or its distinct components including social interaction and wheel-running.

Social enrichment, which increased BDNF protein and mRNA levels, was associated with improved AHN and FC memory in aged APP/PS1 mice [76], while social isolation worsened neurogenesis impairment in aged Tg2576 mice [43].

In one other study [89], environmental enrichment resulted in increased AHN, amelioration of MWM memory and upregulation of hippocampal neurotrophins in APP23 mice, yet these effects were not evident upon wheel-running only. However, one other study on APP/PS1 mice reported MWM rescue after wheel-running [78], thereby suggesting that this specific effect may be modulated by factors including genotype or overall running time.

In a pivotal study, Choi and collaborators [90] dissected the specific contribution of exercise-induced AHN in improving AD symptoms. They found that while genetically or pharmacologically induced AHN had little effect on AD symptoms in 5xFAD mice, running-induced AHN was associated with increased BDNF levels, reduced Aβ load, and improved cognition. Furthermore, combining genetically or pharmacologically induced AHN with BDNF administration was sufficient to rescue cognition, with no impact on Aβ levels. Overall, this study not only confirmed that physical activity can contrast AD symptoms but also demonstrated that BDNF significantly contributes to the therapeutic value of physical activity.

These animal studies are consistent with the reports of positive effects of physical activity in AD patients. In fact, epidemiological evidence indicates that physical activity as well as social and cognitive stimulation can delay dementia in aged individuals. Systematic and longitudinal studies in aged individuals with MCI have confirmed that physical activity can delay AD progression, reduce Aβ deposition and protect the brain from atrophy and temporal lobe volume loss [91, 92]. It has been documented that at least 12 months of mild-to-moderate physical activity (that is 50-70% of maximum cardiac output for 30-40 minutes session) can preserve cognitive function [93, 94].

However, it is yet to be demonstrated that these positive effects are directly mediated by activity-dependent increase of neurogenesis. Evidence rather suggests that the positive impact of physical activity on cognition may be unrelated to neurogenesis. One meta-analysis review indicated that the positive effects of physical activity on cognition are unlikely the results from changes in brain parameters [95]. Some studies evidenced that intense physical activity in humans was associated with an ameliorated pattern separation and mnemonic discrimination [96, 97]. However, these events were too close in time with physical activity to justify a possible involvement of neurogenesis. Our opinion is that

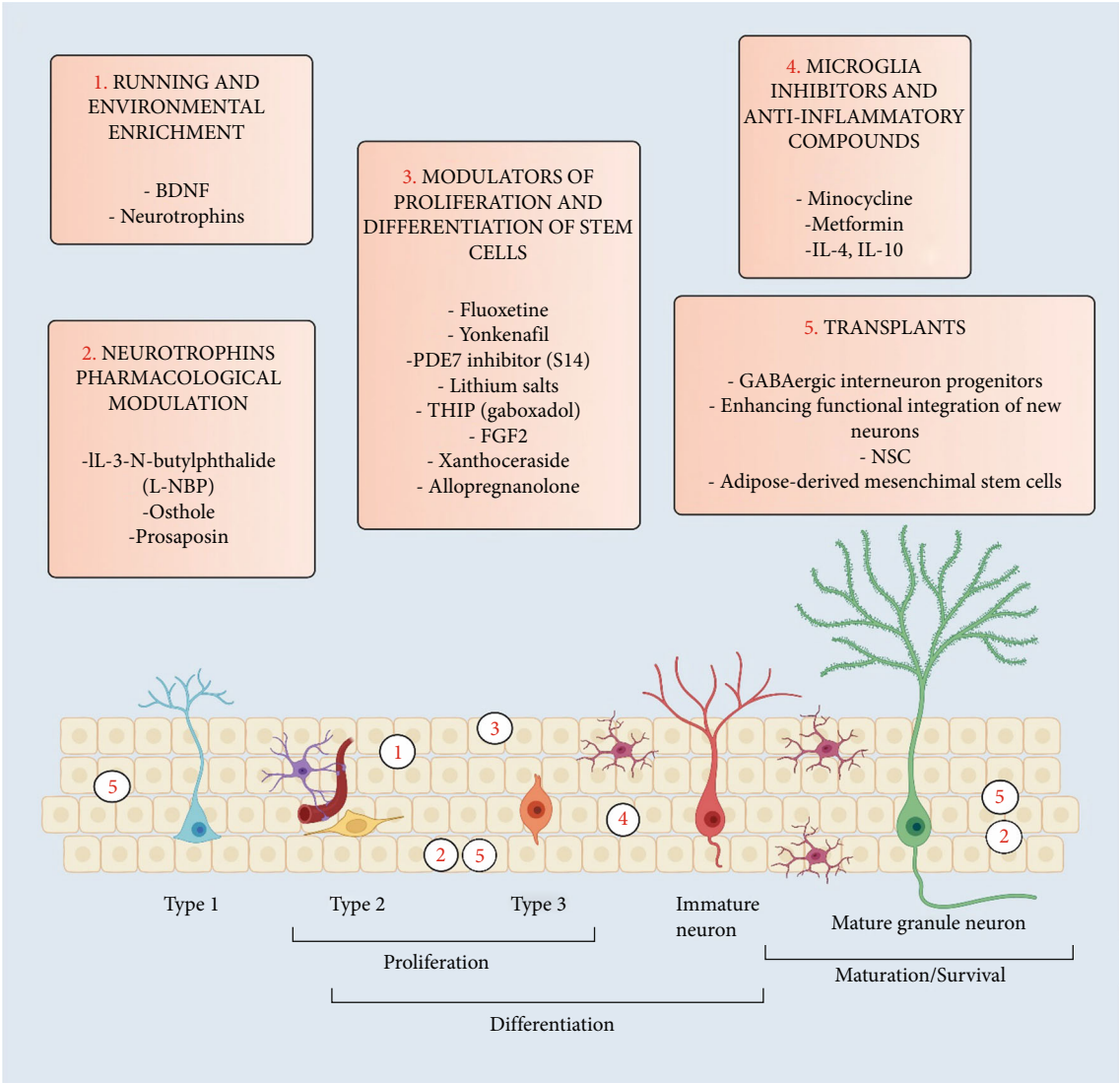

Figure 2: Factors that rescue AHN. Top boxes: factors that increase AHN. (1) Physical activity and environmental enrichment increase neurotrophin levels including BDNF, which in turn stimulate NSC proliferation and differentiation. Consistently, pharmacological modulation of neurotrophins (2) through compounds including L-NBP, Osthole, or Prosaposin can initiate NSC proliferation and also promote survival of newborn neurons. (3) Other modulators of proliferation and differentiation of stem cells can act to potentiate the generation of newborn neurons and their neuron fate specification. (4) Microglia can be found in 3 distinct states, each one affecting AHN through different mechanisms. Resting microglia ensure basal neurogenesis by releasing factors that control neuronal differentiation. Active microglia release proinflammatory cytokines that reduce NSC proliferation and differentiation. Congruent with this, microglia inhibitors and anti-inflammatory compounds rescue AHN in AD mice. Alternatively acting state microglia release anti-inflammatory cytokines including IL-4 and IL-10 that favor differentiation, viral-mediated expression of these cytokines reduces astro/microgliosis and enhances AHN. (5) Transplants of stem cells or progenitors as well as enhancing the functional integration of new neurons can potentiate AHN. Enhancing GABA signaling through transplant of GABAergic progenitors favors the maturation of newborn neurons. Transplanted NSCs in the hippocampus can differentiate in neurons or astrocytes. Mesenchymal stem cell transplantation increases neurogenesis by boosting differentiation and proliferation through mechanisms including the stimulation of alternatively activated microglia. Bottom panel: factors that increase AHN are represented along the stages of spatio-temporal transition of neural stem cell to a mature neuron. Numbers refer to the action of factors reported in top boxes. Please note that numbers position is indicative but not definitive as each factor can act through different mechanisms.

physical activity in humans, by activating neurotrophins [98, 99] and by reducing the levels of inflammatory chemokines [100] could contribute to the rescue of both neurogenesis and memory in AD. Yet, it remains to be determined whether improved AHN can directly drive memory amelioration in AD patients.

4.2.2. Treatments That Enhance AHN. A number of pharmacological approaches have been shown to effectively rescue AHN (Figure 2). Treatments that enhance neurotrophin levels can boost AHN by initiating NSC proliferation or by promoting survival of newborn neurons [101–103]. Among these treatments, the L-3-n-Butylphthalide (L-NPB) [52] or

Osthole [51, 81] improved AHN and MWM performance in APP/PS1 mice. Similarly, administration of Prosaposin, a secreted protein that acts as trophic factor, rescued both loss of AHN and MWM impairment induced by intracranial Aβ1-42 injections in mice [45]. In a clinical trial performed in the 90s, neurotrophic factors were delivered by intracranial injection to the brains of a small group of AD patients. Despite modest cognitive improvement after short-term NGF infusion, this treatment was associated with severe side effects in the long term, making it not suitable for AD therapy [104–106].

Treatments that favor proliferation and differentiation of stem cells also ameliorate AHN and memory in AD mice. Fluoxetine, that increases progenitors' proliferation, restored memory in aged AD mice [43] while administration of phosphodiesterase inhibitors, which promotes cell differentiation, improved AHN and rescued object recognition, Y-maze and MWM in AD mouse models [57, 77]. Furthermore, lithium, which favors proliferation and neuron fate specification of newborn cells, was sufficient to rescue MWM in TgCRND8 mice [74].

Similarly, AAV-mediated expression of FGF2, a neurogenic factor for proliferation and differentiation, enhanced AHN and improved MWM memory in APP/PS1 mice [107]. One other relevant study used viral vectors to express a transcription factor necessary for maturation and survival of adult born cells. By enhancing maturation of granule cells in APP/PS1 mice, the authors found that amelioration in object location task was associated with a recovery of altered dendritic spines [85], indicating that newborn neurons that functionally integrate into hippocampal circuits are likely to support memory recovery. Microglia exert different functions on neurogenic processes, depending on their specific conformations [103]. As mentioned above, microglia at resting state contribute both to maintain RGLs in their quiescent state (qNSCs) and to release factors that promote neuronal differentiation in response to specific neurogenic stimuli [108]. Hence, microglia at this state are necessary to ensure basal neurogenesis. Conversely, active microglia inhibit NSC proliferation and differentiation through the release of proinflammatory cytokines [103]. Congruent with this, microglia inhibitors [109] and anti-inflammatory compounds like metformin [44] rescued both neurogenesis and memory in AD mice. A third state, the alternatively acting microglia, can release anti-inflammatory cytokines including IL-4 and IL-10 that favor differentiation [110, 111]. In line with this, viral-mediated expression of interleukin- (IL-) 4 or (IL-) 10 gene in the hippocampus of APP/PS1 mice resulted in enhanced AHN, reduced astro/microgliosis and Aβ deposition [112], and improved MWM learning [113]. Furthermore, administration of the nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin was associated with memory recovery in AD patients [114].

Transplant of NSCs that differentiate into neurons or astrocytes [115] has been proposed as therapeutic approach to contrast neuronal loss in AD. NSC transplant in the hippocampus APP/PS1 mice enhanced AHN as well as synaptic markers in hippocampal neurons. Interestingly, these changes were accompanied by amelioration in MWM and step-down test, suggesting that new neurons can integrate into synaptic circuits that sustain memory [75]. One other strategy consists in transplanting mesenchymal stem cells (MSCs) that can boost AHN through the expression of neurotrophins [116]. Transplant of adipose-derived MSCs in the hippocampus of APP/PS1 mice resulted in potentiated neurogenesis and novel object recognition ability [117]. As mentioned above, GABA signaling plays a key role in the maturation of adult-born neurons, and GABA imbalance has been found to link AD to impaired neurogenesis [118]. Restoring this signaling has potential therapeutic effects. Pharmacological strengthening of GABAergic function rescued AHN deficit with parallel amelioration of contextual memory in mice [68] and transplant of GABAergic interneuron progenitors in the DG of ApoE4 knock-in mice was sufficient to restore their MWM memory [119].

Hence, studies in AD mouse models have evidenced that treatments that boost AHN can also improve memory, but so far, the few treatments that have been formalized in clinical trials did not lead to the implementation of therapy for AD patients.

5. Conclusions

AHN loss has been reported in AD patients and mouse models before the clinical onset of disease [5, 120], in a preclinical stage commonly characterized by massive Aβ accumulation [121–123].

Here, we reviewed studies in AD animal models and we summarized the factors through which Aβ exerts its effects on AHN, including altered neurotrophins expression and the induction of proinflammatory cytokines. Targeting these factors by specific compounds can both ameliorate AD symptoms and rescue neurogenesis in AD mouse models; however, these results have not been translated into clinical trials.

Nevertheless, physical activity and a healthy lifestyle can restore neurotrophins and cytokine levels. Crucial questions therefore include which are the specific molecules that are modulated by physical activity in AD [124]. It is also of great relevance to investigate the concentrations or the temporal dynamics of such molecules, or what is their role in the amelioration of clinical symptomatology. In a wider perspective, focusing on those specific molecular targets can drive future clinical trials to set therapeutic strategies aimed at mitigating clinical symptoms in AD patients even in conditions that preclude their physical activity.

Despite a significant progress in the study of AD and AHN over the past decades, it still remains to be determined whether AHN loss has a causal role in cognitive decline in AD. Studies in Table 2 sustain this possibility in mouse models, but this scenario cannot be extended to AD patients due to the impossibility to perform similar experimental studies in humans. Furthermore, comparisons between rodents and humans are limited by the fact that the rate of AHN is much higher in rodents than in humans.

Studies in humans should rather demonstrate that pharmacological interventions that clearly restore AHN also delay or contrast cognitive decline. To this aim, researchers

should combine innovative clinical trials, like mesenchymal stem cell transplantation [125], with precise imaging tools that may allow for AHN identification and with specific behavioral studies investigating pattern integration, pattern separation, and memory erasure, the key memory functions associated with AHN.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Neural Plasticity
Volume 2021, Article ID 2522454, 12 pages
https://doi.org/10.1155/2021/2522454

Research Article

Molecular Mechanism for PACAP 38-Induced Neurite Outgrowth in PC12 Cells

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Received 12 April 2021; Revised 8 July 2021; Accepted 17 July 2021; Published 10 August 2021

Academic Editor: Silvia Middei

The present research investigates the molecular mechanism of neurite outgrowth (protrusion elongation) under pituitary adenylate cyclase-activating polypeptide (PACAP) 38 treatments using a rat adrenal-derived pheochromocytoma cell line—PC12. This study specifically looks into the regulation of PACAP38-induced collapsing response mediator protein 2 (CRMP2) previously identified in a mouse brain ischemia model and which could be recovered by PACAP38 treatment. Previously, DNA microarray analysis revealed that PACAP 38-mediated neuroprotection involved not only CRMP2 but also pathways related to glycogen synthase kinase-3β (GSK-3β) and other signaling components. Thus, to clarify whether CRMP2 acts directly on PACAP38 or through GSK-3β as part of the mechanism of PACAP38-induced neurite outgrowth, we observed neurite outgrowth in the presence of GSK-3β inhibitors and activators. PC12 cells were treated with PACAP38 being added to the cell culture medium at concentrations of 10^{-7} M, 10^{-8} M, and 10^{-9} M. Post PACAP38 treatment, immunostaining was used to confirm protrusion elongation of the PC12 cells, while RT-PCR, two-dimensional gel electrophoresis in conjunction with Western blotting, and inhibition experiments were performed to confirm the expression of the PACAP gene, its receptors, and downstream signaling components. Our data show that neurite protrusion elongation by PACAP38 (10^{-7} M) in PC12 cells is mediated through the PAC1-R receptor as demonstrated by its suppression by a specific inhibitor PA-8. Inhibitor experiments suggested that PACAP38-triggered neurite protrusion follows a GSK-3β-regulated pathway, where the AKT and cAMP/ERK pathways are involved and where the inhibition of Rho/Roc could enhance neurite protrusion under PACAP38 stimulation. Although we could not yet confirm the exact role and position of CRMP2 in PACAP38-mediated PC12 cell elongation, it appears that its phosphorylation and dephosphorylation have a correlation with the neurite protrusion elongation through the interplay of CDK5, which needs to be investigated further.

1. Introduction

The physiologically active neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) belonging to the vasoactive intestinal polypeptide (VIP)/glucagon/secretin family as a 27 or 38 amino acid residue (PACAP27 or PACAP38) exhibits a diverse array of biological functions [1–3]. The bioactivity of PACAP is multifunctional, and it is thought to function as a neurotransmitter/regulator in addition to its hormonal action. It is involved in the differentiation and survival maintenance of nerve cells and activation of the neurosecretory system in the central and peripheral nervous systems and regulates nerve synaptic plasticity, differentiation of neural precursor cells, and glucose-

dependent insulin secretion. PACAP has been reported to have a promoting effect, a cell death inhibitory effect during cerebral ischemia, and a cytoprotective effect [2, 4–17]. Generally, the spinal cord and brain, as critical structures of the central nervous system, do not recover once they are damaged, and this is the case for the damaged nerve itself in a variety of diseases resulting from causes such as cerebral infarction, traffic accidents, and spinal cord injury caused by falls. No cure has yet been established to restore this nerve damage and the conditions (diseases/-disorders) arising from it.

PACAP is recognized by receptors in the cell membrane, and like the VIP family members, it shares two common G protein-coupled receptors (GPCRs) VPAC1 R and VPAC2 R. However, PACAP has particular affinity for PAC1-R that is said to be more than 1000 times higher than those of the other two [4, 18, 19]. Studying PACAP and clarifying its function by examining a myriad of downstream (of its receptor) primary and secondary signaling pathways and network linkages may lead to confirmation of the role of PACAP in numerous cellular processes as well as contribute to the development of therapeutic drugs for diseases and disorders [5, 6]. Neuroprotection has attracted a lot of interest in brain research, and our research group has been investigating PACAP effects in the ischemic brain using high-throughput "omics" techniques. One of the high-throughput technologies is the whole genome expression profiling approach. This approach provides a snapshot of almost all (depending on sample quality and experimental design) the molecular events occurring at the level of the gene (i.e., transcriptome) at a particular instance and biological location. Using this genomic approach, we have since established a rigorous and standardized DNA microarray-based protocol to identify with high confidence the transcriptome in a mouse model of permanent middle cerebral artery occlusion (PMCAO) [16]. Additionally, we also utilized a gel-based proteomics approach using the same PMCAO mouse model to identify a collapsing response mediator protein 2 (CRMP2) in the ischemic brain tissue sample following intraventricular administration of PACAP38 [17, 20].

CRMP2, initially called CRMP-62, was first identified in 1995 using the *Xenopus* oocyte expression system [21]. CRMPs are multifunctional adapter proteins/microtubule-related proteins, highly expressed in the brain/central nervous system [22, 23]. In particular, among other functions of CRMP2, it is known to be important for the determination of neuronal polarity and process elongation associated with process injury and neuronal death, neuropathy, and cerebral ischemia [24–30]. These studies led us to hypothesize the yet-to-be-demonstrated role for CRMP2 involvement in the neurite protrusion elongation action of PACAP, including the possible molecular mechanisms underlying such a function if it exists. The CRMP2 induces protrusion formation in the cultured hippocampus, and its action has been reported to be inactivated through phosphorylation by glycogen synthase kinase-3β (GSK-3β) [31, 32]. Furthermore, in addition to GSK-3β, CRMP2 impairs tubulin binding ability by being phosphorylated via kinases such as the cyclin-dependent kinase 5 (Cdk5) [33] and the Rho-associated protein kinase (ROCK) [34]. Previous research has also revealed an involvement of CRMP2 phosphorylation in axon guidance and growth cones [35, 36]. However, little is known about the mechanism governing dendrite guidance and patterns by PACAP and it is unclear how CRMP2 phosphorylation is involved in neurite protrusion elongation.

Therefore, in this report, we specifically aimed to unravel the molecular mechanism behind the neurite protrusion elongation action of PACAP38 and therein the involvement of CRMP2 in a PC12 cell model [37], which are cultured immortalized cells known to differentiate into neurons by NGF and PACAP stimulation. Further, we hoped to find new evidence into the mechanism by which PACAP38 promotes the suppression of the phosphorylation of CRMP2 and thereby gain insight into the pathways that might be involved in the treatment of neuron/nerve damage and for neurodegenerative diseases/disorders.

2. Cell Model and Methods

2.1. Cell Culture. PC12 cells (RCB0009) were obtained from the RIKEN Cell Bank (Japan). Cells were cultivated in RPMI medium 1640 (ATCC Modification, Thermo Fisher) in a CO_2 incubator (37°C, 5%) with 5% fetal bovine serum (FBS) (16140063, Gibco), 10% horse serum (HS) (H1138, SIGMA), and antibiotics (penicillin-streptomycin, P4458, Sigma). Cellmatrix Type IV (Nitta Gelatin Inc.) was used to coat the culture dish. Institutional ethical approval was not required for this study. Experiments as performed below were repeated multiple times, (usually $n = 6$) as indicated in the figure legends, and the data were presented as the mean $\pm$ SD. In the inhibitor experiment, DMSO was adjusted to a final concentration of 0.0001% (v/v) and the medium was used as a control. Except for the inhibitor experiments, SDW was added at 1% of the medium and used as a control.

2.2. Measurement of Neurite Protrusion Elongation. PC12 cells were adjusted to 1×10^3 to 5×10^3 cells/well in a 96-well plate, and after about 6 hours, PACAP (PACAP38, 052-05 Phoenix Pharmaceuticals Inc.) was added to the cell culture medium at concentrations of 10^{-7} M, 10^{-8} M, and 10^{-9} M after confirming complete adhesion to the plate. The cells were observed as a phase-contrast image using an optical microscope BZ-X710 (Keyence) over time at 17, 72, and 144 hours after the addition of PACAP38. Cells with a neurite outgrowth of 20 μm or more from the image were counted as 1. Cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentage of cells with a neurite outgrowth of 20 μm or greater was obtained by dividing by the total number of cells per image. Finally, the results for all fields examined for each condition were averaged ($n = 6$) to calculate the number of neurite outgrowth cells per condition.

2.3. Immunostaining. PC12 cells were seeded in a cover glass chamber (5222-004, Iwaki), and after confirming adhesion, PACAP was added to a working concentration of 10^{-7} M and the cells were cultured for 3 days. After removing the medium, the cells were washed with PBS and fixed with a

Table 1: Primer combinations used for semi-quantitative RT-PCR (*Rattus norvegicus*).

Accession (gene)	Nucleotide sequence (5′-3′)	Nucleotide sequence (5′-3′)	Product size (bp)	Gene name
NM_016989	CTGTTGGTCTACGGGATAAT	CTACAAGTACGCTATTCGGC	498	*PACAP (Adcyap1)*
NM_001270579	TTGCAAGATGTCAGAACTATCCA	GAAGTAACGGTTCACCTTCCAG	259	*RAC1-R (Adcyap1r1)*
NM_012685	AAATGGTCTTCGAACTTGTCGT	GGAGTGTGTCCCTATGAAAAGC	373	*VPAC1 (Vipr1)*
NM_017238	CACTAGTGATGGGTGGTCGG	GCCAGTAGAAGTTCGCCATG	399	*VPAC2 (Virp2)*
NM_017008	CCTGTGACTTCAACAGCAACTC	GGCCTCTCTCTTGCTCTCAGTA	213	*GAPDH*
NM_031144	TGACGGTCAGGTCATCACTATC	GGCAGTAATCTCCTTCTGCATC	229	*Actb*

4% PFA solution. After washing again, the cells were treated with PBS solution containing 0.1% Triton X 100 for 10 minutes. After treatment and washing with PBS, blocking was carried out with PBS solution containing 3% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% Tween 20, followed by incubation using a mouse anti-α-tubulin antibody (Abcam Inc., 1 : 1000) overnight at 4°C. The cells were then washed with PBS and crossreacted with Alexa 594 donkey anti-mouse IgG (Invitrogen, 1: 1000) as a secondary antibody at room temperature for 60 minutes. After washing again with PBS, Alexa Fluor 488-labeled rabbit anti-Neu-N antibody (Abcam, 1 : 50) was crossreacted overnight at 4°C. Postrinsing with PBS, nuclear-specific staining was performed with DAPI (D 1306; Thermo Fisher Scientific, 1: 10000) for 3 minutes at room temperature and the cells were finally washed with PBS and sealed. After drying, the cells were observed under a fluorescence microscope BZ-X 710 (Keyence).

2.4. Total RNA Extraction and RT-PCR. Total RNA was extracted from PC12 cells cultured in a 10 cm culture dish using the RNeasy Mini Kit (74104, QIAGEN). After synthesizing cDNA using the AffinityScript QPCR cDNA Synthesis Kit (600559, Agilent), PCR reaction was performed using EmeraldAmp PCR Master (RR300A, TaKaRa). PCR was carried out at an initial denaturation at 95°C for 5 minutes, and the postfinal cycle extension was performed at 72°C for 10 minutes. The primers and cycling conditions used are shown in Table 1. PCR products were separated on a 1.6% agarose gel and visualized with ethidium bromide staining under UV light.

2.5. PACAP Receptor Inhibition Experiments. PC12 cells were adjusted to 1×10^3 to 5×10^3 cells/well on a 96-well plate and cultured for 24 hours and the PAC1-R inhibitor (PA-8: Professor Ichiro Takasaki, Faculty of Engineering, University of Toyama) [38]. VPAC1-R and VPAC2-R inhibitors (VIP6-28, V4508, Sigma-Aldrich) were added to the cell culture and incubated in a CO_2 incubator (37°C, 5%) for 1 hour. PACAP38 10^{-7} M was added postincubation and cells were observed with or without the addition of inhibitors. A BZ-X710 optical microscope (Keyence) was used to count the total number of cells, and the number of cells with elongated protrusions (20 μm or more) were counted.

2.6. Inhibitor Experiments. PC12 cells were adjusted to 1×10^3 to 5×10^3 cells/well on a 96-well plate and cultured for 24 hours, and 5 μM CHIR99021 (252917-06-9, FUJIFILM Wako Pure Chemical Corporation), 5 μM LY294002 (154447-36-6, FUJIFILM Wako Pure Chemical Corporation), 2.5 μM H89, 2.5 μM U0126, 2.5 μM GF109203X, 10 μM Y27632, and 5 μM purvalanol A were added and placed in a CO_2 incubator (37°C, 5%) for 1 hour, and PACAP 10^{-7} M was added postincubation. After culturing for 3 days, the culture was observed using an optical microscope BZ-X710 (Keyence) and the total number of cells and the number of cells producing protrusions (greater than 20 μm and 20 μm or less) were measured.

2.7. Extraction of Total Soluble Protein. For extracting the proteins, PC12 cells were washed twice with PBS followed by the addition of the LB-TT extraction solution (7 M (w/v) urea, 42 g; 2 M (w/v) thiourea, 15.2 g; 4% (w/v) CHAPS, 4.0 g; 18 mM (w/v) Tris-HCl (pH 8.0), 1.8 mL; 14 mM (w/v) Trizma base, 169.5 mg; 0.2% (v/v) Triton X-100; 0.2 mL 50 mM (w/v) DTT, 771.5 mg; 1% (v/v) pH 3-10 ampholyte, 1 ml; and two EDTA-free proteinase inhibitor (5892791001, Roche) tablets in a total volume of 100 ml) to lyse the cells. One (1) ml of LB-TT was quickly added to the culture dish (diameter 10 cm) and immediately mixed for 1 min at RT. Protein concentration was determined with a Pierce™ 660 nm Protein Assay Reagent (Thermo Fisher Scientific) using bovine serum albumin (BSA) as a standard and a DeNovix DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA).

2.8. Two-Dimensional Gel Electrophoresis and Visualization of the Separated Proteins. Two-dimensional gel electrophoresis was performed according to the ATTO technical manual. Five (5) μg of the protein sample was added to a precast agarose disc gel (pH 5–8, φ2.5 mm × 75 mm; ATTO, Tokyo, Japan), and one-dimensional electrophoresis was performed using a WSE-1500 dicRun-R (ATTO, Tokyo, Japan) at a constant voltage of 300 V for 210 min. After completion of the one-dimensional electrophoresis, agarose disc gels were fixed in a fixative solution (0.25% TCA) for 3 minutes. The gels were washed three times with distilled water for 1 min, replaced with new distilled water, and shaken gently for 2 h, at RT. The distilled water was discarded, and the cells were gently shaken in SDS equilibrium solution (50 mM Tris-HCl (pH 6.8), 1.6% SDS, 0.02% bromophenol blue, 8% Glycerol, and 20 mM DTT) for 10 min, followed by a two-dimensional electrophoresis step. The two-dimensional electrophoresis was performed using e-PAGEL(R) (E-D520L 5-

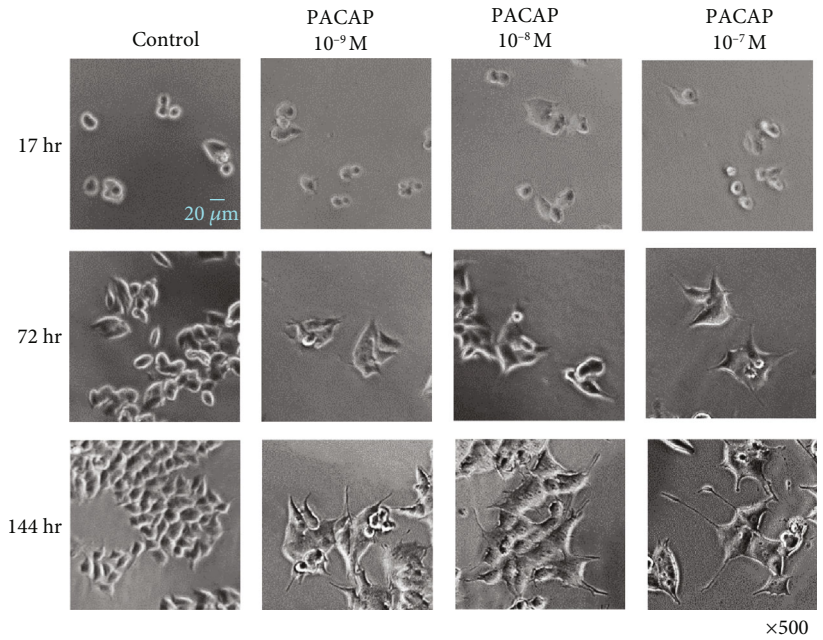


Figure 1: Changes in the PC12 cell neurite outgrowth with different concentrations of PACAP38. Experiments were repeated six times ($n = 6$).

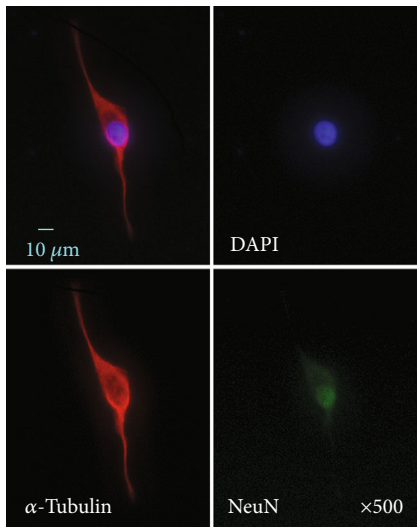


Figure 2: PC12 cell immunostaining. DAPI: blue; α-tubulin: red; NeuN: green. Experiments were repeated six times ($n = 6$).

20%) at a current of 20 mA/gel for 90 min, using a WSE-1150 PageRunAce (ATTO, Tokyo, Japan). Following 2-DE, proteins were transferred onto the PVDF membrane (Trans-Blot Turbo Midi PVDF, 0.2 μM, Transfer Packs Kit; cat. no. 170-4157) using the Trans-Blot Turbo Transfer System (Bio-Rad) following the MIXED MW protocol, 25 V, 2.5 A, 7 min. Following the transfer of proteins on the PVDF membrane (as also confirmed by visualizing all the 10 colored molecular mass standards), it was incubated in 25 ml of blocking solution for 1 h under constant slow shaking at RT. Blocking solution was prepared by dissolving 5.0 g skim milk (Difco) in 100 ml 1X TTBS (10X TTBS: NaCl 80 g; 1 M Tris-HCl (pH 7.5) 200 ml; and Tween-20 10 ml/l). Blocking solution was decanted, and the membrane was washed once in 1X TTBS (5 min), followed by incubation in 10 ml of primary antibody solution (2 μl rabbit anti-CRMP2 (cat. no. ab62661; Abcam), 3 μl mouse anti-CRMP2 phospho T555 (cat. no. ab215742; Abcam), 3 μl rabbit anti-CRMP2 phospho S522 (cat. no. ab193226; Abcam), and 3 μl rabbit anti-CRMP2 phospho T514 (cat. no. ab85934; Abcam) for 1 h, as above). The membrane was then washed five times with 25 ml of 1X TTBS. After decanting the last TTBS wash, the membrane was incubated in 10 ml of secondary antibody solution (0.5 μl of Amersham, ECL anti-rabbit IgG, HRP linked species-specific whole antibody (from Donkey); C= cat. no. NA 934; GE Healthcare) for 1 h, with slow shaking at RT. The 1X TTBS wash step was repeated five times. For image/band development, the luminol/enhancer and peroxide buffer solutions were mixed in a 1 : 1 ratio (Clarity Western ECL Substrate, cat. no. 170-5060, Bio-Rad) and spread over the membrane and incubated at RT for 5 min. Excess solution was drained by touching one end of the membrane on a Kimwipe paper towel, and the signal (crossreacting protein bands) was visualized on a ChemiDoc XRS+ imaging system (Bio-Rad).

2.9. Statistical Analysis. All experiments were repeated multiple times (six times unless stated otherwise). Data are presented as mean ± standard deviation. Microsoft Excel was used to analyze the data. Means were compared using one-way analysis of variance to confirm significance, and the Tukey method was used for multiple comparisons.

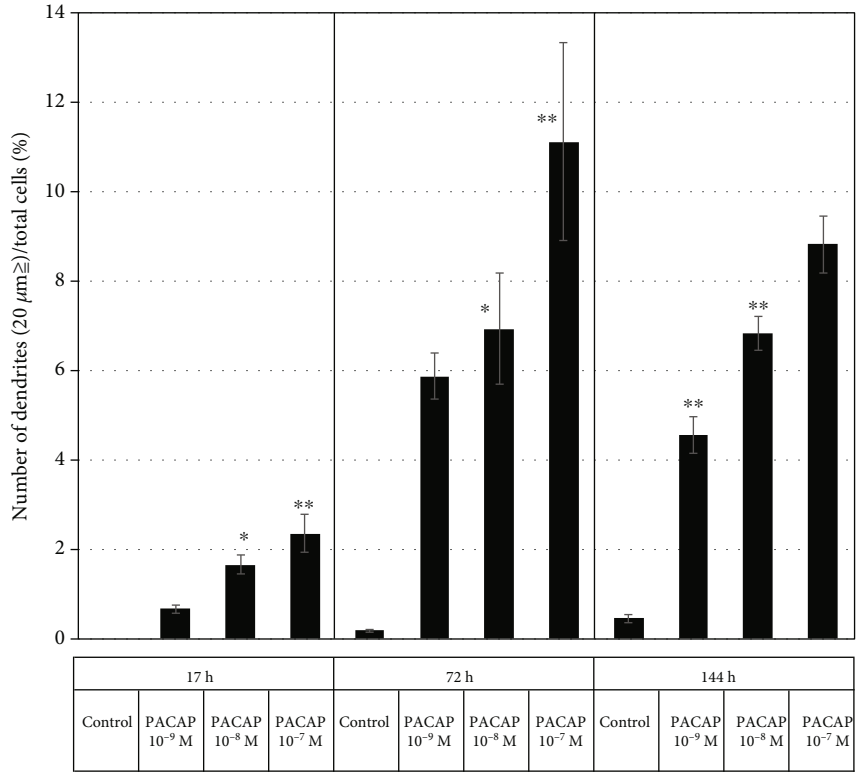


Figure 3: Change in the number of protrusions under different PACAP concentrations in the PC12 cells. Control: PACAP not added ($n = 6$); PACAP: PACAP38 concentrations added as indicated ($n = 6$). $^{*}p < 0.05$ vs Control; $^{**}p < 0.01$ vs Control (Tukey test). Experiments were repeated six times ($n = 6$). Data are presented as the mean ± SD.

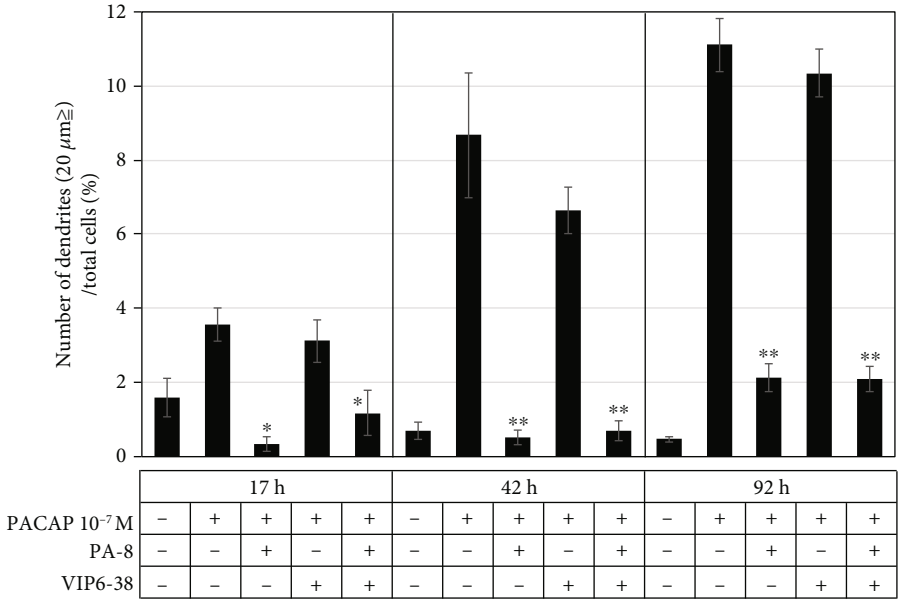


Figure 4: Effect of the PACAP receptor inhibitor on neurite outgrowth in PC12 cells. $^{*}p < 0.05$ vs PACAP 10^{-7} M; $^{**}p < 0.01$ vs PACAP 10^{-7} M (Tukey test). Experiments were repeated six times ($n = 6$). Data are presented as the mean ± SD.

3. Results and Discussion

3.1. Expression of PACAP and Its Receptors in the PC12 Cells. The primary objective here was to confirm the presence or absence of receptor gene expression in PC12 cells; therefore, gene expression analysis of PACAP and its receptors in PC12 cells was examined. Semiquantitative RT-PCR, which is an established method with the right primer design and

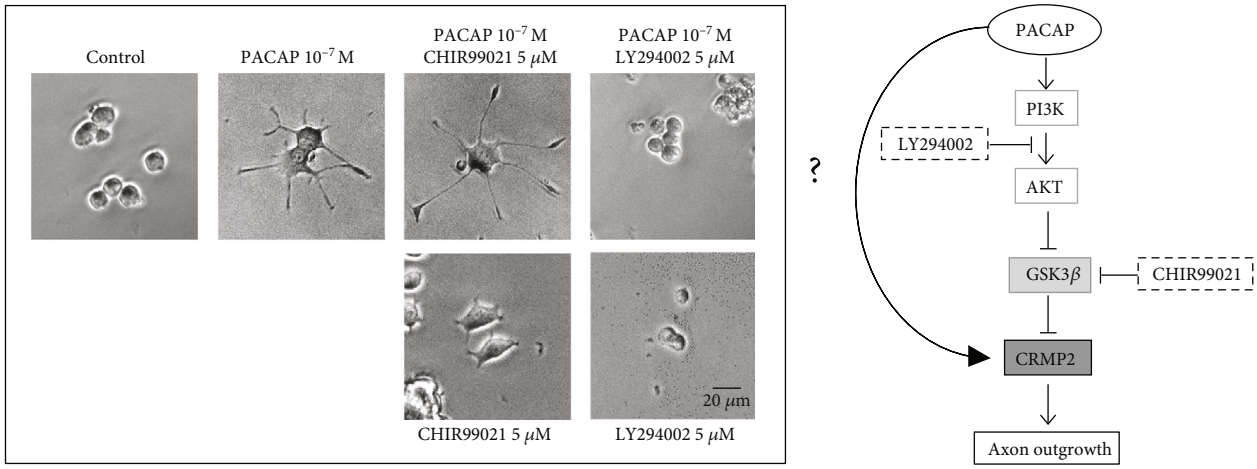


Figure 5: Changes in neurite outgrowth in the PC12 cells due to GSK-3β inhibition and activity. Control: PACAP free; CHIR99021: GSK-3β inhibitor; LY294002: GSK-3β activator. Treatment with PACAP38 was done for 72 h.

visualization of product/band on gel, was used, and after 45 PCR cycles, the PACAP, PAC1-R, VPAC1-R, and VPAC2-R mRNA were shown to be expressed. However, on reducing the PCR reaction time to 36 cycles, only a single band was confirmed for the PAC1-R. This suggested that PAC1-R is the main receptor involved in the neurite protrusion elongation action of PACAP38 in PC12 cells (Table 1). In the case of tissues, the difference in immunostaining can be clearly shown due to the difference in receptors, but in the case of cells, only similar images are obtained, so we confirmed the expression level by quantitative PCR in the present study.

3.2. Effect of PACAP38 Concentration on Neurite Protrusion Elongation. In order to investigate the optimum concentration of PACAP38 under which the PC12 cells show elongate protrusions, various concentrations of PACAP38 were added to PC12 cells and changes in the cells were observed (Figure 1). Immunostaining was used to observe the protrusions in the PC12 cells and their elongation by PACAP38. In the PACAP-added PC12 cells, NeuN specifically localized in the nerve cell nucleus, whereas α-tubulin expression was confirmed in the cell body and processes. This suggests that the protrusions elongated by PACAP38 are indeed the neurite protrusions (Figure 2). By measuring the protrusion elongation cell ratio of PC12 cells at various concentrations of PACAP38 over time, we could confirm the role of PACAP38 in causing protrusion elongation; the number of cells in the PACAP 10^{-7} M addition group confirmed that the protrusion elongation was significantly increased as compared to that in the control group (Figure 3). Results are the mean of different well data, and error bars are shown in SD (Figure 3).

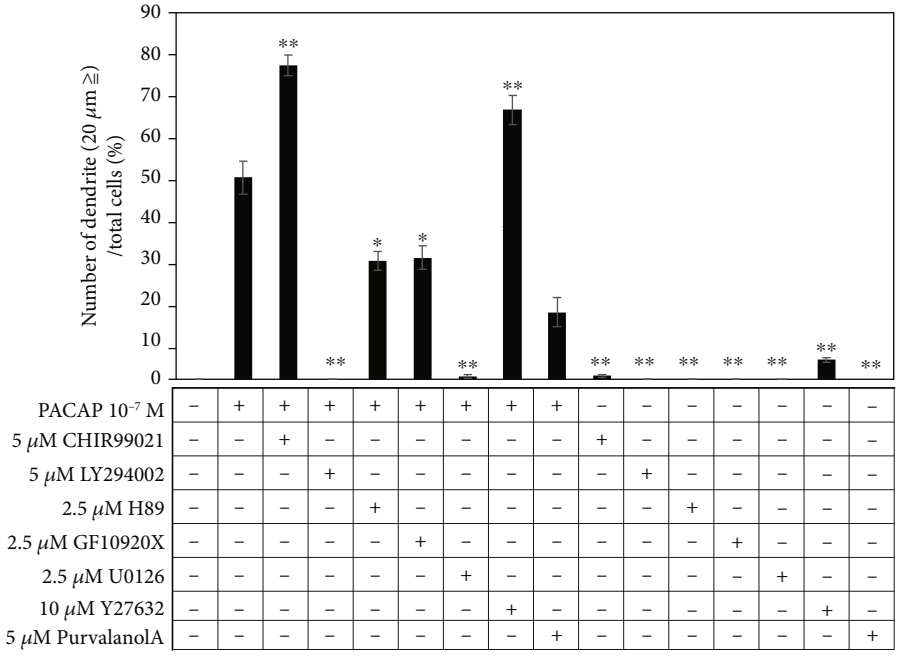
3.3. Changes in Neurite Protrusion Elongation Action by PACAP Receptor Inhibitors. In order to identify the receptors involved in the action of PACAP38 in the PC12 cells, PA-8, which is a specific PAC1-R inhibitor, and VIP6-28, which is a specific VPAC2-R inhibitor, were added and changes in the protrusion elongation action by PACAP38 was observed. The protrusion elongation effect of PACAP38 was significantly suppressed in the presence of PA-8, but not with VIP6-28. This data suggested that the protrusion elongation action of PACAP38 is mediated through the PAC1-R (Figure 4).

3.4. Observation of Neurite Protrusion Elongation Using Inhibitors and Activators

3.4.1. Examination of the GSK-3β Pathway. To clarify whether CRMP2 acts directly on PACAP38 or through GSK-3β as part of the mechanism of PACAP38-induced neurite outgrowth, we observed neurite outgrowth in the presence of GSK-3β inhibitors and activators. The addition of CHIR99021, an inhibitor of GSK-3β, promoted neurite outgrowth induced by PACAP38, while the addition of LY294002, an inhibitor of PI3K (phosphoinositide 3-kinase) upstream of GSK-3β and activator of GSK-β, inhibited neurite outgrowth induced by PACAP38. In this experiment, a neurite outgrowth of less than 20 μm was observed under the conditions where only CHIR99021 or LY294002 was added but most of the neurite outgrowth was greater than 20 μm when only CHIR99021 was added. However, when LY294002 was added, there was almost no elongation of more than 20 μm. These results suggest that the protrusion elongation effect of PACAP38 is mediated by GSK-3β (Figure 5).

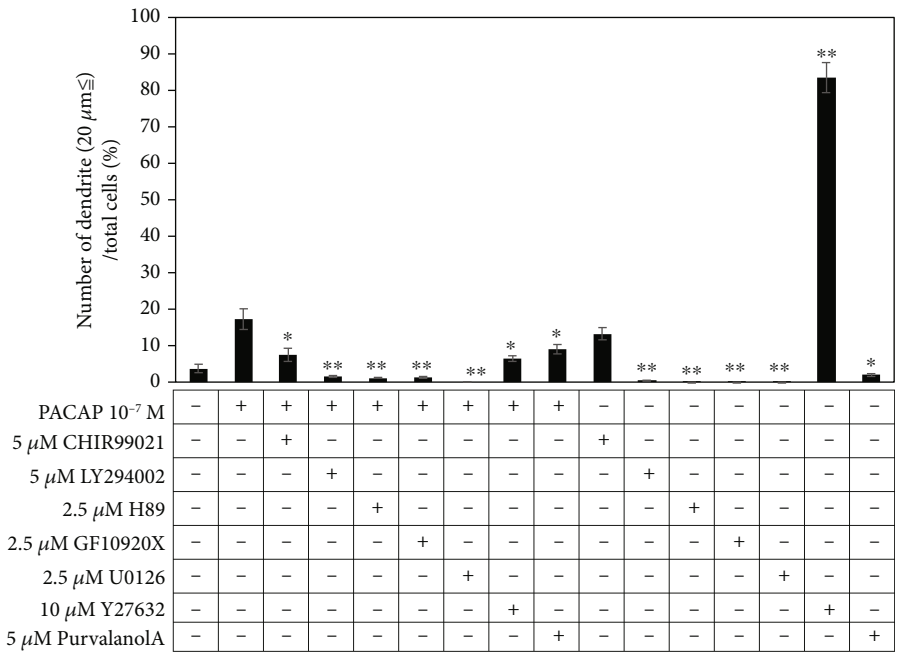
Although PACAP38-induced PC12 neurite outgrowth was thought to be mediated by the GSK-3β pathway, previous studies have shown that the signaling cascade from the PACAP receptor PAC1 moves mainly via the AKT pathway, the cAMP/PKA pathway, the cAMP/ERK pathway, and also the PLC/PKC pathway [39]. CRMP2 is known to be regulated by the PI3 kinase/Akt/GSK-3β signaling pathway [31]. GSK-3β, in addition to the AKT pathway, is also involved in the cAMP/PKA, cAMP/ERK, and PLC/PKC pathways [31, 39]. GSK-3β has been reported to be regulated by the cAMP/PKA, cAMP/ERK, and PLC/PKC pathways in addition to the AKT pathway [40, 41].

From the results of experiments using inhibitors (Figures 6(a) and 6(b)), it was confirmed that the addition of the AKT inhibitor LY294002 and the cAMP/ERK inhibitor



PACAP 10^{-7} M	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
5 μM CHIR99021	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
5 μM LY294002	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−
2.5 μM H89	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−
2.5 μM GF10920X	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−
2.5 μM U0126	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−
10 μM Y27632	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−
5 μM PurvalanolA	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+

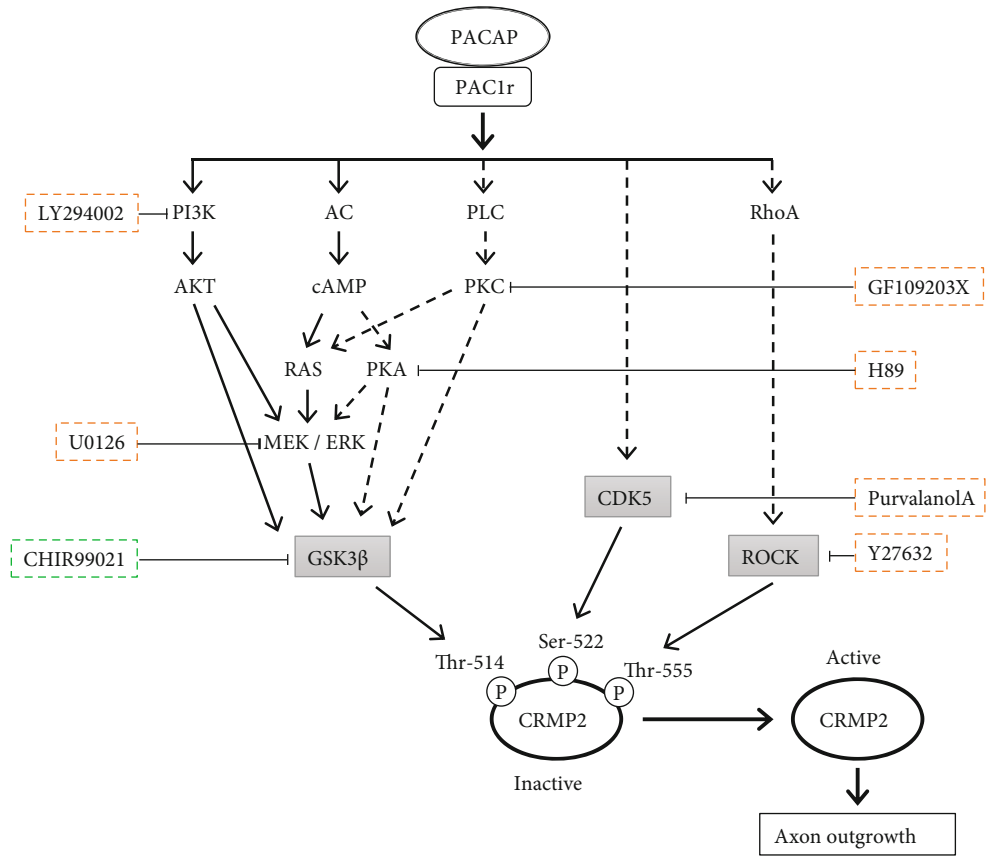
(a)



PACAP 10^{-7} M	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
5 μM CHIR99021	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
5 μM LY294002	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−
2.5 μM H89	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−
2.5 μM GF10920X	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−
2.5 μM U0126	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−
10 μM Y27632	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−
5 μM PurvalanolA	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+

(b)

Figure 6: Continued.



(c)

Figure 6: (a) Examination of pathways involved in protrusion extension (20 μm or more) by PACAP in inhibitor experiments. $^{**}p < 0.01$ vs PACAP 10^{-7} M. Control: PACAP free; CHIR99021: GSK-3β inhibitor; LY294002: GSK-3β activator; H89: PKA inhibitor; U0126: MEK/ERK inhibitor; GF109203X: PKC inhibitor; purvalanol A: CDK5 inhibitor; Y27632: rock inhibitor. (b) Examination of pathways involved in protrusion extension (less than 20 μm) by PACAP in inhibitor experiments. $^{**}p < 0.01$ vs PACAP 10^{-7} M. Control: PACAP free; CHIR99021: GSK-3β inhibitor; LY294002: GSK-3β activator; H89: PKA inhibitor; U0126: MEK/ERK inhibitor; GF109203X: PKC inhibitor; purvalanol A: CDK5 inhibitor and Y27632: rock inhibitor. (c) Examination of the pathways involved in protrusion extension in PC12 cells by PACAP using inhibitor experiments. →: pathways involved in process extension by PACAP; ⇢: pathways that are less involved in PACAP-induced protrusion extension.

U0126 almost completely suppressed the effect of PACAP38 on protrusion elongation. Therefore, under the present experimental conditions, the AKT pathway and the cAMP/ERK pathway were confirmed to be the major processes responsible for the neurite outgrowth effect of PACAP38 in PC12 cells.

3.4.2. Examination of the CDK5 and Rho/ROCK Pathways. In addition to GSK-3β, CDK5 and RhoA have been reported to phosphorylate CRMP2. Sema3A activates Cdk5 and GSK-3β, and Cdk5 phosphorylates CRMP-2 with serine 522 [35, 42]. With regard to the RhoA, activation of RhoA disrupts neurite outgrowth in primary neurons [36, 43] and myelin-related glycoproteins inhibit axon regeneration by a Rho-kinase-dependent mechanism [37, 44]. As a result of conducting experiments with the CDK5 and Rho/Rock pathway inhibitors and confirming the presence or absence of protrusion elongation by PACAP38, it was confirmed that the protrusion elongation action of PACAP was promoted when the Rho/Roc inhibitor Y27632 was added. When only Y27632 was added, many PC12 cells with protrusions of 20 μm or less were observed but protrusion elongation of 20 μm or more was almost negligible. Therefore, it can be suggested that Rho/Rock suppression by Y27632 alone does not cause a protrusion elongation effect. On the other hand, the addition of purvalanol A, a CDK5 inhibitor, slightly suppressed the protrusive elongation effect of PACAP38. From these results, the protrusion elongation action by PACAP38 is involved in both the CDK5 and Rho/Rock pathways but it can be suggested that rather than CDK5 phosphorylating CRMP2, PACAP promotes protrusion elongation via the CDK5 (Figures 6(a) and 6(b)).

From the results of the above inhibitor experiments, it can also be suggested that the main pathways for neurite protrusion elongation by PACAP are the AKT pathway and the cAMP/ERK pathway and that Rho/Roc inhibition was considered to further promote the neurite protrusion elongation action by PACAP38. However, for CDK5, if CRMP2

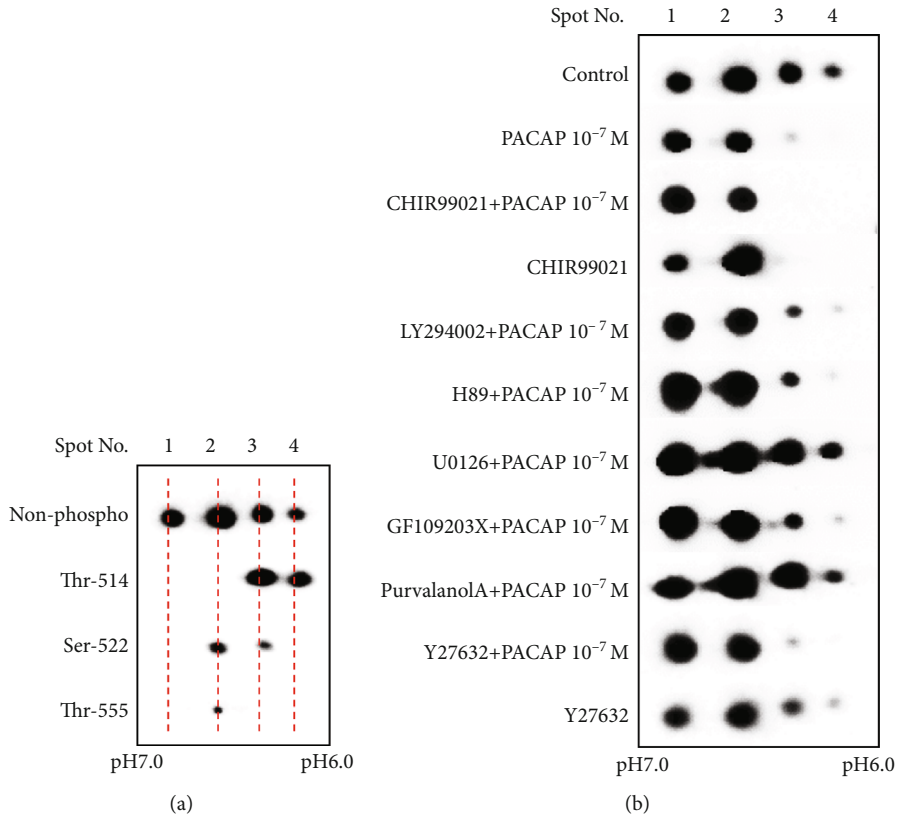


Figure 7: Examination of pathways involved in the CRMP2 phosphorylation and dephosphorylation by PACAP in the inhibitor experiments. (a) Antibody used: non-phosphor: anti-CRMP2 (ab129082); Thr-514: anti-CRMP2 phospho Thr-514 (ab85934); Ser-522: anti-CRMP2 phospho Ser-522 (CK6200); Thr-555: anti-CRMP2 phospho Thr-555 (CK6200). (b) Antibody used: anti-CRMP2 (ab129082); control: PACAP free; CHIR99021: GSK-3β inhibitor; LY294002: GSK-3β activator; H89: PKA inhibitor; U0126: MEK/ERK inhibitor; GF109203X: PKC inhibitor; purvalanol A: CDK5 inhibitor; Y27632: Rock inhibitor. Two-dimensional gel electrophoresis was performed as described in Cell Model and Methods.

phosphorylation by CDK5 acts for the inactivation of CRMP2, the addition of purvalanol A should result in promoting the neurite protrusion elongation action by PACAP38 addition, but in this experiment, the neurite protrusion elongation action was suppressed. Elimination of Cdk5-mediated CRMP2 Ser-522 phosphorylation reduces the density of dendrite spines in hippocampal neurons in the mouse hippocampus [45] and Cdk5-inhibition of CRMP2 phosphorylation in Ser522 in the optic nerve. There are reports that it leads to stabilization and regeneration of axons after nerve damage [46]. In addition, class 3 semaphorins have been reported to mediate dendrite growth in adult neonatal neurons through the Cdk5/FAK pathway [47]. It is implied that the CDK5 activation may be required for the neurite protrusion elongation action by PACAP38 in the PC12 cells (Figure 6(c)).

3.5. Relationship between CRMP2 Phosphorylation and Neurite Protrusion Elongation. Although CRMP2 phosphorylation and dephosphorylation are important for neurite protrusion elongation, the relationship between the phosphorylation/dephosphorylation action of CRMP2 in PACAP38 and neurite protrusion elongation is not clear. In particular, CRMP2 has many phosphorylation sites, and therefore, a two-dimensional gel electrophoresis analysis experiment was conducted to clarify which phosphorylation site might be involved and, thus, is important for the neurite protrusion elongation by PACAP38 (Figure 7).

As a result of the two-dimensional gel electrophoresis analysis (Figures 7(a) and 7(b)), four (4) spots were detected with the CRMP2 antibody. When Thr-514-, Ser-522-, and Thr-555-phosphorylated CRMP2 antibodies were used to confirm crossreacting phosphorylation spots, spot 2 was shown to be Ser-522 and Thr-555 and spot 3 was shown to be Thr-514 and Ser-522. It was confirmed that spot 4 was Thr-514. Spots 2, 3, and 4 abundance reduced with PACAP38 addition compared to the control. With the addition of PACAP38 + CHIR99021, which promoted increased neurite protrusion elongation, spot 2 decreased and spots 3 and 4 disappeared. Spot 4 disappeared in PACAP38 + Y 27632, which also showed more promotion of the neurite protrusion elongation. Furthermore, the addition of PACAP38 + LY294002 and PACAP38 + U0126, which eliminated the protrusion elongation action, did not cause a reduction in spots 3 and 4. Furthermore, spot 4 decreased in PACAP38 + H89, PACAP38 + GF109203*X*, and PACAP 38 + Purvalanol A in which protrusion elongation was slightly suppressed. From the above results, it was considered

that the reduction of spots 3 and 4 is important for promoting neurite protrusion elongation by PACAP38, i.e., the Thy-514 dephosphorylation of CRMP2 is essential. However, although spots 3 and 4 disappeared as in the case upon addition of CHIR99021, neurite protrusion elongation of 20 μm or more could not be confirmed. We cannot explain this observation at present, and a more detailed analysis will be required to understand it better. However, the ratio of spot 1 to spots 2, 3, and 4 was also considered to be important for neurite protrusion elongation of 20 μm or more. It was considered that the lower the ratio of phosphorylated CRMP2, the more that the protrusion elongation of 20 μm or more was promoted.

The neuropeptide PACAP is known to be involved in the neurite protrusion elongation action, but the mechanism of action has not been clarified. Therefore, PC12 cells, which are nerve-like cultured immortalized cells, were used to elucidate the mechanism of action. It was hypothesized that CRMP2 is involved in the protrusion extension action by PACAP38 but it was unclear what pathway was critical. From the results of this inhibition experiment, it was confirmed that the AKT pathway and the cAMP/ERK pathway mediated by the PAC1 receptor are the main pathways in the process elongation action by PACAP38. Furthermore, PACAP38 was found to promote neurite protrusion elongation by promoting dephosphorylation of CRMP2. The main pathways for dephosphorylation of CRMP2 have been reported to be via GSK-3β, Rho/Rock, and CDK5. However, in this study, results showed that the neurite protrusion elongation effect by PACAP38 was further promoted by dephosphorylation of CRMP2 by suppressing GSK-3β and Rho/Rock but the neurite protrusion elongation effect by PACAP38 was suppressed by CDK5 inhibition. CDK5 is said to be involved in the phosphorylation of Ser-522 in CRMP2, but inhibition of CDK5 does not reduce the spots of Ser-522 by two-dimensional electrophoresis, and spot 3 phosphorylation and spot 4 phosphorylation of Thy-514 were increased. CDK5 inhibition results in Ser9 dephosphorylation and in vivo activation of GSK-3β [48, 49]. Cdk5 activity is also associated with axonal and neurite growth [50]. In addition, $Cdk5^{-/-}$ mice have been reported to exhibit defective axonal elongation [51, 52]. From the results of this experiment, GSK-3β activation by CDK5 inhibition and increased phosphorylation of Thy-514 of CRMP2 indicate that CDK5 activity may also be important for the neurite protrusion elongation action by PACAP38.

Aware of the limitations and the need for further research, we are currently investigating the molecular mechanism of CRMP2-mediated neurite protrusion elongation by PACAP38 using DNA microarray and shotgun (LC-MS/MS) proteomic analysis and will further examine whether phosphorylation of CRMP2 by PACAP38 is effective for axon regeneration after injury. In recent years, the inhibition of CRMP2 phosphorylation has been shown to be effective in various diseases such as Alzheimer's disease [53], spinal cord injury [54], amyotrophic lateral sclerosis [55], and optic nerve injury [46]. Studies of PACAP, which inhibits CRMP2 phosphorylation, might pave the way towards the development of new drugs and therapies.

Abbreviations

CRMP2: Collapsing response mediator protein 2
GSK-3β: Glycogen synthase kinase-3β
PACAP: Pituitary adenylate cyclase-activating polypeptide
PC12: Rat adrenal-derived pheochromocytoma cell line
PMCAO: Permanent middle cerebral artery occlusion
VIP: Vasoactive intestinal polypeptide.

Data Availability

All data are publicly available; and data or methods or primers required will be shared fully.

Conflicts of Interest

All authors declare no conflict of interest.

Acknowledgments

This study was supported in part by JSPS KAKENHI numbers 16H02684 and 18H05386 (SS) and JSPS KAKENHI number 20K09262 (RR).

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