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Involvement of the Urokinase Receptor and Its Endogenous Ligands in the Development of the Brain and the Formation of Cognitive Functions

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Studies in recent years have shown that the urokinase receptor (uPAR) and its ligands – urokinase (uPA) and protein SRPX2 – play important roles in the processes underlying the formation and functioning of the brain. Human studies have demonstrated an interaction between a polymorphism of the *uPAR* gene and the development of autism. Patients with autism, incurable epilepsy, verbal dyspraxia, and perisylvian polymicrogyria show changes in uPAR expression. Studies using mice with knockout of the *uPAR* gene showed a tendency to develop epilepsy and impairments to behavioral reactions. Data have also been obtained on the involvement of uPAR ligands – uPA and protein SRPX2 – in the development of pathological brain states associated with autism, cognitive disorders, and speech disorders. The urokinase system has been shown to be able to regulate not only the rate of growth of vessels and nerve fibers by remodeling the matrix and activating neurotrophic and angiogenic factors, but also to function as a system of navigation molecules determining the direction of vessel and nerve growth in tissue regeneration processes. This article summarizes and analyzes results from recent studies of the role of uPAR and its endogenous ligands in the development of the brain and the formation of the cognitive functions.

Keywords: urokinase, urokinase receptor, intracellular signaling, brain, cognitive functions, epilepsy, autism.

Abbreviations

c-met	Hepatocyte growth factor receptor
HGF	Hepatocyte growth factor
HSP	Heat shock protein
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
scuPA	Single-chain urokinase without proteolytic activity
SRPX2	Sushi repeat protein X-linked 2
tPA	Tissue plasminogen activator
uPA	Urokinase-type plasminogen activator, urokinase
uPAR	Urokinase-type plasminogen activator receptor, urokinase receptor
VDD	Verbal dyspraxia of development
GABA	γ-Aminobutyric acid
MRNA	Messenger ribonucleic acid
OCD	Obsessive-compulsive disorder
RNA	Ribonucleic acid
EEG	Electroencephalogram

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General Properties of the Urokinase System. Urokinase (uPA) and the urokinase receptor (uPAR) are components of the fibrinolytic system and play an important role in activating plasminogen and triggering the cascade of proteolytic reactions accompanied by degradation of the extracellular matrix, activation of matrix metalloproteinases, and the release and activation of growth factors (Fig. 1) [9, 10]. The structure of uPAR includes three homologous domains (D1, D2, and D3) and is attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor [9]. uPA is a serine protease – a multidomain protein secreted by many cell

types including cardiovascular and nervous system cells. Binding to cell surfaces via its receptor, uPAR, urokinase cleaves the specific precursor protein plasminogen, converting it into a proteolytically active serine protease with a wide spectrum of substrate specificity. Active plasmin triggers a cascade of proteolytic reactions leading to cleavage of extracellular matrix proteins and release of growth factor deposited in the matrix [9, 85]. Furthermore, acting via limited proteolysis, uPA directly activates a number of proangiogenic growth factors, which in turn promote the proliferation and migration of endothelial cells and their invasion [5, 70]. The directions of these processes are determined on the one hand by the features of uPAR structure, which, lacking transmembrane and cytoplasmic domains, is attached to the membrane via the GPI anchor, promoting high levels of lateral mobility and concentrating molecules on the leading edge of migrating cells. This redistribution of the urokinase complex with its receptor provides local activation of proteolysis of components of the extracellular matrix in the direction of the cell's movement (Fig. 2) [10]. On the other hand, binding of uPA with uPAR leads to activation of a series of signal cascades within the cell [23], triggering rearrangement of the cytoskeleton and supporting targeted cell migration. For example, binding of urokinase with uPAR was shown to induce activation of tyrosine kinases Src and Hsk, p38, p42/44, JAK1, and Tyk2 [29, 53], as well as the small G proteins RhoA and Rac1 [39]. uPA was also able to elicit transdifferentiation of cells – our studies showed that urokinase increased expression of smooth muscle α-actin in fibroblasts in which α-actin is not detected in normal conditions, thus promoting their conversion to myofibroblasts, i.e., cells with a characteristic contractile phenotype [89]. This transformation of fibroblasts into myofibroblasts can enhance the formation of the neoadventitial layer and induce negative remodeling in vessels [57, 62, 64–66, 90], and can also lead to fibrous regeneration of kidney tissue [105]. This mechanism of increasing the expression of smooth muscle α-actin on exposure to uPA remains unclear, though our studies and those of other research groups have identified several signal pathways which may contribute to controlling these processes. Firstly, plasmin, formed by uPA, activates transforming growth factor β-1 (TGF-β1), which in turn stimulates the expression of smooth muscle α-actin in fibroblasts via a transcriptional mechanism [44]. Secondly, increases in the expression of smooth muscle α-actin in renal fibroblasts have been shown to occur on binding of uPA with nicotinic acetylcholine receptors, nAChR [104, 105]. Thirdly, we demonstrated that uPA secreted by cells as a single-chain precursor lacking proteolytic activity (scuPA) can penetrate into cells and reach the nucleus, where it activates the expression of smooth muscle α-actin in fibroblasts [89].

We very recently observed a novel mechanism for the activation of angiogenesis involving urokinase. Our studies showed that scuPA secreted by cells was not in some cases activated by plasmin and penetrated into the nucleus using the transport protein nucleolin [89]. Having reached the nucleus, scuPA binds with a number of transcription factors regulating the promoter activity of the genes encoding vascular endothelial growth factor receptors (VEGFR1 and VEGFR2). We showed that urokinase decreases the repressor activity of transcription factor HHEX/PRH, thus releasing its inhibitory influences on the activity of the promoters of the *VEGFR1* and *VEGFR2* genes, thus increasing the expression of VEGFR1 and VEGFR2 receptors on the surfaces of endothelial cells. Ultimately, this leads to increases in the migration and proliferation of endothelial cells in response to the main growth factor for vessels, VEGF [88].

Data obtained by us and other authors showing that the urokinase system is a powerful stimulator of angioarteriogenesis made it possible to create gene therapy agents for the treatment of ischemia. Expression of urokinase induced by introduction of genetic constructs in experimental ischemia of the lower limb in mice and myocardial infarcts in rats stimulated angioarteriogenesis and restoration of blood flow to the same extent as VEGF expression [93]. The gene therapy agent created in our studies, Yupikor, which contains a plasmid to express urokinase, has undergone successful phase I clinical trials at the Institute of Experimental Cardiology, Ministry of Health of the Russian Federation, and is progressing towards further clinical trials.

Our recent studies in mice with knockout of the urokinase gene, uPA–/– animals, provided the first demonstration that the urokinase system in vessels, apart from the well described stimulation of migration and proliferation of vascular cells, is required for determination of the growth trajectory and capillary branching [81]. These are the first results providing evidence that the urokinase system may have a number of other functions distinct from activation of proteolysis and degradation of the extracellular matrix. Binding of urokinase with uPAR receptors may stimulate angiogenesis, not only facilitating the migration of vascular cells, but also controlling this migration, assisting migrating cells to select the correct direction. This suggestion is supported by data obtained previously in studies of the effects of the soluble form of the urokinase receptor (Fig. 3) on the directed migration (chemotaxis) of activated neutrophils to foci of inflammation [67, 84, 92]. Studies of the signal mechanisms whereby uPAR influences chemotaxis showed that uPAR stimulates targeted cell migration by interacting with the transmembrane receptor binding peptide fMet-Leu-Phe (FPR) [25] and integrins (Fig. 2) [99, 100]. In targeted migration, apart from activating FPR and integrins, uPAR also activates a number of growth factor receptors, including the epidermal growth factor receptor, EGFR, and the platelet-derived growth factor receptor, PDGFR [8, 50, 85]. As the activity of these receptors increases in targeted cell migration [33], their interaction with uPAR may support selection of the growth trajectory and capillary branching [81].

Full regeneration of organs and tissues can only occur when both blood supply and innervation are restored. Despite

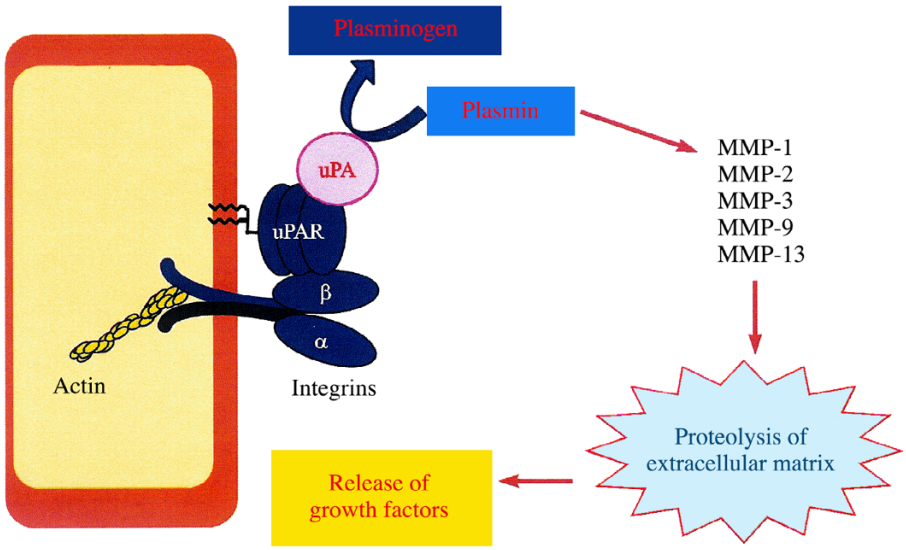

Fig. 1. Mechanism of activation of extracellular proteolysis involving urokinase and the urokinase receptor. Urokinase (uPA), binding its receptor (uPAR), becomes catalytically active and converts plasminogen to plasmin, a protease with a wide spectrum of actions. Plasmin triggers a cascade of proteolytic reactions, activating matrix metalloproteinases (MMP), which carry out the proteolysis of the extracellular matrix and release growth factors. The stability of the uPA/uPAR complex arises from the bond between uPAR and transmembrane heterodimeric integrins (α/β integrin subunits) and the cytoskeleton (actin).

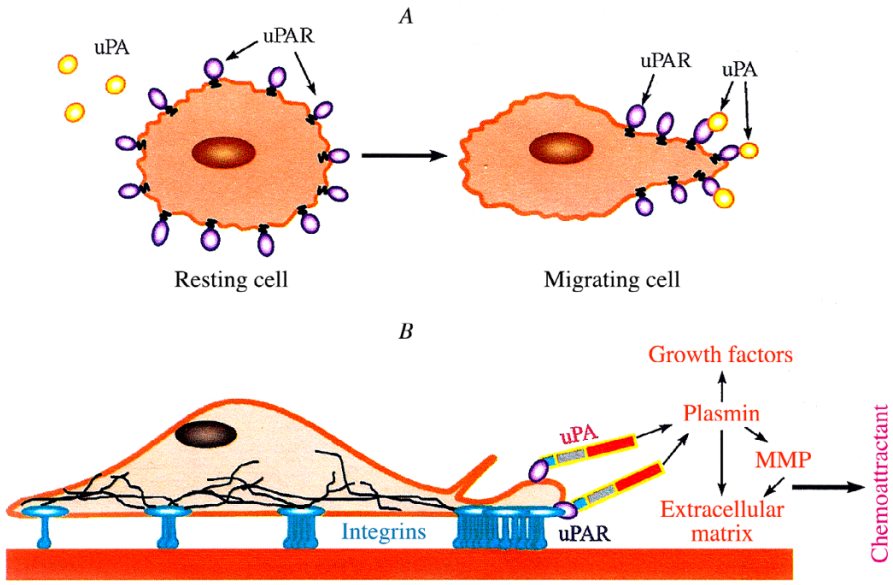

Fig. 2. Involvement of urokinase (uPA) and the urokinase receptor (uPAR) in targeted cell migration. *A*) Resting cells express urokinase receptors, uniformly distributed across the membrane surface. Binding of urokinase with its receptor leads to redistribution of the uPA/uPAR complex to the leading margin of the migrating cell; *B*) this cooperation of the active complex of uPA with uPAR activates plasmin, which carries out local proteolysis of matrix proteins (MMP) and growth factors and remodels the extracellular matrix as vessels and nerves grow. Targeted cell movement is mediated by local proteolysis, reorganization of adhesive contacts, and the involvement of integrins and cytoskeletal dynamics.

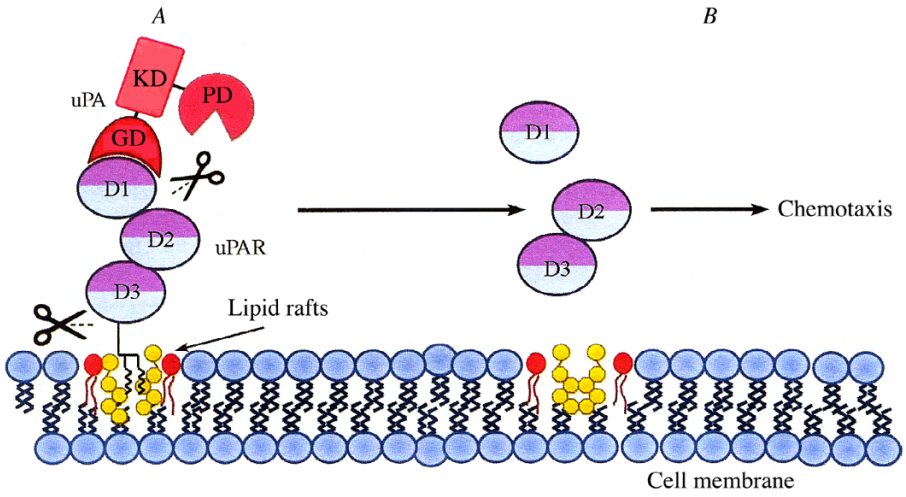

Fig. 3. Diagram showing the membrane-bound (*A*) and soluble (*B*) forms of the urokinase receptor (uPAR). The urokinase receptor is located on the membrane in lipid rafts and is anchored to the membrane via GPI anchors. D1, D2, and D3 are uPAR domains; uPA – urokinase-type plasminogen activator, or urokinase; GD – growth factor domain of urokinase; KD – kringle domain of urokinase; PD – proteolytic domain of urokinase.

functional differences in the blood and nervous systems, there are analogies in their organization: blood vessels and nerves are located alongside each other and have similar growth trajectories during embryogenesis and on regeneration [20, 27, 43, 101]. Regulation of targeted growth and branching of axons involves the same signal mechanisms as the regulation of angiogenesis [3, 86]. In the case of angiogenesis, the roles of uPA and uPAR are quite well understood. However, little is known of the navigational properties of the urokinase system and its involvement in activating signal pathways in neurons, the regulation of targeted growth and branching of axons, or the formation of synapses in the brain. The literature contains only a few studies providing evidence that the urokinase system is involved in forming interneuronal connections and synaptic plasticity, which are important for the morphogenesis of brain structures supporting cognitive functions and memory [14, 15, 28, 30, 48].

The Role of the Urokinase System in the Embryonic Development and Functioning of the Brain. Development and functional maturation during embryogenesis constitute a clearly organized process consisting of a multitude of sequential stages, during which a series of changes programmed in time and space cause neurons and glial cells to acquire defined phenotypic properties. Impairments to the formation of brain structures during embryogenesis and early postnatal development cause formation of neurological pathology and cognitive disorders. Formation of the neocortex is known to involve the migration of immature neurons from active proliferation zones in the telencephalon to the subpallium (subcortical areas) and pallium (cortical structures) [46]. Hepatocyte growth factor (HGF) was identified in the brain as a protein regulating the direction of movement of interneurons from the sites at which they originate (the ganglionic eminence) to the area of the neocortex at which they then differentiate and function [38]. HGF binds with c-met receptors on cell surfaces, these being a transmembrane tyrosine kinase [13, 60]. This interaction activates a intracellular signal cascade stimulating cell proliferation and migration, and also tumor invasion and metastasization [60]. Animals with knockout of the *HGF* gene do not survive to birth; the time course of embryo death corresponds to the period at which interneuron migration starts [95]. Data from studies using explant cultures of brain tissue provided evidence that decreased HGF activity in the ganglionic eminence correlates with decreases in the numbers of neurons reaching the pallium and subpallium [46, 68]. Studies of the mechanism by which HGF regulates interneuron migration showed that despite the fact that exogenous addition of HGF to the culture medium for embryonic tissue explants stimulated interneuron migration, this migration was disordered and followed a trajectory different from the normal one [69]. These results provide evidence that despite the need for HGF expression in brain tissues, there are other proteins or factors regulating HGF expression and activity not only in interneurons, but also in those parts of the brain to which these neurons will subsequently migrate and where they carry out their functions. Data presented below provide evidence that HGF expression and activity are subject to ever more complex regulation than previously suggested.

During embryogenesis, genetically modified mice lacking the *uPAR* gene (uPAR–/–) [26] show decreases in the biological activity of HGF in areas in which interneurons expressing the neurotransmitter γ-aminobutyric acid (inhibitory GABA interneurons) form and to which they migrate. In adult individuals, these impairments lead to 50% decreases in the content of GABA interneurons in the cingulate and parietal areas of the cerebral cortex [46, 68]. Analysis of subpopulations of GABA interneurons showed that the absence of the *uPAR* gene produced greater than 90% suppression of migration and decreased the content of parvalbumin-expressing GABA interneurons in the cortex [68]. The result is that mice lacking the *uPAR* gene are predisposed to epilepsy, both spontaneous (in 3% of cases) and chemically induced (administration of kainic acid into the hippocampus), the latter occurring very severely and not infrequently lethally. Spontaneous epilepsy does not occur in wild-type mice, while induced epilepsy is milder [68]. Electroencephalogram (EEG) data indicate that in uPAR–/– mice, the EEG is characterized by desynchronized periods of low-frequency amplitude. In normal conditions, low-frequency amplitude in the EEG is typical of the γ rhythm, and its generation involves parvalbumin-expressing GABA interneurons. The duration of epileptic seizures induced experimentally by injection of kainic acid into the hippocampus in uPAR–/– mice was significantly longer than that in wild-type mice, with a shorter latent period, and the seizures themselves were characterized by the tonic flexion posture (the neonatal posture) interrupted by movements of the trunk, bilateral clonus of the forelimbs and tail (Straub tail). In control mice, the latent period to the onset of epilepsy was prolonged, while convulsions and changes in body position were less marked [68]. Histological analysis showed that postepileptic neurodegeneration of the hippocampus in uPAR–/– mice was less severe than in wild-type mice and that uPAR–/– mice had an inflammatory reaction in the hippocampus arising as a result of the toxic action of kainic acid, which had a slower course and greater heterogeneity of inflammatory cells [59]. uPAR–/– mice also showed a marked vascular phenotype: as compared with wild-type mice, regeneration of vessels in the damaged hippocampus was less extensive in these animals, which had shorter vessels on histological sections and newly formed vessels with lower levels of maturity. Desynchronization of the γ rhythm, the development of spontaneous epilepsy, and the more severe course of provoked epilepsy in uPAR–/– mice probably occur because of a decrease in the content of GABA interneurons, as administration of HGF to uPAR–/– mice postnatally restored the content of GABA interneurons in the cortex to the normal level and suppressed formation of

foci of epileptic activity [7]. Overall, these data provide evidence that uPAR, controlling HGF expression and activity, is an important factor mediating interneuron migration during the early embryonic development of the brain, and it may be that deficiency of the *uPAR* gene in mice causes impairment to the structure of the brain after epileptic seizures. More detailed studies of the role of uPAR in the pathogenesis of epilepsy will allow uPAR to be used in the future as a potential therapeutic model for the treatment of similar states in humans.

Apart from the predisposition to developing epilepsy, uPAR–/– mice show significant changes in behavior: as compared with wild-type mice, these animals showed increased anxiety, had fear of occupying novel spaces and being in open and illuminated territories [46, 68]. The authors of this study proposed the term "weak socialization," whereby the mouse experienced difficulties in following its instinct to explore the novel space. Wild-type mice easily enter the previously unfamiliar space [46]. Presumably, deviations in behavior in uPAR–/– mice are also associated with deficiency of the inhibition of GABA interneurons in the cerebral cortex, as the role of GABA interneurons in modulating excitatory signals from other neurons has previously been demonstrated, as has a role in regulating the formation of behavioral reactions [32, 68].

Data on the possible role of uPAR in the development of human diseases such as epilepsy and various forms of abnormal behavior have been confirmed in a number of clinical trials. Studies in 25 patients undergoing surgery for incurable anterior frontal lobe epilepsy showed significant increases in uPAR expression in brain samples collected during surgery. Histochemical investigations identified a high levels of expression of uPAR in neurons (NeuN was used as the neuron marker); only minor proportions of uPAR expression were seen in glial cells (GFAP was used as a glial marker) and macrophages (expressing the CD11b marker) [49]. The control group consisted of 15 patients with injuries to the anterior frontal lobe due to acute contusions. Screening of 15 patients with obsessive-compulsive disorder (OCD) showed decreased plasma levels of HGF, uPA, and uPAR in these people. The severity of OCD correlated with the extent of reductions in uPA and uPAR expression, while uPA and uPAR expression levels correlated with the level of expression of HGF [79].

In addition to tendencies to epilepsy and social behavior disorders, uPAR–/– mice have been found to have several features in the organization of the brain characteristic of such pathophysiological states in humans as schizophrenia and autism [1, 59]. Thus, one distinguishing neuroanatomical feature of schizophrenia is a selective decrease in the number of synapses formed between parvalbumin-containing interneurons with calretinin-containing and somatostatin-containing interneurons in the prefrontal cortex of the brain [58]. Despite the fact that the absolute number of interneurons in humans with schizophrenia were not decreased, only the number of synapses showing any reduction, the presence of typical functional impairments and abnormal behaviors in humans with schizophrenia and in uPAR–/– mice suggests a role for uPAR in the etiology of this disease.

The causes of another brain pathology, autism, include impairment to the expression or mutation of the genes influencing maturation of synaptic contacts between neurons and altering the spatial orientation of axons in the cerebral cortex [35]. Despite the absence of a single hypothesis of the origin of autism, there is one indisputable fact: post mortem histological studies of the cerebral cortex generally show impairments to the formation of microcolumns in the prefrontal and temporal areas [11]. These microcolumns consist of several neuron types (GABAergic interneurons and pyramidal and stellate neurons) and form at the levels of layers II–IV of the cerebral cortex. Decreases in the numbers of GABA interneurons lead to impairment to the spatial organization of microcolumns in the cerebral cortex in autism [11]. Insufficiency of GABA interneuron content in the cortex leads to dominance of excitatory signals, with the result that the brain enters a state of constant arousal. It has been suggested that children with autism have characteristic behavior based on a tendency to suppress arousal [11]. These children also have difficulty processing complex information, elevated anxiety, and marked avoidance of social interactions. There are often anomalies in EEG rhythms and 15–25% of cases show spontaneous epilepsy [18]. Modeling of analogous pathologies in animals, especially rodents, is extremely difficult for various reasons, including the absence of speech and higher nervous activity typical of humans. However, the simultaneous presence of epilepsy and behavioral disorders in autism in humans has a number of analogies with the phenotype described in and the abnormal behavior of uPAR–/– mice. These data lead to the hypothesis that the etiology of autism includes a significant role for impairments to information transmission via GABA interneurons, whose migration and ordered organization depend on normal uPAR expression and on the interaction of uPAR with its endogenous ligands.

The involvement of uPAR in the development of autism is evidenced by data showing a high frequency of polymorphism of the rs344781 allele of the *uPAR* gene in patients with autism. Studies within families (to avoid errors associated with individuals from genetically heterogenous populations, $p = 0.006$) and "case-control" studies, in which genotype studies of parents were not carried out, showed that this polymorphism associated with the development of autism in children ($p = 0.007$) and increases in uPAR expression [17]. In addition, the same study also demonstrated a significant interaction between autism and the presence of polymorphisms in other genes, particularly *HGF* rs13238709 and *c-met* rs1858830. Studies of patients with autism showed that the *c-met* polymorphism rs1858830, which decreases c-met expression, is significantly associated with overexpression of uPA, uPAR, and HGF [16].

Thus, it is now known that HGF plays a key role in controlling the migration of interneurons to the cerebral cortex during embryogenesis. HGF expression in turn depends on the level of uPAR expression. The two gene polymorphisms, of *uPAR* and *HGF*, have a combined effect on the development of autism. These data suggest involvement of uPAR in the formation of the cerebral cortex and the development of cognitive functions. The use of experimental models and further studies, using these models, of the role of uPAR in the processes of neuron differentiation, migration, and functioning provide a new view of the mechanisms of development and functioning of the brain in health and disease.

Signal Effects of the Urokinase System in Controlling Axon Growth and Neuron Differentiation in the Brain. The proteolytic degradation of fibrin is an important step in the remodeling of the extracellular matrix for restoration not only of lost blood supply to damaged tissues but also of innervation. Despite elevated uPA, uPAR, and tissue plasminogen activator (tPA) activities in brain trauma, injured nervous tissue still accumulates fibrin, which hinders regeneration, prevents nerve and glial cell migration and targeted axon growth, and synapse formation and maturation [2, 4, 31, 61, 83]. It is now evident that the functions of these proteins in the nervous system are not restricted to degrading fibrin during regeneration. There is extensive evidence that plasminogen activators, triggering remodeling of the extracellular matrix, facilitating nerve cell migration and axon growth [74, 83], also activate intracellular signaling. Activation of intracellular signaling in regeneration is required for the survival and differentiation of neurons, as well as for reorganization of the cytoskeleton and recovery of damaged axons [28, 48, 63].

Expression of uPA, uPAR, and tPA in axon growth cones and the involvement of these proteins in degrading the extracellular matrix to support targeted axon growth to their targets were discovered relatively recently [61, 63]. Suppression of the expression of these proteins increases fibrin accumulation in tissues, which hinders axon growth [74, 83]. However, inhibitor analysis revealed a paradoxical effect – instead of the expected suppression of axon growth, some inhibitors not only had no effect on this process, but in some cases, conversely, stimulated axon growth [63]. Thus, suppression of tPA and uPA with specific synthetic inhibitors correlated with acceleration of axon growth. Antibodies blocking the proteolytic domain of uPA stimulated axon growth twofold, while antibodies binding uPA but not inhibiting its proteolytic activity had no significant effect. Use of time-lapse videomicroscopy to visualize growing neuron processes showed that administration of tPA and uPA inhibitors increased the rate of process formation in most neurons, but simultaneously decreased the formation of lamellopodia at the axon growth cone [63]. The authors of this study were the first to suggest that the urokinase system in neurons not only has well defined proteolytic properties, but also activates intracellular signaling and induces changes to neuron morphology, affecting axon formation, elongation, and branching.

Changes in neuron morphology are tightly linked with changes in the activity of adhesive contacts. For neurons, the formation of adhesive contacts involving integrins and laminins is an important factor in forming the morphological features of the cell body and further axon growth and branching. Neurons mainly express integrins α5-7, β1, and β4 [48, 80, 96]. In in vivo experimental nerve trauma, expression of integrins increases, and the zones of increased integrin expression coincide with the areas in which increased uPA and uPAR expression is seen. It should also be noted that uPAR expression increases sharply in the first hours after nerve damage, while increases in uPA expression occur gradually, reaching a maximum by day 8 after injury [83]. It may be that this time disparity between uPA and uPAR expression is evidence that the function of uPAR in the regeneration of peripheral nerves is not restricted to activation of proteolysis. The literature contains experimental data showing that uPAR can not only bind uPA to support localized proteolysis, but can also take part in activation of intracellular signaling due to direct interaction with integrins [48]. In particular, retinal neuron axon growth was shown to occur along the uPA gradient. Neurite elongation was characterized for the formation of numerous spines – future neurite branch points – and the activity of this process depends on the uPA concentration. Binding with uPA, uPAR redistributes on the axon membrane and forms a supramolecular complex with integral α5/β1 heterodimers. Further colocalization of integrins with uPAR leads to triggering of intracellular signaling involving FAK and Src kinases, inducing rearrangement of the cytoskeleton at the axon cone [48]. Thus, uPA and uPAR in neurons coordinate several processes simultaneously: firstly, they activate targeted intracellular proteolysis and remodel the matrix, facilitating axon growth; secondly, they reorganize adhesive contacts by means of assembly of integral heterodimers; thirdly, they trigger the intracellular signaling required for forming the structure of the cytoskeleton.

Another important function of uPAR in neurons is that of maintaining them in the differentiated state. RNA interference studies showed that "exclusion" of the *uPAR* gene blocks cell differentiation in neurons despite the presence in the culture medium of the neurotrophic factor NGF [28]. Morphologically, this is apparent as a decrease in the formation of neuron processes, while at the intracellular signaling level it leads to loss of expression of sodium channel proteins and COX-1 protein, which are important markers for NGF-dependent neuron differentiation [28]. This uPAR-dependent neuron differentiation presumably involves transcription factors. The first candidates for this role are transcription factors Fos and Jun, which contain regions interacting with the promoter of the *uPAR* gene [19] and also regulate NGF expression [97]. In the absence of uPAR,

there is a decrease in the activity of the Fos/Jun transcription factor heterodimer, which is followed by a drop in the promoter activity of the *NGF* gene and a decrease in the expression of NGF itself by cells. Deficiency of NGF in the culture medium leads to neuron death [28].

Apart from uPAR, uPA can also carry out a neuroprotective function. Thus, modeling of epilepsy in mice by administration of kainic acid into the hippocampus showed that premedication with recombinant uPA decreased further excitotoxic neuron death as compared with the control group [21]. Subsequent studies of the mechanism of this phenomenon showed that the protective effect of uPA involves glutamate receptors, which respond to prior administration of uPA by reducing negative effects on neuron survival after toxic exposure to kainic acid [21].

Along with results providing evidence that uPA and uPAR are important for survival and maintenance of the differentiated state of neurons, there are directly contradictory data indicating that high levels of uPA expression can induce neuron death [91]. Thus, there is a sharp increase in uPA activity in injured neurons in stroke, which stimulates the release of matrix metalloproteinase-9 (MMP9), which activates astrocytes. This activation of astrocytes induces the expression of β-interleukin-1 in them, this substance demonstrating toxicity to neurons and also stimulating the inflammatory response. Administration of the uPA inhibitors amiloride and PAI-I decreases MMP9 contents and pathological activation of astrocytes, decreasing the expression of β-interleukin-1 in them, thus reducing neurotoxicity [91].

Thus, there is extensive evidence that uPA and uPAR are involved not only in remodeling the extracellular matrix as an important component in the recovery of the brain, but that they also mediate intracellular signaling in neurons activated in response to changes in their morphology, differentiation, and survival. Overall, these data allow the roles of the uPA/uPAR system in brain development to be reassessed and the strategy used for the treatment of ischemic or inflammatory brain damage to be altered.

Endogenous uPAR Ligands in the Central Nervous System and Their Role in the Development of the Brain and Cognitive Functions. uPA was long regarded as the main, if not the only, ligand of uPAR, mediating its functions, including those in the brain [9]. It might be expected that as in the case of the lack of uPAR, the absence of uPA would induce similar, if not identical, impairments to the development and functioning of the brain. However, the absence of uPA in knockout mice (uPA–/–) does not produce changes similar to the uPAR–/– phenotype in relation to the brain. uPA–/– mice are not predisposed to developing epilepsy, the migration of GABA interneurons is not impaired, and HGF activity in embryogenesis is not decreased during period when the pallium and subpallium form. In addition, the behavior of uPA–/– mice is not abnormal [12, 73]. Overall, these data suggest that there may be an alternative ligand for uPAR in the brain, different from uPA, which mediates its functions during brain development in health and pathology.

Sushi Repeat Protein, X-linked 2 (SRPX2) (Genbank NP_055282), a secreted three-domain protein-proteoglycan, has been shown to have three so-called Sushi motifs and is able to bind uPAR [54, 78]. This interaction was demonstrated in vitro using a two-hybrid yeast system using a coimmunoprecipitation method and surface plasmon resonance [78]. Histologically, expression of SRPX2 coincides with uPAR expression in different areas of the brain, including the cortex and subcortical structures [78]. Mutations of the *SRPX2* gene or impairments to its expression correlate with cognitive disorders and speech impairments associated with diseases such as Rolandic epilepsy (Sylvian epilepsy or "language" syndrome), Rolandic epilepsy combined with speech impairments (so-called RESDX syndrome), developmental verbal dyspraxia (problems with motor functions and speech development in children), and bilateral perisylvian polymicrogyria (a malformation consisting of excessively smooth relief and insufficient depth of sulci in the cortex) [14, 75, 77]. The p.N327S mutation in the *SRPX2* gene (MIM 300643), impairing its glycosylation, leads to delayed mental development, the formation of foci of epileptic activity in the Rolandic sulcus of the brain, and the development of speech dyspraxia [77]. Another mutation in the *SRPX2* gene, p.Y72S (MIM 30642), leads to the development of more severe defects in the development of the cerebral cortex – so-called bilateral perisylvian microgyria, speech deficiency, and mental weakness. This p.Y72S mutation produced a 5.8-fold increase in the ability of SRPX2 protein to bind with uPAR. It is of note that this same mutation in the *SRPX2* gene, p.Y72S (MIM 300388), develops a rather different disease in males, leading to the development of Rolandic epilepsy and perisylvian polymicrogyria, though it does not produced delayed mental development [75].

Sushi sequences are encountered not only in SRPX2 protein, but also in various other proteins forming the extracellular matrix in the brain. The literature contains various data showing that uPAR can interact with both SRPX2 and other Sushi-containing proteins [14]. It can be suggested that proteins whose sequences contain Sushi repeats may be necessary in the brain for the correct membrane localization and synaptic mobility of the two B1 subunits of the GABA receptor [34]. Sushi sequences are also present in Sez-6 protein (Seizure-related 6 homolog protein), which is a multidomain transmembrane protein for which mutations are known to interact with the development of febrile convulsions. Sez-6 protein is a candidate for a major role in the etiology of epilepsy and cognitive dysfunctions in bipolar disorder [104]. Furthermore, Sushi sequences are present in the B1a subunit of the GABA receptor, which have been shown to have an important role in the development of epilepsy [71]. Interaction of SRPX2 protein with uPAR is seen only in the brain. The SRPX2-uPAR interaction plays an important stimulatory role in angiogenesis on activation of

endothelial cell migration [54]. Activated endothelial cells coexpress SRPX2 and uPAR, while "switching off" the *SRPX2* gene by RNA interference suppress the migration of these cells and their formation of capillary-like structures [54]. Overall, these data suggest that the interaction of uPAR with SRPX2 mediates not only the development of cortical structures, but also the formation and remodeling of vessels in the brain.

SRPX2 protein can interact with the D1 domain of uPAR and with the D2 and D3 domains. The D1, D2, and D3 domains of uPAR have sequences typical of the so-called group of Ly-6/uPAR neurotoxic proteins, many of which, like uPAR, are GPI-anchored [9]. These proteins have a wide spectrum of action and are involved in the activation of intracellular signaling, including that with the involvement of the nicotinic acetylcholine receptor, nAChR [72, 102]. Studies of Ly-6/uPAR-dependent activation of intracellular signaling involving nAChR showed that direct neurotoxin-containing proteins do not activate the receptor though, operating as laterally nAChR-associated adapter proteins, they are able to alter the sensitivity of nAChR to its known agonists, such as acetylcholine and nicotine [51, 56]. Proteins of the Ly-6-uPAR superfamily, known as SLURP2 (or LYNX1) and having one Ly-6-like domain, conversely, operate as nAChR suppressors, not altering nAChR function as an ion channel (i.e., neither agonist nor antagonist), but altering its metabotropic effects, suppressing cell proliferation in response to addition of acetylcholine [51, 52]. Different types of nAChR are known to participate in forming the cognitive functions of the brain, including learning and memory formation processes, and also in the development of a number of convulsive disorders [56]. Considering that uPA can bind with nAChR [104, 105], it is possible that limited proteolysis of uPAR by uPA, with release of the soluble D1 domain (Fig. 3), may lead to its association with nAChR, which might affect nAChR-dependent signaling, like the action of SLURP2/LYNX1, and regulate the formation of the motor and cognitive functions of the brain.

Relatively recent studies identified a further two potential ligands of uPAR, belonging to the heat shock proteins group (HSP) – these are protein MRJ (or DNAJB6) and protein HSP70 [24, 47], which may mediate the effects of uPAR in the brain. MRJ protein functions in association with chaperone protein HSP70, which in turn is involved in controlling signaling to support the protection of neurons from injury on activation of inflammatory processes and ischemia [37]. The protective mechanism is based on suppression of glial cell activation and the release of proinflammatory cytokines [37]. MRJ protein determines the substrate specificity of HSP70 protein on binding with proteins at different stages of their maturation, assembly, and transmembrane transport/secretion [36]. Binding of uPAR with MRJ and HSP70 proteins significantly increases cell adhesion to vitronectin. Vitronectin is an important component of the extracellular matrix for neurons, regulating the formation of adhesive contacts of neurons, which involves integrin heterodimers, and taking part in regulating the formation of the brain during embryogenesis, as well as the functioning of the brain [24, 55]. Mice lacking the *MRJ gene (MRJ–/–)* are characterized by impairments to the formation and maturation of the neural tube at the early stages of embryogenesis. In addition, MRJ protein is important for neuron proliferation and the regeneration of nervous tissue involving neural stem cells [98]. High levels of MRJ expression in the brain prevent aggregation of polyglutamine proteins, which play the defining role in the development of Huntington's chorea – a severe neurodegenerative pathology with progressive cognitive disorders and dementia [22]. Further study of the interaction of uPAR with MRJ is important not only from the biological point of view for studies of the mechanisms of development of the brain and cognitive functions, but also for a deeper understanding of the pathogenesis of neurological and psychiatric disorders and the possible involvement of uPAR in these pathologies.

Thus, several endogenous ligands of uPAR able to mediate the functions of this receptor in the brain are now known. The role of ligand can be taken by adapter proteins which interact laterally with uPAR in the axon membrane, for example integrins, nAChR, Sez-6 protein, and MRJ, which mediate the signal influences of uPAR, and proteins forming the extracellular matrix for brain neurons, among which the most important is SRPX2. As existing data provide evidence that mutations and polymorphisms of the *SRPX2* and *uPAR* genes influence the formation of brain structures and induce severe developmental pathology, with speech defects and cognitive disorders, it becomes apparent that studies of the expression and activities of these genes in neurons are important for our understanding of the development and functioning of these parts of the brain.

Mutual Regulation of the Activity of uPAR Genes and SRPX2 Protein at the Level of FOXP2 Transcription Factor. One of the most complex tasks in contemporary neurobiology is that of explaining the mechanisms coordinating the operation of neurons and speech at the molecular level. Little is known of which intracellular processes underlie the formation of speech. Scientific attention on solving this point is currently focused on protein SRPX2, discussed in the preceding section, and transcription factor FOXP2 (synonyms: TNRC10, SPCH1), which is regarded as a potential candidate for the role of a regulatory factor involved in the development and coordination of speech at the molecular and genetic levels. Transcription factor FOXP2 is a member of the large FOX family of transcription factors. In normal conditions, FOXP2 expression regulates the development of brain, lung, and intestine tissues [76, 82]. In the brain, the *FOXP2* gene is associated with the development of language skills [40, 41]. Mutations in the *FOXP2* gene in humans are known to be combined with very rare cognitive disorders of speech, so-called "specif-

ic speech disorder of type 1" (speech-language disorder 1; OMIM 602081). This group includes quite severe diseases such as childhood apraxia of speech, developmental verbal dyspraxia (VDD), and VDD with disorders of the orofacial dyspraxia type [41, 42, 76, 87].

SRPX2 protein has been identified as an important factor in speech development. Mutations in the *SRPX2* gene inducing developed impairments to the brain lead to the development of Rolandic (Sylvian) epilepsy ("language syndrome"), VDD, and bilateral perisylvian polymicrogyria. These diseases are associated with severe speech dysfunctions [14]. It has been suggested that mutations in the *SRPX2* gene trigger pathophysiological processes underlying impairments of its interaction with uPAR [6]. Use of a chromatin precipitation method identified a region within the promoter of the *uPAR* gene which interacts with transcription factor FOXP2 [76]. Use of computer modeling and a method for determining molecular surface interactions identified the regions of the interaction between FOXP2 and the promoters of the *SRPX2* and *uPAR* genes. In health, FOXP2 maintains SRPX2 and uPAR expression at low levels; experimental overexpression of FOXP2 in neurons leads to decreases in the activity of the *SRPX2* (by 80%) and *uPAR* (by 77%) promoters and decreases in the contents of SRPX2 (by 43%) and uPAR (by 38%) mRNAs. The mutant form of transcription factor FOXP2, p.R553H, induces the development of VDD [95], and is unable to interact effectively with the promoters of the *SRPX2* and *uPAR* genes and, in contrast to the native of FOXP2, has no suppressive influence on the promoters of these genes, which at the protein level is apparent as an increase in SRPX2 and uPAR expression [76]. Another type of FOXP2 mutation, p.M406T, in the domain responsible for dimerization of this protein, was seen in patients with polymicrogyria of the left Rolandic sulcus and children with autism [45]. This type of mutation only partly impairs the interaction of the transcription factor with the promoter of the *SRPX2* gene, though it has no influence on the activity of the promoter of the *uPAR* gene.

Thus, in health, transcription factor FOXP2 suppresses the activity of the *uPAR* and *SRPX2* genes, while mutations in the *FOXP2* gene release its suppressive action on uPAR and SRPX2 expression, resulting in an increase in the interaction of these proteins. At the cellular level, these changes lead to impairment to the interaction of uPAR located on the membrane with the extracellular matrix containing SRPX2. Impairment to the normal trajectory of axon growth and branching is the cause of the abnormal formation of neural networks, which is apparent as neurological and mental disorders [11]. The interaction between FOXP2 activity and the expression of uPAR and SRPX2 proteins is evidence that uPAR is involved in the development of speech and cognitive functions. This is now regarded as the most probable mechanism explaining the role of uPAR in these processes, clarifying all stages from impairments to the activity of transcription factor FOXP2, which affects uPAR expression, to impairment of the interaction of uPAR with the extracellular matrix containing neurons.

Conclusions. uPA and uPAR are known to regulate vital processes in cells, such as proliferation, adhesion, migration, and metastasis. uPAR, apart from activating extracellular proteolysis, triggers an intracellular signaling cascade and modulates cell adhesion, interacting with integrins in the membrane and vitronectin in the extracellular matrix. The literature contains data on the involvement of uPAR in regulating the processes of the development and functioning of the central nervous system. The diversity of the functions of uPAR reflects its multidomain structure, its ability to move rapidly within the membrane due to its GPI anchor, its interaction with a wide spectrum of ligands, and its ability to undergo limited hydrolysis, which releases individual domains and screens additional epitopes required for interaction with other molecules. Studies of the mechanisms of functioning of uPAR and its interaction with partner proteins in nervous tissues may play an important role in our understanding and successful correction of various pathologies. The key finding in studies of the role of uPAR in embryogenesis is the fact that uPAR controls the expression and activity of HGF, which is one of the main factors mediating the migration of GABA interneurons during the period of early embryonic development. Phenotypically, uPAR–/– mice show not only a predisposition to epilepsy, but also impairments to behavior typical of schizophrenia and autism in humans. A correlation is known to exist between a high frequency of the T-allele polymorphism of the *uPAR* gene, rs344781, and autism. Changes in uPAR expression are also seen in patients suffering from epilepsy, verbal dyspraxia, and perisylvian polymicrogyria.

The novel uPAR ligand found in brain tissues – SRPX2 protein – mutations of which are associated with Rolandic epilepsy, the development of verbal dyspraxias, perisylvian polymicrogyria, and general disorders of speech and behavior, point to a link between uPAR and the manifestations of neuroepileptic disorders and impairments to brain development. Nonetheless, data presented in the scientific and medical literature to date do not identify the mechanisms by which the ligand of SRPX2 or other potential ligands could influence the processes of uPAR-dependent intracellular signaling in neural cells, including the axon growth and navigation and synapse formation. Indeed, it is known that these processes ultimately influence the development of the pathologies listed above, which are associated with mutations in the *SRPX2* and *uPAR* genes.

An interaction between the levels of expression of SRPX2 and uPAR at the level of transcription factor FOXP2 has been demonstrated. Impairments to the expression of or mutations in the *SRPX2* or *FOXP2* genes have the same adverse implications for the functioning of the brain as deviations in the expression of or mutations in the *uPAR* gene, and are apparent as predisposition to epilepsy, development of dyspraxia, and perisylvian polymicrogyria, as well as

general speech and behavioral disorders. The relationship between uPAR expression and the activity of transcription factor FOXP2 provides a tighter link between uPAR function and pathologies of nervous system development and the manifestations of nervous and epileptic disorders.

The results of studies seeking to explain the role of the urokinase system in model experiments on animals with knockout of the *uPAR* gene, along with studies of polymorphisms of or mutations in the *SRPX2*, *uPAR*, and *FOXP2* genes in populations of humans with abnormalities of the development and functioning of the brain allows us to take a new view of the previous concept of the formation of cognitive disorders to select a novel potential strategy for the therapeutic correction of these pathologies.

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