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REVIEW

Axon guidance molecules in liver pathology: Journeys on a damaged passport

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Abstract

Background and Aims: The liver is an innervated organ that develops a variety of chronic liver disease (CLD). Axon guidance cues (AGCs), of which ephrins, netrins, semaphorins and slits are the main representative, are secreted or membrane-bound proteins that can attract or repel axons through interactions with their growth cones that contain receptors recognizing these messengers. While fundamentally implicated in the physiological development of the nervous system, the expression of AGCs can also be reinduced under acute or chronic conditions, such as CLD, that necessitate redeployment of neural networks.

Methods: This review considers the ad hoc literature through the neglected canonical neural function of these proteins that is also applicable to the diseased liver (and not solely their observed parenchymal impact).

Results: AGCs impact fibrosis regulation, immune functions, viral/host interactions, angiogenesis, and cell growth, both at the CLD and HCC levels. Special attention has been paid to distinguishing correlative and causal data in such datasets in order to streamline data interpretation. While hepatic mechanistic insights are to date limited, bioinformatic evidence for the identification of AGCs mRNAs positive cells, protein expression, quantitative regulation, and prognostic data have been provided. Liver-pertinent clinical studies based on the US Clinical Trials database are listed. Future research directions derived from AGC targeting are proposed.

Conclusion: This review highlights frequent implication of AGCs in CLD, linking traits of liver disorders and the local autonomic nervous system. Such data should contribute to diversifying current parameters of patient stratification and our understanding of CLD.

KEYWORDS
autonomic nervous system, axonal guidance cues, chronic liver disease, neurons

Abbreviations: AGC, axonal guidance cue; CLD, chronic liver disease; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; LSEC, liver sinusoidal endothelial cells; NASH, non-alcoholic steatohepatitis; NCT, national clinical trial; SRGAP, SLIT and ROBO GTPase-activating protein; UPR, unfolded protein response.

1 | INTRODUCTION: LIVER NEURONS, WOUND HEALING PROCESSES AND NERVE SPROUTING

Hepatic innervation accompanies and intensifies during embryonic development,[1] leading to a highly innervated organ, the functions of which are modulated by different nerves. Indeed, the liver is innervated by branches of the splanchnic and vagal nerves, which comprise sympathetic and parasympathetic efferent nerves of the autonomic nervous system (ANS), respectively, and sensory afferent nerves.[2] Their branches innervate the parenchyma via the portal triads, running along the hepatic artery, the portal vein, and bile ducts.[1] The involvement of the ANS in physiological regeneration and wound healing has been repeatedly evidenced over the last decades. Interestingly, on a systemic and developmental basis, most nerves are essential for tissue growth[3] and wound healing,[4–7] and may in the latter case be accompanied by anarchic nerve sprouting and hyperinnervation, determining long-term pathological sensations close to the site of injury, well-known, for instance, after the amputation of a limb. Of importance, liver regeneration is also dependent on neural output.[8] Nerves thus influence tissue repair and regeneration, both phenomena largely linked to carcinogenesis if altered or dysregulated.

There is a permanent connection between the brain and the liver via afferent and efferent nerves. The hepatic branch of the sympathetic system regulates glucose release, hepatic regeneration,[9] fibrosis,[10] angiogenesis,[11] and portal venous tension.[12] The parasympathetic system controls glucose storage, hepatic metabolism,[2] and inflammation.[13] Importantly, it was shown that after partial hepatectomy, liver regeneration was severely impaired in rats following vagotomy (i.e., the removal of part of the vagus nerves).[14] Moreover, a correlation between degeneration of sympathetic nerves and non-alcoholic steatohepatitis (NASH), a causative agent for CLD, was reported.[15] These findings suggest that at least a subset of liver nerves rely on axon guidance cues (AGCs) as regulating factors for their own redeployment during CLD, thus modulating disease development. While AGCs are historically known as neural regulators,[16,17] the link between these factors and liver neurons as part of CLD alterations has seldom been highlighted. In addition to improving our current understanding of the implication of AGCs in liver pathology, this review also proposes to consider liver pathologies as potential neuron-regulated targetable conditions.

We will first tackle notions pertaining to AGCs in neurology and development. We will then consider these proteins in the context of CLD, before describing their role in HCC. Special attention will be paid to distinguishing correlative and causal data in such datasets in order to streamline data interpretation. We will present all past and ongoing clinical trials involving AGCs, few of which are currently conducted on liver, based on the US ClinicalTrials.gov database. Finally, we will try to describe pertinent future research directions in this interesting field.

Key points

- While AGCs are historically identified as neural regulators, links between AGCs and liver neurons are seldom highlighted for interpretation.
- Expression of AGCs often impact fibrosis and immune processes, is of adverse prognosis in the majority of instances, including HCC, suggesting pertinence for further research and in vivo targeting.
- Several clinical trials are ongoing, including in liver pathology.
- Research perspectives include mechanistic and testing in animal models updated to the currently evolving epidemiology of CLD and HCC.

2 | AXON GUIDANCE: GENERAL CONSIDERATIONS

2.1 | Development

AGCs, including ephrins, netrins, semaphorins, and slits (see Figure 1 for structural features), are crucial for the development of neural circuits linking the 80 billion neurons of the human post-natal central nervous system (CNS).[16,17] Most AGCs are bifunctional and determine attractive and repulsive events, navigating the axons through pre-existing tissues to find target cells, in health and disease.[18] These signals can be soluble or membrane-bound, operating over large or short distances, and are also restricted by interactions with certain components of the extracellular matrix (ECM) or expressed through gradients with likely diverse spatio-temporal dynamics.[16,17]

AGCs also operate outside the nervous system, where they play an important role in cell migration and cell–cell communication during somitogenesis, vascular development, and organogenesis of various organs, including the heart, liver, kidney, lung, mammary gland and bone.[19,20] Moreover, as will be presented below, these proteins are also involved in pathological functions, such as reconfiguring the innervation of diseased organs.

2.2 | Adulthood

The following sections will initially present the role of the different types of AGCs in adulthood physiology. Their expression ensures neuronal plasticity, and is triggered following nervous system injury in rodents and during neural pathologies in humans.[18] However, studies on intracellular pathways have largely focused on neurons, while the intracellular impact of AGCs on non-neural tissues that display distinct kinetics and functioning from neurons warrants further investigation.

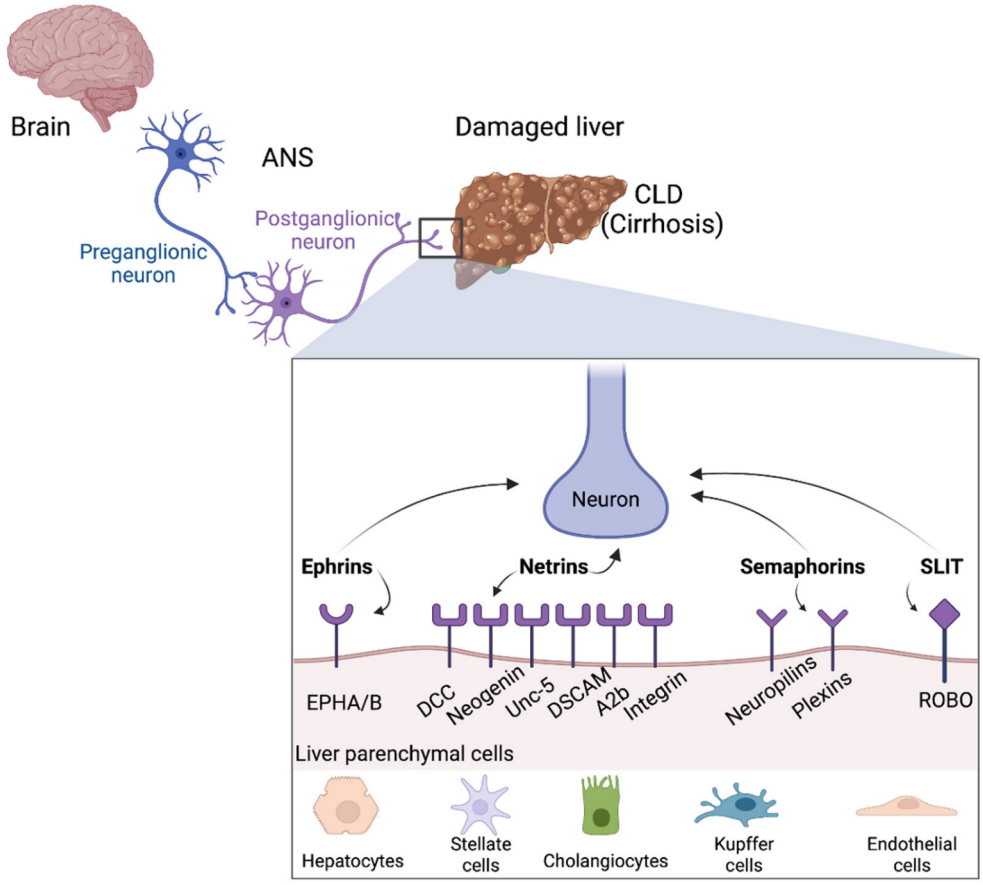

FIGURE 1 General functional scheme implicating axonal guidance cues (AGCs) as mediators between the central nervous system, the autonomic nervous system (ANS) and the liver. The brain, the ANS and the liver signal both ways through the implication of AGCs as functional hubs enabling redeployment of neurons topography and functions during hepatic wound healing processes in chronic liver disease.

2.2.1 | Physiological roles of EPHs and ephrins

Ephrins (also known as ephrin ligands or eph family receptor-interacting proteins) are a family of proteins that serve as the ligands of the so-called 'EPH' receptor. The eight ephrin ligands identified in humans are divided into two subclasses based on their structure and linkage to the cell membrane. The five GPI-anchored ephrin-A ligands (ephrin A1-5) interact with nine EPHA receptors (EPHA1-8 and EPHA10), and the three transmembrane ephrin-B ligands (ephrin-B1-3) interact with five EPHB receptors (EPHB1-4 and EPHB6) and one EPHA receptor (EPHA4), providing a rich, uncharted, combinatory repertoire of downstream events. Through the contact between receptor-expressing cells and ligand-expressing cells, ephrins and EPHs are capable of eliciting 'bidirectional triggering' where EPHs mediate forward signalling and ephrins reverse signalling, using a flip-flopped head-to-tail configuration with respect to the cell of interest.[21]

2.2.2 | Physiological roles of netrins

Orthologues of netrin-1 play a highly conserved role as guidance cues from archaic bilaterians to humans for midline crossing. Three secreted netrins (netrin-1, -3 and -4) have been identified in mammals, in addition to two GPI-anchored membrane proteins, netrin-G1 and -G2. Of note, netrin-1, -3, and -4 bear a laminin VI domain, a notion of interest with respect to ECM-accumulating properties in CLD. Secreted netrins attract axons via the Deleted in Colorectal Cancer (DCC) family of receptors, including DCC and neogenin, but repulse them through the UNC5 family (UNC5A, UNC5B, UNC5C and UNC5D) of receptors.[22] The Down Syndrome Cell Adhesion Molecule (Dscam) and the adenosine A2B receptor have also been implicated as additional netrin-1 receptors. Netrin-G1 and netrin-G2, on the other hand, selectively bind to the transmembrane proteins NGL1 and NGL2, respectively.

Netrin-1 is mainly associated with membranes and ECM.[23] Importantly, with respect to CLD, netrins also influence chemotropic

cell migration, morphogenesis and angiogenesis,[24,25] inflammation,[24] and cell–cell and cell-matrix adhesion.[26]

2.2.3 | Physiological roles of semaphorins

Semaphorins (SEMA, 30 members including 21 in mammals) are secreted and membrane-resident proteins that were also originally identified as axon growth cone guidance molecules, signalling attractively or repulsively through multimeric receptor complexes.[27] Semaphorins are divided into eight classes; the first seven are ordered by number, from class 1 to class 7, whereas the eighth group is class V, that is specific to viruses[28] with no obvious link with the liver. Classes 3–7 are found in vertebrates. Each semaphorin is characterized by a specific domain of about 500 amino acids called the 'sema' domain. Semaphorins are ubiquitous[29] and signal through engagement of two main families of receptors, the Neuropilins (NP1 and NP2) and the Plexins (PLEXA1-4, PLEXB1-3, PLEXC1 and PLEXD).[27,29] Secreted class 3 semaphorins (except SEMA3E) mainly signal through heterocomplexes of neuropilins and class A and D plexins. Combination of such edifices likely provides specificity for binding and transducing signals from different class 3 semaphorins.[30] Membrane-bound class 4–7 semaphorins and SEMA3E bind and directly activate Plexins. The class 7 semaphorin (SEMA7A) can also use integrins as its receptor suggesting its ability to alter cell-ECM crosstalks in ECM accumulating tissues. A variety of neurons, including sympathetic ones respond to semaphorins.[27,29] Class 3 semaphorins modulate axon regrowth, re-vascularization, re-myelination and the immune response after trauma in the CNS,[31] indicating their potential involvement in other injured innervated tissues, such as the liver.

2.2.4 | Physiological roles of SLITs

SLITS belong to a family of secreted ECM proteins which play an important signalling role in the neural development of most bilaterians (hence including humans), which possess three homologues: SLIT1, SLIT2 and SLIT3.[32] SLIT proteins acts as midline repellents, preventing the crossing of longitudinal axons through the midline of the CNS.[33] A major feature of SLITS is a β-sandwich domain similar to laminin-G,[34] allowing stable embedding into the ECM, a notion, as for netrins, of interest in CLD. Though non-exclusive, Robos are the principal receptors for SLITs ligands. There are three Robos expressed in neural cells (ROBO1-3), and the fourth Robo receptor (ROBO4) is expressed in endothelial cells. SLIT/ROBO signalling regulates gonadal physiology, pancreatic islet β-cells morphogenesis[35] and function[36] and, of note for this review, peripheral nerve regeneration upon injury.[37]

Importantly, evidence suggests that signalling of ephrin, netrin, semaphorin and slit AGCs intersect to regulate different physiological functions, in organogenesis, but also in angiogenesis, cell proliferation and stem cell regulation,[38] all these functions being relevant to the liver, especially in the context of CLD and HCC.

For several years, different scientific communities have separately been investigating the neural and non-neural roles of AGCs, in the context of development or pathology. Given the richness of the datasets obtained in both settings, it is now possible to provide a more unified view of this knowledge when focusing on a given organ, such as the liver. Accordingly, AGCs would therefore, as many factors high jacked during tumorigenesis, initially play a role as master neural regulators and turn into deleterious signalling cues during CLD and HCC.

3 | AXON GUIDANCE MOLECULES IN CLD

As in many organs during gastrulation, hepatic neurogenesis, although limited in utero, is synchronous to hepatic organogenesis.[1] Hepatic regenerative processes, which are reactivated during CLD and HCC, are functionally related to earlier developmental processes and are accompanied and regulated by neural remodelling.[8,15,39] These neural events are known to be regulated by the above mentioned semaphorins, slits, netrins and eph/ephrins[16–18] (Figure 2). Hepatic neural ablation highlighted the importance of autonomic innervation in both physiological and pathological contexts, including liver regeneration[2] which further emphasizes the role of neural factors in CLD. Both sympathetic and parasympathetic branches of the ANS influence nutrient metabolism, immune processes, haemodynamics, as well as bloodstream and hormone homeostasis. Their implication in liver repair and regeneration in the context of CLD, including cirrhosis, have been experimentally established.[15,40] Indeed, increased sympathetic neuropathy was observed in hepatic steatosis patients,[15] while sympathetic hyperstimulation progressively induced the metabolic syndrome, fatty liver disease and steatohepatitis in mice on normal diet, the latter being reversible by chemical sympathectomy.[41] In addition, increased activity of sympathetic fibres in the liver was reported in obese mice fed a high-fat diet, another model of fatty liver disease, and sympathetic disorganization was identified in steatotic mice fed a western diet.[42] Interestingly, this sympathetic overactivity was reversible, which suggests the possibility of therapeutic intervention as proposed in the prostate setting.[43]

Hepatic fibrogenesis is characterized by increased and altered deposition of ECM and represents the most common and pathogenic hallmark of CLD. Although several other cell types may also participate in the onset and development of liver fibrosis, hepatic stellate cells (HSC) are recognized as the primary cells responsible for ECM deposition, and are responsive to neural inputs,[10,44] suggesting a likely contributive if not causal impact of AGCs on the activation of HSCs, perhaps both ways.

3.1 | EPHs and ephrins as involved in hepatic viral and pro-fibrotic events

Although mostly expressed during development, the ephrin-A/ephA duos are reactivated in adulthood in pathological contexts. Indeed,

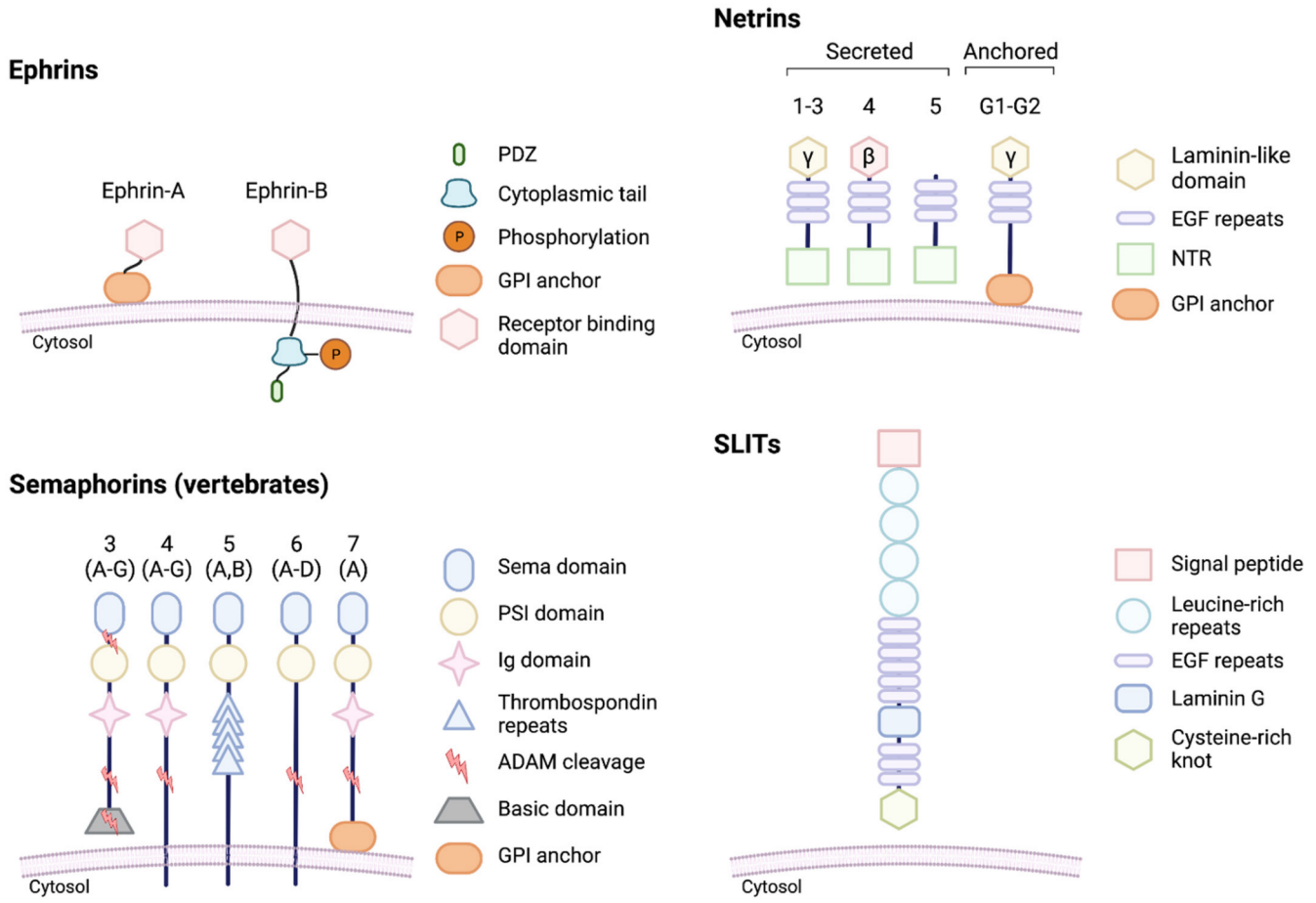

FIGURE 2 Structural features of human axonal guidance cues. Ephrins-A and -B encode for a receptor-binding domain linked to a GPI anchor or a transmembrane domain, respectively, ensuring their attachment to the plasma membrane. Secreted netrins encode for a laminin domain followed by three EGF-like repeats upstream of an NTR domain, endowed with zinc metalloproteinase inhibitory function. Vertebrate semaphorins encode for several common sequences across all types: their specific Sema domain linked to a PSI domain, a cysteine-rich module frequently found in extracellular fragments of signalling proteins, itself attached to a immunoglobulin-like C2-type domain. Other domains that are closer to the plasma membrane foster attachment to the 'mother' cell in most cases. SLITs encode for several leucine-rich repeats prior to several EGF-like repeats in which is intercalated a laminin G-like domain. A C-terminal cystine knot domain terminates the chain.

ephrins and ephs regulate fundamental biological processes, such as cell migration, myofibroblast activation, angiogenesis and tissue remodelling, which are involved in fibrosis. Increased expression of ephrin-B2 was observed in patients with cardiac,[45] kidney,[46] lung,[47] skin[48] and in malaria- and CCl_4-related liver fibrosis.[49,50] In the liver, platelet-derived growth factor signalling through ephrin-B2 encourages hepatic angiogenesis.[51] Ephrin-B2 was shown to modulate HSC activation in vitro and enhance pathological sinusoidal remodelling and portal hypertension in vivo by stimulating VEGF production by HSC.[49,50] Concomitantly, downregulation of ephrin-B2 in patients with nodular regenerative hyperplasia of the liver was observed.[52] These data introducing a possible proliferation repressive activity of this protein. Association of ephrin receptor A3 (*EPHA3*) gene polymorphism with susceptibility to chronic severe hepatitis B[53] was also identified, a dataset in accordance with the ability of ephrins to modulate T lymphocyte functions.[54–56] Finally, ephA2, regulated transcriptionally via the receptor TR4,[57] was identified as a host factor for the entry of hepatitis C virus,[58] providing evidence of the important role of this type of protein in chronic hepatitis C development, a major aetiology of CLD. Conversely, neural consequences of the increased occupancy rate of ephA2 by HCV particles are unknown.

3.2 | Netrin-1 as a near universally induced AGC in CLD

Netrin-1, besides its AGC status, was also characterized as interacting with a dependence receptor in cancer, that is a receptor transducing death signals when left unbound.[59] Several studies document the equivocal role of netrin-1 in the liver. Netrin-1 is the sole netrin member for which information is found in CLD. In primary biliary cholangitis, *NTN1* is correlated with immune response pathways that influence pathogenesis of primary biliary cholangitis,[60] though perturbation data were lacking in this study. In patients, netrin-1 was

upregulated and its receptor UNC5A was downregulated in HBV- and HCV-positive hepatic lesions at all stages of fibrosis and in HCC,[61,62] providing HCV with a feedforward loop, beneficial for both the virus and the related hepatitis.[61] A study showed the liver-protective and anti-inflammatory action of the neuroimmune netrin-1 through its receptor A2B.[63] Netrin-1 also attenuated hepatic steatosis via UNC5b/PPARγ-mediated suppression of inflammation and ER stress.[64] In non-steatotic conditions, hepatic inflammation triggered by viral and bacterial motifs elicits production of netrin-1 through an atypical mechanism, that is exclusive activation of translation, where netrin-1 displays macrophage-dependent pro-inflammatory activity.[65] Netrin-1 also protects hepatocytes against cell death through sustained translation during the hepatic unfolded protein response (UPR),[66] a hallmark of CLD that fosters, on a long-term basis resistance to cell death. Consistently, and in line with its neurotrophic status, netrin-1 promotes liver regeneration possibly by facilitating vagal nerve repair after partial hepatectomy in mice.[67,68] While netrin-1 seems to be induced, in several instances translationally through STAU1 and LARP1,[61,65] in a variety of liver conditions and in vivo models, it is still challenging to unequivocally assign to netrin-1 a specific impact on a chronic basis on liver histology itself, aside from its anti-apoptotic and regenerative roles, which could have carcinogenic consequences in genetically unstable and histologically injured livers. While in rare cases formally introduced as AGC in liver studies,[63] hepatic netrin-1 has to date mainly been considered as a mere pro-survival agent, following cancer-oriented views.

3.3 | Semaphorins as fibrotic regulators in CLD

Causal hepatic data on semaphorins are scanter than those on netrins, albeit in favour of a pathogenic role in CLD. SEMA3C, a new marker of HSC activation, was overexpressed in fibrotic patient livers and exacerbates TGF-β-mediated myofibroblast activation and liver fibrosis in vivo.[69] In addition, inhibition of its receptor NRP2 reduced liver fibrosis.[69] Interestingly, serum concentrations of SEMA3C, SEMA5A and SEMA6D were associated with pathogenesis of viral hepatitis and progression of fibrosis in HCV genotype 1 and 3-infected patients. Moreover, causal status of viral replication was assessed after antiviral treatments, and verified.[70] SEMA3C and SEMA5A were overexpressed in HCV cirrhotic liver and HCC tissues.[70] Of note, SEMA3E was showed to play a causal role in HSC activation, LSEC pathological regeneration, and the progression of liver fibrosis in CCl_4-treated mice.[71] Finally, SEMA7A was correlated with hepatic steatosis[72] and evolutive fibrosis in mice and patients.[73]

3.4 | Slits as profibrotic or angiogenic factors in CLD

SLIT2/ROBO1-2 couples were shown to be overexpressed in patients with liver fibrosis. ROBO2 was detected in septa of fibrotic livers and, interestingly, on the surface of HSCs in experimental models of fibrosis in vivo, where SLIT2 increases the expression of pro-fibrotic mediators, such as TGF-β1, CTGF, as well as collagen-1, a major ECM component accumulating in CLD, in vitro[74] and in vivo.[75] SLIT2, which activates HSCs in vivo,[76] also mediates profibrotic and angiogenic effects of liver myofibroblasts, the conditioned medium of which promoted survival of HSCs and tubulogenesis of endothelial cells.[77] Ductular reaction that promotes periductular fibrosis and inflammatory cell recruitment in CLD, also enhances intrahepatic angiogenesis through SLIT2-ROBO1 signalling.[78] Of note, as was the case with netrin-1 on inflammation[65] and SEMA3E on fibrosis,[71] an anti-ROBO1 neutralizing antibody was reported to inhibit the progression of liver injury and fibrosis in the mouse CCl_4 model.[75]

A summary of the functions of axon guidance molecules in liver pathology is provided in Table 1. Such data were enriched by a search performed on single cell RNAseq and IHC databases, indicating frequent RNA expression in fibroblasts (likely activated HSCs) and antibody-validated evidence for protein expression (Table 2). Of note, despite the wealth of available data described above, regulation of AGC transcripts was found limited to *SEMA4C* only upon comparison between chronic hepatitis and normal livers (GSE89377 dataset, gathering a continuum of samples from normal liver to HCC), suggesting that frequent and unadressed post-transcriptional regulations may account for this unexpected result. Taken together, datasets pertaining to all four classes of AGCs highlight their HSC activating and ECM participating roles, and prompt for further investigations on their targetability in CLD in the clinic.

4 | AXON GUIDANCE MOLECULES IN HCC

AGCs triggered interest as cancer contributors and anticancer targets for the first time in the years 2000. Many AGCs were also shown to control the development of the vasculature and may thus control angiogenesis in tumours,[96] even prompting a general review on the topic, entitled 'The brain within the tumor'.[97] Given their implication in physiological and pathological conditions in various organs, neuronal guidance molecules have thus been the focus of research in oncology,[98,99] as well as in HCC through the studies depicted below. However, as already mentioned, their original functions remain seldom considered in this field, in which theoretical prerequisites linking AGCs to cancers tend to be more directly cell death-related.[98]

4.1 | EPHs and ephrins as HCC aggressiveness factors

The 'eph' receptor tyrosine kinase was identified from a HCC cell line in 1987.[100] EphA1, subject to ADAM12-mediated cleavage,[101] and ephA2 were found overexpressed in HCC patient samples and associated with the absence of tumour capsule, portal vein invasion, lower differentiation,[63] advanced TNM stage and poor prognosis of HCC[102] as did ephrin-A3.[82] Ephrin-A1 expression gradually

TABLE 1 Functions of AGCs in liver pathology.

AGC name	Context	Function and experimental model	References
Ephrin-B2	CLD	Augments malaria- and CCl_4-related liver fibrosis	49,50
	CLD	Modulates HSC activation in vitro	49,50
	CLD	Enhances pathological sinusoidal remodelling and portal hypertension in vivo in malaria- and CCl_4-related liver fibrosis	49,50
EphA2	CLD	Entry factor for HCV in vitro	58
Netrin-1	CLD	Enhances HCV entry and viral translation in vitro	61
	CLD	Participates in resolution programs of liver inflammation in the mouse zymosan model	68
	CLD	Attenuates hepatic steatosis in high fat diet fed mice	64
	CLD	Displays macrophage-dependent proinflammatory activity in TLR-driven hepatic acute inflammation in mice	65
	CLD	Protects hepatocytes against cell death during the UPR in tunicamycin-treated mice	66
	CLD	Promotes liver regeneration after partial hepatectomy	68
SEMA3C	CLD	Exacerbates TGF-β-mediated myofibroblast activation and liver fibrosis in CCl_4/high-fat diet/fructose-palmitate-cholesterol mice	69
SEMA3E	CLD	Activates HSC, LSECs regeneration, and progression of liver fibrosis in CCl_4-treated mice	71
SLIT2	CLD	Increases the expression of pro-fibrotic mediators, such as TGF-β1, CTGF, as well as collagen-1 in vitro and in CCl_4-treated mice	74,75
	CLD	Activates HSCs in bild duct-ligated mice	76
	CLD	Promotes survival of HSCs	77
SLIT2-ROBO1	CLD	Enhances liver injury and fibrosis in the CCl_4 model	75
	CLD	Enhances hepatic angiogenenesis upon ductular reaction triggered in mice by DDC	78
Ephrin-A1	HCC	Contributes to several malignant characteristics of HCC cells in vitro	79
	HCC	Promotes SDF-1 secretion and recruitment of endothelial progenitor cells to HCC in coculture and HCC cells xenografts	80
Ephrin-A2	HCC	Promotes tumorigenicity via NF-κB in HCC cells xenografts	81
Ephrin-A3	HCC	Enhances cancer stemness in hypoxic HCC cells	82
Ephrin-A5	HCC	Sustains the viability of HCC cells via MAP kinases in HCC cells xenografts	83
Ephrin-B1	HCC	Neovascularization in HCC cells xenografts	84
EphA2	HCC	Enhances invasion of HCC cells	85
Netrin-1	HCC	Skews hepatic UPR towards pro-survival outcomes in vitro	86
	HCC	Migration of liver cancer cells in 3D cell culture	87,88
	HCC	Induces epithelial-mesenchymal transition of HCC cells in vitro	88
SEMA3A	HCC	Promotes HCC growth, invasion, and metastasis in HCC cells xenografts	89
	HCC	Macrophage-mediated angiogenesis and growth in HCC cells xenografts	89
SEMA3D	HCC	Impedes growth through inactivating PI3K/AKT signalling in HCC cells xenografts	90
SEMA3F	HCC	Promotes HCC propagation by activating focal adhesion pathway	91
SEMA4C	HCC	HCC xenograft promoting via lncRNA CYTOR	92
NRP1 (SEMA receptor)	HCC	Tumour growth- and vascular remodelling-promoting role in SV40 HCC-bearing transgenic mice	93
ROBO1 (SLIT receptor)	HCC	Promotes tumour angiogenesis and tumour growth in HCC cells xenografts	94
SRGAP2	HCC	Trigger EMT in vitro	95

Abbreviations: AGCs, axonal guidance cues; CLD, chronic liver disease; HCC, hepatocellular carcinoma.

increases from normal liver to cirrhosis to HCC. It is strongly correlated with α-foetoprotein ($r=0.87$, a value rarely encountered in pathology).[79] Ephrin-A1 also contributes to several malignant characteristics of HCC cells.[79] Ephrin-A1-induced EphA1 activation was reported to promote SDF-1 secretion and chemotaxis of endothelial progenitor cells to HCC through the SDF-1/CXCR4 signalling pathway. Perturbation studies abolished tube formation in vitro and decreased tumour size and angiogenesis due to inhibition of endothelial progenitor cell homing to the tumour tissue.[80] Ephrin-A1/ephA1 inhibition in vitro attenuated neo-angiogenesis

TABLE 2 AGC/AGC receptors expressing cell types and their regulation from cirrhosis-to-HCC. Data were retrieved from the Single Cell RNAseq portal (Broad Institute), the ProteinAtlas database (Karolinska Institute) and the publicly available GEO bulk RNAseq dataset GSE124535 ($n = 35$ F4/HCC pairs, Log_2 fold-changes of TPM normalized values are shown after GEO2R analyses using default settings). For IHC, ProteinAtlas staining certainty scores increase from *uncertain* to *supported* to *approved* to *enhanced*.

AGC and receptor names	Positivity by ssRNAseq in CLD	Evidence for protein signal in HCC	Frequency of medium or strong staining in HCC by IHC	Expression changes (F4 to HCC) Log_2FC; $p_{adj} < 0.05$
EphA2	Hepatocytes, cholangiocytes	Analysis in progress	N/A	+0.9
Ephrin-A1	Hepatocytes, cholangiocytes, endothelial cells, fibroblasts, smooth muscle cells	Approved	1/12	ns
Ephrin-A2	Hepatocytes, cholangiocytes	Analysis in progress	N/A	ns
Ephrin-A3	Hepatocytes, immune cells	Uncertain	11/12	+0.67
Ephrin-A5	Cholangiocytes, T cells, smooth muscle cells	Approved	7/11	ns
Ephrin-B1	Endothelial cells, fibroblasts	Uncertain	11/11	ns
Ephrin-B2	Endothelial cells, fibroblasts, smooth muscle cells	Supported	9/23 Discrepancies between tested antibodies	+0.5
Netrin-1	Hepatocytes, smooth muscle cells	Approved	3/11	ns
NRP1 (SEMA receptor)	Fibroblasts, endothelial cells, macrophages	Approved	14/23	ns
ROBO1 (SLIT receptor)	Fibroblasts, endothelial cells, macrophages	Uncertain	7/11	+2.8
SEMA3A	Smooth muscle cells	Approved	12/12	+1.8
SEMA3C	Macrophages, Kupffer cells, B cells	Analysis in progress	N/A	+1.1
SEMA3D	Smooth muscle cells	Uncertain	0/12	+2.1
SEMA3E	Cholangiocytes, macrophages, B cells, endothelial cells	Uncertain	12/12	ns
SEMA3F	Endothelial cells, smooth muscle cells, fibroblasts	Approved	5/12	+0.78
SEMA4C	All liver cell types	Approved	3/12	ns
SLIT2	Endothelial cells, fibroblasts, cholangiocytes, Kupffer cells	Uncertain	18/34	+0.73
SRGAP2	All liver cell types	Approved	9/20	+0.45

Abbreviations: AGC, axonal guidance cue; HCC, hepatocellular carcinoma; ns, non-significant.

and tumour growth in HCC in vitro and in vivo.[80] The ephrin-A3/ephA2 axis is known to regulate cellular metabolic plasticity to enhance cancer stemness in hypoxic HCC. Mechanistically, the ephrin-A3/ephA2 axis promote maturation of the SREBP1 transcription factor. Expression of its target, ACLY, was significantly associated with the expression of ephrin-A3 and hypoxia markers in clinical cohorts.[82] Conversely, the TR4 nuclear receptor suppresses HCC cell invasion via downregulating EphA2 expression.[85] Analysis of tumour tissue from 304 patients with HCC having undergone surgical resection generated a methylation-based prognostic signature in which ephrin-B2 was also considered a candidate 'epi' driver of HCC,[103] although causality was not checked. Ephrin-A2 promotes tumorigenicity through the Rac1/Akt/NF-κB signalling pathway[81] and is a potential biomarker for HCC.[63] Ephrin-A5, conditioned by miR-96 and -182 regulation,[104] participates in a kinase complex that sustains the viability of HCC cells through downstream protein kinase B-dependent, extracellular signal-regulated kinase-dependent, and p38-dependent signalling pathways.[83] Peritumoral small ephrin-A5 isoform level predicts postoperative survival in hepatocellular carcinoma,[105] suggesting that subtle isoform complexities may enrich the diversity of actions of AGCs. Finally, expression of ephrin-B1 in HCC cells enhanced in vivo aggressiveness and endothelial migration and proliferation in vitro,[84] an interesting finding due to HCC's targetability by VEGF inhibitors and susceptibility to microvascular invasion, a strong and adverse event.

4.2 | Netrin-1 as an anti-apoptotic factor in HCC

Netrin-1 is the sole netrin member for which information is found in HCC. Interestingly, data featuring cirrhosis- and HCC-associated depletion of the netrin-1 and the death receptor Uncoordinated Phenotype-5A (UNC5A)[62] also featured its causal role in skewing the hepatic UPR towards pro-survival outcomes.[86] Netrin-1 promotes the collective migration of liver cancer cells in a 3D cell culture model[87] as well as the promotion of cell migration and invasion by down-regulation of BVES (a novel adhesion molecule enabling tight

junction formation) expression in human HCC cells.[106] Finally, while able to protect hepatocytes from cell death upon the UPR, a HCC relevant phenomenon, as depicted earlier,[66] netrin-1 also induces epithelial-mesenchymal transition and promotes invasiveness of HCC cells[88] in a context where the epidermal growth factor receptor (EGFR) is also activated by netrin-1 following HCV infection.[61]

Although some occasional data pertaining to netrin-1 in cancer may identify this factor as endowed with anticancer properties,[107] all of the literature in the liver indicate that netrin-1 and its receptors (through their inhibition[59] as dependence receptors) promote or aggravate the pathogenic features of HCC cells. Nevertheless, to our knowledge, documented clinical correlative data and in vivo causal data determining the actual ability of netrin-1 to trigger similar phenotypes are to date lacking.

4.3 | Semaphorins as pro- or anti-HCC players

Apart from ephrins and netrin-1, semaphorin-3A seems to represent the only well documented pathogenic type of semaphorin with respect to HCC. However, other semaphorins such as semaphorin-3B and 3F seem to bear antitumoral properties, suggesting functional switches between them during disease progression. The semaphorin receptor neuropilin-1 (NRP1) is upregulated in HCC compared to normal liver tissue in a miR-148b-dependent manner.[108] To evaluate the therapeutic potential of targeting NRP1 in HCC, SV40-driven HCC transgenic mice were treated with peptide N, an NRP1-binding recombinant protein and competitive inhibitor, enabling the authors to demonstrate that this semaphorin receptor promotes tumour growth and vascular remodelling.[93] The expression of SEMA3A was also elevated in HCC patients and conditioned by the microRNA miR-192-5p.[109] SEMA3A promotes HCC growth, invasion, and metastasis in vivo, by increasing galectin-3, enolase-2 and EpCAM.[89] In another study, SEMA3A was shown to promote tumour progression and was identified as a factor of poor prognosis.[110] Importantly, SEMA3A also binds to NRP-plexA receptor complexes on tumour-associated macrophages, guiding them to hypoxic regions of the tumour, where they further promote angiogenesis and tumour growth.[89] This phenomenon was notably reversed via *SEMA3A* RNA interference or macrophage-specific NRP inactivation in vitro and in vivo in lung cancer.[111] A study demonstrated that SEMA3D was able to restrain the progression of HCC by inactivating PI3K/AKT signalling.[90] In an in silico study using the TCGA cohort, *SEMA3F* was correlated with invasiveness, metastasis and activation of focal adhesion kinase transcripts,[91] while SEMA4C was instrumental to the HCC-promoting lncRNA CYTOR to exert its pathogenic role.[92]

4.4 | Slits are candidate contributors to HCC through their ROBO receptors

Few causal orthotopic data exist on SLIT proteins in HCC, prompting the authors to consider their ROBO receptors. ROBO1 is overexpressed in HCC patient samples compared to normal and adjacent peri-tumoral tissues, with a strong enrichment in poorly differentiated HCC compared to well-differentiated and moderately differentiated samples.[112] ROBO1 promotes tumour angiogenesis and tumour growth in HCC in vivo and its targeting with a neutralizing monoclonal antibody showed anti-tumour activity in a HCC xenograft model.[94] Considering now SLIT, under epigenetic regulation in HCC,[113] and ROBO GTPase-activating proteins (SRGAPs), one study reported their strong correlation with HCC onset in public databases, as well as its ability to trigger EMT in vitro.[95]

Although warranting more basic investigations on the role of AGC in HCC, these informations are in favour of a pathogenic reactivation of AGC-related processes in primary liver cancer. Causally identified functions of axon guidance molecules in HCC are provided in Table 1. Most pathophysiologically relevant data are depicted in Figure 3. Cells of origin of AGCs or of their receptor(s) together with experimental evidence for protein expression and localization in liver tissues are provided in Table 2 using publicly available databases and datasets.

5 | AXON GUIDANCE MOLECULES AS THERAPEUTIC TARGETS IN CANCER: POTENTIAL AVENUES IN HCC

Analysing cirrhosis/HCC paired tissues, the majority of AGCs and receptors transcripts, except Ephrin-A5, were found up-regulated from F4 to HCC (Table 2, GSE124535 dataset). Also, in HCC, data mining from the *Protein Atlas* database indicates that a majority of axon guidance receptors are associated with a significantly unfavourable prognosis at 5-year, whereas the ligands are correlated to both favourable and unfavourable prognoses (Figure 4). While this difference in prognosis orientation of ligand and receptors deserves further investigations, it can be hypothesized that tumour-derived functional modulation of receptors of AGCs could participate in subverting the activities of such ligands, which advocates for their future study and eventual targeting in HCC.

CLD and HCC represent highly heterogeneous diseases leaving researchers and clinicians with currently moderate hope for breakthroughs in patient stratification enabling treatment of patients with pertinent drugs. The liver is innervated by afferent and efferent nerves. Axon guidance molecules mediate, *in fine*, CNS-liver interactions both ways through modulation of the hepatic ANS. These cellular/tissular structures linking the general pathophysiology of the patient with HCC may be of interest, as they are patient-specific and may allow novel ways of defining stratification criteria. Several recent studies highlighted the relevance of studying cancer neurosciences of peripheral organs. In that context, pathological innervation and ANS involvement or dysregulation have been identified in ovarian, prostate, gastric, pancreatic and lung cancers,[114–119] nurturing cancer cells and tumour microenvironment and conferring stronger tumorigenic properties. Ephrins (NCT02252211 and NCT04180371 in ephrin-A2 positive cancer patients, NCT03076372 in a panel of

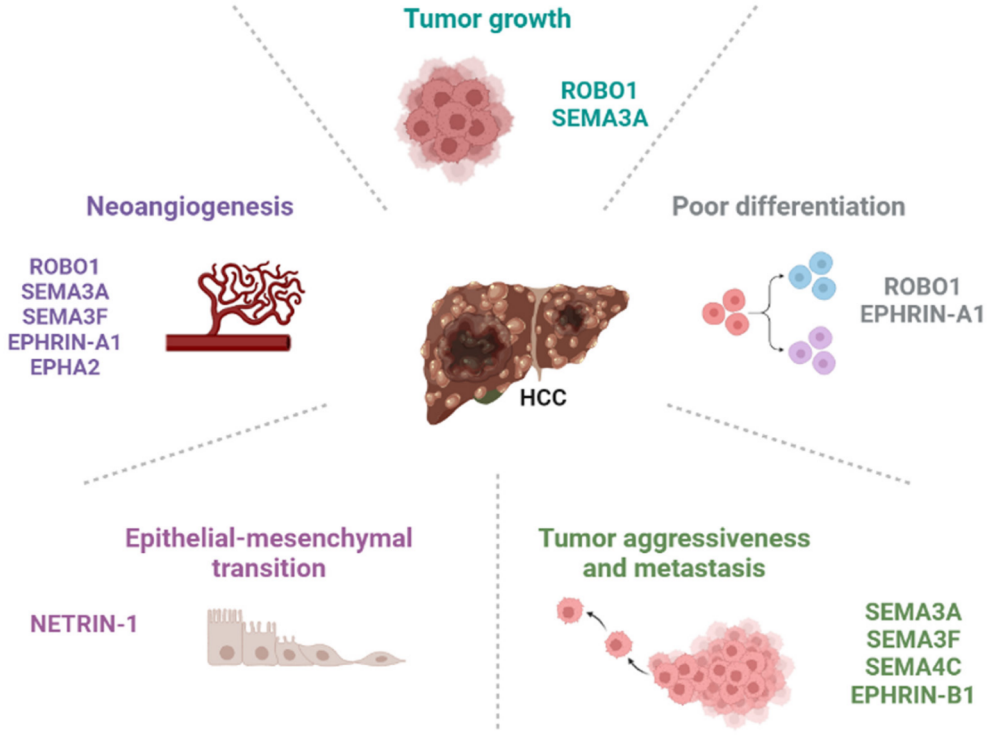

FIGURE 3 Main HCC-relevant processes regulated by axonal guidance cues.

solid tumours, NCT02575261 in glioma patients, NCT00364780 in lung cancer patients), netrin-1 (NCT02977195 in another panel of solid tumours) and semaphorins (NCT04573543, NCT02820285 in NASH) have been considered as potential targets in several clinical trials. SLIT targeting does not seem to have entered clinical trials so far. The reason for which no data pertaining to clinical trials on most axonal guidance cues and HCC could be identified so far is unknown. Hence, one can propose that in addition to their known impact on tumour cells, HCC data and current clinical trials targeting ephrins, netrin-1, semaphorins and SLITs in other conditions than HCC would also benefit from being reinterpreted in light of their ability to target them as axon guidance molecules in the next future.

6 | FUTURE PERSPECTIVES

Despite growing evidence on the likely pathogenic involvement of guidance cues in the liver, little is known about how they influence their environment.

To provide a better comprehensive overview of AGC impact on fibrosis, CLD-to-HCC transition and HCC progression a panel of studies will be needed to (i) elucidate the ligand/receptor couples at play in various pathological contexts, (ii) identify the cell types targeted by the different cues as well as their cell of origin, (iii) assess the consequences of changes in their expression in in vivo models pertinent to the current changes in the epidemiology of CLD, (iv) determine their cognate signalling pathways, and (v) examine the cross-talk between the main representatives of the four axon guidance families. Interestingly, most ligands and receptors show prognostic value. Therefore, they should be studied in large cohorts of CLD and HCC patients, in correlation with disease stage, liver function and clinical outcome, if possible at the protein level given the secreted character of ligands and hence their likely post-transcriptional regulation.

Datasets described herein indicate that most axon guidance molecules are likely pathogenic in CLD and HCC. Linking their historical functions of neural regulators and spatiotemporal orienteers of neurons to the phenotypes caused by these proteins in the liver may open new avenues for more integrated research. This review raises questions such as: Do AGCs mediate or dampen neural control of the diseased liver? Is this neural control pathogenic or compensatory with respect to liver disease progression? What are the neural targets of each class of AGCs—namely, do they preferably interact with sympathetic or parasympathetic branches of the liver ANS?

Potentially novel therapies targeting either axon guidance ligands or receptors are mostly being studied at early clinical phases I and II, evaluating their tolerance and safety profile. More data are therefore expected to arise from such studies focusing on these intriguing molecules that constitute the interface between neurons and their liver parenchymal targets, which are seldom recognized as such in hepatology.

AUTHOR CONTRIBUTIONS

Ievgeniia Chicherova provided initial drafts of the main text and of some figures. **Charlotte Hernandez** enriched the manuscript and

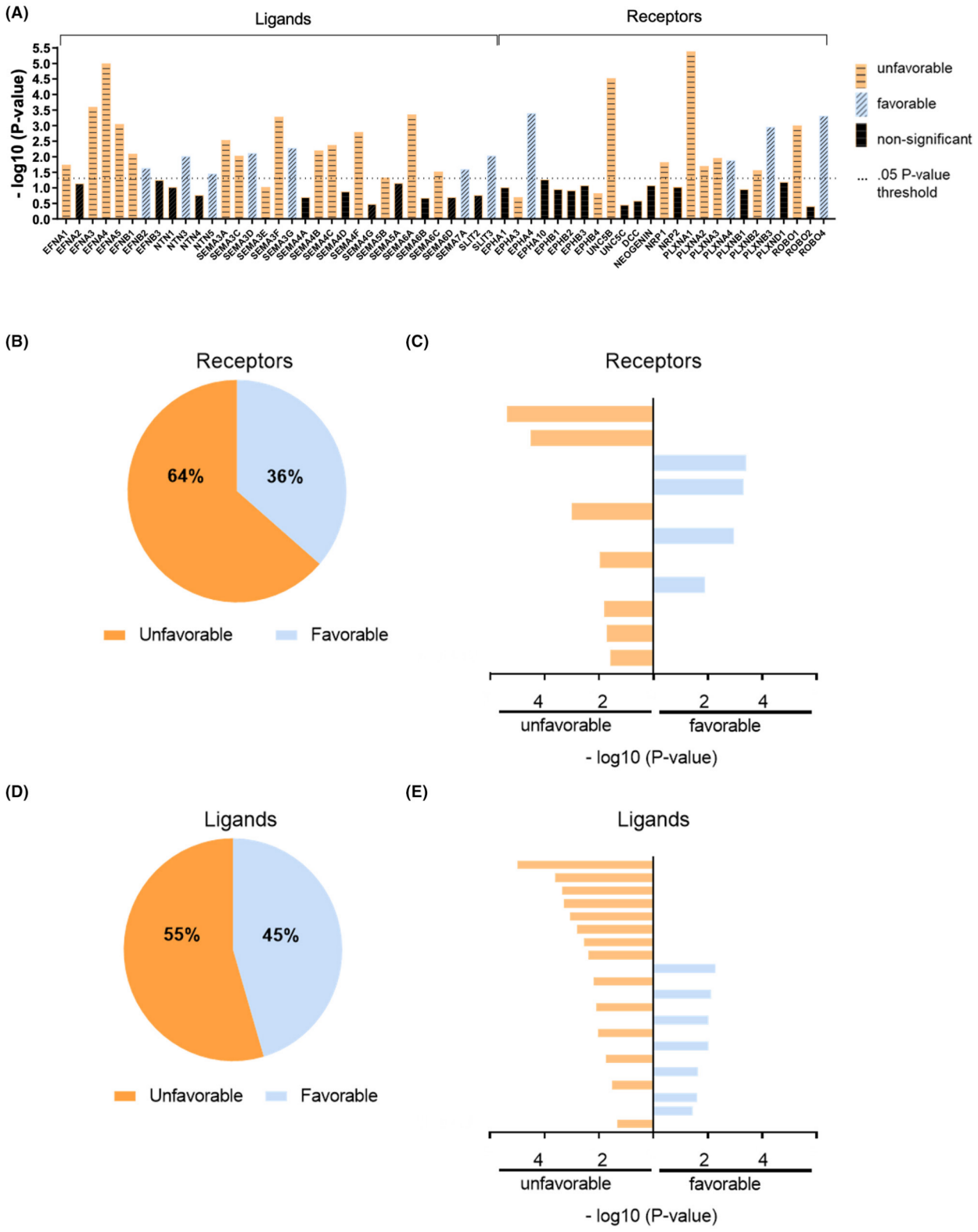

FIGURE 4 Prognostic orientation and associated value of axonal guidance cues (AGCs) transcripts in HCC TCGA RNA samples and *Protein Atlas* data samples. (A) AGCs and and AGCs receptors transcripts prognostic values at 5-year mortality rate (FPKM levels, Log-rank test, $p < 0.05$, derived from TCGA data). (B, D) Distribution of unfavourable and favourable prognostic markers. (C, E) Associated degree of certainty (p value) of favourable and unfavourable AGCs. Were included only statistically significant markers. Panels (B–E) were derived from www.proteinatlas.org (accessed 7 February 2023).

provided additional figures. **Fanny Mann** provided specific neural expertise throughout. **Fabien Zoulim** provided clinical expertise throughout and critically amended the manuscript. **Romain Parent** designed the study, co-wrote and finalized the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial or private relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study were derived from the following resources available in the public domain: Protein Atlas, https://www.proteinatlas.org—Gene Expression Omnibus, https://www.ncbi.nlm.nih.gov/geo/.

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