Chemogenetic approaches to unravel circuit wiring and related behavior after spinal cord injury

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1. Introduction

The spinal cord, together with the brain, forms the central nervous system (CNS) which controls all body parts through a wide variety of neuronal pathways and connections. Proper connectivity of these neurons is established early on during development through synaptic pruning which ensures that only functional connections remain in place. In the spinal cord, these neuronal connections transmit descending motor information to the body and ascending sensory information to the brain. After spinal cord injury, many spinal tracts are interrupted and fail to regenerate, often leading to loss of motor and sensory input below the lesion site (Wilson, Cadotte, and Fehlings, 2012). To regain motor functionality or sensation, current research aims at improving the regenerative capacity of the CNS or at boosting the formation of detour pathways via systemic ligand injections. Apart from neurons, non-neuronal cells such as microglia and astrocytes also contribute to the formation of neuronal connections (Bar and Barak, 2019; Farhy-Tselnicker and Allen, 2018; Matejuk and Ransohoff, 2020; Perez-Catalan, Doe, and Ackerman, 2021; Reemst, Noctor, Lucassen, and Hol, 2016; Szepesi, Manouchehrian, Bachiller, and Deierborg, 2018; Wake and Miyamoto, 2013). Here again, chemogenetic tools are advantageous to manipulate distinct cell types as a means to understand their exact contribution to circuit formation. This review provides an overview on commonly used chemogenetic approaches based on metabotropic receptors and their use to improve our understanding of circuit wiring following spinal cord injury.
2. Chemogenetic strategies

The development and optimization of a variety of genetically encoded tools has facilitated neuroscience research and has contributed tremendously to our understanding of neuronal circuitry. Chemogenetic strategies, allowing researchers to intervene with cellular activity, are a valuable tool not only to understand the formation of neural networks during development, disease or after injury but also to understand causal relationships between neuronal networks and behavior (Alexander et al., 2009; Atasoy, Nicholas Betley, Su, and Sternson, 2012; Becnel et al., 2013; Garner et al., 2012; Krakauer, Ghanzanfar, Gomez-Marín, Maclver, and Poopepl, 2017; Krashes et al., 2011a; Roth, 2016; Whissell, Tohyama, and Martin, 2016). Chemogenetic proteins are engineered macromolecules that have the capacity to regulate cellular signal transduction and are therefore capable of activating or silencing neurons in vivo upon the systemic delivery of small molecules. Current chemogenetic tools are modified ligand-gated ion channels, kinases, non-kinase enzymes but most commonly used chemogenetic constructs are based on native G protein-coupled receptors (GPCRs) (Alexander et al., 2009; Armbruster, Li, Pausch, Herlitze, and Roth, 2007; Armbruster and Roth, 2005; Roth, 2016; Vardy et al., 2015; Wacker, Stevens, and Roth, 2017). Using random and site-directed mutagenesis, these macromolecules can no longer bind their endogenous chemical actuators, but rather bind synthetic molecules which can be administered in a non-invasive manner. These receptors then only activated by high affinity exogenous compounds and these ligands should not bind to other native receptors. As these two conditions are crucial to exclude any off-target effects, the development and optimization of new chemogenetic constructs has mainly aimed at developing new receptors with increased potency and specificity of exogenous ligands.

2.1. Development of chemogenetic constructs

Because of the regulatory role of GPCRs on neuronal signal transduction and gene regulation, their molecular structure served as a primary base for the development of functional chemogenetic proteins (Allen and Roth, 2011). In 1991, Strader et al. (Strader et al., 1991), developed the first chemogenetic construct by substituting a single amino acid residue on the β2-adrenergic receptor. This mutation ensured that the new receptor lacked the ability to bind endogenous adrenaline but could bind to the synthetic molecule L-185,870. Due to the low potency of the ligand to activate the receptor, a second generation of chemogenetic constructs were introduced by Coward et al. (Coward et al., 1998). RASSLs (Receptors Activated Solely by a Synthetic Ligand) demonstrated an improved activation potential by exogenous ligands with limited interaction of the receptors with endogenous ligands. The synthetic compounds used for activation of RASSLs, such as spiradoline - for the engineered inhibitory k-opioid receptor - were however non-specific as they were also binding to the endogenous receptors leading to various off-target effects (Coward et al., 1998; Vonoigtlander and Lewis, 1988). This made it nearly impossible to detect any cell-specific effects or causal relationships with behavioral outcomes (Conklin et al., 2008). Thus, a third generation of chemogenetic tools has been established called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), that are used in combination with potent ligands exhibiting reduced endogenous activity (Alexander et al., 2009; Armbruster, Li, Pausch, Herlitze, and Roth, 2007; Armbruster and Roth, 2005). DREADDs are currently the most commonly used chemogenetic constructs, commercially available in a variety of plasmids, viral constructs and transgenic mice, facilitating their use in a wide spectrum of (neuro)scientific applications (Alexander et al., 2009; Farrell et al., 2013; Zhu et al., 2014).

2.2. DREADD-based constructs

Commonly used DREADD-based constructs are derived from the human muscarinic receptor and K-opioid receptor which are activated by clozapine-N-oxide (CNO) or salvinorin B respectively (Fig. 1) (Conklin et al., 2008; Vardy et al., 2015). Both ligands are inert metabolites derived from the antipsychotic drug clozapine and psychoactive compound salvinorin A. DREADDS can be classified based on their association with the G protein complex consisting of Gαs, Gαi and Gq proteins. Upon ligand binding to a GPCR, a conformational change allows the GPCR to function as a guanine nucleotide exchange factor, substituting bound GDP to GTP on the associated Gαi subunit. Gq then dissociates from Gβγ to alter intracellular signaling pathways. Depending on the type of Gαi subunits the GPCR interacts with (for DREADDs Gi, Gq, and Gs), distinct pathways can be activated (Farrell and Roth, 2013).

2.3. Activating DREADDs Gq and Gi

Excitatory Gq-coupled DREADDS (hM1Dq, hM3Dq, hM5Dq) or less commonly used Gi-coupled DREADDs refer to DREADD constructs that activate Gq or Gi signaling respectively. Dissociation of both G proteins upon ligand binding of the GPCR, results in distinct cellular signal transduction. Gq activation leads to increased phospholipase C, cleaving its substrate phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (Berridge, 1984; Epand, 2017). Formation of secondary messenger IP3 which diffuses to the cytosol consecutively leads to the release of intracellular calcium stores as it binds calcium channels on the endoplasmic reticulum. DAG on the other hand remains at the plasma membrane and activates protein kinase C (PKC), inducing additional signaling pathways. Ultimately, their combined effects lead to increased neuronal firing (Alexander et al., 2009; Armbruster, Li, Pausch, Herlitze, and Roth, 2007). The exact working mechanism of Giq DREADDs are however complex and still not entirely untangled (Atasoy and Sternson, 2018). Gs signaling is linked to separate intracellular signaling pathways. Upon activation, Gi leads to activation of adenylate cyclase, followed by increased cyclic adenosine monophosphate (cAMP), consecutive activation of multiple ion channels and protein kinase A (PKA), finally altering gene expression (Farrell et al., 2013; Guettier et al., 2009a). Activation of Giq and Gicoupled DREADDs does therefore not directly induce neuronal firing but rather lowers the threshold for propagating action potentials.

2.3.1. Inhibitory DREADDS Gi and KORD

Inhibitory Gi-coupled DREADDS such as hM4Di and KORD, depending on a muscarinic or K-opioid backbone respectively, are efficient in silencing neurons. Gi DREADDs were developed by mutating homologous residues of hM3Dq in hM4Di and hM2Di (Armbruster, Li, Pausch, Herlitze, and Roth, 2007; Armbruster and Roth, 2005). Reduced electrical activity is achieved by decreasing cAMP production and activation of G protein inward-rectifying potassium (GIRK) channels, ultimately followed by membrane hyperpolarization. Apart from inhibiting electrical activity, hM4Di DREADDS are shown to inhibit synaptic release from axonal projections which would thus be the main mode of silencing neuronal activity (Armbruster, Li, Pausch, Herlitze, and Roth, 2007; Stanchniak, Ghosh, and Sternson, 2014). Apart from the human muscarinic receptor, other GPCRs can also be a basis to the development of new DREADDs. In particular, the development of KORD, was a new milestone in chemogenetic research. Aside from its capabilities to inhibit neuronal electrical activity and synaptic release, it allows bidirectional control of cell populations as it allows activation, using CNO, and inhibition, using salvinorin B, of neuronal subpopulations in a single animal (Vardy et al., 2015). To this end however, it should be noted that both ligands display distinct temporal activation patterns in vivo. Systemic administration of CNO will lead to increased neuronal firing within 5–10 min, with peak activity around 45 min, but exhibits lasting effects up to 9 h post administration (Alexander et al., 2009; Guettier et al., 2009a). Salvinorin B on the other hand inhibits neuronal activity fast, within several minutes and maintains its inhibiting activity for only up to an hour due to the short half-life of Salvinorin B in vivo.
Fig. 1. Overview of the most commonly used DREADD constructs and their respective ligands. The three main DREADD constructs are based on the human muscarinic (hM) and kappa-opioid receptor (KORD), activated by clozapine or clozapine-N-Oxide (CNO) and salvinorin B (SALB) respectively. Binding of the ligand initiates an intracellular signaling cascade through Gq coupling (left panel), or Gi coupling, leading to neuronal silencing (right panel). PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-biphosphate; DAG, diacylglycerol; IP3, inositol-1,4,5-triphosphate; PKC, protein kinase C; GIRK, G-protein coupled inwardly rectifying potassium channel; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A.

(Vardy et al., 2015).

2.4. Benefits of chemogenetic tools in vivo

To modulate cellular activity in vivo, two other regularly used methods, apart from chemogenetic tools, can be employed. On the one hand, electrostimulation, a technique that directly alters neuronal activity by external electrical stimulation, can be implemented and on the other hand, optogenetic constructs, based on photosensitive proteins, can be employed (Boyden, Zhang, Bamberg, Nagel, and Deisseroth, 2005; Deisseroth, 2015; Gradinaru, Thompson, and Deisseroth, 2008; Grosenick, Marshel, and Deisseroth, 2015; Ponce, 2014). While electrostimulation directly stimulates the targeted cells surrounding the electrode, optogenetics is a genetic approach based on light sensitive ion channels (rhodopsins) which can be introduced to subpopulations of neurons, similar to chemogenetic tools. When these neurons are subsequently illuminated by specific wavelength, the channels open leading to depolarization. As each of these techniques has its own benefits and limitations, they can often be used as complementary tools to validate a specific research question. Here, we discuss the main benefits of implementing chemogenetic strategies in vivo.

2.5. Spatial resolution

As a genetically encoded tool, expression of DREADDs in vivo can be directed towards distinct cellular populations using gene-based promoters, or local injections targeting anatomical projections. Many distinct promoters have been tested that allow expression of proteins in specified cell types, human synapsin (hSyn) is often used for targeting neurons while for instance glial fibrillary acidic protein (GFAP) can be implemented to guide expression in astrocytes (Sjulson, Cassataro, Dagupta, and Miesenböck, 2016). When probing the formation of novel circuitry patterns or their causal relationship with animal behavior, expression based on marker genes might not provide sufficient accuracy. To this end, combined viral approaches (also using retrograde viruses e.g. retroAAV) with cre recombinase-expressing vectors or mice provide an easy way to manipulate specific cells based on anatomical projections. Specifically, for the complex but spatially organized circuitry in the spinal cord, this combinatorial approach can be used to deliver DREADDs only to subsets of motor or sensory tracts, to distinct anatomical areas or even different classes of propriospinal neurons (Bradley et al., 2019; B. Chen et al., 2018; Engmann et al., 2020; Hilton et al., 2016; Wang, Maunze, Wang, Tsoufas, and Blackmore, 2018). The development and commercialization of DREADD constructs in several AAV serotypes and generation of floxed DREADD mice further facilitated the implementation of DREADDs to fully understand CNS circuitry (Akhmedov et al., 2017; Scioli et al., 2016; Zhu, Olsen, Swearingen, and Roth, 2016). Finally, even more advanced CNS circuitry-related questions can be addressed by using local CNO injections in order to target anatomic projections of neurons rather than systemic administration (Mahler et al., 2014; Stuchnik, Ghosh, and Sterrinson, 2014). Especially for inhibitory DREADDs, which silence neurons by inhibiting synaptic release, local CNO application along the axon can bring new information about neuronal wiring. Optogenetic strategies also allow precise manipulation with cellular resolution but require an optical probe close to the modulation site and is therefore not ideal when stimulating larger or multiple regions simultaneously. Electrostimulation on the other hand lacks the capacity to modulate neurons with cellular precision as it modulates all neurons and non-neuronal cells located within the applied electric field around the electrode. This potential off-target effect also poses a great limit for the use of functional electrostimulation (Davis and Gaitanis, 2020; Liu et al., 2018; Singh and Richmond, 2000; Turner, Loeser, Deyo, and Sanders, 2004).

2.5.1. Non-invasive ligand administration

Since the activating ligands for chemogenetic receptors can be administered systemically, chemogenetic control is considered to be non-invasive. Compared to other techniques such as electrostimulation and optogenetic control, where either an electrode or optical probe has to be inserted, the ease of activating cellular subpopulations with chemogenetics tremendously facilitates neuromodulation. In particular when unravelling cell populations or circuitry in deeper tissue layers or large and topical distinct anatomical regions have to be activated or silenced, chemogenetics are the primary choice as this is often not feasible utilizing electro- or optogenetic activation. Apart from these considerations, chemogenetic control also allows for chronic activity
modulation. As systemic administration is non-invasive, cell populations can be silenced or activated multiple times without the need for surgical interventions.

2.5.2. Modulation of distinct cell types

Apart from stimulating or silencing neuronal populations, DREADDs can also be used in a wide variety of non-neuronal cells as they alter intracellular pathways rather than relying on electrical excitability. DREADDs have been used in an extensive range of applications from hepatocytes, pancreatic β-cells, T-lymphocytes, breast cancer cells and many more (Guettier et al., 2009b; Jain et al., 2013; Li et al., 2013; Park et al., 2014; Yagi et al., 2011). More interestingly related to this review, however, is the modulation of non-neuronal cells that are a crucial part of the spinal cord such as astrocytes and microglia. DREADDs have been extensively used in the CNS to alter intracellular signaling of both cell types in vivo and are major contributors for neuronal circuit formation and maintenance (Agulhon et al., 2013; Bonder and McCarthy, 2014; Bull et al., 2014; Grace et al., 2016; Phillijens, Turnbull, Thedy, Moon, and Kim, 2020; Scafﬁeld et al., 2015; Sweeney, Qi, Xu, and Yang, 2016; Sweger, Casper, Scharf-Levie, Conklin, and McCarthy, 2007; Yang, Qi, and Yang, 2015).

2.6. Caveats and challenges

2.6.1. Ligand specificity

The inert properties of the activating DREADD ligands are a perquisite to study cell-speciﬁc effects and behavior. Yet, a common problem amongst especially the ﬁrst developed chemogenetic tools is the lack of speciﬁcity of the synthetic compounds used to activate the modiﬁed receptors. Although the latest generation of chemogenetic constructs require CNO as a ligand for DREADD-based constructs, which shows limited activation of endogenous receptors at low doses, it was shown that a small fraction of the administered CNO can revert-back metabolize to Clozapine, which could directly activate endogenous receptors (Chang et al., 1998; Gomez et al., 2017; Jendryka et al., 2019; M W and Y W, 1994; MacLaren et al., 2016; Manvich et al., 2018). However, the concentration used and behavioral effect studied is of utmost importance to control for these off-targets effects. Average CNO doses range from 0.1 up to 1 mg/kg, which appears to be biologically inert in mice (Alexander et al., 2009; Farrell et al., 2013; Krashes et al., 2011b). For behavioral testing, MacLaren et al. (MacLaren et al., 2016) have shown for example that relatively high doses of CNO (1 mg/kg) can alter speciﬁcally the startle response to a loud stimulus but does not alter spontaneous locomotion up to 5 mg/kg. Currently, it is not yet entirely clear whether these off-target effects are directly relatable to CNO activation or linked to possible reverse-metabolism of CNO to clozapine. The latter is further supported by the debate on whether CNO itself can cross the blood-brain-barrier (Gomez et al., 2017; Jendryka et al., 2019; Ji et al., 2016). To ensure, both for potential off target activation at the cellular level as well as to interpret behavioral effects, proper controls are crucial in every study utilizing DREADD-based chemogenetic strategies. It has been suggested to use Clozapine directly rather than CNO to activate DREADDs (Gomez et al., 2017). However, as clozapine is taken up efﬁciently in the brain, there is a high chance for binding to endogenous dopamine and serotonin receptors leading to a high chance of off-target effects (Gomez et al., 2017; MacLaren et al., 2016; Manvich et al., 2018). Recently, new synthetic ligands, such as compound 21 were developed with similar potency to activate the activating hM3Dq DREADD but lower chance of reverse-metabolism (Chen et al., 2015; Jendryka et al., 2019). These new ligands also require lower doses for DREADD activation, therefore, especially in studies where high doses of CNO administration are required, it might be advantageous to switch to newly developed ligands. Further research is however needed to fully classify these new compounds and compare them with CNO as a ligand for DREADD constructs. Interestingly, while the application of potential non-speciﬁc exogenous xenogenous ligands has always been described as a limiting factor for chemogenetics, prolonged optogenetic control by light has also been shown to induce off-target and adverse changes in neuronal function (Herman, Huang, Murphey, Garcia, and Arenkiel, 2014; Mahn, Prigge, Ron, Levy, and Yizhar, 2016). Caution should therefore always be taken when utilizing genetic methods to modulate neuronal activity.

2.6.2. Temporal resolution

While optogenetic constructs and electrostimulation can be applied at millisecond time resolution, chemogenetic activation leads to hour-long activation of these neurons, leaving limited space to improve stimulation timelines (Alexander et al., 2009; Guettier et al., 2009b). Therefore, chemogenetic control is limited by the poor temporal resolution. The reason for this small window of temporal modulation lies within the working mechanism of DREADDS as compared with optogenetic tools and electrostimulation. Rather than directly depolarization, chemogenetic approaches lower or increase the threshold for neuronal activation through altered cellular signaling by implementing activating or silencing constructs respectively, reducing the risk to induce non-physiological hyperpolarization (Guru, Post, Ho, and Warden, 2015; Kravitz and Bonei, 2013).

2.6.3. Chronic administration: Desensitization and expression trade-off

As with many endogenous GPCRs (DeWire, Ahn, Lefkowitz, and Shenoy, 2007; Kelly, Bailey, and Henderson, 2008), desensitization could also occur when chronically activating DREADDs. Following repetitive application of the synthetic ligand, reduced to almost no response can be observed owing to receptor internalization and down-regulation upon repetitive ligand induced activation. Desensitization however also depends on expression levels of these receptors (Roth, 2016). When expression levels are increased, less ligand is required to reach a maximal response and these receptors, which form the "receptor reserve" will never become saturated. Therefore, increased expression reduces the sensitivity to repeated dosing. On the other hand, high expression levels could also induce constitutive GPCR activity, without administration of CNO (Roth, 2016), although this has so far not been shown for virally or transgenically expressed DREADDs and might not occur for most DREADD applications (Alexander et al., 2009; Krashes et al., 2011a). Similar to possible off-target ligand effects, ideally controls are included to assess desensitization upon chronic ligand administration and receptor activation.

3. Implementing chemogenetics for spinal cord research

The spinal cord is composed of various descending motor and ascending sensory tracts, and contains a multitude of neuronal sub-populations which through functional networks ensure proper motor behavior and sensation. While the understanding of spinal neuronal wiring is relatively established, the functional connections and behavioral correlate in the context of SCI remain poorly understood. The use of chemogenetic strategies in the ﬁeld of SCI can be two-fold: On the one hand, chemogenetic activation and silencing can be used to decipher newly established connections after SCI, either spontaneously formed circuits or stimulated by therapeutic interventions (Fig. 2). On the other hand, as the development of neuronal connections relies on neuronal activity (Arakawa et al., 2014; Buffelli et al., 2003b; Flavell and Greenberg, 2008b), chemogenetic tools can also be used to modulate neuronal activity after SCI and specifically guide new connectivity patterns and synaptic contacts (Fig. 3).

3.1. Neuronal targets

As SCI is complex and functional impairment highly depends on which circuits are damaged, it is initially crucial to study which circuits are responsible for distinct functional correlates. Furthermore, as neuronal re-wiring is one of the key mechanisms of functional recovery after SCI, chemogenetic approaches can allow the modulation of
neuronal activity in order to understand spontaneously formed or therapeutically induced circuit remodeling after SCI.

### 3.1.1. Unravelling the function of established, remodeled and induced circuits

One of the main motor circuit relaying information from the motor cortex to the spinal cord is the corticospinal tract (CST). Originating in layer 5 in the cortex, the CST is spatially organized in 3 compartments in rodents: the main dorsal CST harvesting up to 96% of the CST projections, the dorsolateral CST containing 3% of the CST axons and the minor ventral CST (Rasmussen and Carlsen, 2016). Depending on the type of injury, the entire CST or parts of the CST are injured which lead to functional impairments (Hilton et al., 2013; Raineteau and Schwab, 2001; Rasmussen and Carlsen, 2016). Interestingly however, while the innate capacity for regeneration of the CNS is limited, spontaneous functional recovery does occur, e.g. via remodeling of the CST tracts (Bareyre et al., 2004; Courten et al., 2008; Jacobi and Bareyre, 2015; Raineteau and Schwab, 2001; Rasmussen and Carlsen, 2016; Rosenzweig et al., 2010; Zörner et al., 2014a). In an attempt to understand the functional correlate of CST projections, Wang et al. (Wang, Maunze, Wang, Tsoulfas, and Blackmore, 2018) set out to clarify how silencing of neuronal subpopulations, such as upper CST motoneurons or interneurons in the cervical spinal cord, would affect motor behavior. By combining retroAAV-expressing CRE and local hM4Di injections, they silenced the forelimb CST and the general neuron population in the cervical spinal cord. They found that general silencing of spinal interneurons led to gross locomotion deficits while the silencing of CST upper neurons led to more subtle changes in motor behavior that were only evident on a horizontal ladder rung. As the CST is important in the supraspinal control of fine paw placement (Bareyre et al., 2004), this study effectively demonstrates how DREADDs can be used to link spinal circuitry with functional outcomes.

One of the first studies implementing chemogenetics to understand the functional wiring after SCI was performed by Hilton et al. (Hilton et al., 2016) in 2016. In this study, the authors aimed at understanding whether spontaneous recovery after specific injury of the main dorsal CST at cervical level (C3-C4) was dependent on re-wiring severed vs spared axons. By using AAVs expressing silencing hM4Di DREADDs and stereotactic injections of AAV-expressing CRE, they were able to silence the spared dorsolateral CST connections and assess if the improvement of motor function after spontaneous recovery was abrogated. They found that acute silencing of the dorsolateral tract after spontaneous recovery significantly abolished recovery, providing evidence for the prominent role of the uninjured dorsolateral CST for spontaneous recovery. In line with the use of DREADDs to understand the functional importance of circuit wiring after SCI was a study involving the gigantocellular reticular nucleus (NGR) after incomplete SCI (Engmann et al., 2020). Here the authors studied spontaneous recovery of reticulospinal circuits (ReSt), descending motor pathways originating in the brain stem. Spontaneous recovery linked to these tracts after unilateral cervical SCI has been described by two mechanisms, either local remodeling around the lesion site or compensatory outgrowth of the spared axons (Zörner et al., 2014b). Using DREADDs to silence both distinct populations, they were able to study their respective contribution to spontaneous recovery. They found that both populations are required for functional recovery but that each is responsible for specific aspects of the motor function. While spontaneous re-wiring of spinal tracts has been shown at different spinal levels and distinct lesion types, these studies employed chemogenetic tools to link the functional recovery with anatomical re-wiring.

Chemogenetic tools can also advance our understanding of functional wiring after SCI by shedding light on how distinct treatment paradigms affect wiring and their functional outcome. A study by Sun et al. (Sun et al., 2020) has used gabapentin, a blocker of the α2δ subunit of voltage-gated calcium channels, as a treatment for CST fibers to facilitate functional recovery after cervical SCI. By silencing DREADDs in the forelimb CST three months after treatment, they could show that the functional recovery was abolished. Utilizing chemogenetic tools therefore provides extra information on how therapeutic interventions lead to functional improvements.

### 3.1.2. Manipulating circuits to understand the mechanisms driving axonal rewiring after SCI

As the establishment of neuronal connections is highly dependent on neuronal activity during development, tools to modulate activity like DREADDs could be advantageous to alter circuit wiring after SCI as damaged neurons share some transcriptional similarities with developing neurons (Poplawski et al., 2020). Several studies have used DREADDs in different models of SCI to assess whether and how the modulation of neuronal activity could influence functional recovery. Using staggered lateral hemisections in mice at T7-T10, Chen et al. studied how reducing excitability of inhibitory interneurons around the lesion site could affect functional recovery (Chen et al., 2018). Implementing silencing DREADDs in inhibitory neurons and daily administration of CNO, they show that mice gain hindlimb-specific functional skills while interestingly activation of DREADDs in excitatory neurons did not show any motor improvements. This study indicates that neuronal activity could indeed serve as a therapeutic tool, but that

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**Fig. 2.** Spontaneous formation of spinal detour circuits and local rewiring following spinal cord injury. A- The hindlimb corticospinal tract (CST) is a descending tract, relaying information from the motor cortex to lumbar spinal motor neurons. The CST is mainly localized in the dorsal column in rodents, while a smaller portion runs dorsolaterally and through the ventral white matter. B- After incomplete spinal cord injury, a detour circuit is formed spontaneously. The injured CST axons form new connections onto spared long propriospinal interneurons (LPSNs), which in turn relay the information to the motor neurons, ultimately leading to functional recovery. C- Another type of remodeling can occur by local rewiring of the CST itself, either via injured or spared CST projections.
A. Acute treatment

Intact circuit functionality

B. Chronic treatment

Detour circuit formation

Local rewiring

C. Modulation of non-neuronal cells

D. Altered behaviour

Ladder Rung
Catwalk
Kinematics

Fig. 3. Use of DREADDs in the context of spinal cord injury and possible functional assessments to study their effect. DREADDs have been used via AAV-mediated delivery in three main ways. A- Study of intact circuitry and correlated behavior, for instance by specifically silencing the hindlimb CST. B- DREADDs have been used to modulate neuronal activity and study the effect on the formation of either detour circuits or local rewiring after injury. C- DREADDs have also been used to study how neuronal activity in the spinal cord can influence the cellular response after injury of non-neuronal cells such as glial cells. D- Specific motor tasks are often implemented to understand the impact of neuronal activity manipulation. The ladder rung, either regularly or irregularly spaced, allows for assessment of fine paw placement, while catwalk analysis provides information on gait parameters. Finally, the use of a treadmill for kinematic analysis can also be used, where many kinematic parameters can be extracted for a detailed overview of changes at the anatomical level.
the subpopulations of neurons targeted, and the level of activity are critical to improve motor behavior. In one of our own studies, we set out to study if neuronal activity is also relevant for the formation of a detour circuit (Bradley et al., 2019). Previously, it was shown that apart from local remodeling around the lesion site, the formation of new circuits bypassing the lesion can be formed after incomplete SCI which leads to spontaneous functional recovery (Bareyre et al., 2004; Jacobi et al., 2015; Loy et al., 2018, 2021). Rather than re-establishing functional synapses between the CST tract and motor neurons, this type of rewiring relays information via long propriospinal interneurons (LPSNs). Upon thoracic bilateral hemisection, the injured dorsal CST sprouts into the cervical spinal cord and forms synaptic contacts onto ventral LPSNs of which the axonal projections are spared. These projections in turn contact motor neurons and form a new functional circuit that leads to functional recovery. In our study, we have silenced these interneurons, either specifically in LPSNs by combining retroAAV-expressing CRE injections in the lumbar part and cre-dependent silencing DREADDs at the cervical level or by silencing all excitatory neurons in the cervical spinal cord by local injection of cre-dependent silencing DREADDs of Vglut2-Cre mice (Bradley et al., 2019). We found that overall silencing of excitatory neurons led to decreased CST sprouting into the ventral spinal cord, diminished formation of synaptic contacts and ultimately decreased functional recovery. Neuronal activity is therefore crucial to establish new detour circuits to enhance functional recovery.

Another set of studies have used chemogenetic tools to foster functional recovery at an acute timescale, meaning that they study the acute effects of stimulation rather than the long-term effects which would be based on circuit remodeling rather than direct neuronal activation. hM3Dq DREADDs have been used to excite glutamatergic V2a neurons of the ventral cervical spinal cord after a cervical C2 hemisection (Jensen, Allain, and Crone, 2019). A lesion at this level leads to paralysis of the diaphragm as measured with electromyography (EMG) recordings. Upon activation of the excitatory DREADDs, an acute restoration of rhythmic burst activity was detected while the nonlesioned side retained its normal rhythmic breathing level. Silencing of activity in V2a neurons however led to slow and aberrant breathing. A similar study was performed where activation of all mid-cervical excitatory neurons led to acute promotion of respiratory function (Satku-nendrarajah, Karadimas, Laliberte, Montandon, and Fehlings, 2018). A recent publication by Brommer et al. (Brommer et al., 2021) focused on how activity modulation around the lesion site of a thoracic T8 complete spinal cord injury could mediate acute functional motor effects. Utilizing either hM3Dq expression in Vglut2-Cre mice, or hM4Di in Vgat-Cre mice, they have shown that acute activation of either DREADD in both experimental designs, led to a marked acute improvement of motor skills and stepping kinetics.

### 3.2. Non-neuronal effects upon chemogenetic manipulation

SCI does not only affect neuronal subtypes but induces a widespread change in distinct cell types such as microglia, astrocytes and oligodendrocytes (Burdar and Sofroniew, 2014a, 2014b; Duncan et al., 2020; O’Shea, Burda, and Sofroniew, 2017). These cells however do not operate completely autonomously but depend on neuronal activity patterns (Aguado, Espinosa-Parrilla, Carmona, and Soriano, 2002; Gautier et al., 2015; Habas, Hahn, Wang, and Margeta, 2013; Hasel et al., 2017; Liu et al., 2015; Umpierre and Wu, 2020). Therefore, chemogenetic strategies have been proven useful to unravel the effects of neuronal activity on non-neuronal cells after SCI. Another research area focusing on effects of non-neuronal targets is how exogenous stem-cell based grafts can contribute to functional recovery after SCI. Also here, chemogenetics allows to further understand the exact role of these grafted cells, both for circuit formation as well as for motor behavior. In this final part, we review SCI studies that have used chemogenetic approaches to either alter neuronal activity in view to understand the indirect effects on non-neuronal cell populations or to directly modulate activity in non-neuronal cells such as grafted stem cells.

#### 3.2.1. Indirect cellular responses upon neuronal activity modulation

As many other cell types co-exist and sustain a functional neuronal network via direct and indirect communication with neurons, the modulation of neuronal activity could also affect other cell types in an acute and chronic manner. Mitew et al. (Mitew et al., 2018) have shown that chemogenetic activation of neurons could for instance alter myelination in the spinal cord. Specifically, stimulation of somatosensory axons led to proliferation and differentiation of oligodendrocyte progenitor cells (OPCs), therefore leading to an increased likelihood of being myelinated. Therefore, increasing neuronal activation could also improve re-wiring or even functional restoration by facilitating myelination of newly grown axonal sprouts. Recently a new study reinforces the role of oligodendrocytes upon activation of neurons (Luo et al., 2021). Upon a mild contusion injury at thoracic T10 level, upper motor neurons were chemogenetically activated leading to proliferation and maturation of OPCs and improvement in functional outcomes. Another study however shows that the indirect effects of modulating neuronal activity can be more widespread (Ueno, Ueno-Nakamura, Niehaus, Popovich, and Yoshida, 2016). After SCI, brainstem control can be retained or lost depending on whether the lesion occurred higher or below cervical level C5 respectively. When lesions occur at high cervical levels and brainstem control is removed, systemic immune suppression follows due to splenic atrophy and leucopenia (Zhang et al., 2013). Upon silencing of spinal interneurons, Ueno et al. found that the immune suppressive autonomic reflex is attenuated after SCI (Ueno, Ueno-Nakamura, Niehaus, Popovich, and Yoshida, 2016). While currently limited, it is crucial to understand whether functional outcomes upon neuronal activity modulation are direct or indirect as they could also give rise to new downstream therapeutic targets.

#### 3.2.2. Chemogenetic approaches to modulate non-neuronal cells in SCI

Another application of chemogenetics related to SCI is the use of grafted stem cells, which has gained significant interest over the past decade (Barnabe-Heider and Frisén, 2008; Coutts and Keirstead, 2008; Cummings et al., 2005; Dell’Anno et al., 2018; Iwamani et al., 2005; Liau et al., 2020; Lu, Jones, Snyder, and Tuszynski, 2003; Paul Lu et al., 2012; Tewarie, Hurtado, Bartels, Grotenhuis, and Oudega, 2009; Tsuji et al., 2010). While grafting differentiated stem cells in distinct SCI models has been shown to improve locomotion, chemogenetic modulation of these grafted cells could provide new insights to determine whether and how they are involved in the formation of new circuits. A study by Dell’Anno has implemented silencing hM4Di DREADDs to show that grafted human neuroepithelial stem (NES) cells are able to form new connections and are essential for functional recovery (Dell’Anno et al., 2018). They show that upon transplantation into the lesion after dorsal hemisection, elongation of both the grafted as well as the endogenous axonal projections occurs, ultimately leading to improved locomotion. To further understand whether this newly established relay circuit is also responsible for functional recovery, silencing DREADDs were introduced to the grafted NES cells. Upon acute activation with CNO, they found that mice showed a significant decline in locomotor skills, while they regained motor skills after a wash-out period of 24 h post-CNO. This data convincingly showed that the grafted cells do not only form a new detour circuit but that they are integrated in a new functional network that is crucial for functional recovery.

### 4. Current limitations of chemogenetics implementation for SCI

As previously demonstrated, chemogenetic tools clearly help shedding light on spinal circuitry in injured and uninjured conditions and can be used to guide neuronal plasticity after SCI. However the readout of such experiments can be challenging as often validation of the chemogenetic approaches to either alter neuronal activity in view to understand the indirect effects on non-neuronal cell populations or to directly modulate activity in non-neuronal cells such as grafted stem cells.
the actual behavioral assays. For example, as chemogenetic tools do not act by directly stimulating neuronal populations but rather function through altered cellular signaling, validation of these approaches is required in diverse neuronal populations and at distinct ligand concentrations (Pati et al., 2019). A wide array of techniques can be implemented not only to assess whether silencing or excitatory DREADDs influence neuronal firing but also provide further insight into how distinct neuron populations fire in terms of frequency, amplitude and network activity. Apart from immunohistochemistry for c-fos, genetic tools such as calcium or voltage indicators and finally electrophysiology can be used to assess the capacity and extend of DREADDs to alter neuronal activity. An early example of this was published by Wahl et al. (Wahl et al., 2014) that used acute chemogenetic silencing of midline-crossing CST fibers after stroke to demonstrate that these axonal projections were required for spontaneous recovery. Utilizing EMG recordings after intracortical microstimulation, they confirm the efficacy of their silencing paradigm as the EMG signal was significantly reduced 30 min post CNO administration but was maintained in animals without CRE-induced expression of silencing DREADDs. A second limitation of implementing chemogenetic tools for SCI is the complexity of understanding correlated behavioral recovery (Touadé, Hurd, and Magnusson, 2013). Similarly to other treatment-based SCI studies, one of the main outcomes to assess the extent of a treatment effect is motor recovery. With the spontaneous formation of detour circuits, it can be challenging to carefully dissect the exact contribution of neuronal plasticity. Recovery can be either be based on an identical movement patterns as in uninjured conditions or on new compensatory patterns. To address these questions, more detailed behavioral tests and motion analysis such as kinematics are required. Development of new AI-based tools such as DeepLabCut (Mathis et al., 2018) and DANNCE (Dunn et al., 2021) in combination with easy to use analysis toolboxes such as ALMA (Aljovic, Zhao, Chahin, and Val, C. de la R. del, Steenbergen, V. van, Kerschensteiner, M., and Bareyre, F. M., 2021), facilitate the implementation of kinematic analysis and will significantly improve our understanding of motor recovery after SCI.

5. Conclusion

The development of DREADDs opens up new possibilities for different research areas, particularly for functional neuroscience. The ability to acutely or chronically modulate neuronal activity allows not only to study neuronal circuitry but also behavioral correlates both in health and disease. As neuronal wiring after SCI is complex with the formation of detour circuits and compensation mechanisms, the use of chemogenetic approaches is already shining new light on the functionality of these circuits and the role of neuronal activity after SCI. It also opens new possibilities for future translational and clinical applications as chemogenetic tools have also been shown to not only understand but to directly intervene with the formation of new circuits. While significant efforts are made to modulate neuronal activity after SCI, there is also a need to study how chemogenetic modulation of non-neuronal cells such as microglia could play a role in the cellular response to SCI. Therefore, the use of chemogenetic approaches should not be limited solely to neurons but expanded to other cellular players involved in the cellular response to SCI. Altogether, the use of chemogenetic tools will improve our understanding of circuit wiring after SCI and advance the development of new treatment paradigms that could be of great use for translational and clinical research.

Author contributions

VVS and FMB wrote the paper. All authors approved the final version of the paper.

Declaration of Competing Interest

None.

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